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Oligogenic inheritance of optineurin (*OPTN*) and *C9ORF72* mutations in ALS highlights localisation of OPTN in the TDP-43-negative inclusions of *C9ORF72*-ALS.

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Abstract

Amyotrophic lateral sclerosis (ALS) is characterised by motor neurone loss resulting in muscle weakness, spasticity and ultimately death. Whilst most cases are sporadic, 5-10% are caused by inherited mutations, most commonly *C9ORF72*, *SOD1, TARDBP* and *FUS.* Rarer genetic causes of ALS include mutation of optineurin *(OPTN)*. Furthermore, the optineurin protein has been localised to the ubiquitylated protein aggregates in several neurodegenerative diseases, including ALS.

This study 1) used sequencing to investigate the frequency of *OPTN* mutations in ALS patients from the North of England, 2) characterised the clinical and neuropathological features of ALS associated with a mutation of *OPTN*, 3) investigated optineurin neuropathology in *C9ORF72*-related ALS (*C9ORF72*-ALS).

Screening of 42 familial and 47 sporadic ALS cases identified a heterozygous p.E322K missense mutation in exon 10 of *OPTN* in one familial ALS patient who additionally had a mutation of *C9ORF72*. This patient had bulbar, limb and respiratory symptoms and signs without obvious cognitive problems. Neuropathological examination revealed motor neurone loss, TDP-43-positive neuronal and glial cytoplasmic inclusions together with the TDP-43-negative neuronal cytoplasmic inclusions in extra motor regions that are characteristic of *C9ORF72*-ALS. We have demonstrated the novel neuropathological finding that both TDP-43-positive and negative inclusion types had positive staining for optineurin by immunohistochemistry. We went on to show that optineurin was present in TDP-43-negative cytoplasmic extra motor inclusions in *C9ORF72*-ALS cases that do not carry *OPTN* mutations.

We conclude that: 1) *OPTN* mutations appear to be associated with ALS; 2) Optineurin protein is present in a subset of the extramotor inclusions of *C9ORF72*-ALS; 3) It is not uncommon for multiple ALS-causing mutations to occur in the same patient and 4) Studies

of optineurin are likely to provide useful data regarding the pathophysiology of ALS in particular and neurodegenerative disease in general.

Keywords:

Amyotrophic Lateral Sclerosis, optineurin, C9ORF72, multifactorial inheritance, inclusion bodies.

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common form of motor neurone disease (MND). It is characterised by relentless degeneration of vulnerable neuronal cell populations, principally upper and lower motor neurones of the precentral gyrus, brainstem and spinal cord. This results in muscle weakness, atrophy and spasticity, leading to widespread paralysis that culminates in respiratory failure, typically within 2 to 3 years from the point of diagnosis[1]. ALS has an incidence of approximately 2 individuals per 100,000[2] with a slight male preponderance[3]. Pathologically, classical ALS is characterised by motor neurone loss with ubiquitylated neuronal cytoplasmic inclusions of TDP-43[4].

Approximately 5-10% of ALS cases are classified as familial (fALS), where ALS is documented in a first or second degree relative, whereas the remainder are deemed sporadic (sALS), appearing to occur without a relevant family history[5]. The first ALS gene found to cause fALS was *SOD1* in 1993[6] and since the discovery that *TARDBP* mutations can cause ALS in 2006[7], the number of known of ALS-causing genes has grown rapidly to over 20 (alsod.iop.kcl.ac.uk). We now know the genetic aetiology of approximately two thirds of familial cases and 11% of sporadic cases[8], following the identification of the GGGGCC repeat expansion in the *C9ORF72* gene in 40-50% of fALS cases[9, 10].

Several mutations have been identified within the optineurin (*OPTN*) gene on chromosome 10 that are reported to cause ALS, first in Japanese, then in European and Scandinavian populations[11-16]. However, a number of studies have failed to find *OPTN* mutations in ALS patients[17-20], suggesting this to be a rare occurrence.

OPTN mutations have long been known to cause the neurodegenerative disorder primary open angle glaucoma (POAG)[21], and, more recently have been associated with Paget's disease of bone[22]. The *OPTN* gene spans 37kb on chromosome 10, has 3 non-coding exons and 13 exons that encode a 577 amino acid, 67kDa protein which functions as a dimer[23]. It shares a 53% similarity with the NFκB essential modulator (NEMO) at both the primary sequence and domain organisation levels[24]. It has two coiled-coils, a ubiquitin binding domain, a ubiquitin-binding zinc finger, a microtubule associated protein 1 light chain 3 (LC3)-interacting motif and a zinc finger[25]. It is a ubiquitously expressed cytosolic protein that primarily localises to the perinuclear region of the cell where it associates with the Golgi apparatus and is known to interact with a plethora of molecules including the type 1a metabotropic glutamate receptor and huntingtin[26, 27]. It is involved in multiple cellular functions and signalling pathways, including down regulation of the NFκB pathway, protection against oxidative stress-induced apoptosis, membrane trafficking and Golgi organisation, protein secretion and regulation of mitotic progression[23].

The aim of this study was to establish the contribution of *OPTN* mutations as a genetic cause of fALS and sALS in a Caucasian cohort from the North of England. On finding a case with simultaneous *OPTN* and *C9ORF72* mutations, we proceeded to characterise the neuropathology, and elucidated the degree of optineurin incorporation into the TDP-43-negative ubiquitylated neuronal cytoplasmic inclusions of *C9ORF72*-ALS.

Materials and Methods

Genetic screening

Our cohort consisted of 42 familial (35 classical ALS, 3 PMA, 1 PLS, 1 PBP, 1 ALS+FTD and 1 ALS+PD) and 47 sporadic (39 classical ALS, 1 PMA, 1 PBP, 5 ALS+FTD and 1 ALS+MSA) cases in the Sheffield MND Blood DNA and Brain Tissue Banks. Control samples (n=375) were recruited from neurologically normal, age and gender matched healthy volunteers. Informed written consent was obtained from all participants prior to the collection of blood or from their immediate families prior to autopsy. The study was approved by the South Sheffield Research Ethics Committee.

Patients (n=89), 48 male and 41 female (M:F ratio 1.17:1), were diagnosed between June 1990 and December 2008 with suspected, probable or definite ALS according to the 1998 revised El Escorial Rating criteria defined by the World Federation of Neurology. Age at symptom onset ranged from 27 to 88 years with a respective mean disease duration of 28 and 41 months for familial and sporadic cases respectively. Onset occurred focally in at least 90% of patients. Of these, approximately two thirds presented with asymmetric upper or lower limb dysfunction, one third presented with bulbar symptoms and <2% exhibited clinical evidence of cognitive impairment.

Genomic material isolated from whole venous blood (n=34) or snap frozen cerebral cortex (n=55) was extracted using the Nucleon® BACC3 (Tepnel Life Sciences PLC, UK) and Soft Tissue (GE Healthcare Ltd, UK) kits according to the manufacturers guidelines.

Subjects had previously been screened for mutations in *TARDBP*[28], *SOD1*[29], *FUS*[30] and were subsequently screened for *C9ORF72*[31].

The promoter region, 3 non-coding exons, 13 coding exons, intron/exon boundaries and splice acceptor/donor sites of the *OPTN* gene (Ensembl transcript ID: ENST00000378748) were screened using previously published primer sequences (Eurofins MWG Operon,

Germany)[11]. PCR reactions were performed in a total volume of 20µl as described previously[32]. Post optimization, an initial denaturation step of 95^0C for 5 min was followed by 35 cycles of 95^0C (30 sec), 52^0C (30 sec) and 72^0C (45 sec) followed by a final extension of 72^0C for 10 min for exons 5, 7, 9 and 13. The remaining exons were amplified using an annealing temperature of 54^0C. The size of the PCR products were verified by agarose gel electrophoresis prior to purification using Exonuclease (New England Biolabs, UK) and shrimp alkaline phosphatase (SAP) (USB Corporation, UK). Sequencing was performed in house at the University of Sheffield Core Genomics Facility. Samples were run on a BigDye® v3.1 Terminator machine and ABI 3730 capillary analyser (Applied Biosystems, UK). Potential mutations or coding polymorphisms were confirmed by bi-directional sequencing in a second PCR reaction.

Control subjects were screened for the non-synonymous c.293T>A (p.M98K) polymorphism in exon 5 by restriction enzyme digest using *Stu1* (New England Biolabs, UK), whilst direct sequencing determined the frequency of the potential missense mutation in exon 10 c.964G>A (p.E322K). *C9ORF72* screening and southern blotting methods have been described elsewhere[31, 33].

Neuropathology

The brain and spinal cord from all cases assessed for neuropathology were donated to the Sheffield Brain Tissue Bank with the consent of the next of kin and with ethics committee approval. The CNS tissue handling, dissection and histological preparation with OPTN immunohistochemistry performed using a rabbit polyclonal antibody (Abcam ab79110, 1:50 dilution; antigen retrieval at pH9) were performed using standard protocols for our laboratory[31].

Optineurin immunohistochemistry was also performed on the spinal cord from the proband, 1 neurologically healthy control, 3 cases of sporadic ALS, 1 case of *FUS*-ALS (p.R524W, previously reported as case 1[30]), 1 case of *SOD1*-ALS (p.I114T), 6 cases of *C9ORF72*-ALS

and the hippocampus from 12 cases of *C9ORF72*-ALS. Immunohistochemistry for ubiquitin (antigen retrieval at pH9 in A. Menarini Access Super RTU in pressure cooker; Dako Z0458 primary antibody used at 1:400 dilution) and optineurin was performed on adjacent sections of spinal cord from the proband. Immunohistochemistry for p62, TDP-43 and poly-GA dipeptide repeat was performed as described elsewhere[31, 34]. We are grateful to Prof Dieter Edbauer of the German Centre for Neurodegenerative Diseases, Munich for the kind gift of the poly-GA antibody.

Adjacent sections of spinal cord from

Results

Mutation Screening

Screening analysis of the 16 exons of *OPTN*, including intron/exon boundaries, in 42 fALS and 47 sALS index cases identified two non-synonymous substitutions in exons 5 and 10 and one novel intronic substitution. In addition, sequencing also identified seventeen previously identified single nucleotide polymorphisms (SNPs) including two upstream of the coding region, eleven intronic variants as well as two 5'UTR and two synonymous substitutions (Table 1).

A heterozygous c.964G>A (p.E322K) missense mutation (rs523747) was identified in exon 10 of one fALS individual (Figure 1a; the proband), which was absent from 375 unrelated neurologically normal control subjects (Figure 1b). The negatively charged glutamic acid, which is substituted to a positively charged lysine in the coiled coil II domain, is highly conserved across a diverse range of species, including mammals, chicken, zebrafish and treefrog (Figure 2). The p.E322K mutation is described as a missense variant by Ensembl and is predicted to be pathogenic according to Polyphen (probably damaging) and SIFT (deleterious; Ensembl predicted consequences; Table 2). Our screening of 375 controls failed to detect any carriers, and the worldwide minor allele frequency reported by the 1000

Genomes Project Phase 3 is 0.01, having sequenced 2504 individuals. The mutation is listed as having an allele frequency of 0.001 in the European population (having screened 502 Europeans in the 1000 Genomes Phase 3), due to a single individual having being identified in the UK out of 90 individuals screened. Whilst it is reported to be found more frequently in the African population (0.023%), it is absent from East and Southern Asian populations and only seen in 2 individuals of 692 individuals screened from the Americas (Colombia/Mexico/Puerto Rica origin) (1000 Genomes Project Phase 3). We therefore believe this variant to be a mutation.

Interestingly, the proband was subsequently found to carry a hexanucleotide repeat expansion in *C9ORF72*. Southern blotting on DNA extracted from venous blood, cerebellum and frontal cortex revealed somatic heterogeneity, but in all tissues repeat length was >600 units.

The heterozygous non-synonymous c.293T>A (p.M98K) change in exon 5 (rs11258194) was detected in six fALS cases and one sALS patient. The methionine to lysine substitution occurs within the binding site of Rab8 and is situated in the coiled coil I domain of optineurin. This nucleotide change was also found in 4.15% of controls screened, a frequency similar to that of 4.5% seen in the 1000 Genomes Phase 1 data for Europe. Consistent with the presence of the allele within the normal population, in silico analysis using PolyPhen and SIFT suggests the alteration is non-pathogenic.

The novel intronic polymorphism was not predicted to alter any splice sites according to Flybase analysis. Of the other SNPs identified, the frequencies of these within our ALS cohort were comparable to those reported within the 1000 Genomes Project Phase1.

Clinical Phenotype

The proband (II:5) was a Caucasian female who in her past had suffered with migraine, a ruptured ovarian cyst and had undergone a hysterectomy for menorrhagia. Of note she had a significant family history of ALS. Her father (I:4) was diagnosed with bulbar onset ALS and

dementia and died aged 74 years. Additionally a male paternal cousin (II:3) also developed MND and died aged 34 years (DNA was not available from this individual for analysis). The proband presented with a 6 month history of dysphagia and dysarthria. She subsequently progressed to develop limb weakness 7 months into the illness, resulting in falls and significant hand weakness, which impaired her ability to perform activities of daily living. She also reported troublesome emotional lability.

On examination she exhibited mixed upper and lower motor signs in the bulbar and limb regions. The tongue was wasted with prominent fasciculations. There was significant weakness of her arm muscles, in particular of abductor pollicus brevis bilaterally. Tone was increased in the legs and all of the tendon reflexes were brisk. The limb weakness and bulbar symptoms deteriorated with rapid progression. The patient did not have glaucoma.

Investigations included magnetic resonance imaging (MRI) of the brain and cervical spine which was unremarkable. Electromyography demonstrated widespread neurogenic changes fulfilling the El Escorial electrodiagnostic criteria for ALS.

During the disease course, weakness progressed to the point the patient required hoisting for transfers. A gastrostomy was performed 23 months into the illness due to severe dysphagia and malnutrition. Respiratory function was relatively maintained. However, she died following a cardiorespiratory arrest, approximately 30 months after symptom onset.

Neuropathology

Macroscopically the brain and spinal cord were unremarkable externally and on slicing. Microscopy revealed classical ALS neuropathology (Figure 3): For the motor system, the spinal cord and medulla showed depleted numbers of lower motor neurones, whilst the motor cortex showed mild (predominantly layer II) vacuolation with negligible neuronal loss. Immunohistochemistry revealed glial and neuronal (both skein-like and compact) ubiquitylated cytoplasmic inclusions in residual motor neurones that were identified on p62 and TDP-43 immunohistochemistry. Long, pyramidal tract degeneration was highlighted by

pallor of myelin staining in the lateral tracts of the spinal cord. Immunohistochemistry for FUS showed normal, predominantly nuclear staining.

Superimposed on this classical ALS neuropathology, was the extra-motor pathology that is the hallmark of *C9ORF72*-ALS. Thus, in the hippocampus, there were multiple ubiquitylated (p62-positive, TDP-43-negative; henceforth TDP-43-negative inclusions). These were also seen in the frontal neocortex and cerebellum, in common with other *C9ORF72*-ALS cases from our collection[35].

Immunohistochemistry for optineurin in neurologically healthy control tissue revealed expression in neuronal cytoplasm (including dendritic processes) and neuropil with a small amount of glial (predominantly astrocytic) expression (figure 4a). Spinal cord from sporadic ALS cases revealed a similar pattern of staining (figure 4b), with the additional labelling of neuronal cytoplasmic inclusions (both compact and skein-like) as described by previous authors[11, 36].

Optineurin immunopositivity in neuronal cytoplasmic inclusions in the spinal cord anterior horn was also present in the index case (figure 4c). Immunohistochemistry for ubiquitin or optineurin on adjacent sections revealed neurones that either had cytoplasmic inclusions positive for both optineurin and ubiquitin; or had no inclusions on either preparation (supplementary figure 1). Interestingly, there was also labelling of some of the TDP-43 negative inclusions, most readily apparent in the CA3-4 subfield of the hippocampus. These were much harder to discern in the cerebellum, but were present (figures 4e, f). Fluorescence immunohistochemistry with double staining for optineurin and poly-(Gly-Ala)-(GA) dipeptide repeat protein was performed on the cerebellum. For optineurin, the cytoplasmic inclusions had signal intensity far in excess of background. Approximately 5% of inclusions staining for poly-(Gly-Ala)-(GA) dipeptide repeat protein also had intense expression of optineurin (Figure 4g).

Having demonstrated optineurin labelling of the TDP-43-negative inclusions in the index case with an *OPTN* mutation, we hypothesised that the protein would also be present in the TDP-43-negative inclusions seen in cases of *C9ORF72*-ALS without mutations of *OPTN*. This was investigated by performing immunohistochemistry on the hippocampus of 12 such cases. Optineurin-positive neuronal cytoplasmic inclusions were seen in CA3-4 in 10 (83%) of these cases. In general, the number of Optineurin-positive inclusions was much smaller than the number of p62-positive inclusions. Co-localisation of poly-GA and optineurin was also demonstrated in these cases. There was no obvious relationship between the number of optineurin-positive inclusions and the age of onset, duration of disease, site of symptoms onset or presence/absence of cognitive symptoms in the *C9ORF72*-ALS cases.

Optineurin immunohistochemistry also labelled neuronal cytoplasmic inclusions in the cases of ALS due to mutations of *FUS* and *SOD1*. This was as has been described previously for *FUS* mutations[37]. In contrast with the existing literature[11], optineurin-positive inclusions were distinct from hyaline conglomerate inclusions, which were unlabelled.

Discussion

In this study, we have screened a cohort of cases of ALS from the North of England, and identified a p.E322K mutation in *OPTN*. The amino acid substitution is predicted to be pathogenic and has been previously reported as a cause of primary open angle glaucoma (POAG)[23]. The amino acid is highly conserved throughout the species and was not found in 750 chromosomes screened in age-matched controls from the same geographical region; only one other variant has been identified within a screen of 1004 European chromosomes, establishing it as a very rare variant, which we proposed to be associated with ALS. The individual was subsequently shown to carry a hexanucleotide expansion of *C9ORF72*. Reports of oligogenic inheritance in ALS are increasingly being published; mutations in *C9ORF72* and *SOD1*, *FUS*, *TARDBP*, *VAPB* and other rarer variants have been identified, as well as mutations in angiogenin (*ANG)* along with *SOD1*, *FUS*, *TARDBP* and *VAPB*

mutations and other double rare variants[38-41]. The presence of additional genetic variants has been demonstrated to be associated with an earlier age of onset, suggesting additive or synergistic effects, in disease pathogenesis[40].

The p.E322K mutation described here is rare in the UK and European population and high levels of conservation are observed in multiple different species. However, despite this mutation having been associated with POAG, the pathogenicity of this OPTN mutation in relation to ALS needs to confirmed experimentally, particularly given that this patient was subsequently shown to carry a hexanucleotide repeat expansion of *C9ORF72* [9, 10, 31].

The patient with mutations in both *OPTN* and *C9ORF72* had fairly classical clinical features of ALS. Neuropathology of this case revealed 1) classical features of ALS, including TDP-43-positive neuronal cytoplasmic inclusions with 2) superimposed features *C9ORF72*-ALS, namely TDP-43-negative ubiquitylated neuronal and glial cytoplasmic inclusions in extra motor regions. We then proceeded to demonstrate that some of both the TDP-43-positive and TDP-43-negative inclusions showed positive immunoreactivity for optineurin.

Following this, optineurin immunolabelling was demonstrated in the extra motor, TDP-43-negative, ubiquitylated neuronal cytoplasmic inclusions in the hippocampus of 10/12 *C9ORF72*-ALS cases that did not have a mutation of *OPTN*. Immunofluorescence experiments also demonstrated these optineurin-positive inclusions in the cerebellum and demonstrated colocalisation with transcribed non-ATG-dependent dipeptide repeat protein poly-GA.

ALS-causing GGGGCC, hexanucleotide repeat mutations of *C9ORF72* were first described in 2011[9, 10] and account for the largest burden of both sporadic (~7%) and familial (~40%) ALS[42]. Pathologically, this subtype of ALS is characterised by classical TDP-43-positive neuronal and glial cytoplasmic inclusions in the motor system. Superimposed on this pathology are ubiquitylated cytoplasmic inclusions in extra motor regions that are TDP-43-negative[31, 43]. The absence of TDP-43 raised the question as to what the protein

constituents of these inclusions were. Subsequent studies have revealed these to contain dipeptide repeat proteins that have been translated from sense and antisense RNA transcripts in a non-ATG dependent manner[44, 45]. Here we demonstrate the novel finding that many of these inclusions also contain optineurin.

Optineurin has also been detected in proteinaceous inclusions in contexts outside of ALS including other neurodegenerative diseases such as neurofibrillary tangles and dystrophic neurites of Alzheimer's disease, Lewy bodies of Parkinson's disease, glial cytoplasmic inclusions of multiple system atrophy, Pick bodies in Pick disease, nuclear inclusions of polyglutamine diseases and Marinesco bodies[46-48]. Thus, suggests that optineurin appears to be involved, at some level, in the general aetiology of many neurodegenerative proteinopathies. That mutations of *OPTN* can cause neurodegenerative disease suggests that its role may be fundamental.

Our findings demonstrate similarities between *OPTN,* ubiquilin 2 (*UBQN2*)[49] and sequestosome 1 (*SQSTM1*), encoding p62[50]: Firstly these genes, when mutated, can cause ALS. Secondly, these genes all have a role in protein degradation via both the autophagy and ubiquitin proteasome systems. Thirdly, the protein products of these genes are present in both the TDP-43-positive neuronal cytoplasmic inclusions as well as the TDP-43-negative neuronal cytoplasmic inclusions of *C9ORF72*-ALS. Finally, the protein products are also present in ubiquitylated inclusions of neurodegenerative diseases other than ALS. This stresses the role of protein catabolism in ALS specifically, and neurodegeneration more generally, when one considers the presence of such proteins in inclusions in conditions outside the ALS spectrum. In support of this, it appears that optineurin facilitates protein aggregate[51] and mitochondrial[52] autophagy, the former function being disrupted by ALS-causing *OPTN* mutations[53].

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Figure and table legends

Table 1 *OPTN* nucleotide substitutions identified in Sheffield fALS and sALS cases.

All mutations and polymorphisms listed have been reported according to the Human Genome Variation Society guidelines. Numbering is taken from the Ensembl transcript ID ENST00000378748 and NM_001008211. Genotype frequencies given are those for Europe provided by the 1000 Genomes Project Phase1.

Table 2 In silico analysis summary of mutation effects.

Figure 1 a Chromatograph readout showing the c.964G>A nucleotide substitution identified in the proband (II:5). **b** Pedigree diagram of the fALS family in which the mutation has been identified in exon 10 of the *OPTN* gene. Index case is indicated by an asterisk. WT = wild type, heterozygous for p.E322K.

Figure 2 ClustalW2 multiple alignment of the optineurin protein sequence showing the highly conserved p.E322K amino acid in human (*Homo sapiens:* ENSP00000368022), chimpanzee (*Pan troglodytes:* ENSPTRP00000040385), mouse (*Mus musculus:* ENSMUSP00000110648), cow, (*Bos taurus* ENSBTAP00000021808), chicken (*Gallus gallus:* ENSGALP00000022287), zebrafish (*Danio reno*: ENSDARP00000099052) and African clawed frog (*Xenopus laevis:* ENSXETP00000019986). Location of the p.E322K mutation identified in the index case is boxed.

Figure 3: Neurohistology from the spinal cord of the proband reveals skein-like (arrow) and compact (arrowhead) ubiquitylated neuronal cytoplasmic inclusions in lower motor neurones evident on p62 (a, bar=100μm) and TDP-43 (b, bar=100μm) immunohistochemistry. Degeneration of the pyramidal tract is revealed by myelin pallor of the lateral tracts on luxol fast blue staining in the spinal cord (c, bar=1mm) and the presence of ubiquitylated neuronal (arrow) and glial (arrowhead) cytoplasmic inclusions in the motor

cortex (d, bar=50μm). Superimposed on this are TDP-43-negative neuronal cytoplasmic inclusions (arrows) in extra motor regions, e.g. the hippocampus (e, bar=50μm) and the cerebellum (f, bar=50μm).

Figure 4: Optineurin immunohistochemistry showing neuronal cytoplasmic staining in a neurologically healthy control (a, bar=100μm), sporadic ALS patient (b, bar=100μm) and the proband with *OPTN* and *C9ORF72* mutations (c, bar=100μm). Optineurin intensely labels neuronal cytoplasmic inclusions (arrows) in sporadic ALS (b) and the proband (c). Optineurin also labels a subset of TDP-43-negative extra motor inclusions (arrows) in the hippocampus (d, bar=50μm) and cerebellum (e, bar=50μm) as well as a case carrying a *C9ORF72* but not an *OPTN* mutation (f, hippocampus, bar=50μm). Double labelling immunohistochemistry of the cerebellum in the index case shows colocalisation of optineurin and poly-GA dipeptide repeat (g).

Supplementary figure 1: Adjacent sections stained for optineurin or ubiquitin reveal neuronal cytoplasmic inclusions that are positive for both optineurin and ubiquitin in the same cells (black arrows) or cells that contain neither ubiquitylated nor optineurin-positive inclusions (white arrows; bar = 50μm).