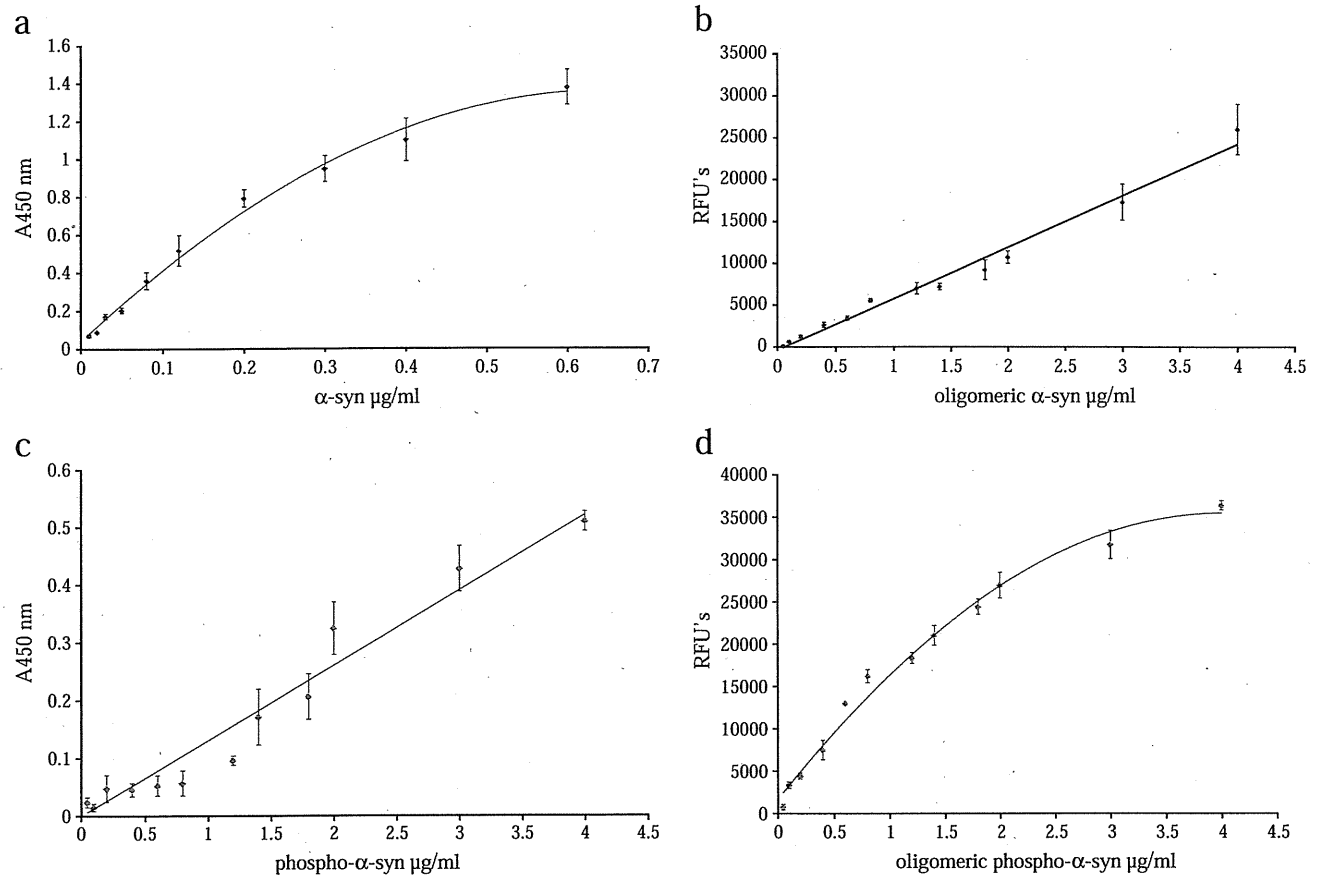

Fig. 1. Standard curves for immunoassays. Total α-syn (a), oligomeric α-syn (b), phosphorylated α-syn (c) and oligomeric phosphorylated α-syn (d).

selected for immunoblotting in order to characterise the molecular properties of α-syn within CSF.

For SDS-PAGE, these samples were run on 12.5% polyacrylamide gels and the separated proteins were electrotransferred onto nitrocellulose membranes (0.45 µm, Invitrogen), at 25 V, 125 mA for 75 min, which were then blocked with 5% powdered, skimmed milk dissolved in PBS-T for 1 h. Membranes were incubated overnight with (a) polyclonal antibody, anti-α/β/γ-synuclein (FL-140) (Santa Cruz, USA), dilution 1:3000 or (b) phosphorylated anti-α-synuclein pS129 (Epitomics, USA), dilution 1:3000. The membranes were washed three times in PBS-T, followed by incubation with HRP-conjugated rabbit anti-mouse or goat anti-rabbit (Sigma), as appropriate, at 1:5000 in PBS-T, for 1 h. The protein bands were visualised using ECL reagents (Pierce, Rockford, IL) as described by the manufacturer.

Haemoglobin assays

Because previous studies have suggested that contamination of CSF samples by blood, either at lumbar puncture or at post mortem, might contribute through lysed red cells to α-syn measures (Hong et al., 2010), we assayed our CSF samples for haemoglobin levels using an immunoassay. The haemoglobin levels in CSF samples were measured using a Human haemoglobin ELISA Quantitation Kit from Bethyl Lab Inc (Montgomery, TX, USA) according to the manufacturer's instructions.

Histological methods

Wax sections were immunostained for α-syn pathology using the rabbit polyclonal antibody #1175 with microwave pretreatment in 0.1 M citrate buffer pH 6.0. This antibody recognises both phosphorylated and non-phosphorylated forms of α-syn, but in PD and DLB generates identical patterns of immunostaining as pSer129 — an antibody specific to forms of α-syn phosphorylated at Ser129 (Obi et al., 2008).

The severity of α-syn pathology (ie overall density of Lewy bodies and Lewy neurites) within each brain region was rated on a 4 point scale (0 = absent, 1 = occasional/mild, 2 = common/moderate, 3 = numerous/severe), and a composite score across all 5 regions obtained by summation of individual scores, with a maximum score of 20 possible.

Statistical analysis

All data analysis was performed using SPSS v 16.0. For normally distributed data, two-sample Student's *t*-test for independent samples or one-way ANOVA were applied in comparing means of CSF α-syn measures between two or more groups, respectively. Alternatively, for non-normally distributed data, Kruskal–Wallis analysis of variance with post hoc Mann Whitney *U* test was used. Similarly, when testing the correlations between CSF α-syn measures and age at onset or death, or duration of illness, or haemoglobin concentration Spearman's first rank correlation or Pearson's correlation tests were used as appropriate. All levels of significance were two-tailed and set at $p<0.05$.

Results

There were no significant differences in mean CSF levels of total α-syn (Fig. 2a), oligomeric α-syn (Fig. 2b) or phosphorylated α-syn (Fig. 2c) between PD, DLB, PSP, MSA and control groups ($F_{4,89}=1.36$, $p=0.255$, $F_{4,89}=1.37$, $p=0.249$, $F_{4,87}=1.21$, $p=0.313$, respectively) (Table 2). In contrast, mean CSF levels of oligomeric phosphorylated α-syn (Fig. 2d) were highly significantly different between PD, DLB, PSP, MSA and control groups ($F_{4,84}=22.4$ $p<0.001$) (Table 2). Post

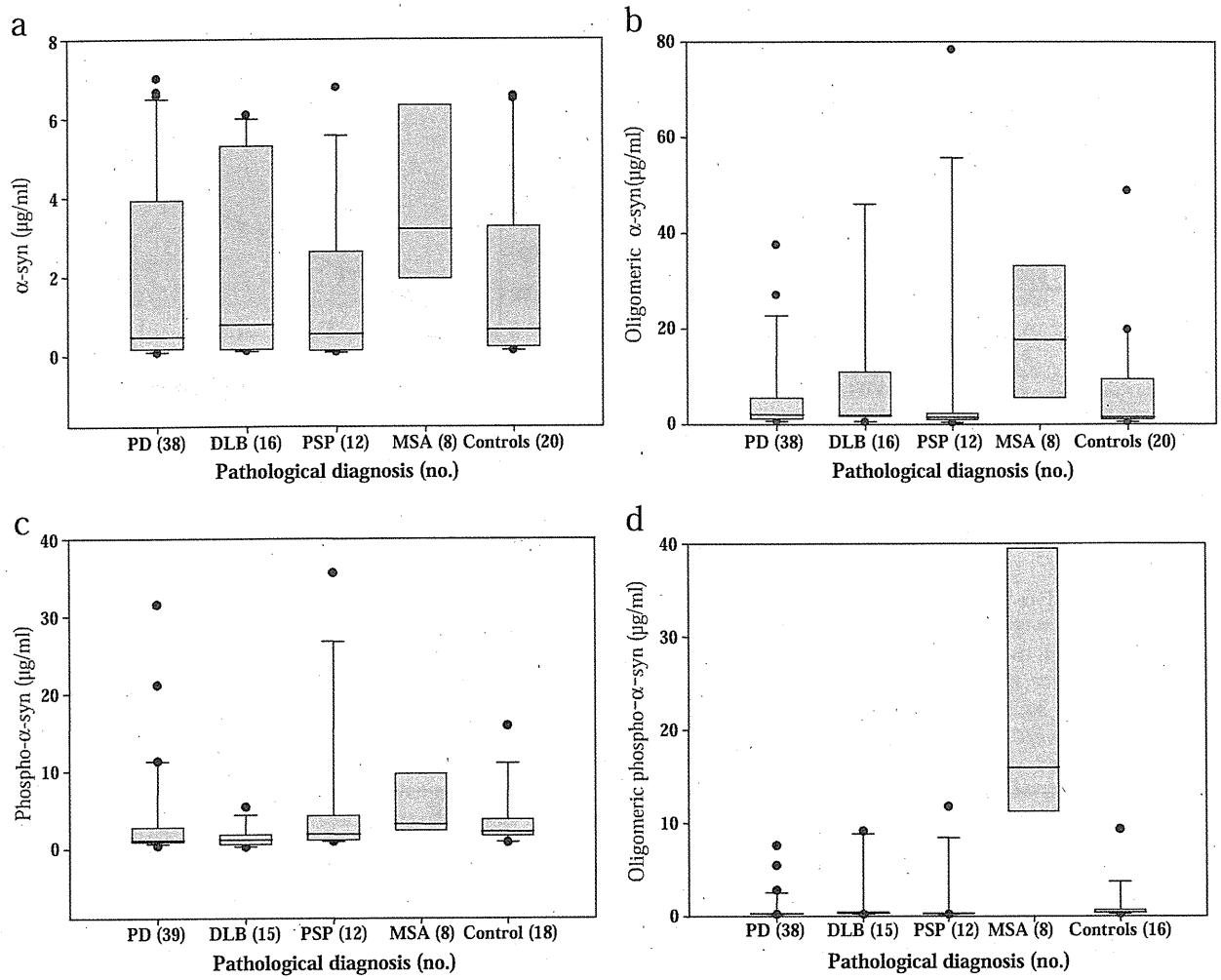

Fig. 2. Box-whisker plots for the CSF α-synuclein immunoassay results. The length of each box represents the interquartile range (75–25%) of the sample, the solid line drawn across the box the median, and outliers are denoted by dots. Immunoassay results are presented for each pathological diagnostic group for (a) total α-syn levels, (b) oligomeric α-syn levels, (c) phosphorylated α-syn levels and (d) oligomeric phosphorylated α-syn levels.

hoc Tukey test showed highly significant differences ($p<0.001$) in mean CSF levels of oligomeric phosphorylated α-syn between the MSA and all of the other diagnostic groups (Table 2). There were no significant differences in mean CSF levels of total α-syn, oligomeric α-syn, total phosphorylated α-syn or oligomeric phosphorylated α-syn between PD, PD (Cog) and PD (Dem) groups ($F_{2,37}=1.23$, $p=0.303$, $F_{2,37}=0.77$, $p=0.468$, $F_{2,38}=0.53$, $p=0.596$, $F_{2,37}=1.67$, $p=0.202$, respectively) although it is noted that the numerical levels of oligomeric phosphorylated α-syn increased progressively from PD through PD (Cog) to PD (Dem) groups (Table 2).

Table 2
Mean (±SD) CSF levels of α-syn (μg/ml) as determined by each immunoassay in patients with PD (non-demented (nonD), cognitively impaired (Cog) and demented (Dem), DLB, PSP, MSA and normal control individuals.

	Total α-syn (μg/ml)	Oligo α-syn (μg/ml)	pS α-syn (μg/ml)	Oligo pS α-syn (μg/ml)
PD (n=39)	1.93±2.49	7.04±1.64	3.43±6.18	0.77±1.51
PD (nonD) (n=13)	1.34±2.16	11.11±2.58	4.41±8.68	0.26±0.03
PD (Cog) (n=10)	1.47±2.10	2.35±2.02	1.76±1.02	0.68±0.78
PD (Dem) (n=16)	2.67±2.83	6.37±9.91	3.67±5.73	1.28±2.27
DLB (n=16)	2.31±2.51	9.47±2.09	1.63±1.42	1.60±3.02
PSP (n=12)	1.45±1.97	7.91±2.21	5.14±9.73	1.25±3.32
MSA (n=8)	3.80±2.40	22.49±.19	7.14±9.19	19.56±1.66*
Control (n=20)	1.87±2.29	6.78±1.14	3.58±3.85	1.05±2.23

*Indicates significantly different ($P<0.001$) from assay value for patients with PD (overall, and nonD, Cog and Dem subgroups), DLB, PSP and normal control individuals.

There were no significant correlations between CSF levels of total α-syn, oligomeric α-syn, phosphorylated α-syn or oligomeric phosphorylated α-syn, and pathology scores, expressed either as total pathology score or as severity scores for each individual area, either across all 71 patients or within the PD and DLB patients, separately or combined. Similarly, there were no significant correlations between CSF levels of total α-syn, oligomeric α-syn, phosphorylated α-syn or oligomeric phosphorylated α-syn, with age at onset of disease or duration of illness within PD and DLB patients, either as single or combined groups.

Immunoblotting

Immunoblots of α-syn within CSF, of PD, DLB, MSA, PSP and control cases with relatively high and low absorption α-syn values are shown in Fig. 3. Immunoblots using the polyclonal anti-α/β/γ-synuclein antibody FL-140 (Fig. 3a), showed in most/all samples irrespective of diagnostic status, an immunoreactive band at ~46–48 kDa, which was strongly present in all samples with high α-syn CSF levels (lanes C, E, G and I) but was less strongly present in those with low CSF α-syn levels (lanes B, D, F and H). In two samples with high CSF α-syn levels there was an additional α-syn species at 16 kDa which represents the monomeric protein (lanes C and G) and was not present in those with low CSF α-syn levels. Using the phosphorylated anti-α-synuclein pS129 antibody (Fig. 3b), only the 46–48 kDa species was detected, again this being strongly present in

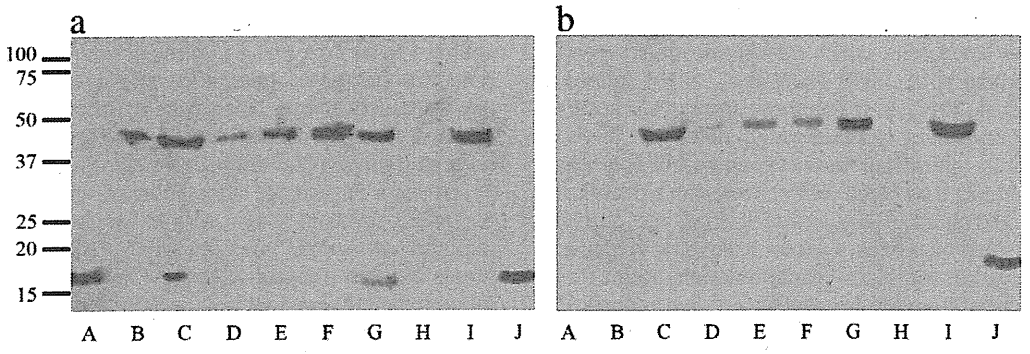

Fig. 3. Immunoblots of α-syn (a) and pS-α-syn (b) within CSF of PD, DLB, MSA, PSP and control cases with relatively high and low α-syn immunoassay levels (lanes C, E, G, I and B, D, F and H, respectively) are shown, along with recombinant non-phosphorylated standard (lane A) and recombinant oligomerised, phosphorylated standard (lane J). The immunoblot using polyclonal anti-α/β/γ-synuclein antibody FL-140 (Santa Cruz) (Figure a), shows in most/all samples irrespective of diagnostic status, an α-syn species at ~46–48 kDa, which is strongly present in all samples with high CSF α-syn levels (lanes C, E, G and I) but is less strongly present in those with low CSF α-syn levels (lanes B, D, F and H). In two samples with high CSF α-syn levels there is an additional α-syn species at 16 kDa (lanes C and G) which is not present in those with low CSF α-syn levels, but has the same molecular mass as the recombinant protein (lane A). Using the phosphorylated anti-α-synuclein pS129 antibody (Figure b), only the 46–48 kDa species is detected, again this being more strongly present in all samples with high CSF α-syn levels (lanes C, E, G and I) but less strongly present in those with low CSF α-syn levels (lanes). Recombinant α-syn (lane A) is not detected by the phosphorylation specific α-syn antibody (lane A).

all samples with high CSF α-syn levels (lanes C, E, G and I) but less strongly present in those with low CSF α-syn levels (lanes B, D, F and H). Recombinant α-syn at 16 kDa (lane A) was only detected by the FL-140 antibody, and not the phosphorylation dependent α-syn antibody pS129 (see Fig. 3a, lane A). Inspection of the immunoblots (Fig. 3) suggests, therefore, that this 46–48 kDa α-syn species might represent an oligomerised and phosphorylated form of α-syn (the estimated molecular mass would suggest a trimer). Minor quantities of non-phosphorylated (monomeric) α-syn were also present, but these were only detectable in those cases with high CSF α-syn levels.

Confounding factors

It has been suggested from previous studies (Mollenhauer et al., 2008) that levels of total α-syn within CSF may progressively increase with increasing post mortem delay time. However, we found no correlation between levels of α-syn within CSF and post mortem delay time for any of the 4 immunoassays, either when all 96 patients were considered as a group, or separately according to diagnosis (data not shown). Moreover, mean post mortem delay time did not differ significantly between any of the diagnostic groups ($F_{4,89} = 1.90$, $p = 0.118$).

Furthermore, because, some of the CSF samples were obviously contaminated with blood, and because previous studies have suggested that such contamination of CSF samples might contribute to α-syn measures through lysed red cells (Hong et al., 2010), we assayed the CSF samples for haemoglobin. Mean haemoglobin levels within CSF samples did not differ significantly between the various diagnostic groups (PD = 4.6 ± 2.3 μg/ml; DLB = 5.1 ± 2.6 μg/ml; PSP = 5.8 ± 2.2 μg/ml; MSA = 4.4 ± 2.0 μg/ml; Controls = 6.1 ± 3.2 μg/ml; $F_{4,73} = 1.01$, $p = 0.408$), nor was there any correlation between CSF haemoglobin level and any one of the 4 measures of α-syn (total α-syn $r = 0.168$, $p = 0.148$; oligomeric α-syn $r = 0.156$, $p = 0.178$, phosphorylated α-syn $r = 0.094$ $p = 0.418$, oligomeric phosphorylated α-syn $r = 0.027$ $p = 0.818$).

Discussion

In the present study, we have shown by immunoblotting that both phosphorylated and non-phosphorylated forms of α-syn can be detected in CSF of patients with PD, DLB, PSP, MSA, and also in control individuals, and that the levels of these can be measured by immunoassay. Consequently, we have not only employed conventional immunoassays to measure total levels of α-syn, as many other groups have done previously, but we have developed new assays based on the detection of phosphorylated and/or oligomerised forms of α-syn, since these may have more relevance in targeting and indexing the pathological species of α-syn that is accumulated in the brains of patients with these disorders (Fujiwara et al., 2002; Anderson et al., 2006; Obi et al., 2008).

The main finding to emerge from this study is the observation that measurement of oligomeric phosphorylated forms of α-syn in CSF can differentiate patients with MSA from all of the other diagnostic groups. Although this finding is based on a relatively small number of MSA cases, and may therefore be considered preliminary, the distinction between MSA and other patient groups was robust, and higher α-syn levels were consistently seen across all 4 assays in MSA compared to PD/DLB and other non-synucleinopathies. Recent findings by Hirohata et al. (2011) showing that an unidentified factor in CSF promotes the *in vitro* aggregation of α-syn, and that CSF from patients with MSA was more effective in this respect than CSF samples from patients with PD, would be consistent with our findings.

There have been a few other recent studies looking at biomarkers in MSA. Aerts et al. (in press) compared total α-syn in lumbar CSF from 47 patients with MSA with those from 58 patients with PD, 3 with DLB, 10 with PSP and others with CBS and vascular parkinsonism, but detected no significant differences in mean level between any of the diagnostic groups. Similarly, Shi et al. (2011) did not find any differences in mean α-syn levels between 32 patients with MSA and 126 with PD, though in both instances such levels were significantly lower than 137 control subjects and 50 patients with Alzheimer's Disease (AD). Mollenhauer et al. (2011) also reported α-syn levels to be decreased in patients with MSA (as well as in those with PD and DLB) compared to patients with AD, and ones with other neurological disorders. As seen in these other studies, we also found that measurements of total α-syn did not clearly distinguish patients with MSA from those with PD (and other parkinsonian disorders).

Our present data therefore suggest that raised levels of phosphorylated forms of α-syn, rather than total α-syn, might provide a test for not only distinguishing MSA from normal individuals, but perhaps more importantly from other synucleinopathies. However, it is accepted that these are very preliminary data and will need verification in larger sample cohorts, especially in samples taken from living patients earlier in the course of their illness before it is possible to categorically state the value of this as a diagnostic test.

There are several potential confounders in our study which we have taken into consideration, leading us to believe that our present findings are genuine.

Firstly, as compared to lumbar puncture where this is less of a problem, it is almost inevitable that the practicalities of taking CSF

samples at post mortem will inadvertently include contamination of some samples by red blood cells, which themselves are a potential source of α-syn (Barbour et al., 2008; Hong et al., 2010). Indeed, a recent study (Hong et al., 2010), based on the analysis of CSF samples obtained at lumbar puncture by Luminex assays, noted that levels of α-syn were significantly lower than those of controls, but only when those samples contaminated by blood (with haemoglobin concentrations greater than 200 ng/ml) were excluded from the analysis. Consequently, we assayed our CSF samples for haemoglobin to assess whether varying degrees of blood contamination might have contributed to the output from our immunoassays. Our findings of a lack of correlation between CSF haemoglobin level and any of the α-syn measures, either over all the patients and controls, or within any of the diagnostic groups, makes us confident that the assay results presented here are reliable and have not been influenced to any great extent by contamination by red blood cells. Moreover, it is known that α-syn within platelets is not phosphorylated at serine 129 (Shults et al., 2006), and therefore contamination of CSF samples by blood may not, in any case, be expected to influence the output from the immunoassays for phosphorylated forms of α-syn.

Secondly, it has been suggested from previous studies (Mollenhauer et al., 2008) that levels of total α-syn within CSF may progressively increase with increasing post mortem delay time and hence, the variations in α-syn measured in this study might simply reflect group differences in post mortem delay times. However, we found no correlation between levels of α-syn within CSF and post mortem delay time for any of the 4 immunoassays, either when all 96 patients were considered as a group, or separately according to diagnosis (data not shown). Moreover, mean post mortem delay time did not differ significantly between any of the diagnostic groups. Therefore, we have no reason to believe that the findings presented here are not reliable. To our knowledge, there have been no other studies except that of Mollenhauer et al. (2008) indicating a rise in levels of α-syn within CSF at post mortem, and so it is still uncertain as to whether these levels do indeed change after death.

There have been a number of previous studies investigating whether measurement of total α-syn can also be employed as a biomarker for PD, though these have not always provided consistent results. For example, an earlier study by Tokuda et al. (2006) found levels of total α-syn on average to be lower in samples of CSF taken from a group of patients with PD compared with a group of normal or neurological controls. However, in a later study (Tokuda et al., 2010) no such clear differences were seen. Elsewhere, Mollenhauer et al. (2008, 2011) have similarly reported a lowering of total α-syn levels between PD patients and controls, though this finding has not been replicated by others (Ohrfelt et al., 2009; Spies et al., 2009; Reesink et al., 2010; Aerts et al., in press). In the present study, using a similar immunoassay protocol to Tokuda et al. (2006, 2010), we also found no significant differences in total α-syn levels between PD patients and controls, or between DLB patients and controls. Hence, it remains unclear whether measuring total α-syn levels in CSF has any utility in discriminating between patients with LBD (i.e. PD and DLB) and control subjects.

Because recent studies have suggested that oligomeric forms of α-syn may be the toxic species that induce neuronal cell death, it has been suggested that measurement of these particular forms of α-syn might be better biomarker for PD than total α-syn (Tokuda et al., 2010). Indeed, it has been shown that soluble oligomeric forms of α-syn are elevated in brain homogenates of patients with PD and DLB (Sharon et al., 2003, Paleologou et al., 2009), supporting such an argument. In keeping with this hypothesis, El-Agnaf and colleagues have found elevated levels of oligomeric α-syn in plasma (El-Agnaf et al., 2006) and CSF (Tokuda et al., 2010) of PD patients compared to controls. However, again using similar methodologies, we were unable to replicate these findings with our results showing no clear distinctions between PD or DLB patients and controls with respect to oligomeric forms of α-syn.

It is not clear from a methodological standpoint why we have obtained results dissimilar to those of Tokuda et al. (2010), though it is notable that these latter authors employed CSF samples obtained at lumbar puncture from living patients most of whom were sampled within 5 years of onset of illness. In the present study we employed post mortem samples from end-stage PD and DLB patients dying 11–44 years on average after onset of illness. Hence, increases in oligomeric forms of α-syn early in the course of the disease could diminish with time. This same kind of situation has been seen in Motor Neurone Disease where CSF levels of TDP-43 protein were found to be increased within the first 11 months of illness, but after this time fell and became not significantly different from control subjects (Kasai et al., 2009).

In summary therefore, we present some new methods of assessing α-syn levels in CSF from patients with parkinsonian disorders, and show that those assays based on the detection of phosphorylated oligomeric forms of α-syn may have utility in differentiating patients with MSA from other parkinsonian disorders in which the underlying pathology is also α-syn based (i.e. PD and DLB) or is tau-based (i.e. PSP).

Supplementary materials related to this article can be found online at doi:10.1016/j.nbd.2011.08.003.

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References

Aerts, M.B., et al., in press. CSF α-synuclein does not discriminate between parkinsonian disorders. Neurobiol Aging. doi:10.1016/j.neurobiolaging.2010.12.001.
Angot, E., Brundin, P., 2009. Dissecting the potential molecular mechanisms underlying alpha-synuclein cell-to-cell transfer in Parkinson's disease. Parkinsonism Relat. Disord. 15 (Suppl. 3), S143–S147.
Anderson, J.P., et al., 2006. Phosphorylation of Ser-129 is the dominant pathological modification of α-synuclein in familial and sporadic Lewy body disease. J. Biol. Chem. 281, 29739–29752.
Barbour, R., et al., 2008. Red blood cells are the major source of alpha-synuclein in blood. Neurodegener. Dis. 5, 55–59.
Blennow, K., Hampel, H., 2003. CSF markers for incipient Alzheimer's Disease. Lancet Neurol. 2, 605–613.
El-Agnaf, O.M., et al., 2003. Alpha-synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma. FASEB J. 17, 1945–1947.
El-Agnaf, O.M., et al., 2006. Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease. FASEB J. 20, 419–425.
Emmanouilidou, E., et al., 2010. Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. J. Neurosci. 30, 6838–6851.
Foulds, P., et al., 2010. Progress towards a molecular biomarker for Parkinson disease. Nat. Rev. Neurol. 6, 359–361.
Fujiwara, H., et al., 2002. Alpha-synuclein is phosphorylated in synucleinopathy lesions. Nat. Cell Biol. 4, 160–164.
Hirohata, M., et al., 2011. Cerebrospinal fluid from patients with multiple system atrophy promotes in vitro α-synuclein fibril formation. Neurosci. Lett. 491, 48–52.
Hong, Z., et al., 2010. DJ-1 and α-synuclein in human cerebrospinal fluid as biomarkers of Parkinson's disease. Brain 133, 713–726.
Hughes, A.J., et al., 1992. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J. Neurol. Neurosurg. Psychiatry 55, 181–184.
Hughes, A.J., et al., 2001. Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease. Neurology 57, 1497–1499.
Kasai, T., et al., 2009. Increased TDP-43 protein in cerebrospinal fluid of patients with amyotrophic lateral sclerosis. Acta Neuropathol. 117, 55–62.
Lee, H.J., et al., 2005. Intravesicular localization and exocytosis of alpha-synuclein and its aggregates. J. Neurosci. 25, 6016–6024.
Mollenhauer, B., et al., 2008. Direct quantification of CSF α-synuclein by ELISA and first cross-sectional study in patients with neurodegeneration. Exp. Neurol. 213, 315–325.
Mollenhauer, B., et al., 2011. α-synuclein and tau concentrations in cerebrospinal fluid of patients presenting with parkinsonism: a cohort study. Lancet Neurol. 10, 230–240.

Obi, K., et al., 2008. Relationship of phosphorylated α-synuclein and tau accumulation to Ab deposition in the cerebral cortex of dementia with Lewy bodies. Exp. Neurol. 210, 409–420.
Ohrfelt, A., et al., 2009. Cerebrospinal fluid α-synuclein in neurodegenerative disorders: a marker of synapse loss? Neurosci. Lett. 450, 332–335.
Paleologou, K., et al., 2009. Detection of elevated levels of soluble {αlph}-synuclein oligomers in post mortem brain extracts from patients with dementia with Lewy bodies. Brain 132, 1093–1101.
Poewe, W., Wenning, G., 2002. The differential diagnosis of Parkinson's disease. Eur. J. Neurol. 9, 23–30.
Reesink, F.E., et al., 2010. CSF α-synuclein does not discriminate Dementia with Lewy bodies from Alzheimer's disease. J. Alzheimer's Dis. 22, 87–95.
Sasakawa, H., et al., 2007. Ultra-high field NMR studies of antibody binding and site-specific phosphorylation of α-synuclein. Biochem. Biophys. Res. Commun. 363, 795–799.
Schlossmacher, M.G., Mollenhauer, B., 2010. Biomarker research in Parkinson's disease: objective measures needed for patient stratification in future cause-directed trials. Biomarkers 4, 647–650.
Sharon, R., et al., 2003. The formation of highly soluble oligomers of α-synuclein is regulated by fatty acids and enhanced in Parkinson's disease. Neuron 37, 583–595.
Shi, M., et al., 2011. Cerebrospinal fluid biomarkers for Parkinson disease diagnosis and progression. Ann. Neurol. doi:10.1002/ana.22311.
Shults, C.W., et al., 2006. Alpha-synuclein from platelets is not phosphorylated at serine 129 in Parkinson's disease and multiple system atrophy. Neurosci. Lett. 25, 223–225.
Spies, P.E., et al., 2009. Cerebrospinal alpha synuclein does not discriminate between dementia disorders. J. Alzheimer Dis. 16, 363–369.
Spillantini, M.G., et al., 1997. Alpha-synuclein in Lewy bodies. Nature 388, 839–840.
Spillantini, M.G., et al., 1998. Alpha-synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc. Natl. Acad. Sci. U.S.A. 95, 6469–6473.
Tokuda, T., et al., 2006. Decreased α-synuclein in cerebrospinal fluid of aged individuals and subjects with Parkinson's disease. Biochem. Biophys. Res. Commun. 349, 162–166.
Tokuda, T., et al., 2010. Detection of elevated levels of α-synuclein oligomers in CSF from patients with Parkinson disease. Neurology 75, 1766–1772.

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Molecular Dissection of TDP-43 Proteinopathies

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Abstract TDP-43 has been identified as a major component of ubiquitin-positive tau-negative cytoplasmic inclusions in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and in amyotrophic lateral sclerosis (ALS). We raised antibodies to phosphopeptides representing 36 out of 64 candidate phosphorylation sites of human TDP-43 and showed that the antibodies to pS379, pS403/404, pS409, pS410 and pS409/410 labeled the inclusions, but not the nuclei. Immunoblot analyses demonstrated that the antibodies recognized TDP-43 at ~45 kDa, smearing substances and 18–26 kDa C-terminal fragments. Furthermore, the band patterns of the C-terminal fragments differed between neuropathological subtypes, but were indistinguishable between brain regions and spinal cord in each individual patient. Protease treatment of Sarkosyl-insoluble TDP-43 suggests that the different band patterns of the C-terminal fragments reflect different conformations of abnormal TDP-43 molecules between the diseases. These results suggest that molecular species of abnormal TDP-43 are different between the diseases and that they propagate from affected cells to other cells during disease progression and determine the clinicopathological phenotypes of the diseases.

Keywords Propagation · Phosphorylation · Tau · α-Synuclein · Prion · Cancer

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Introduction

TAR DNA-binding protein of M_r=43 kDa (TDP-43) is a nuclear factor that functions in regulating transcription and splicing. It is structurally characterized by two RNA recognition motifs and the C-terminal tail containing a glycine-rich region, and resembles a heterogeneous ribonucleoprotein (hnRNP) (Ayala et al. 2005). It has been shown to interact with several nuclear ribonucleoproteins (RNP), including hnRNP A and B and survival motor neuron protein, inhibiting alternative splicing (Buratti et al. 2005; Bose et al. 2008). In 2006, TDP-43 was identified as a major component of ubiquitin-positive inclusions in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS) (Arai et al. 2006; Neumann et al. 2006). Subsequent immunohistochemical examination demonstrated abnormal accumulation of TDP-43 in neurodegenerative disorders other than FTLD-U and ALS, including ALS/parkinsonism–

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dementia complex of Guam (Geser et al. 2007; Hasegawa et al. 2007), Alzheimer's disease (AD) (Amador-Ortiz et al. 2007; Higashi et al. 2007; Arai et al. 2009), dementia with Lewy bodies (DLB) (Higashi et al. 2007; Nakashima-Yasuda et al. 2007; Arai et al. 2009), Pick's disease (Arai et al. 2006; Freeman et al. 2008; Lin and Dickson 2008), argyrophilic grain disease (Fujishiro et al. 2009) and corticobasal degeneration (Uryu et al. 2008). These diseases with TDP-43 pathologies are now referred to as TDP-43 proteinopathies. In 2008, mutations in the TDP-43 gene (*TARDBP*) were discovered in familial and sporadic cases of ALS (Yokoseki et al. 2008; Gitcho et al. 2008; Sreedharan et al. 2008; Kabashi et al. 2008; Van Deerlin et al. 2008; Barmada and Finkbeiner 2010; Pesiridis et al. 2009), FTD-MND (Benajiba et al. 2009) and FTD (Borroni et al. 2009), clearly indicating that abnormality of TDP-43 protein causes neurodegeneration.

Identification of Abnormal Phosphorylation Sites of TDP-43

Biochemical analyses of the detergent-insoluble fraction extracted from brains of patients afflicted with FTLD-TDP and ALS show that TDP-43 accumulated in these pathological structures is phosphorylated and cleaved (Arai et al. 2006; Neumann et al. 2006). By producing antibodies against synthetic phosphopeptides containing 36 different phosphorylation sites from among the 56 serine/threonine residues of TDP-43, five abnormal phosphorylation sites were identified at serine residues in the C-terminal region (Hasegawa et al. 2008). The antibodies to pS379, pS403/404, pS409, pS410 and pS409/410 strongly stain abnormal neuronal cytoplasmic and dendritic inclusions in FTLD-U, and skein-like and glial cytoplasmic inclusions in ALS spinal cord, with no nuclear staining, and thus permit easier and more sensitive detection of abnormal TDP-43 accumulation in neuropathological examinations (Hasegawa et al. 2008). Immunoblotting of the Sarkosyl-insoluble fraction from control, FTLD-U and ALS cases using these phospho-specific antibodies clearly demonstrated that hyperphosphorylated full-length TDP-43 at ~45 kDa, smearing substances and fragments at 18–26 kDa are the major species of TDP-43 accumulated in FTLD-U and ALS (Hasegawa et al. 2008).

Cellular Models of TDP-43

To establish cellular models of TDP-43 proteinopathies, several deletion mutants of human TDP-43 in SH-SY5Y cells were expressed and the accumulation of TDP-43 was analyzed by use of the phospho-TDP-43 antibodies and ubiquitin. Wild-type (WT) full-length TDP-43 was localized to nuclei and no inclusions were observed, whereas in cells transfected with C-terminal fragments as GFP fusions, round cytoplasmic inclusions with intense GFP fluorescence were formed (Nonaka et al. 2009b). In addition, a deletion mutant lacking the nuclear localizing signal (NLS) and six amino acids similar to the NLS also formed aggregates in cells without any treatment (Nonaka et al. 2009a). These inclusions are strongly positive for antibodies to phosphorylated TDP-43 and ubiquitin. Using these cellular models, the effect of pathogenic mutations of the TDP-43 gene was analyzed. Of 14 mutants examined, seven mutants showed a significantly higher number of aggregates than the WT C-terminal fragment, strongly suggesting that these mutations of TDP-43 accelerate aggregation of the C-terminal fragments (Nonaka et al. 2009b). In addition, when GFP-tagged C-terminal fragments were co-expressed with DsRed-tagged full-length TDP-43, cytoplasmic inclusions with both GFP and DsRed signals were formed, suggesting that exogenous full-length TDP-43 is trapped in cytoplasmic inclusions formed by C-terminal fragments. This may explain why normal nuclear staining of TDP-43 is lost in neuronal cells with inclusions in diseased brains (Nonaka et al. 2009b). Furthermore, we identified two cleavage sites of TDP-43 deposited in FTLD-U by mass spectrometric analysis, and confirmed that expression of these fragments as GFP fusions also afforded cytoplasmic inclusions positive for ubiquitin and phosphorylated TDP-43 (Nonaka et al. 2009b). The cleavage sites identified in the 23-kDa C-terminal fragment of FTLD were different from that of caspase-3, suggesting that caspase is not the enzyme responsible for generating the 23-kDa fragment (Nonaka et al. 2009b). These cellular models recapitulate many of the features of TDP-43 in patients, and therefore, should be useful for screening small molecules for activity to inhibit TDP-43 aggregate formation. We tested whether or not methylene blue and dimebon have the ability to suppress formation of pathological TDP-43 inclusions. Compared to controls, a 50% reduction in the number of inclusions with 0.05 μM methylene blue, a 45% reduction with 5 μM dimebon and an 80% reduction with the combination of 0.05 μM methylene blue and 5 μM dimebon were observed (Yamashita et al. 2009). The effects were statistically significant and the results were also confirmed by Western blotting. These results suggest that these two compounds may be effective in the therapy of ALS, FTLD-U and other TDP-43 proteinopathies.

TDP-43 C-Terminal Fragments

Based on neuropathological studies, TDP-43 proteinopathies have been classified into 4 subtypes (Cairns et al.

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2007). Type 1 is characterized by dystrophic neurites (DNs) with few neuronal cytoplasmic inclusions (NCIs) and no neuronal intranuclear inclusions (NIIs), Type 2 has numerous NCIs with few DNs and no NIIs, Type 3 has numerous NCIs and DNs and occasional NIIs and Type 4 has numerous NIIs and DNs with few NCIs, a pattern which is specific for familial FTLD-U with mutations of VCP gene. There appears to be a strong relationship between other subtypes of TDP-43 pathology and clinical phenotype. Type 1 is associated with semantic dementia, type 2 with FTLD with motor neuron disease (MND), ALS or clinical signs of MND, and type 3 with progressive non-fluent aphasia or FTD with mutation in the progranulin gene. Recent studies of ALS have clarified the wide distribution of neuronal and glial TDP-43 pathology in multiple areas of the central nervous systems (Geser et al. 2008; Nishihira et al. 2009), suggesting that ALS does not selectively affect only the motor system, but rather is a multisystem neurodegenerative TDP-43 proteinopathy affecting both neurons and glial cells.

By immunoblot analyses of the Sarkosyl-insoluble fractions from FTLD-U and ALS patients, we found that the band patterns of the C-terminal fragments of phosphorylated TDP-43 corresponded to the neuropathological subtypes. Type 1 FTLD-U showed two major bands at 23 and 24 kDa and two minor bands at 18 and 19 kDa, while type 2 ALS showed three major bands at 23, 24 and 26 kDa and two minor bands at 18 and 19 kDa. Type 3 FTD with mutation in the progranulin gene showed an intermediate pattern between those two. These results clearly indicate that TDP-43 proteinopathies subclassified by neuropathological differences can also be distinguished biochemically. This strong association between the neuropathology and the biochemistry is critical for understanding the molecular pathogenesis of TDP-43 proteinopathies.

Biochemical Analysis of TDP-43 in FTLD-U and ALS

The biochemical differences of TDP-43, as shown in the different band patterns of TDP-43 C-terminal fragments, are closely linked to the morphologies of inclusions. The properties of the abnormal TDP-43 may determine the neuropathological and clinical phenotypes of TDP-43 proteinopathies. Similar biochemical and neuropathological differences have been reported in tau between PSP and CBD. Both PSP and CBD are tauopathies with deposition of 4-repeat tau isoforms; however, distinct types of C-terminal fragments are detected, i.e., a 33-kDa band in PSP and ~3-kDa bands in CBD (Arai et al. 2004).

So, what do the different band patterns mean? It is clear that the fragments are produced by cleavage at multiple sites of TDP-43. The band patterns also suggest that the cleavage sites are slightly altered between the diseases. Based on these observations, it is likely that the event may occur after the assembly or aggregation of abnormal TDP-43, and represent relatively protease-resistant domains of TDP-43, which form beta-sheet structure. That is, the different band patterns in TDP-43 proteinopathies represent different conformations of abnormal TDP-43 in the diseases.

To test this idea, we performed protease treatment of the abnormal TDP-43 recovered in the Sarkosyl-insoluble pellets, and analyzed the protease-resistant bands. Proteins can be easily cleaved by proteases if they are denatured or unstructured, but domains that have rigid structures such as beta-sheet structure, or that are structurally buried or interacting with other molecules, are highly resistant to proteases. Figure 1 shows the result of immunoblot analysis of abnormal TDP-43 from two ALS and two FTLD-U cases before and after protease treatment. Before treatment, hyperphosphorylated full-length TDP-43 at 45 kDa, smearing substances and 18–26 kDa C-terminal fragments were detected by pS409/410. The band patterns of the C-terminal fragments are different between FTLD-U with type 1 pathology and ALS with type 2 pathology. Upon trypsin or chymotrypsin treatment, the full-length 45-kDa band and smearing substance of TDP-43 disappeared and protease-resistant core fragments appeared at 16–26 kDa (Fig. 1). As expected, the protease-resistant band pattern of ALS is different and clearly distinguishable from that of FTLD-U. Doublet bands at ~16 kDa and a band at 25 kDa were detected in ALS, but only a single broad band at ~16 kDa was detected in FTLD-U with type 1 pathology after trypsin treatment (Fig. 1). Similarly, multiple protease-resistant bands were detected at 16–25 kDa after chymotrypsin treatment and the band patterns were different between ALS and FTLD-U (Fig. 1). These results strongly support the idea that the different band patterns of the C-terminal fragments reflect different conformations of abnormal TDP-43 molecules between ALS and FTLD-U.

TDP-43 in Different Brain Regions

Similar protease-resistant bands and differences in the band patterns have been reported in prion diseases, CJD and BSE (Collinge et al. 1996). Protease-resistant prion from new-variant CJD cases showed a different characteristic pattern from that in sporadic CJD cases, and the band pattern is indistinguishable from that of mice infected with BSE prion. This is biochemical evidence that the BSE agent has been transmitted from bovine to human.

Applying this to TDP-43 in TDP-43 proteinopathies, it is possible to determine whether there is any difference between the abnormal TDP-43 accumulated in cortex and that in spinal cord by analyzing the band patterns of the C-

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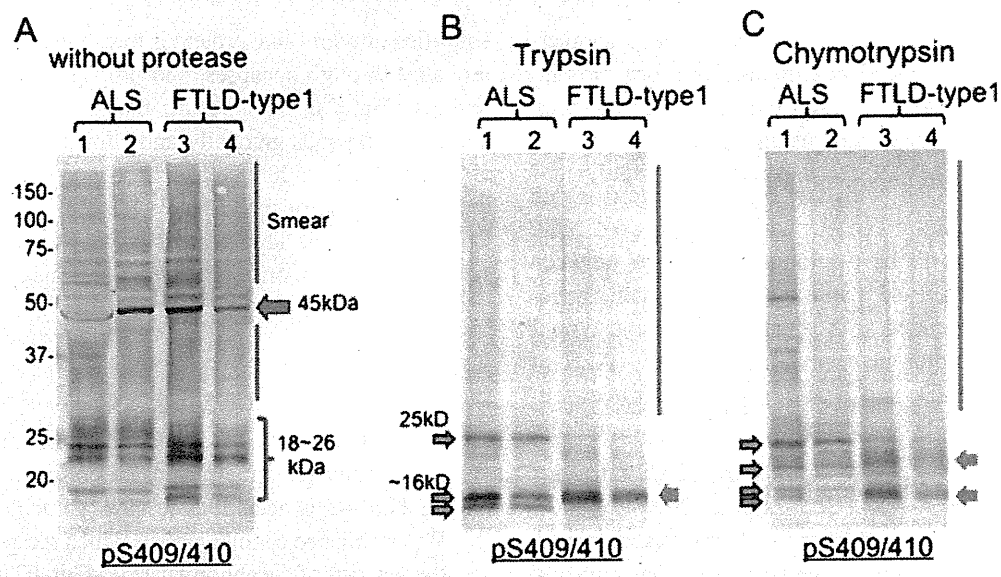

Fig. 1 Immunoblot analysis of abnormal TDP-43 from two ALS and two FTLD-U cases before and after protease treatment with a phosphorylation dependent anti-TDP-43 rabbit polyclonal antibody (pS409/410). **a** Hyperphosphorylated full-length TDP-43 at 45 kDa, smearing substances and 18–26 kDa C-terminal fragments were detected by pS409/410 before treatment. The band patterns of the C-terminal fragments are different between FTLD-U with type 1 pathology and ALS with type 2 pathology. **b** Upon trypsin treatment, the full-length 45 kDa band and smearing substance of TDP-43 disappeared and protease-resistant core fragments appeared at 16~26 kDa. Doublet bands at ~16 kDa and a band at 25 kDa were seen in ALS, but a single broad band at ~16 kDa was detected in FTLD-U with type 1 pathology after trypsin treatment. **c** Multiple protease-resistant bands were detected at 16~25 kDa after chymotrypsin treatment and the band patterns were different between ALS and FTLD-U

terminal fragments of TDP-43. Thus, we have prepared Sarkosyl-insoluble fractions from cortex and spinal cords of three ALS cases, immunoblotted them with pS409/410 and compared the results. In all three cases, type 2 C-terminal fragments of TDP-43 were detected, and there was no significant difference between the band pattern in cortex and that in spinal cord (data not shown). This strongly suggests that the same form of abnormal TDP-43 molecule is deposited in different brain regions. Similar results were also obtained from the analysis of the C-terminal band pattern of TDP-43 in FTLD-U. It seems highly unlikely that the same conformational change would occur synchronously in different brain regions. Instead, it seems more likely that abnormal protein produced in cells is transferred to different regions and propagated. These biochemical data obtained from the brains of patients provide biochemical evidence that abnormal species of TDP-43 are transmitted from cell to cell and propagated in vivo.

Discussion

Amyloid-like protein deposition is a common neuropathological feature of many neurodegenerative diseases. Hyperphosphorylated tau in Alzheimer's disease and related tauopathies, hyperphosphorylated alpha-synuclein in Parkinson's disease and other alpha-synucleinopathies, and expanded polyglutamines in polyglutamine diseases have been identified. Importantly, the extent of the abnormal protein pathologies is closely correlated with the disease progression (Braak and Braak 1991; Braak et al. 2003; Saito et al. 2003). The proteins or protein fibrils deposited in cells in these diseases have been shown to have a common structural feature. Cross-beta structure, which is the same as in abnormal prion protein, has been demonstrated in filaments or fibrils composed of tau (Berriman et al. 2003), alpha-synuclein (Serpell et al. 2000) or expanded polyglutamines (Perutz 1999). It has not been demonstrated in TDP-43 yet, but we have shown by electron microscopy that phosphorylated TDP-43 in motor neurons of ALS patients has a fibrous structure (Hasegawa et al. 2008), suggesting that TDP-43 is also an amyloid-like protein.

For the assembly of amyloid fibrils, nucleation-dependent protein polymerization has been proposed. This comprises nucleation and elongation phases, and nucleation is the rate-limiting step. It takes a long time to form the first aggregated seed from the monomer, but once the seed is formed, the elongation step proceeds relatively quickly. More importantly, by addition of amyloid-seed, proteins are often converted to the same conformation as that of the seed. For example, WT monomeric alpha-synuclein is converted to A30P-type amyloid fibrils when it is incubated with a small amount of fibril-seeds formed with A30P mutant alpha-synuclein (Yonetani et al. 2009). Differences in the conformations of the amyloid fibrils are detected based on the differences in the protease-resistant band

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patterns, as in the typing of prion proteins. There is another example of nucleation-dependent amyloid fibril formation in cultured cells. We developed a novel method for introducing amyloid seeds into cultured cells using lipofectoamine, and presented experimental evidence of seed-dependent polymerization of alpha-synuclein, leading to the formation of filamentous protein deposits and cell death (Nonaka et al. 2010). Overexpression of alpha-synuclein itself in cells does not generate abnormal inclusions, but if fibril seeds formed with alpha-synuclein are introduced into cells, abundant filamentous alpha-synuclein aggregates positive for PSer129 and ubiquitin are developed, and cells with inclusions undergo cell death. This was also clearly demonstrated in cells expressing different tau isoforms by introducing the corresponding tau fibril seeds (Nonaka et al. 2010).

The above results obtained from biochemical analyses of abnormal proteins in patients strongly suggest that intracellular amyloid-like proteins, including TDP-43, propagate from cell to cell and this propagation is the cause of disease progression, analogously to metastasis of cancer cells to multiple different tissues in cancer progression. From this point of view, we have proposed as a hypothesis that neurodegenerative diseases with amyloid-like proteins can be regarded as "protein cancers." The term prion, coined in 1982 by Stanley B. Prusiner, describes an agent transmissible among humans and a variety of mammals. On the other hand, the term "protein cancers" describes diseases that involve the spreading or propagation of abnormal proteins in tissues or individuals, even though the mechanism of propagation is basically the same as that of prions. Amyloid-like protein interacts with normal protein and converts it to the same abnormal conformation, and the amplified amyloid-like protein is transmitted from cell to cell, probably through synapses, and propagates to various brain regions (Fig. 2). As a result, the same abnormal protein pathology expands gradually, and clinical manifestations that are associated with affected brain regions become more marked because of the transmission and propagation of the abnormal protein. Therefore, it is important to regulate the propagation of abnormal proteins for clinical therapy.

Conclusions

1. In ALS, FTLD-U and other TDP-43 proteinopathies, abnormally phosphorylated, ubiquitinated, and truncated TDP-43 is accumulated in a filamentous form.
2. We established cellular models which recapitulate many of the features of the abnormal TDP-43 in FTLD-U and ALS
3. ALS-related pathogenic mutations of the TDP-43 gene accelerate aggregate formation by the C-terminal fragments.
4. The band pattern of the TDP-43 C-terminal fragments is different between diseases with different clinicopathological phenotypes, and it represents different conformations of the abnormal TDP-43 between the diseases.
5. The C-terminal band patterns in several brain areas and spinal cord in each individual case of sporadic ALS are indistinguishable.
6. These and other results suggest that abnormal TDP-43, tau and alpha-synuclein are transmitted and propagated from cell to cell in different regions during disease progression. It is important to find drugs that can block the propagation of abnormal proteins for clinical therapy.

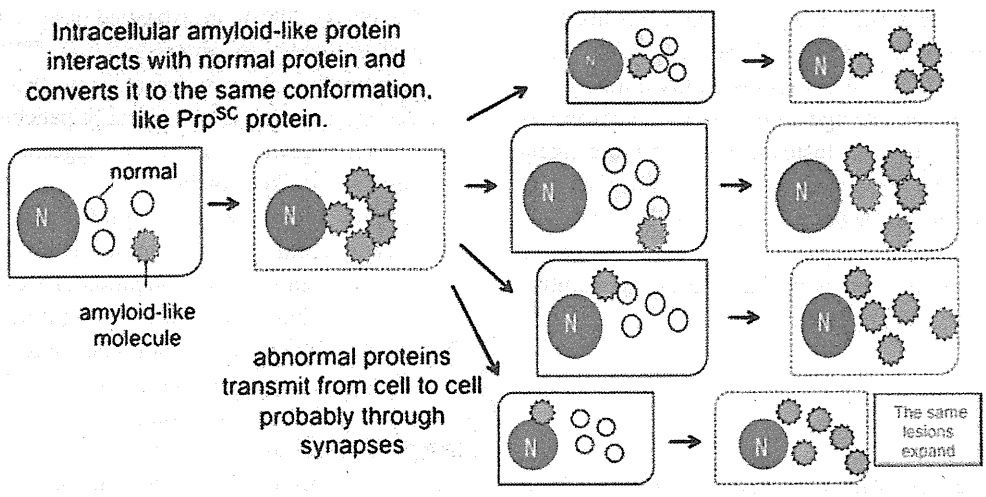

Fig. 2 Schematic representation of prion-like conversion of normal protein into amyloid-like protein and its propagation in neurodegenerative diseases. Intracellular amyloid-like protein interacts with normal protein and converts it to the same abnormal conformation. Amplified abnormal amyloid-like protein is transmitted from cell to cell, probably through synapses, and propagates to various brain regions. As a result, the same abnormal protein pathology expands gradually, and clinical manifestations that are associated with affected brain regions become more marked because of the transmission and propagation of the abnormal protein. From this point of view, neurodegenerative diseases with amyloid-like proteins can be regarded as 'protein cancers"

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References

Amador-Ortiz C, Lin WL, Ahmed Z et al (2007) TDP-43 immunoreactivity inhippocampal sclerosis and Alzheimer's disease. Ann Neurol 61:435–445

Arai T, Ikeda K, Akiyama H, Nonaka T, Hasegawa M, Ishiguro K et al (2004) Identification of amino-terminally cleaved tau fragments that distinguish progressive supranuclear palsy from corticobasal degeneration. Ann Neurol 55:72–79

Arai T, Hasegawa M, Akiyama H et al (2006) TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochem Biophys Res Commun 351:602–611

Arai T, Mackenzie IR, Hasegawa M et al (2009) Phosphorylated TDP-43 in Alzheimer's disease and dementia with Lewy bodies. Acta Neuropathol 117:125–136

Ayala YM, Pantano S, D'Ambrogio A et al (2005) Human, *Drosophila*, and *C. elegans* TDP43: nucleic acid binding properties and splicing regulatory function. J Mol Biol 348:575–588

Barmada SJ, Finkbeiner S (2010) Pathogenic TARDBP mutations in amyotrophic lateral sclerosis and frontotemporal dementia: disease-associated pathways. Rev Neurosci 21:251–272 (Review)

Benajiba L, Le Ber I, Camuzat A, Lacoste M et al (2009) TARDBP mutations in motoneuron disease with frontotemporal lobar degeneration. Ann Neurol 65:470–473

Berriman J, Serpell LC, Oberg KA et al (2003) Tau filaments from human brain and from in vitro assembly of recombinant protein show cross-beta structure. Proc Natl Acad Sci USA 100:9034–9038

Borroni B, Bonvicini C, Alberici A et al (2009) Mutation within TARDBP leads to frontotemporal dementia without motor neuron disease. Hum Mutat 30:E974–E983

Bose JK, Wang IF, Hung L, Tarn WY, Shen CK (2008) TDP-43 overexpression enhances exon 7 inclusion during the survival of motor neuron pre-mRNA splicing. J Biol Chem 283:28852–28859

Braak H, Braak E (1991) Neuropathological staging of Alzheimer-related changes. Acta Neuropathol 82:239–259

Braak H, Del Tredici K, Rub U, de Vos RA et al (2003) Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 24:197–211

Buratti E, Brindisi A, Giombi M, Tisminetzky S, Ayala YM, Baralle FE (2005) TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail. J Biol Chem 280:37572–37584

Cairns NJ, Bigio EH, Mackenzie IR et al (2007) Neuropathologic diagnostic and nosologic criteria for frontotemporal lobar degeneration: consensus of the Consortium for Frontotemporal Lobar Degeneration. Acta Neuropathol 114:5–22

Collinge J, Sidle KC, Meads J, Ironside J, Hill AF (1996) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature 383:685–690

Freeman SH, Spires-Jones T, Hyman BT, Growdon JH, Frosch MP (2008) TAR-DNA binding protein 43 in Pick disease. J Neuropathol Exp Neurol 67:62–67

Fujishiro H, Uchikado H, Arai T et al (2009) Accumulation of phosphorylated TDP-43 in brains of patients with argyrophilic grain disease. Acta Neuropathol 117:151–158

Geser F, Winton MJ, Kwong LK et al (2007) Pathological TDP-43 in parkinsonism-dementia complex and amyotrophic lateral sclerosis of Guam. Acta Neuropathol 115:133–145

Geser F, Brandmeir NJ, Kwong LK et al (2008) Evidence of multisystem disorder in whole-brain map of pathological TDP-43 in amyotrophic lateral sclerosis. Arch Neurol 65:636–641

Gitcho MA, Baloh RH, Chakraverty S et al (2008) TDP-43 A315T mutation in familial motor neuron disease. Ann Neurol 63:535–538

Hasegawa M, Arai T, Akiyama H et al (2007) TDP-43 is deposited in the Guam parkinsonism-dementia complex brains. Brain 130:1386–1394

Hasegawa M, Arai T, Nonaka T et al (2008) Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Ann Neurol 64:60–70

Higashi S, Iseki E, Yamamoto R et al (2007) Concurrence of TDP-43, tau and alpha-synuclein pathology in brains of Alzheimer's disease and dementia with Lewy bodies. Brain Res 1184:284–294

Kabashi E, Valdmanis PN, Dion P et al (2008) TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. Nat Genet 40:572–574

Lin WL, Dickson DW (2008) Ultrastructural localization of TDP-43 in filamentous neuronal inclusions in various neurodegenerative diseases. Acta Neuropathol 116:205–213

Nakashima-Yasuda H, Uryu K, Robinson J et al (2007) Co-morbidity of TDP-43 proteinopathy in Lewy body related diseases. Acta Neuropathol 114:221–229

Neumann M, Sampathu DM, Kwong LK et al (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science 314:130–133

Nishihira Y, Tan CF, Hoshi Y et al (2009) Sporadic amyotrophic lateral sclerosis of long duration is associated with relatively mild TDP-43 pathology. Acta Neuropathol 117:45–53

Nonaka T, Arai T, Buratti E, Baralle FE, Akiyama H, Hasegawa M (2009a) Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells. FEBS Lett 583:394–400

Nonaka T, Kametani F, Arai T, Akiyama H, Hasegawa M (2009b) Truncation and pathogenic mutations facilitate the formation of intracellular aggregates of TDP-43. Hum Mol Genet 18:3353–3364

Nonaka T, Watanabe ST, Iwatsubo T, Hasegawa M (2010) Seeded aggregation and toxicity of alpha-synuclein and tau: cellular models of neurodegenerative diseases. J Biol Chem 285:34885–34898

Perutz MF (1999) Glutamine repeats and neurodegenerative diseases. Brain Res Bull 50:467

Pesiridis GS, Lee VM, Trojanowski JQ (2009) Mutations in TDP-43 link glycine-rich domain functions to amyotrophic lateral sclerosis. Hum Mol Genet 18:R156–R162

Saito Y, Kawashima A, Ruberu NN, Fujiwara H et al (2003) Accumulation of phosphorylated alpha-synuclein in aging human brain. J Neuropathol Exp Neurol 62:644–654

Serpell LC, Berriman J, Jakes R et al (2000) Fiber diffraction of synthetic alpha-synuclein filaments shows amyloid-like cross-beta conformation. Proc Natl Acad Sci USA 97:4897–4902

Sreedharan J, Blair IP, Tripathi VB et al (2008) TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. Science 319:1668–1672

Uryu K, Nakashima-Yasuda H, Forman MS et al (2008) Concomitant TAR-DNA-binding protein 43 pathology is present in Alzheimer disease and corticobasal degeneration but not in other tauopathies. J Neuropathol Exp Neurol 67:555–564

Van Deerlin VM, Leverenz JB, Bekris LM et al (2008) TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis. Lancet Neurol 7:409–416

Yamashita, M., Nonaka, T., Arai, T et al (2009) Methylene blue and dimebon inhibit aggregation of TDP-43 in cellular models. FEBS Lett 583:2419–24

Yokoseki A, Shiga A, Tan CF et al (2008) TDP-43 Mutation in familial amyotrophic lateral sclerosis. Ann Neurol 63:538–542

Yonetani M, Nonaka T, Masuda M et al (2009) Conversion of wild-type alpha-synuclein into mutant-type fibrils and its propagation in the presence of A30P mutant. J Biol Chem 284:7940–7950

ARTICLE ADDENDUM

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In vitro recapitulation of aberrant protein inclusions in neurodegenerative diseases

New cellular models of neurodegenerative diseases

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Aberrant protein aggregates in affected brain cells of patients with neurodegenerative diseases are a well-known hallmark, but although the formation of these inclusions is an important pathogenic event, the mechanism involved remains unclear. We have recently established a simple method to introduce protein fibrils into cultured cells as seeds for protein aggregation, and we showed that intracellular soluble α-synuclein or tau can aggregate in cultured cells dependently upon seeds introduced in this way. Seeded aggregation of α-synuclein induced necrotic cell death, which was suppressed by the addition of various polyphenols. Our cellular models are expected to be valuable tools not only for elucidating the molecular mechanisms of onset of neurodegenerative diseases, but also for drug discovery.

Key words: alpha-synuclein, tau, intracellular aggregates formation, transduction of protein fibrils, cell death

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In patients with neurodegenerative disorders, intracellular aberrant protein inclusions are often found in the brain, including neurofibrillary tangles in Alzheimer's disease or Lewy bodies in Parkinson's disease and dementia with Lewy bodies. These aberrant protein aggregates are often observed in the most affected regions of diseased brains, suggesting they may cause neuronal cell death, leading to onset of these diseases. Tau and α-synuclein are well-known cytosolic proteins that are the main components of neurofibrillary tangles and Lewy bodies, respectively. They are soluble and natively unfolded proteins, and it remains unclear how they become aggregated in neuronal cells. Indeed, intracellular aggregate formation of these proteins does not occur when cultured cells are transfected with expression plasmids encoding these proteins. On the other hand, many in vitro studies using recombinant proteins, such as Abeta, tau, α-synuclein or poly glutamine-containing protein, have shown that these proteins are readily aggregated into fibrils in the presence of seeds for aggregation. These findings prompted us to examine whether seeds-dependent aggregation would occur in cultured cells. Thus, we aimed to introduce protein fibrils into cultured cells as seeds for aggregation.

Transfection of plasmid DNA into cultured cells is conducted routinely by the use of liposomes of polycationic and neutral lipids in water, based on the principle of cell fusion. Several commercially available reagents such as Lipofectoamine, Lipofectamine 2000 or FuGENE6 are available to efficiently transfect plasmid DNA into cultured cells. We tested whether these transfection reagents could transfect cultured SH-SY5Y cells not only with plasmid DNA, but also with protein fibrils. After much trial and error, we finally succeeded in transfecting α-synuclein fibrils into these cells using Lipofectamine reagent. We found that the introduced recombinant α-synuclein fibrils are phosphorylated at Ser129 in cultured cells, indicating that they had been introduced by Lipofectamine.[1] Interestingly, monomeric and oligomeric α-synuclein could not be introduced by the use of Lipofectamine. We applied for a patent covering the use of Lipofectamine for transduction of recombinant protein fibrils into cultured cells in 2005 (patents

pending in the United States: 12/086124, the European Union: 06834541.2 and Japan: 2007-549210). Recently, other groups have also reported introduction of fibrillar protein into cultured cells with or without specific reagents.[2-5]

Next, we examined whether intracellular α-synuclein can be aggregated dependently upon introduced seeds. When α-synuclein fibrils mixed with Lipofectamine were introduced into cells transiently expressing α-synuclein, phosphorylated and ubiquitinated α-synuclein inclusions (~10 μm in diameter) were observed by means of confocal laser microscopy, indicating that plasmid-derived soluble α-synuclein formed aggregates in the presence of exogenous α-synuclein fibrils in cells, and these inclusions resembled Lewy bodies in diseased brains. Others have also reported that α-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells.[4] On the other hand, introduced tau fibrils were also shown to act as seeds for intracellular aggregation of plasmid-derived soluble tau protein. Interestingly, we found that fibrils composed of 3-repeat tau isoform serve as seeds for intracellular aggregation of soluble 3-repeat tau, but not soluble 4-repeat tau and fibrils of 4-repeat tau seed serve as seeds for aggregation of soluble 4-repeat tau, but not soluble 3-repeat tau. Likewise, introduction of α-synuclein fibrils did not elicit intracellular tau aggregation in cells and soluble α-synuclein did not form intracellular aggregates in the presence of any tau fibrils. These results clearly indicate that intracellular protein aggregation is highly dependent on the species of protein fibril seeds. Now, we are examining whether detergent-insoluble fractions prepared from several diseased brains can be introduced into cells by Lipofectamine and can serve as seeds for intracellular aggregate formation of soluble α-synuclein, tau or TDP-43.

Does the formation of these inclusions lead to cell death or toxicity? The answer is yes. We observed non-apoptotic cell death in cells harboring these inclusions. In these cells, proteasome activity was found to be significantly reduced. This suppression may be related to the cause of cell death. Furthermore, we showed that cell death in cells with α-synuclein inclusions is effectively suppressed by the addition of various small molecules to the culture medium; polyphenols such as exifone and gossypetin were the most effective, suggesting that these compounds may be possible new drugs for the treatment of neurodegenerative diseases.

Our study strongly supports a seed-dependent mechanism for the formation of the intracellular protein aggregates. Recently, the intercellular transfer of inclusions made of tau,[3,6] α-synuclein[2,7,8] and huntingtin[9] has been reported, suggesting the existence of mechanisms reminiscent of those by which prions spread through the nervous system. It remains to be clarified whether the incorporation of amyloid seeds into neurons or glial cells, as shown in our study, also occurs in vivo, but our results strongly suggest that extracellular aggregates may be taken up into neurons by endocytosis or under certain specific conditions. Therefore, it may be crucial to inhibit not only the production of intracellular amyloid seeds, but also their spread into extracellular space and their propagation. Vaccination against α-synuclein[10] or tau may be an effective treatment, together with the inhibition of intracellular aggregates formation with small-molecular compounds, for the therapy of neurodegenerative diseases.

References

1. Nonaka T, Watanabe ST, Iwatsubo T, Hasegawa M. Seeded aggregation and toxicity of α-synuclein and tau: cellular models of neurodegenerative diseases. J Biol Chem 2010; 285:34885-98.
2. Desplats P, Lee HJ, Bae EJ, Patrick C, Rockenstein E, Crews L, et al. Inclusion formation and neuronal cell death through neuron-to-neuron transmission of α-synuclein. Proc Natl Acad Sci USA 2009; 106:13010-5.
3. Frost B, Jacks RL, Diamond MI. Prion-like mechanisms in neurodegenerative diseases. J Biol Chem 2009; 284:12845-52.
4. Luk KC, Song C, O'Brien P, Stieber A, Branch JR, Brunden KR, et al. Exogenous α-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells. Proc Natl Acad Sci USA 2009; 106:20051-6.
5. Waxman EA, Giasson BI. A novel, high-efficiency cellular model of fibrillar α-synuclein inclusions and the examination of mutations that inhibit amyloid formation. J Neurochem 2010; 113:374-88.
6. Clavaguera F, Bolmont T, Crowther RA, Abramowski D, Frank S, Probst A, et al. Transmission and spreading of tauopathy in transgenic mouse brain. Nat Cell Biol 2009; 11:909-13.
7. Li JY, Englund E, Holton JL, Soulet D, Hagell P, Lees AJ, et al. Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. Nat Med 2008; 14:501-3.
8. Kordower JH, Chu Y, Hauser RA, Freeman TB, Olanow CW. Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. Nat Med 2008; 14:504-6.
9. Ren PH, Lauckner JE, Kachirskaia I, Heuser JE, Melki R, Kopito RR. Cytoplasmic penetration and persistent infection of mammalian cells by polyglutamine aggregates. Nat Cell Biol 2009; 11:219-25.
10. Masliah E, Rockenstein E, Adame A, Alford M, Crews L, Hashimoto M, et al. Effects of alpha-synuclein immunization in a mouse model of Parkinson's disease. Neuron 2005; 46:857-68.

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C-Jun N-terminal kinase controls TDP-43 accumulation in stress granules induced by oxidative stress

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Abstract

Background: TDP-43 proteinopathies are characterized by loss of nuclear TDP-43 expression and formation of C-terminal TDP-43 fragmentation and accumulation in the cytoplasm. Recent studies have shown that TDP-43 can accumulate in RNA stress granules (SGs) in response to cell stresses and this could be associated with subsequent formation of TDP-43 ubiquinated protein aggregates. However, the initial mechanisms controlling endogenous TDP-43 accumulation in SGs during chronic disease are not understood. In this study we investigated the mechanism of TDP-43 processing and accumulation in SGs in SH-SY5Y neuronal-like cells exposed to chronic oxidative stress. Cell cultures were treated overnight with the mitochondrial inhibitor paraquat and examined for TDP-43 and SG processing.

Results: We found that mild stress induced by paraquat led to formation of TDP-43 and HuR-positive SGs, a proportion of which were ubiquitinated. The co-localization of TDP-43 with SGs could be fully prevented by inhibition of c-Jun N-terminal kinase (JNK). JNK inhibition did not prevent formation of HuR-positive SGs and did not prevent diffuse TDP-43 accumulation in the cytosol. In contrast, ERK or p38 inhibition prevented formation of both TDP-43 and HuR-positive SGs. JNK inhibition also inhibited TDP-43 SG localization in cells acutely treated with sodium arsenite and reduced the number of aggregates per cell in cultures transfected with C-terminal TDP-43 162-414 and 219-414 constructs.

Conclusions: Our studies are the first to demonstrate a critical role for kinase control of TDP-43 accumulation in SGs and may have important implications for development of treatments for FTD and ALS, targeting cell signal pathway control of TDP-43 aggregation.

Keywords: TDP-43, stress granules, JNK, kinases, oxidative stress, paraquat, hnRNP

Background

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disease in which the function of motor neurons in the spinal cord and brain progressively deteriorates. ALS is by far the most prevalent form of motor neuron disease. Patients with ALS rarely survive more than 3-5 years after diagnosis with respiratory failure the most common cause of death [1]. Approximately 5% of patients with ALS have a positive family history of the disorder. The first pathological mutations identified in ALS were in superoxide dismutase 1 (SOD1) and account for around 20% of familial ALS cases [2]. That discovery has been the basis for most ALS research in the past decade, and animal models containing SOD1 mutant transgenes have provided important insights into SOD1-mediated neurotoxic effects. However, SOD1 mutations only account for 1-2% of all ALS cases [3].

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Frontotemporal dementia (FTD) is the second most common cause of presenile dementia, affecting people in their 50s and 60s [4,5]. There are several clinical phenotypes and the historical neuropathological classification included either frontotemporal lobar degeneration with tau positive (FTLD-tau) or ubiquitin-positive (FTLD-U) inclusions [4,5]. The observation that some ALS patients developed cognitive deficits with frontal lobe degeneration resembling FTLD-U has led to the belief that ALS and FTD with FTLD-U might involve a clinical spectrum of neurodegenerative illnesses [5].

In 2006, TAR DNA binding protein 43 (TDP-43) was identified as the major protein constituent of ubiquitinated neuronal inclusions in FTLD-U and in non-SOD1 ALS cases [6,7]. This led to the re-classification of FTLD-U to FTLD-TDP-43, and TDP-43-positive ALS and FTLD-TDP-43 cases are now referred to collectively as primary TDP-43 proteinopathies [8]. These findings also provided further support for the concept of FTD and ALS as diseases within the same broad clinical spectrum. Subsequently, TDP-43-positive inclusions have been identified in a number of neurodegenerative diseases. In these cases, the TDP-43 identification is referred to as a secondary TDP-43 proteinopathy [8]. While the role of abnormal TDP-43 accumulation in both primary and secondary TDP-43 proteinopathies is not yet fully understood, the identification of TDP-43 mutations associated with ALS and FTD (~40 at present) has provided clear evidence that altered TDP-43 processing can be a primary cause of neurodegeneration and is not just a secondary phenomenon [9,10].

TDP-43 is a 414 amino acid protein of the heterogeneous nuclear ribonucleoprotein (hnRNP) family and consists of two RNA recognition motifs and a C-terminal glycine rich region [8,11]. It has a number of reported roles including transcription, pre-mRNA splicing, and transport and stabilization of mRNA [8]. The protein is normally localized to the nucleus and has a classical bipartite nuclear localization sequence [12]. TDP-43 contains two caspase 3 consensus cleavage sites leading to formation of C-terminal fragments (CTFs) of 35 kDa and 25 kDa that are excluded from the nucleus [8]. The majority of TDP-43 mutations occur in the C-terminal region and CTFs are commonly identified in ALS and FTD inclusions.

In post-mortem tissue from ALS and FTD, the hallmark neuropathological features include loss of TDP-43 expression in the nucleus together with accumulation of TDP-43 in cytoplasmic inclusions. These inclusions are enriched in ubiquitinated and hyperphosphorylated (phospho-Ser409/410) TDP-43 and there can be substantial enrichment of CTF-TDP-43 [8,11]. Recent cell studies have shown that transfection with CTF-TDP-43 can accurately re-capitulate the histopathological findings of ALS and FTD with accumulation of cytosolic ubiquitinated and phosphorylated CTF-TDP-43 aggregates [13-15]. In addition, transfection with these constructs can result in neurotoxicity and cell death although the pathways involved are not known [14].

However, while these studies have recapitulated findings of post-mortem disease tissue, they have told us little of the early disease processes associated with abnormal TDP-43 metabolism, particularly in sporadic TDP-43 proteinopathies which account for > 90% of ALS (and FTD) cases. A new insight into TDP-43 accumulation is developing through studies identifying TDP-43 association with RNA stress granule proteins [16,17]. Stress granules (SGs) are cytoplasmic sites of stalled mRNA pre-initiation complexes induced by oxidative changes, heat shock or osmotic stress where the cell stalls mRNA translation of non-critical proteins to shift energy expenditure to key repair and survival proteins [18]. Recent studies have shown that under stress, TDP-43 is recruited to SGs in a variety of cells [16,17,19,20]. Initially Moisse et. al. [21] reported that TDP-43 localized to SGs after axotomy in mice. Subsequently, studies in cells revealed that acute cell stress induced TDP-43 SG association and this was dependent on residues 216-315 and the first RNA recognition motif [19]. While the same group reported a lack of TDP-43 association with SG markers in ALS tissues, subsequent work by Volkening et al. [22] reported an association between TDP-43 and staufen in ALS spinal cord tissue. TDP-43 SG co-localization in ALS and FTLD-U has since been reported by Liu-Yesucevitz et al., [17] and FUS, another hnRNP protein associated with ALS, has also been identified in ALS SGs [23,24]. Liu-Yesucevitz et al. [17] also reported that TDP-43 may associate with SGs through interaction with SG proteins such as TIA-1 and this has been supported by studies on TDP-43 association with a number of SG proteins [20,25]

However, while these studies have advanced our understanding of the early stages of TDP-43 aggregation, the majority of this research has been performed in cells exposed to acute and highly toxic treatment with sodium arsenite, the standard means of inducing SGs [17,19,20]. In addition, much of our knowledge has been gained through generation of CTF-TDP-43 over-expression in transfected cells. There is a lack of understanding about the processes involved in endogenous TDP-43 aggregation during chronic oxidative stress. As the majority of ALS and FTD cases involve no known mutation in TDP-43 and the slow disease process characteristic of neurodegeneration involves chronic oxidative and nitrosative stresses [2,26], it is critical to determine how these factors affect TDP-43 SG cytosolic accumulation. Moreover, SG proteins have a high propensity to aggregate and over-expression of highly aggregating CTF

fragments may not accurately re-capitulate the underlying mechanistic processes involved in endogenous TDP-43 aggregation and association with SGs during chronic stress. Therefore, we investigated the effects of mild, chronic oxidative and nitrosative stress on endogenous TDP-43 in neuronal-like cell cultures. Our findings revealed that in contrast to acute stress, chronic oxidative stress induced several features consistent with TDP-43 proteinopathies including loss of nuclear TDP-43, accumulation of diffuse TDP-43 in the cytosol, formation of a 35 kDa C-terminal fragment and accumulation of TDP-43 in SGs, some of which revealed ubiquitination. Importantly, our findings revealed that TDP-43 localization to SGs was controlled by c-Jun N-terminal kinase (JNK). Inhibition of JNK also modulated TDP-43 accumulation in SGs induced by sodium arsenite and in cells transfected with CTF-TDP-43 constructs. Our data also indicated that the aggregation of TDP-43 may be associated with JNK modulation of hnRNP-TDP-43 interactions and SG localization.

Results

To investigate the effects of chronic stress on TDP-43 metabolism, we first determined optimal concentrations of oxidative and nitrosative stress inducers in SH-SY5Y neuronal-like cultures. Cells were treated overnight with each compound at a range of concentrations and the cell viability was determined by MTT assay and cell death was measured using an LDH assay (not shown). Additional File 1 shows the selected concentrations used for further investigation. The concentrations shown in Additional File 1 induced mild but significant reductions in cell viability overnight. However, except for 2 mM paraquat (24 ± 3.2% cell death) and 75 μM rotenone (32 ± 4.6% cell death), no change to LDH release was observed compared to untreated controls. These doses were used to mimic sub-lethal chronic stress conditions relevant to brain or spinal cord neurons during disease *in vivo*.

Nitrosative stress inducers mediate altered TDP-43 processing

Treatment of SH-SY5Y cells with inducers of nitrosative stress resulted in changes to sub-cellular distribution of TDP-43. Compared to untreated controls (Figure 1A-C), SIN-1, a peroxynitrite donor caused a frequent, evenly distributed, diffuse accumulation of TDP-43 in the cytosol of treated cells (Figure 1D-F). In contrast, paraquat, an inhibitor of the mitochondrial electron transport chain and inducer of superoxide/peroxynitrite stress (a common feature in neurodegeneration), induced substantial and varied cytoplasmic accumulation of TDP-43 including aggregates of TDP-43 resembling RNA SGs (Figure 1G-I). Arginine (nitric oxide precursor) had no consistent effect (Figure 1J-L).

Paraquat induces a robust cell model of TDP-43 proteinopathy

Further examination of TDP-43 in paraquat-treated cells revealed multiple features reported for human TDP-43 proteinopathies. Paraquat-treated cells frequently showed clear loss of nuclear TDP-43 (Figure 2D-F), accumulation of diffuse TDP-43 in the cytosol (Figure 2G-I) and formation of cytoplasmic aggregates. Interestingly, these changes were not always observed in the same cells suggesting that loss of nuclear TDP-43 expression and accumulation in the cytosol may been caused by different stress-mediated processes. To determine if the cytosolic aggregates of TDP-43 induced by paraquat were SGs, cells were co-stained for the SG marker, HuR. The majority of TDP-43 aggregates co-localized with HuR although there were also additional HuR-positive SGs that lacked TDP-43 (Figure 2N-Q). Quantitative analysis revealed that 66 ± 8% of paraquat-induced SGs that were positive for HuR were also positive for TDP-43 and that SG formation correlated to increasing toxicity of paraquat (Additional File 2). TDP-43 also frequently co-localized with the SG marker, TIA-1 (data not shown). We examined the time course of TDP-43 SG formation and found that TDP-43 only accumulated into SGs between 8 and 20 hr after exposure to paraquat. This is in contrast to the rapid accumulation of TDP-43 into SGs reported for arsenite or osmotic stress [19,20]. Our findings were also observed in retinoic-acid differentiated SY5Y neuronal-like cells, confirming that these changes can occur in non-dividing SY5Y cells (Additional File 2H-K).

We extended the investigation of this model further by examining if TDP-43-positive SGs revealed presence of the protein aggregate marker ubiquitin, also a hallmark feature of the ubiquitinated inclusions in ALS and FTLD-U in FTD. Interestingly, our study revealed that a number of the TDP-43-positive SGs co-localized with ubiquitin (Figure 3F and 3J). 24 ± 6% of TDP-43-positive SGs were also positive for ubiquitin indicating that only a portion of the SGs may progress to ubiquitinated protein aggregates (Figure 3). Diffuse TDP-43 did not consistently co-localize with ubiquitin (Figure 3J). Whether the ubiquitination of the SGs was associated directly with the TDP-43 or ubiquitination of alternative SG proteins is uncertain. Due to the relatively low numbers of cells containing ubiquitinated SGs and lack of a method for purifying SGs, it was not possible to determine if the ubiquitinated protein in the SGs was specifically TDP-43.

Interestingly, we did not observe phosphorylated TDP-43 associated with the SGs (Additional File 3A-F). This was confirmed by Western blot analysis that detected no increase in phosphorylated TDP-43 or phosphorylated CTF-TDP-43 post-exposure to paraquat

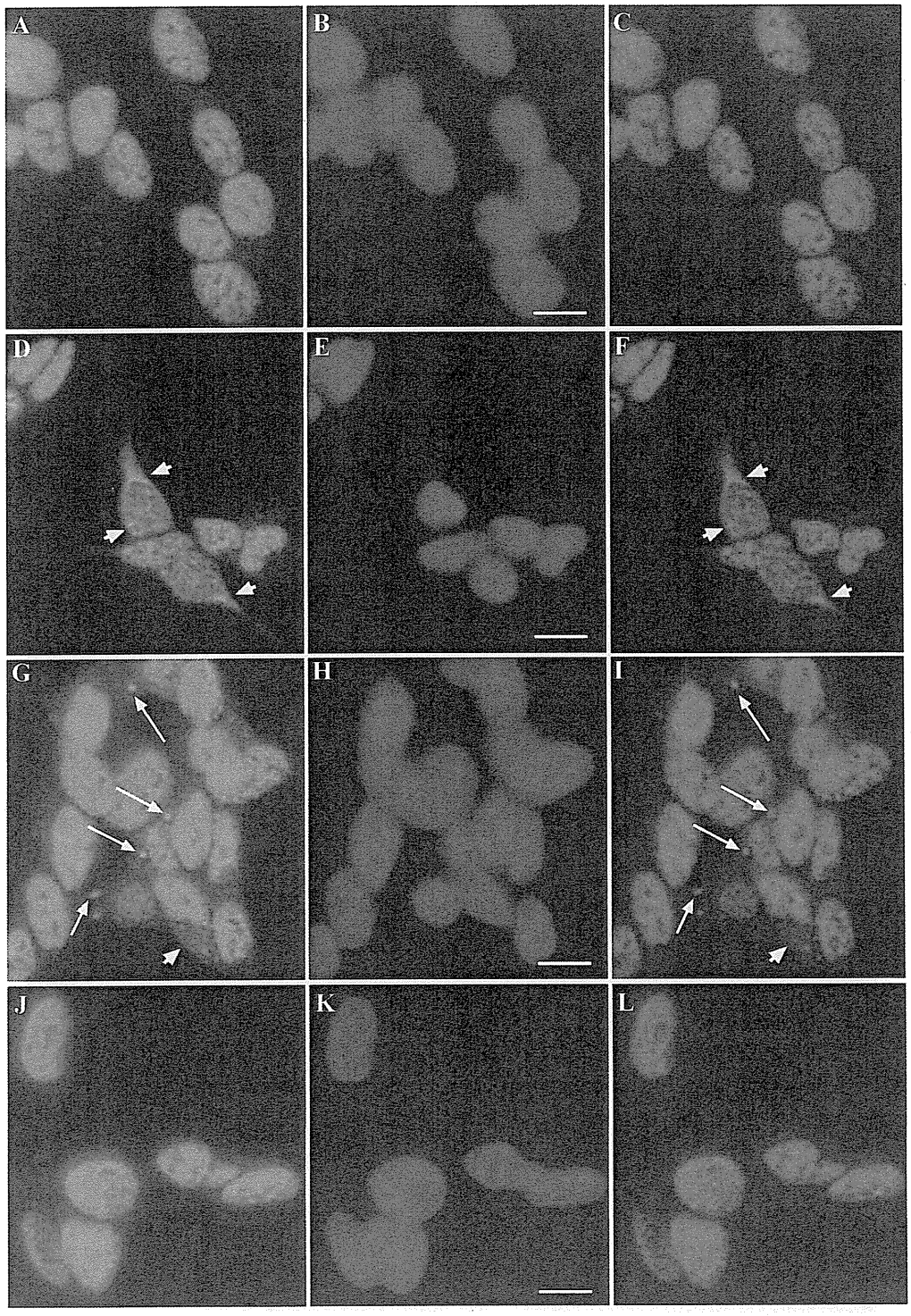

Figure 1 Effect of SIN-1, paraquat and arginine on TDP-43 localization in SH-SY5Y cells. Cells were exposed overnight with 0.1 mM SIN-1, 1 mM paraquat or 1 mM arginine and TDP-43 localization was examined by immunofluorescence. **A-C**: untreated, **D-F**: SIN-1, **G-I**: paraquat, **J-L**: arginine. Green = TDP-43, blue = DAPI. Right-hand panel = merged images of TDP-43 and DAPI. Arrowheads show diffuse cytosolic TDP-43. Arrows show aggregated cytosolic TDP-43. Bar = 10 μm. Representative images from three separate experiments performed in duplicate or triplicate.

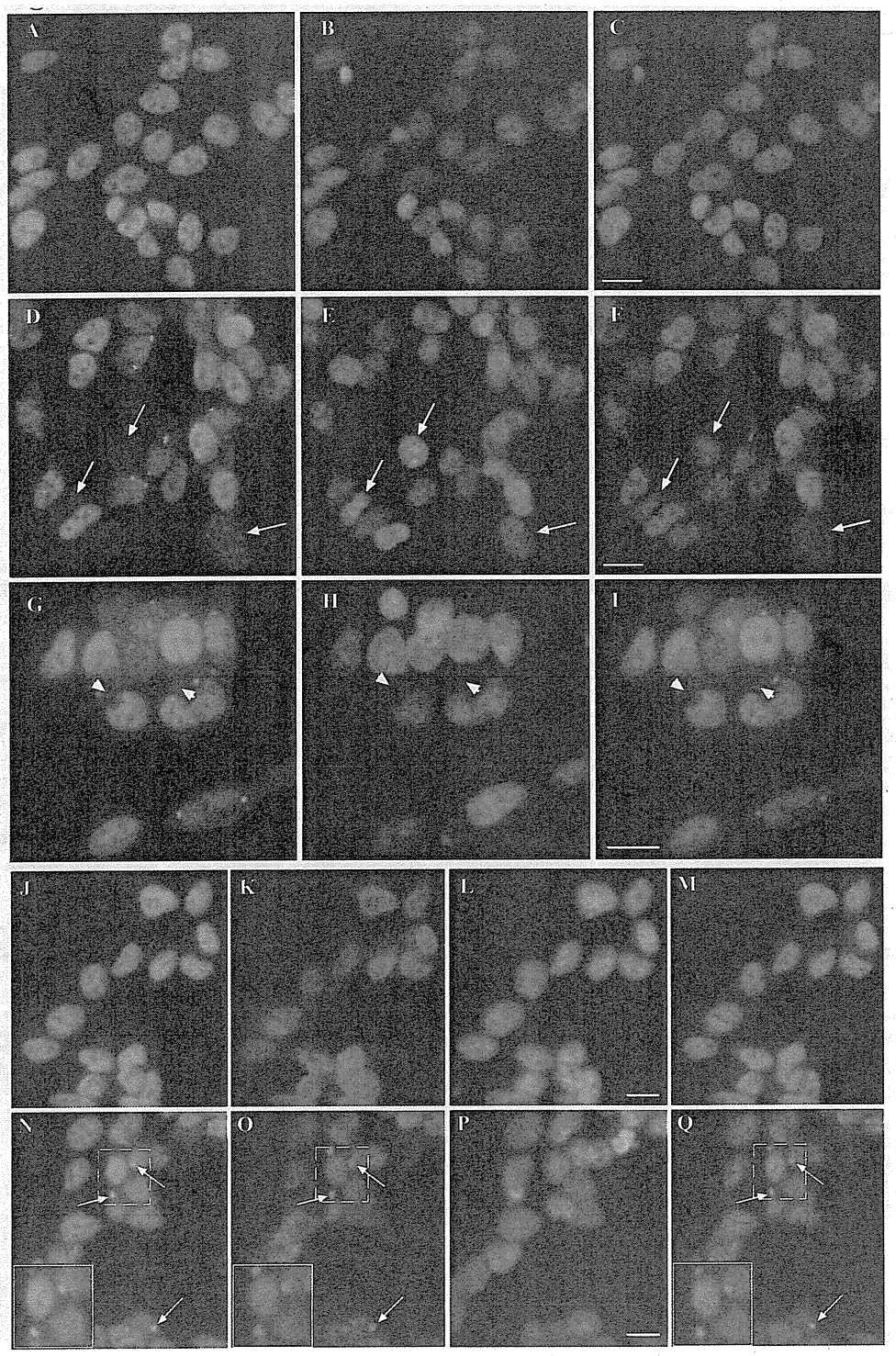

Figure 2 Induction of cytosolic TDP-43 accumulation and SGs by paraquat in SH-SY5Y cells. Cells were exposed overnight to 1 mM paraquat and TDP-43 localization was examined by immunofluorescence. **A-C**: untreated, **D-I**: paraquat treated. Arrows show loss of nuclear TDP-43. Arrowheads show diffuse cytosolic TDP-43. Green = TDP-43, blue = DAPI. Right-hand panels = merged images. **J-Q:** Cells were exposed to 1 mM paraquat overnight and TDP-43 and HuR localization was measured by immunofluorescence. **J-M**: untreated, **N-Q**: paraquat treated. Green = TDP-43, Red = HuR, Blue = DAPI. **M** and **Q** are merged images from TDP-43 and HuR panels. Arrows indicate stress granules. Inset shows higher magnification of TDP-43 and HuR positive SGs. Bar = 10 μm. Representative images from four separate experiments performed in duplicate or triplicate.

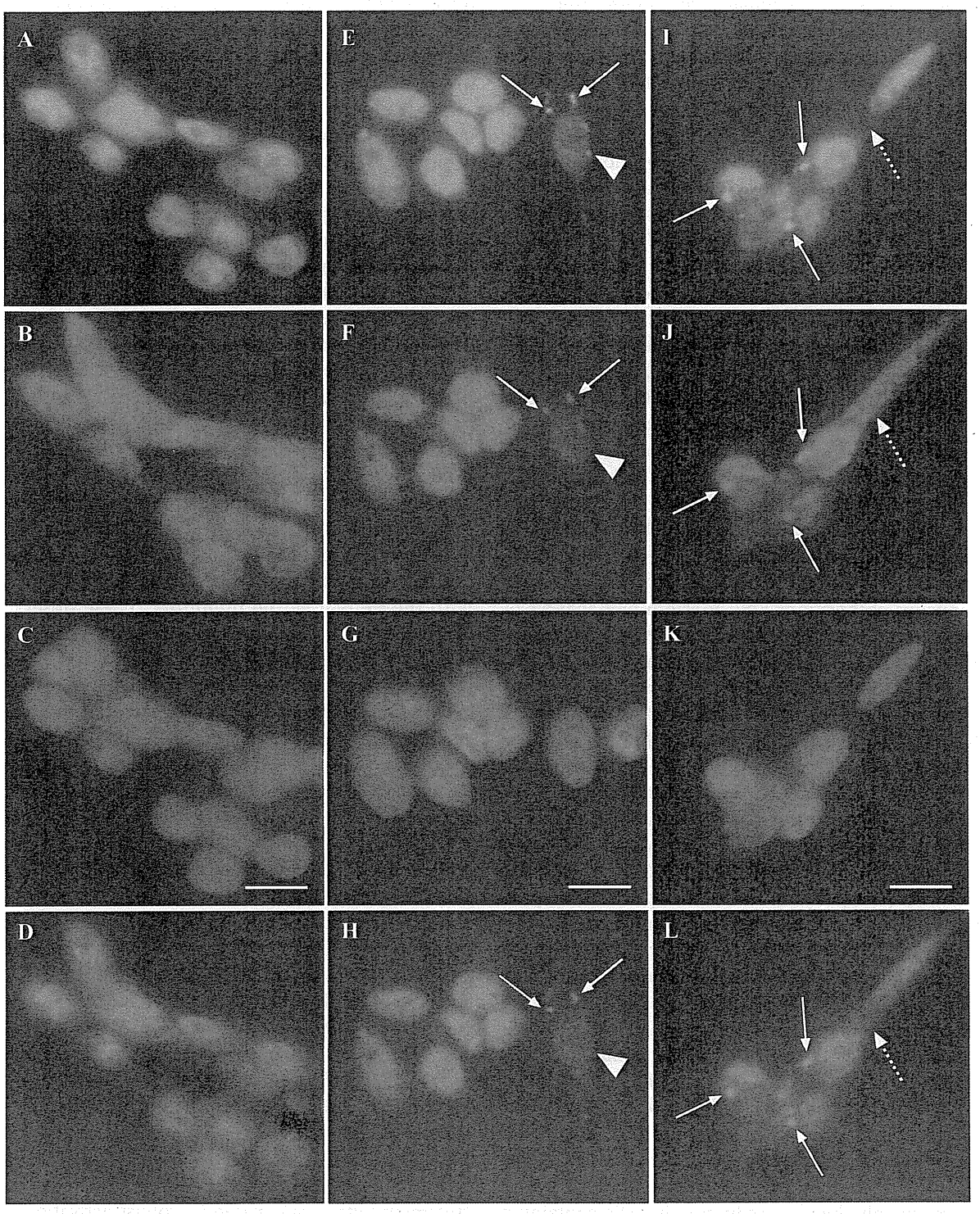

Figure 3 Treatment of SH-SY5Y neurons induces co-localization of TDP-43 and ubiquitin in SGs. Cells were treated overnight with 1 mM paraquat and localization of TDP-43 and ubiquitin was determined by immunofluorescence. **A-D**: untreated, **E-L**: paraquat treated. Green = TDP-43, red = ubiquitin, blue = DAPI. **D**, **H** and **L** represent merged images of panels above. Solid arrows indicate co-localization of TDP-43 and ubiquitin in SGs. Arrowhead indicates lack of co-localization of TDP-43 and ubiquitin in one SG in panel **F**. Dotted arrow indicates lack of co-localization of diffuse TDP-43 and ubiquitin. Bar = 10 μm. Representative images from three separate experiments performed in duplicate or triplicate.

(Additional File 3G). It is possible that more prolonged treatment of cells is required to induce phosphorylation or that the correct cellular kinases are not present or not-localized to SGs. Alternatively the TDP-43 may be phosphorylated on sites different to the 409/410 site. However, the combination of clear nuclear loss of TDP-43, diffuse cytosolic accumulation, aggregation and ubiquitination under mild stress provided a unique model for investigating the early processes in abnormal TDP-43 processing associated with ALS and FTD.

Paraquat induces formation of caspase-dependent and caspase-independent TDP-43 SGs

One of the hallmark neuropathological features of TDP-43 proteinopathies is the formation of C-terminal TDP-43 fragments (CTF-TDP-43), often of 35 or 25 kDa in mass [6,8]. Cell studies have re-capitulated features of end-stage TDP-43 proteinopathies through expression of these fragments which aggregate and co-localize with SG proteins [13,27]. Therefore, we examined if our paraquat model also induced CTF-TDP-43. The SH-SY5Y cells revealed basal expression of a 35 kDa TDP-43 band even in untreated cultures. This is consistent with previous observations [28]. Western blot analysis of paraquat-treated cells revealed the increased expression of this 35 kDa CTF-TDP-43 (Figure 4A). Interestingly, none of the additional mitochondrial inhibitors or nitrosative stress inducers tested significantly elevated 35 kDa CTF-TDP-43 (Figure 4A and 4B). This was despite inducing a similar loss of cell viability (Additional File 1). These findings suggested that formation of TDP-43 SGs may be specifically associated with CTF-TDP-43 as previously supported by studies involving transfection of cells with TDP-43 CTF constructs [16]. Co-treatment of cells with paraquat and the broad-spectrum caspase inhibitor, Z-VAD-fmk, resulted in a complete inhibition of increased 35 kDa CTF-TDP-43 expression (Figure 4C). This supported previous studies demonstrating that 35 kDa TDP-43 CTFs are generated by caspase-cleavage at a DETD consensus site within the NLS of TDP-43 [29,30]. However, we found that while inhibiting CTF-TDP-43 generation with Z-VAD-fmk partially inhibited TDP-43 SG formation (Figure 4H), the effect was not complete. Treatment of cultures with Z-VAD-fmk reduced the number of cells containing TDP-43-positive SGs from 18 ± 8% to 8 ± 2% ($P < 0.05$). This inhibitory effect was mainly due to a reduction in cells containing smaller TDP-43-positive SGs as there was no loss of large (≥ 1 μm) TDP-43 SGs in Z-VAD-fmk treated cells, despite a complete inhibition of enhanced 35 kDa CTF formation. In our cultures, no change was observed to a faint 25 kDa CTF-TDP-43 (Figure 4C), ruling out involvement of this fragment in TDP-43 SG formation. Nishimoto et al. have also reported that the 25 kDa form is not involved in TDP-43 SG formation [30]. These findings strongly suggest that while paraquat treatment enhanced 35 kDa CTF-TDP-43 formation, this was not sufficient for TDP-43 SG formation.

Induction of cytosolic TDP-43 accumulation by paraquat is not mediated through mitochondrial inhibition

As paraquat is a mitochondrial electron transport chain inhibitor, we compared paraquat treatment with alternative inhibitors of cellular respiration to determine if mitochondrial impairment induced TDP-43 SGs. Figure 5P-R shows that only paraquat induced cytosolic accumulation and formation of TDP-43-positive SGs after an overnight treatment. Other mitochondrial inhibitors including rotenone (Figure 5D-F), 3-NP (Figure 5G-I), MPP+ (Figure 5J-L) and sodium azide (Figure 5M-O) had no effect on TDP-43 despite being applied at concentrations that induced the same or increased level of mild cell toxicity (Additional File 1). We then determined if the alternative mitochondrial respiration inhibitors induced HuR-positive SGs that lacked TDP-43. However, as shown in Additional File 4, no HuR-positive SGs were observed in cells after overnight treatment with the mitochondrial inhibitors. These observations showed that the ability of paraquat to induce loss of nuclear TDP-43, cytosolic accumulation and SGs is not solely attributable to its ability to inhibit mitochondrial activity per se. These findings suggest that the effects of paraquat on TDP-43 are more likely associated with specific pathways of oxidative or nitrosative stress induction that differ from the other mitochondrial inhibitors.

JNK controls TDP-43 localization to SGs during oxidative stress

It has been reported previously that kinases can control cytoplasmic localization and SG accumulation of hnRNPs such as hnRNP A1 and hnRNP K. This includes p38, JNK and ERK-dependent modulation of hnRNP sub-cellular accumulation [31-36]. Of these, JNK has been clearly established as a critical stress-activated kinase [37] and is central to toxic effects of paraquat [38,39]. Therefore, we examined if modulation of JNK activity affected TDP-43-positive SG formation. Initially, we determined if paraquat induced activation of JNK and p38 as previously reported [38]. After overnight treatment with 1 mM paraquat robust activation of JNK and ERK was observed with weaker p38 activity (Figure 6A). A time course of activation revealed elevated JNK and ERK phosphorylation after 30-60 min with maximal activation at 2 hr (Figure 6A). No early activation of p38 was observed (data not shown). Subsequent co-treatment of cultures with paraquat and the JNK inhibitor, SP600125, resulted in almost complete inhibition of