ALS-associated protein FIG4 is localized in Pick and Lewy bodies, and also neuronal nuclear inclusions, in polyglutamine and intranuclear inclusion body diseases

（ALS 関連タンパク質である FIG4 は、ピック小体、レヴィ小体およびポリグルタミン病と核内封入体病における核内封入体に局在する）

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Abstract

FIG4 is a phosphatase that regulates intracellular vesicle trafficking along the endosomal-lysosomal pathway. Mutations of *FIG4* lead to the development of Charcot-Marie-Tooth disease type 4J and amyotrophic lateral sclerosis (ALS). Moreover, ALS-associated proteins (TDP-43, FUS, optineurin, ubiquilin-2, CHMP2B and valosin-containing protein) are involved in inclusion body formation in several neurodegenerative diseases. Using immunohistochemistry, we examined the brains and spinal cords of patients with various neurodegenerative diseases including sporadic TDP-43 proteinopathy (ALS and frontotemporal lobar degeneration). TDP-43 proteinopathy demonstrated no FIG4 immunoreactivity in neuronal inclusions. However, FIG4 immunoreactivity was present in Pick bodies in Pick's disease, Lewy bodies in Parkinson's disease and dementia with Lewy bodies, neuronal nuclear inclusions in polyglutamine and intranuclear inclusion body diseases, and Marinesco and Hirano bodies in aged control subjects. These findings suggest that FIG4 is not incorporated in TDP-43 inclusions and that it may have a common role in the formation or degradation of neuronal cytoplasmic and nuclear inclusions in several neurodegenerative diseases.

Key words: endosomal-lysosomal pathway, FIG4, Lewy body, nuclear inclusion, Pick body.

INTRODUCTION

Factor-Induced-Gene 4 (FIG4), also known as *SAC3*, was first cloned from a human immature myeloid cell line in 1996.[1,2] The protein encoded by *FIG4* is a phosphatase that regulates phosphatidylinositol 3,5-bisphosphate, a molecule critical for intracellular vesicle trafficking along the endosomal-lysosomal pathway.[3] Previous studies have shown that FIG4 is abundantly expressed during neural development in mice and rats; FIG4 is expressed in neurons and myelin-forming cells in the central and peripheral nervous systems, particularly in spinal ganglia sensory neurons and Schwann cells.[4] Although FIG4 protein and mRNA levels are markedly diminished in neurons of the adult central nervous system, spinal cord injury induces upregulation of FIG4 in the adult spinal cord, and this is associated with accumulation of lysosomes in neurons and glia.[4] FIG4 knockout mice and rats result in spongiform neurodegeneration with enlarged lysosomal vesicles, defective myelination and juvenile lethality.[5,6] These findings suggest that expression of FIG4 is required for neural development and is necessary to prevent neurodegeneration. Mutations of *FIG4* cause Charcot-Marie-Tooth disease type 4J (CMT4J; MIM 611228), a severe form of peripheral neuropathy.[6,7] Mutations of *FIG4* may also lead to the development of familial and sporadic amyotrophic lateral sclerosis (ALS) (ALS11; MIM 609390).[8] However, the localization of FIG4 in the human nervous system has not yet been investigated immunohistochemically.

Abnormal accumulation and aggregation of disease-specific proteins are common features of several neurodegenerative diseases.[9] Impairment of the endosomal-lysosomal and autophagy-lysosomal pathways is one of the common

pathomechanisms of various neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD) and polyglutamine diseases.[10] Recently, several investigators have reported that familial ALS-associated proteins (TDP-43,[11-14] FUS,[15,16] optineurin,[17,18] ubiquilin-2,[19,20] CHMP2B[21,22] and valosin-containing protein[23]) are involved in inclusion body formation in various neurodegenerative diseases. These reports prompted us to investigate whether FIG4 is involved in a variety of neurodegenerative diseases including TDP-43 proteinopathy (sporadic ALS and frontotemporal lobar degeneration). Using immunohistochemistry, we therefore examined the brains and spinal cords of patients with various neurodegenerative diseases and control subjects using anti-FIG4 antibody. Here we report that FIG4 is not incorporated in TDP-43 inclusions and that FIG4 immunoreactivity is present in Pick bodies in Pick's disease, Lewy bodies in PD and dementia with Lewy bodies (DLB), and neuronal nuclear inclusions (NNIs) in polyglutamine and intranuclear inclusion body diseases.

MATERIALS AND METHODS

Subjects

Seventy-four autopsy cases were investigated in this study; these included cases of sporadic ALS (n = 5), frontotemporal lobar degeneration with TDP-43-positive inclusions (FTLD-TDP type B; n = 5),[24] AD (n = 5), Pick's disease (n = 4), progressive supranuclear palsy (PSP; n = 4), corticobasal degeneration (CBD; n = 4), argyrophilic grain disease (AGD; n = 4), PD (n = 5), neocortical-type DLB (n = 5), multiple system atrophy (MSA; n = 5), dentatorubral-pallidoluysian atrophy (DRPLA; n = 3),

Huntington's disease (HD; n = 5), spinocerebellar ataxia type 1 (SCA1; n = 3), SCA2 (n = 1),[13] SCA3 (n = 5), intranuclear inclusion body disease (INIBD; n = 5) and normal controls (aged 48-84 years, average 63.8 years, n = 6). All the diagnoses had been confirmed by neuropathological examinations using immunohistochemistry for tau, β-amyloid, α-synuclein, TDP-43, polyglutamine and ubiquitin. This study was approved by the Institutional Ethics Committee of Hirosaki University Graduate School of Medicine.

Immunohistochemistry

Immunohistochemical analysis was carried out using formalin-fixed, paraffin-embedded sections from the frontal cortex, hippocampus, basal ganglia, midbrain, pons, medulla oblongata, cerebellum, spinal cord, and sympathetic and spinal ganglia of normal controls. In other cases, multiple sections taken from the affected regions were immunostained; the frontal cortex and hippocampus in FTLD-TDP, AD, Pick's disease, CBD, DLB, SCA1 and INIBD, the amygdaloid nucleus and hippocampus in AGD, the basal ganglia in HD and SCA2, the midbrain in PSP, PD and DLB, the pons in MSA, DRPLA and SCA3, and the motor cortex and spinal cord in ALS. The sections were initially subjected to heat retrieval for 10 minutes in 10 mmol/L citrate buffer (pH 6.0) using an autoclave, and then subjected to immunohistochemical processing using the avidin-biotin-peroxidase complex method with diaminobenzidine. The primary antibody used was a rabbit polyclonal anti-FIG4 antibody (CAB017823 in The Human Protein Atlas; Novus Biologicals, Littleton, CO, USA; 1:300).

Double immunofluorescence analysis was performed to detect overlapping expression of FIG4 and phosphorylated tau, phosphorylated α-synuclein, polyglutamine

or ubiquitin. Paraffin sections from the hippocampus of patients with Pick's disease and DLB, the midbrain of patients with PD, the pons of patients with DRPLA and SCA3, and the frontal cortex of patients with INIBD were processed for double-label immunofluorescence. De-paraffinized sections were blocked with donkey serum and then incubated overnight at 4°C with a mixture of polyclonal anti-FIG4 (1:100) and monoclonal anti-phosphorylated tau (AT8; Innogenetics, Ghent, Belgium; 1:200) for Pick's disease, anti-phosphorylated α-synuclein (#64; Wako, Osaka, Japan; 1:1000) for PD and DLB, anti-polyglutamine (1C2; Chemicon, Temecula, CA, USA; 1:40) for DRPLA and SCA3, or anti-ubiquitin (1B3; MBL, Nagoya, Japan; 1:400) for INIBD. The sections were then rinsed and incubated with anti-rabbit IgG tagged with Alexa Fluora 488 (Invitrogen, Carlsbad, CA, USA; 1:1000) or anti-mouse IgG tagged with Alexa Fluora 594 (Invitrogen; 1:1000) for 1 h at 38°C. The sections were mounted using ProLong gold antifade reagent with DAPI (Invitrogen) and examined with a confocal microscope (EZ-Ci; Nikon, Tokyo, Japan). The proportion of FIG4-positive inclusions relative to the total number of inclusions positive for phosphorylated tau, phosphorylated α-synuclein, polyglutamine or ubiquitin was calculated in each case. Values were expressed as the mean for each diagnostic group.

RESULTS

FIG4 immunoreactivity in normal controls

In normal controls, anti-FIG4 antibody immunolabeled the neuronal cytoplasm in a diffuse granular pattern throughout the central nervous system, including the cerebral cortex (Fig. 1A), hippocampus (Fig. 1B), basal ganglia (Fig. 1C), brainstem (Fig. 1D-F),

cerebellum (Fig. 1G) and spinal cord (Fig. 1H). The cytoplasm of astrocytes and oligodendrocytes was also weakly immunostained with anti-FIG4 (Fig. 1I, J). Although axons and presynaptic nerve terminals were barely immunolabeled, or unstained, mossy fiber terminals (axon terminals of dentate granule cells) were intensely immunolabeled (Fig. 1K). In the sympathetic and spinal ganglia, the cytoplasm of ganglion cells, satellite cells and Schwann cells was immunostained (Fig. 1L, M). Neuronal and glial nuclei were not stained with anti-FIG4 antibody.

FIG4 immunoreactivity in neurodegenerative diseases

Although TDP-43-positive neuronal and glial cytoplasmic inclusions were found in the cerebral cortex in FTLD-TDP and the upper and lower motor neuron systems in ALS, no FIG4-immunoreactive inclusions were noted in these areas (data not shown).

In AD, dystrophic neurites in senile plaques were positive for FIG4 (Fig. 2A). In Pick's disease, Pick bodies were intensely immunostained with anti-FIG4 (Fig. 2B). However, no FIG4 immunoreactivity was found in neurofibrillary tangles in AD, PSP and CBD, argyrophilic grains in AGD, tufted astrocytes in PSP, or astrocytic plaques in CBD.

In PD and DLB, the majority of brainstem-type Lewy bodies were positive for FIG4 (Fig. 2C). A small fraction of cortical Lewy bodies were also positive for FIG4 (Fig. 2D). Both brainstem-type and cortical Lewy bodies showed intense staining in their central portion, whereas the peripheral portion was not stained with anti-FIG4. Pale bodies, which have been considered precursors of Lewy bodies,[25] and intraneuritic Lewy bodies (Lewy neurites) were negative for FIG4. In MSA, glial cytoplasmic inclusions, glial nuclear inclusions, neuronal cytoplasmic inclusions, neuronal nuclear

inclusions, and swollen neurites were intensely immunolabeled with anti-phosphorylated α-synuclein.[26] However, these structures were FIG4-negative.

Immunohistochemistry for ubiquitin and polyglutamine revealed NNIs were in all of the cases of polyglutamine diseases examined. NNIs in DRPLA and SCA3, but not in HD, SCA1 and SCA2, were immunolabeled with anti-FIG4 (Fig. 2E, F).

In INIBD, ubiquitin-positive nuclear inclusions were found in both neurons and glial cells. FIG4 immunoreactivity was present in nuclear inclusions in neurons (Fig. 2G), but not in glial cells.

In aged normal controls and patients with neurodegenerative diseases, Marinesco bodies were observed in the nuclei of substantia nigra pigmented neurons, and were strongly positive for FIG4 (Fig. 2H). In addition, Hirano bodies in the hippocampus were FIG4 positive (Fig. 2I).

There was no apparent difference in the staining intensity of neuronal cytoplasm with and without inclusions between patients with neurodegenerative diseases and normal controls.

Double immunofluorescence analysis

Double immunofluorescence analysis revealed co-localization of FIG4 and phosphorylated tau in Pick bodies (Fig. 3A-C) and neuropil threads (Fig. 3D-F) in Pick's disease, the latter corresponding to small Pick bodies in the neurites.[27,28] The average proportion of FIG4-positive Pick bodies relative to the total number of inclusions was 88.7%. In both brainstem-type and cortical Lewy bodies, FIG4 immunoreactivity was concentrated in the central portion and α-synuclein immunoreactivity was more intense in the peripheral portion (Fig. 3G-L). The average

proportion of FIG4-positive brainstem-type and cortical Lewy bodies relative to the total number of inclusions was 88.9% and 45.3%, respectively. Co-localization of FIG4 with polyglutamine or ubiquitin was demonstrated in NNIs in DRPLA (Fig. 3M-O), SCA3 (Fig. 3P-R) and INIBD (Fig. 3S-U). The FIG4 positivity rate of NNIs in DRPLA, SCA3 and INIBD was 19.5%, 19.7% and 28.6%, respectively. Almost all Marinesco bodies (99.8%) were positive for FIG4.

DISCUSSION

In rodents, FIG4 is abundantly expressed in neurons and myelin-forming cells in the central and peripheral nervous systems during neural development, and is markedly diminished in neurons of the adult central nervous system.[4] In the present study, we demonstrated that FIG4 immunoreactivity was present in neuronal cytoplasm in the brain, spinal cord and peripheral ganglia of adult humans. Schwann cells in the peripheral nervous system were also strongly immunolabeled with anti-FIG4, whereas oligodendrocytes and astrocytes in the central nervous system were weakly positive. These findings suggest that FIG4 is widely expressed in neurons and glial cells throughout the adult human nervous system.

In the present study, no FIG4 immunoreactivity was found in a variety of neuronal and glial inclusions in sporadic TDP-43 proteinopathy (ALS and FTLD-TDP type B). Although TDP-43-positive neuronal and glial cytoplasmic inclusions have been found in a previous case of SCA2,[13] no FIG4-immunoreactive inclusions were noted in that case. Our data indicate that FIG4 is not incorporated into TDP-43 inclusions.

We further demonstrated that the majority of Pick bodies were immunopositive for FIG4. Considering that dentate granule cells are one of the sites where Pick bodies accumulate preferentially, it is important to note that the cytoplasm and axon terminals of dentate granule cells were strongly positive for FIG4. Previous immunohistochemical studies have shown that Pick bodies are immunoreactive for synaptic proteins.[29] These findings suggest that the proteins synthesized in neuronal perikarya might be entrapped within the filamentous structure of Pick bodies. In the present study, however, Pick bodies present inside and outside the dentate gyrus were intensely immunolabeled with anti-FIG4. Moreover, co-localization of FIG4 and phosphorylated tau was seen in the neuropil, which corresponds to small Pick bodies in the neurites.[27,28] It seems likely that incorporation of FIG4 into Pick bodies is a pathological event, and does not simply reflect to entrapment of the protein.

Lewy bodies consist of a dense core and a peripheral halo, which correspond ultrastructurally to zones of densely compacted circular profiles and zones of filaments, respectively.[30] It is well known that the constituent filaments of Lewy bodies are composed of α-synuclein. However, little is known about the components of the central core of Lewy bodies. In the present study, the cores of brainstem-type and cortical Lewy bodies were immunolabeled intensely by anti-FIG4 antibody, but their peripheral portions were only weakly stained or unstained. This localization implies that FIG4 is involved in formation of the central core of Lewy bodies and that FIG4 may not interact with α-synuclein.

In polyglutamine diseases, NNIs in DRPLA and SCA3, but not in HD, SCA1 and SCA2, were immunopositive for FIG4. NNIs in INIBD were also positive for FIG4. In addition to the cytoplasm, FIG4 is reportedly localized in the nuclear pore, being

required for efficient export of nuclear signal-containing reporter protein.[31] This interaction is thought to be important for the regulation of gene expression or DNA systhesis.[30] In polyglutamine diseases, NNIs may affect nuclear function and recruitment other proteins, possibly resulting in loss of the physiological function of recruited proteins, and subsequent neuronal dysfunction.[32] Similar mechanisms may occur in the pathogenesis of INIBD, although the major component of nuclear inclusions in this disease is uncertain. It is possible to consider that FIG4 translocates from the cytoplasm to the nucleus in order to protect cells from cytotoxic events. However, it is unclear why only two polyglutamine diseases (DRPLA and SCA3) showed FIG4 immunoreactivity in NNIs. The evidence suggests that the mechanism of inclusion body formation may differ among the various polyglutamine diseases.

In the present study, Marinesco bodies were also immunoreactive for FIG4. The frequency of Marinesco bodies is significantly higher in nigral neurons with Lewy bodies than in those without.[33] The melanin content of nigral neurons containing Marinesco bodies is lower than that of nigral neurons lacking Marinesco bodies.[34] The available evidence suggests that Marinesco bodies may play a pathogenic role in certain neurodegenerative disorders, and that the formation and disaggregation of Marinesco bodies are features common to the disease process of neurodegenerative conditions characterized by the presence of intranuclear inclusions.[35]

Mutations of *FIG4* result in the accumulation of enlarged vesicles derived from the endosomal-lysosomal pathway in the central and peripheral nervous systems of FIG4 mutated mice.[6] A similar phenomenon is evident in fibroblasts from patients with CMT4J, suggesting impaired trafficking of intracellular organelles due to physical obstruction by vacuoles.[7] FIG4 has not been directly implicated in autophagy, whereas

a role for phosphatidylinositol-3-phosphate, which is both a metabolic precursor and a product of phosphatidylinositol 3,5-bisphosphate, is involved in autophagy.[36] This implies the involvement of FIG4 in both the endosomal-lysosomal and autophagy-lysosomal pathways.[37] Lázaro-Diéguez *et al*. have reported that in a variety of mammalian cells the reversible formation of filamentous actin-enriched aggresomes is generated by the actin toxin jasplakinolide.[38] Notably, these aggresomes resemble Hirano bodies observed in the human brain in many respects. Moreover, Hirano bodies are immunopositive for ubiquilin-1.[39] The available evidence suggests that ubiquilin-1 exerts a cytoprotective role by targeting polyubiquitinated proteins for proteasomal degradation or the action of autophagosomes, or by sequestering aggregated proteins to aggresomes.[40-44] The above findings suggest that Hirano bodies may represent autophagy- and/or aggresome-related structures.

In conclusion, we have demonstrated for the first time that FIG4 immunoreactivity is present in Pick bodies in Pick's disease, Lewy bodies in PD and DLB, and NNIs in polyglutamine and intranuclear inclusion body diseases. These findings suggest that FIG4 may have a common role in the formation or degradation of neuronal cytoplasmic and nuclear inclusions in several neurodegenerative diseases.

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FIGURE LEGENDS

Fig. 1 FIG4 immunoreactivity in the normal human nervous system. The neuronal cytoplasm in the frontal cortex (A), dentate gyrus (B), putamen (C), substantia nigra (D), pontine nucleus (E), inferior olivary nucleus (F), cerebellar cortex (G) and anterior horn of the lumbar cord (H) showing FIG4 immunoreactivity. The cytoplasm of astrocytes in the periaqueductal white matter (I) and oligodendrocytes in the pontine base (J) showing weak FIG4 immunoreactivity. Strong FIG4 immunoreactivity is evident in mossy fiber terminals in the hippocampal CA4 region (K). Ganglion cells and satellite cells in the spinal ganglia (L) and Schwann cells in the peripheral nerves (M) showing FIG4 immunoreactivity. Bars = 40 μm.

Fig. 2 FIG4 immunoreactivity in neurodegenerative diseases. Neuritic plaques in the hippocampus of Alzheimer's disease (A). Pick bodies in the dentate granule cells of Pick's disease (B). Lewy bodies in the substantia nigra of Parkinson's disease (C) and temporal cortex of dementia with Lewy bodies (D). (E-G) Neuronal nuclear inclusions in the pons of dentatorubral-pallidoluysian atrophy (DRPLA) (E) and spinocerebellar ataxia type 3 (SCA3) (F), and in the frontal cortex of intranuclear inclusion body disease (INIBD) (G). Marinesco bodies in the substantia nigra (H) and Hirano bodies (arrows) in the hippocampus (I) of control subjects. Bars = 100 μm (A), 40 μm (B, I), 10 μm (C-H)

Fig. 3 Double-labeling immunofluorescence demonstrating co-localization of FIG4 and phosphorylated tau (p-Tau) in Pick bodies (A-C) and neuropil threads (D-F)

(arrows) in the dentate gyrus in Pick's disease, FIG4 and phosphorylated α-synuclein (p-α-Syn) in nigral (G-I) and cortical (J-L) Lewy bodies, FIG4 and polyglutamine (PolyQ) or ubiquitin (UBQ) in neuronal nuclear inclusions in DRPLA (M-O), SCA3 (P-R) and INIBD (S-U). FIG4 appears *green* (A, D, G, J, M, P, S) and p-Tau (B, E), p-α-Syn (H, K), PolyQ (N, Q) or UBQ (T) appears *red*. Overlap of FIG4 with p-Tau, p-α-Syn, PolyQ or UBQ appears *yellow* (merge). Bars = 10 μm.

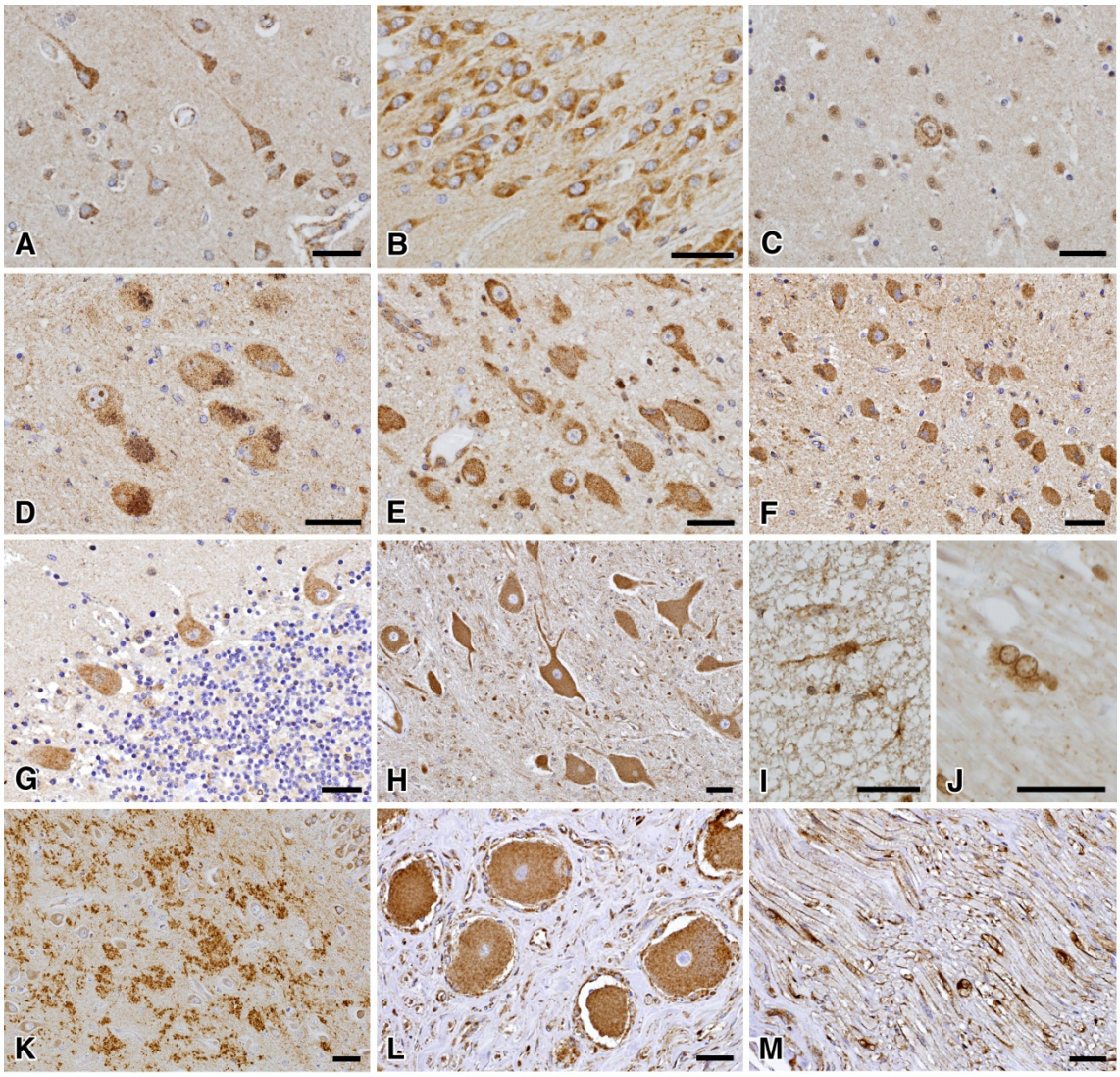

Fig.1

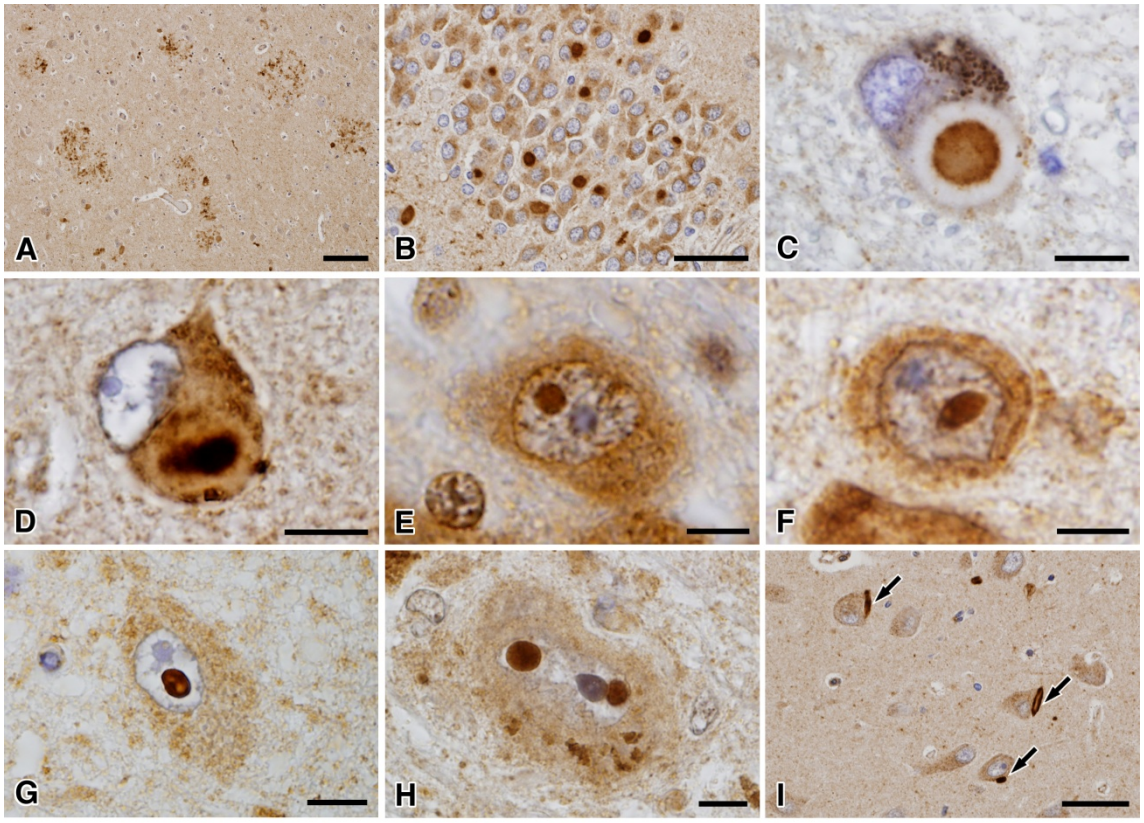

Fig. 2

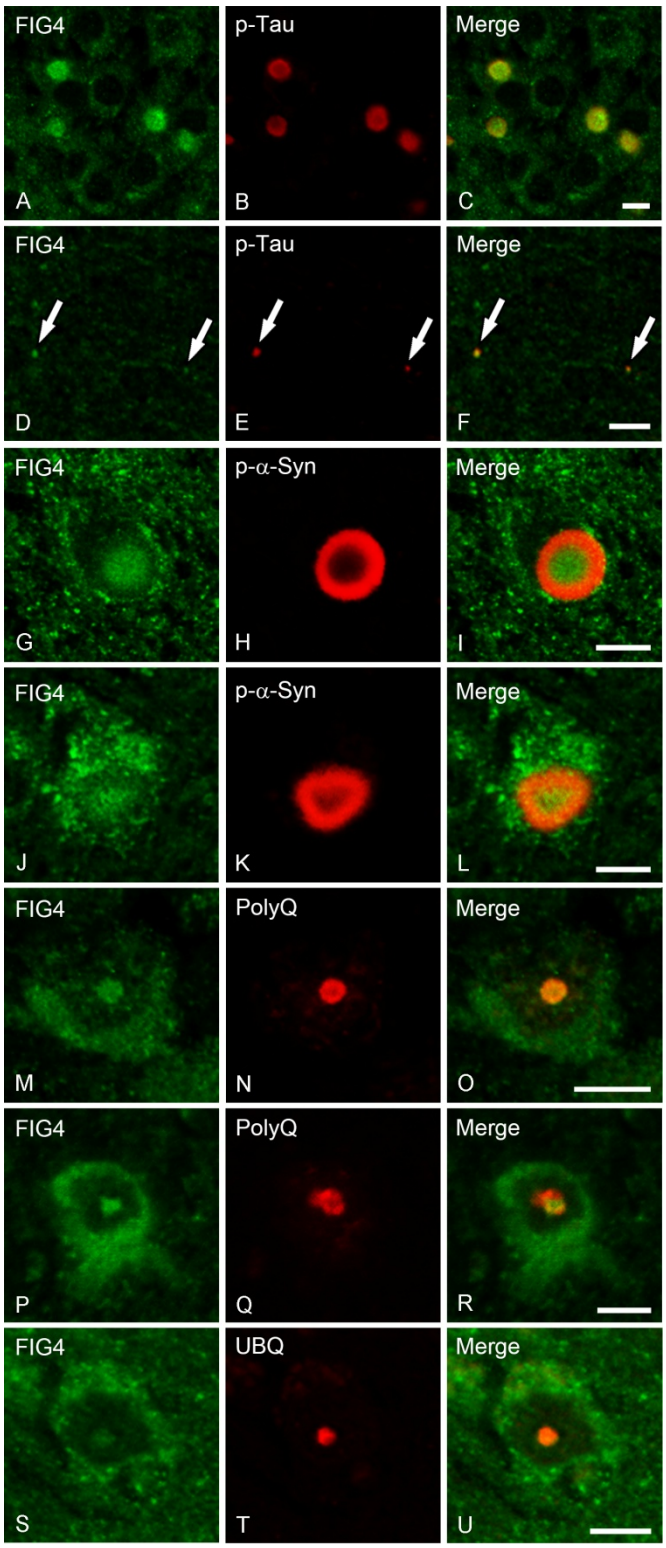
FIG4
p-Tau
Merge
A
B
C
FIG4
p-Tau
Merge
D
E
F
FIG4
p-α-Syn
Merge
G
H
I
FIG4
p-α-Syn
Merge
J
K
L
FIG4
PolyQ
Merge
M
N
O
FIG4
PolyQ
Merge
P
Q
R
FIG4
UBQ
Merge
S
T
U

Fig. 3