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Differential expression of molecular motors in the motor cortex of sporadic ALS

Maria Pantelidou, a, Spyros E. Zographos, a,1 Carsten W. Lederer, a Theodore Kyriakides, b Michael W. Pfaffl, c and Niovi Santamaa,a,*

aDepartment of Biological Sciences, University of Cyprus and Cyprus Institute of Neurology and Genetics, P.O. Box 20537, 1678 Nicosia, Cyprus
bThe Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus
cPhysiology-Weihenstephan, Center of Life and Food Sciences, Technical University of Munich, Germany

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The molecular mechanisms underlying the selective neurodegeneration of motor neurons in amyotrophic lateral sclerosis (ALS) are inadequately understood. Recent breakthroughs have implicated impaired axonal transport, mediated by molecular motors, as a key element for disease onset and progression. The current work identifies the expression of 15 kinesin-like motors in healthy human motor cortex, including three novel isoforms. Our comprehensive quantitative mRNA analysis in control and sporadic ALS (SALS) motor cortex specimens detects SALS-specific down-regulation of KIF1Bβ and novel KIF3Aβ, two isoforms we show to be enriched in the brain, and also of SOD1, a key enzyme linked to familial ALS. This is accompanied by a marked reduction of KIF3Aβ protein levels. In the motor cortex KIF3Aβ localizes in cholinergic neurons, including upper motor neurons. No mutations causing splicing defects or altering protein-coding sequences were identified in the genes of the three proteins. The present study implicates two motor proteins as possible candidates in SALS pathology.

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Keywords: Real-time RT-PCR; Motor neuron disease; Kinesin-like proteins; KIF3A; KIF1Bβ

Abbreviations: ALS, amyotrophic lateral sclerosis; CMT 2A, Charcot–Marie–Tooth motor neuronopathy type 2A; FALS, familial amyotrophic lateral sclerosis; KLPs, kinesin-like proteins; ORF, open reading frame; Real-time RT-PCR, real-time reverse transcription-polymerase chain reaction; RG, reference gene; SALS, sporadic amyotrophic lateral sclerosis; SNP, single nucleotide polymorphism; SOD1, superoxide dismutase 1

* Corresponding author. Fax: +357 22 350557.
E-mail address: santama@ucy.ac.cy (N. Santama).
1 Present address: Institute of Organic and Pharmaceutical Chemistry, National Hellenic Research Foundation, Athens, Greece.

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Introduction

Amyotrophic lateral sclerosis (ALS) is an invariably fatal, adult-onset neurological disorder characterized by upper and lower motor neuron degeneration in motor cortex, brainstem, and spinal cord, leading to progressive atrophy and paralysis of skeletal muscles. Approximately 10% of ALS is familial (FALS) and about 20% of FALS cases are associated with mutations in the copper/zinc superoxide dismutase 1 (SOD1) gene (Deng et al., 1993; Rosen et al., 1993). The remaining 90% of ALS are without family history and therefore considered sporadic (SALS) (Al-Chalabi and Leigh, 1998). Although the molecular mechanisms of motor neuron death are still unclear, several models have been put forward to explain the pathogenesis of ALS. These include oxidative stress (Ferrante et al., 1997), glutamate excitotoxicity (Lin et al., 1998; Howland et al., 2002), but also see Meyer et al., 1999), excitotoxic influx of Ca2+ through AMPA receptors (Kawahara et al., 2004; Kuner et al., 2005), neurofilament aggregation (Williamson and Cleveland, 1999), mitochondrial dysfunction (Kirkinezos et al., 2005; Manfredi and Xu, 2005), apoptosis (Estevez et al., 1999; Martin, 2000), and others.

Recent breakthroughs have illustrated that the intracellular transport machinery of neurons is a major cellular target for the initiation or progression of neurodegeneration in motor neuron disease (reviewed by Goldstein, 2003; Levy and Holzbaur, 2006). Key players of the machinery that mediates cytoskeleton-based, bidirectional transport are a large number of “molecular motors”, otherwise known as motor proteins, together with adaptors, effectors and regulators of transport complexes as well as diverse interacting proteins and cellular cargoes. Motor proteins have been implicated in the pathogenesis of motor neuron disorders by a number of studies. Early evidence indicated that mutations in the fast axonal transport motor protein kinesin in Drosophila cause organelle jams that disrupt fast axonal transport bidirectionally, leading to defective action potentials, dystrophic terminals, reduced transmitter secretion, and progressive distal paralysis.
(Hurd and Saxton, 1996), all typical manifestations of motor neuron disorders. More recent work revealed that a loss-of-function mutation in the motor protein KIF1Bβ, which transports synaptic vesicle precursors along the axon, is a cause of the most common inherited human peripheral neuropathy, Charcot–Marie– Tooth (CMT) disease type 2A (Zhao et al., 2001). Furthermore, hereditary spastic paraplegia (SPG10) was found to be associated with a missense mutation in the motor domain of the neuronal kinesin heavy chain KIF5A gene (Reid et al., 2002). Intriguingly, targeted disruption of the KIF5A heavy chain in transgenic mice causes abnormal neurofilament transport (Xia et al., 2003), a hallmark cytopathic manifestation of motor neuron disease. Along the same line, functional inhibition or mutations of the motor dynein/dynactin complex, involved in retrograde transport, cause a late-onset progressive motor neuron degeneration in mouse models that phenocopies human motor neuron disease (LaMonte et al., 2002; Puls et al., 2003; Hafezparast et al., 2003). Surprisingly, a mutation in dynein in the SOD1 G93A mutant background of a FALS mouse model rescues axonal transport defects and prolongs survival (Kieran et al., 2005). Therefore, independent and diverse studies have established a tantalizing link between defective motor proteins that compromise axonal transport and the pathogenetic mechanisms of different types of motor neuron disorders.

Mindful of the possible involvement of motor proteins in ALS pathology, we therefore set out to assess their expression in motor neurons of SALS patients. To achieve this goal we performed an initial step the first, to our knowledge, systematic identification of kinesin-like motor proteins expressed in cultured rat motor neurons and normal human motor cortex. We then conducted a comprehensive analysis of the mRNA expression of the human motors in post-mortem motor cortex specimens of SALS patients, using isoform-specific quantitative real-time RT-PCR. For a novel isoform of KIF3A, KIF3Aβ, showing the highest significant mRNA down-regulation (<50% of normal values), this analysis was complemented by quantitative protein expression analysis and immunolocalization in the motor cortex. We also quantified mRNA expression for differentially expressed candidate genes in many human tissues and assessed by genomic sequencing the possibility that mutations in these genes might be involved in SALS pathology.

Materials and methods

Human samples

Precentral gyrus sections from post-mortem human brains were used for genomic DNA and RNA extraction. All brain samples used in this study were procured following ethical procedures pertaining to the collection, transport and use of human tissue and protection of personal data and with the informed written consent of donors.

Transgenic mice

Mouse strains B6SJL-Tg(SOD1-G93A)1Gur (an ALS mouse model carrying the disease-causing G93A mutation in an SOD1 human transgene) and B6SJL-TgN(SOD1)2Gur (a reference strain carrying a wild-type SOD1 human transgene), originally obtained from the Jackson Laboratory, were verified by PCR genotyping of tail material and used for real-time RT-PCR. The animals used were 60 days old without ALS-like symptoms, 90 days old displaying the onset of neurodegenerative symptoms, and end-stage animals of 126 days of age.

RNA extraction and cDNA preparation

Total RNA was isolated from human brain tissue (~100 mg), using the TRIzol reagent according to the manufacturer’s protocol (Invitrogen) with the following modifications: after the phase separation step of the protocol, one volume of 70% ethanol was added and each sample transferred to a mini column (RNeasy mini kit, Qiagen). RNA extraction and DNase I treatment were conducted according to the RNeasy mini kit instructions, RNA from other human tissues (Fig. 2) was purchased from Ambion Inc. First strand cDNA was synthesized from 1 μg of RNA using the ProtoScript Synthesis kit (New England Biolabs), according to the manufacturer’s protocol. Rat spinal motor neuron culture cDNA was kindly donated by Dr. Giampietro Schiavo (Molecular Neuropathology Laboratory, Cancer Research, UK).

Oligonucleotides

All oligonucleotides used in this work were synthesized by MWG Biotech (Germany) (Tables S1 and S2). They were designed in a manner that would allow the specific amplification of individual isoforms within each motor family, and their specificity was confirmed prior to use.

Real-time RT-PCR

Real-time RT-PCR was performed on the LightCycler (Roche Molecular Biochemicals) using the primers listed in Table S1. Oligonucleotides were designed to amplify short diagnostic cDNA fragments, appropriate for real-time RT-PCR and specific for each of the 13 cDNAs tested. Resulting amplicons were assessed by agarose electrophoresis, which revealed in each case a single product of the predicted size (data not shown).

Each 20-μl reaction included 2 μl LightCycler FastStart DNA Master SYBR Green I, 4 mM of MgCl₂, 0.5 μM each of forward and reverse primer, and 2 μl of 1:2.5 (v/v) diluted cDNA (made from 1 μg total RNA). In each experiment an internal standard curve was used, which consisted of five serial dilutions of a cDNA mix in duplicate. The experiment was performed according to the following protocol: (i) denaturation at 95 °C for 10 min, (ii) amplification (30–40 cycles) at 95 °C for 15 s, 55–65 °C for 10 s, depending on primer sequence (Table S1), and 72 °C for 10–28 s with a single fluorescence measurement (521 nm), depending on amplicon size (Table S1), (iii) melting at 60–75 °C for 15 s with continuous fluorescence measurement up to 95 °C, and (iv)
coiling at 40 °C for 30 s. All experiments included two no-template controls and all samples were analyzed independently between two and four times. Crossing Point (CP) determination and quantification were achieved using the Second Derivative Maximum Method of the LightCycler Software 3.5 (Roche Molecular Biochemicals). For data normalization, the expression of the following reference genes (RGs) was also quantified: beta-2-microglobulin (B2M), ribosomal protein L19 (RPL19), and pumilio homolog 1 transcript variant 2 (PUM1). Melting curve analysis was performed at the end of each experiment to determine the specificity of the amplification.

**Statistical analysis**

The data for real-time RT-PCR were analyzed by REST-384© (November 2005), a Microsoft-Excel-based software which uses permutation analysis, specifically a Pair Wise Fixed Reallocation Randomization Test©, to compare the relative quantification between two groups and to determine the significance of the results (Pfaffl et al., 2002). The geometric mean of the CPs from all three RGs employed in this study was used for gene expression data normalization, and 10,000 permutations were used for statistical evaluation.

The multi-tissue quantitative analysis was based on the calculated concentrations provided by the LightCycler Software 3.5 using the Second Derivative Maximum Method. The calculated concentration of RPL19 reference gene in each type of tissue was used for gene expression data normalization and the standard deviations were calculated from three independent experiments for each gene.

**Antibodies and immunofluorescence with frozen sections**

A mouse monoclonal antibody, raised to the C-terminal amino acids (aa) 563–671 of human KIF3A, (611508, BD Biosciences) was used at 1:250 dilution in Western blotting and at 1:50 for immunofluorescence for the detection of KIF3A isoforms. An antibody to choline acetyl transferase (ChAT) (AB114P, BD Biosciences) was used at 1:25 for immunofluorescence. Alexa Fluor 488-labeled donkey anti-mouse IgG (A11057, Molecular Probes) and Alexa Fluor 568-labeled donkey anti-goat IgG (A21202, Molecular Probes) secondary antibodies were used at 1:2000 and 1:1500, respectively. For the detection of α-tubulin in Western blotting, a mouse monoclonal antibody (T5168, Sigma) was used at 1:8000 dilution and HRP-conjugated secondary antibody to mouse IgG (NA931, Amersham Pharmacia Biotech) was used at 1:10,000 dilution.

For immunofluorescence, frozen motor cortex slices were fixed in acetone at −20 °C and blocked with 5% BSA/0.5% Triton X-100 in PBS for 1 h. Incubation with both primary antibodies in 0.05% Tween/PBS was performed overnight at 4 °C followed by incubation with secondary antibodies at room temperature for 1 h. All washes were done with PBS. To quench lipofuscin autofluorescence, slices were incubated in Autofluorescence Eliminator Reagent (Chemicon) for 20 min and washed with 70% ethanol. Nuclei were stained with Hoechst33342 and mounted for microscopy in 10% Mowiol, 1%, 1,4-Diazabicyclo[2.2.2]octane, and 25% glycerol in 0.1 M Tris buffer (pH 8). Images were acquired using an Axiovert 200M microscope with the Axiovision 4.5 image acquisition software (Carl Zeiss Inc.).

**SDS-PAGE, Western blotting and quantification**

Following SDS-PAGE as per standard methods, Western blotting was carried out with the semi-dry method (transfer buffer was 10 mM NaHCO₃, 3 mM NaCO₃, 20% methanol, pH 9.9). Visualization of immunoreactive bands was performed by the enhanced chemiluminescence system (Amersham Pharmacia Biotech). Quantification of signals was carried out with the Scion Image software 4.0.3.2 (Scion Corporation). Statistical analysis of protein expression levels was performed using the individual averages for each triplicate sample and comparing patient and control samples by a heteroscedastic two-tailed t-test, i.e. a two-sample t-test not assuming equal variance.

**Human genomic DNA extraction, amplification and sequencing**

Extraction of human genomic DNA from peripheral blood (nineteen donors; H11–H16 and D12–D24) was carried out according to a modified protocol based on Miller et al. (1988) and from frozen motor cortex specimens (eleven donors; D1-D11) using the TRizol reagent according to the manufacturer’s instructions (Invitrogen).

In human, the KIF1B gene is located on chromosome 1 (genomic contig NT086572) and consists of 47 exons, of which exons 1–11 encode the motor domain of the protein. The gene for KIF3A is located on chromosome 5 (genomic contig NT086668) and consists of 17 exons, of which exons 2–8 encode the motor domain. The gene for SOD1 is on chromosome 21 (genomic contig NT011512) and consists of 5 exons that encode its entire ORF. To screen for mutations, PCR fragments corresponding to exons 1–11 of the KIF1B gene, exons 2–8 of the KIF3 gene and exons 1–5 of SOD1 were amplified. The PCR primers were designed to bracket the exons at sufficient distance from their respective 5‘ and 3‘ ends to include intronic sequences containing the characteristic intron/exon and branch site consensus sequences (Table S2). Amplification of selected sequences from all 30 samples was carried out by PCR, using 500 ng genomic DNA, 0.8 μM of each oligonucleotide and 1 U of Taq polymerase for 33 cycles. Purification and sequencing of PCR products were carried out by MWG Biotech (Germany).

**Other molecular biology techniques**

All other molecular biology techniques used for KLP identification in rat motor neurons and human motor cortex (standard PCR, restriction digests, ligations, blue/white bacterial colony screening, plasmid purification, Southern blotting, etc.) were performed according to standard procedures. T/A cloning vector PCR2.1 was purchased from Invitrogen and used according to the manufacturer’s instructions.

**Results**

**Identification of kinesin-like motor proteins expressed in rat spinal motor neurons and human motor cortex**

Despite the wealth of information about the identification and expression of kinesin-like motors (KLPs) in many human tissues, little is known about their specific expression in motor neurons in human or other mammals, a shortcoming addressed in the present study. The obvious starting point of our analysis was the identification of distinct KLP motors in motor neurons. In the absence of
a human equivalent for routine analyses, we used rat spinal motor neuron cultures as the source of initial identification. The results of the analysis in rat were then verified, expanded, and refined with the use of human motor cortex tissue, enriched in motor neurons.

For the purpose of KLP identification in rat motor neurons, a pair of degenerate oligonucleotide primers was designed (primer pair #1, Table S1) (Santama, 2001) specific for the extremely well-conserved “signature motifs” shared by the motor domains of all known KLPs (Hirokawa et al., 1998). A PCR using this degenerate primer pair with cDNA synthesized from a homogeneous primary culture of rat spinal motor neurons as template resulted in a mixed product of 500 bp that was subcloned in the T/A vector pCR2.1. A total of 372 recombinant clones, carrying partial motor protein cDNAs, were analyzed by a combination of restriction digest mapping, Southern blotting and nucleotide sequencing. This revealed the presence of seven distinct types of KLP cDNAs: KIF1A, KIF1Bβ3, KIF2, KIF2C, KIF3A, KIFC3, and KIFC5A (Table 1). Additionally, we specifically tested for the expression of KLPs that were not picked up in our screen but were known to be important in the nervous system, namely KIF5A (neuronal kinesin heavy chain) (Niclas et al., 1994), KIF5B (conventional kinesin heavy chain) (Navone et al., 1992), the motor neuron-enriched KIF5C (Kanai et al., 2000) and the KIFC5B isoform (Novolanic and Sperry, 2000). Of those, KIF5A and KIFC5 were found to be abundantly expressed, while KIF5B and KIFC5B were barely detectable. In conclusion, the combination of these approaches led to the identification of nine KLPs in rat spinal motor neurons (Table 1).

Having identified KLPs in rat spinal motor neurons, oligonucleotide primers for their human orthologues, their variants and for other human KLPs possibly relevant to ALS pathology were designed and used to identify KLPs in a human motor cortex specimen (healthy donor H8) by RT-PCR. Systematic PCR analysis of partial cDNAs, and eventual full-length cloning of open reading frames (ORFs) combined with cDNA sequencing, confirmed the expression of fifteen kinesin-like motors in healthy human motor cortex (Table 1). These KLPs included members of the kinesin-1 family (KIF5A and KIF5C/KIAA531), the kinesin-2 family (KIF3A, KIF3Aβ, KIF3B and KIF3C), the kinesin-3 family (KIF1A, KIF1Bβ, KIF1Bβ), KIAA5911/KIF1Bβ and KIF1C), the kinesin-14 family (C-terminal kinesin KIFC3), and also KLPs KIF13A2, KIF21A variant, and KIF21B variant (Table 1). Among these novel KLP isoforms, namely KIF3Aβ, KIF21A variant, and KIF21B variant (deposited to the EMBL database with accession numbers AM177178–80, respectively).

Analysis by real-time RT-PCR in healthy and SALS motor cortex shows significant down-regulation of mRNAs for KLPs KIF1Bβ, KIF3Aβ, and for SOD1

Of 15 KLP motors detected in the human motor cortex (Table 1), 13 cDNAs could be reliably quantified in post-mortem specimens of fresh-frozen motor cortex tissue of eleven clinically diagnosed SALS patients and nine age- and sex-matched control subjects (Table 2A) by real-time RT-PCR. Our analysis also included SOD1, whose mutations are a causative event in 20% of FALS and in a small proportion of SALS. Two of the KLPs expressed in motor neurons were not included in the real-time RT-PCR analysis, either because of insufficient specificity of multiple primer sets tested (KIF21Bβ) or because the abundance of the amplicon was consistently low, indicating low-level expression of the particular cDNA in the motor cortex (KIF21B variant). To allow the normalization of candidate gene expression (Bustin, 2002; Dheda et al., 2005) we also performed the quantification of multiple reference genes, whose expression does not vary across the experimental conditions, specifically B2M, RPL19, and PUM1 (Vandesompele et al., 2002; Pfaffl et al., 2004).

The results of relative quantification and statistical analysis are listed in Table 3 and expressed as the fold-change of normalized expression levels between diseased and healthy samples. Of the 13 KLP cDNA tested, two motors were statistically significantly down-regulated (p<0.05) in human SALS motor cortex tissue (Fig. 1). Specifically, KIAA5911/KIF1Bβ was down-regulated by a factor of 0.686 (or 68.60±1.56% of normalized average control value) and the novel isoform KIF3Aβ by a factor of 0.425 (42.49±2.35%) (Fig. 1). The expression levels of the remaining KLPs showed no statistically significant difference, although all members of the kinesin-2 (KIF3) family showed marked down-regulation. Intriguingly, the expression of SOD1 in SALS patients was also significantly down-regulated by a factor of 0.713 (71.3±2.80%) (p<0.05) (Table 3, Fig. 1).

KIF3Aβ and KIAA5911/KIF1Bβ are isoforms particularly enriched in the human central nervous system including the motor cortex

We next wanted to test the gene expression pattern of the two motors that appeared to be significantly down-regulated in human...
Expression of 13 KLPs and of SOD1 was assessed in the human motor cortex of SALS individuals. The results from two or three independent experiments listed in this table are expressed as normalized fold change of diseased versus control (geometric average of all measurements per specimen±standard deviation). Highlighted cDNA species were found to be significantly down-regulated \((p<0.05)\) in SALS patients. A more detailed presentation of data can be found in Table S3.

**Table 3. Quantitative results of real-time RT-PCR for human subjects**

<table>
<thead>
<tr>
<th>KLP Name</th>
<th>EMBL Acc. No.</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIF1A</td>
<td>NM004321</td>
<td>0.935</td>
</tr>
<tr>
<td>KIF1Bβ (KIAA1448)</td>
<td>NM138416</td>
<td>0.910</td>
</tr>
<tr>
<td>KIAA0591/KIF1Bβ</td>
<td>AB017133</td>
<td>0.686 ± 0.016 ((p &lt; 0.05))</td>
</tr>
<tr>
<td>KIF1C</td>
<td>NM006612</td>
<td>0.911</td>
</tr>
<tr>
<td>KIF3A</td>
<td>NM007054</td>
<td>0.553</td>
</tr>
<tr>
<td>KIF3Aβ</td>
<td>AM177178</td>
<td>0.425 ± 0.023 ((p &lt; 0.05))</td>
</tr>
<tr>
<td>KIF3B</td>
<td>NM004798</td>
<td>0.687</td>
</tr>
<tr>
<td>KIF3C</td>
<td>NM002254</td>
<td>0.517</td>
</tr>
<tr>
<td>KIF3D</td>
<td>NM005550</td>
<td>0.814</td>
</tr>
<tr>
<td>KIF5A</td>
<td>NM004964</td>
<td>0.937</td>
</tr>
<tr>
<td>KIF5C</td>
<td>AB011103</td>
<td>0.767</td>
</tr>
<tr>
<td>KIF13A2</td>
<td>AJ291579</td>
<td>0.791</td>
</tr>
<tr>
<td>KIF21A variant</td>
<td>AM177179</td>
<td>0.641</td>
</tr>
<tr>
<td>SOD1</td>
<td>NM000454</td>
<td>0.713 ± 0.028 ((p &lt; 0.05))</td>
</tr>
</tbody>
</table>

Expression of 13 KLPs and of SOD1 was assessed in the human motor cortex of SALS individuals. The results from two or three independent experiments listed in this table are expressed as normalized fold change of diseased versus control (geometric average of all measurements per specimen±standard deviation). Highlighted cDNA species were found to be significantly down-regulated \((p<0.05)\) in SALS patients. A more detailed presentation of data can be found in Table S3.

**Fig. 1.** mRNA levels of motor proteins KIF1Bβ and KIF3Aβ and SOD1 are statistically significantly down-regulated in SALS motor cortex \((p<0.05)\). The quantitative results of real-time RT-PCR for SALS specimens are expressed as a percentage of the normalized average control values per cDNA. Asterisk denotes statistical significance. Error bars correspond to standard deviation of three independent experiments.
Having established that both mRNA and protein levels of KIF3Aβ are statistically significantly down-regulated in SALS patients, we next examined its cellular expression in the motor cortex. We therefore conducted double immunofluorescence experiments on frozen sections representing layers I–VI of the motor cortex (Fig. 5A, panel A) using antibodies to ChAT (a marker of presynaptic cholinergic neurons) and the anti-KIF3A/KIF3Aβ antibody. Cell nuclei were marked with the fluorescent

Cellular localization of KIF3Aβ in the human motor cortex

Having established that both mRNA and protein levels of KIF3Aβ are statistically significantly down-regulated in SALS patients, we next examined its cellular expression in the motor cortex. We therefore conducted double immunofluorescence experiments on frozen sections representing layers I–VI of the motor cortex (Fig. 5A, panel A) using antibodies to ChAT (a marker of presynaptic cholinergic neurons) and the anti-KIF3A/KIF3Aβ antibody. Cell nuclei were marked with the fluorescent

Fig. 2. KIAA0591/KIF1Bβ and KIF3Aβ mRNAs are highly enriched in the human central nervous system, including the motor cortex, among the tissues tested, whereas KIF3A is ubiquitously expressed across most of the fifteen tissues or brain areas tested. Quantitative analysis by real-time RT-PCR of the levels of KIF3A, KIF3Aβ, and KIAA0591/KIF1Bβ cDNAs was carried out using identical amounts of RNA isolated from the human tissues shown. For the motor cortex a sample from a healthy individual (H3) was used for the analysis. Error bars correspond to standard deviation of three independent experiments. AU: arbitrary units.

Fig. 3. Predicted probability of coiled-coil formation and protein domains in KIF3A and KIF3Aβ. Prediction of coils (KIF3A in blue and KIF3Aβ in black) was based on the algorithm of Lupas et al. (1991) and domain analysis was performed using the Predict Protein Server (Rost and Liu, 2003). Sketches of the protein overall organization with the 27-aa sequence uniquely present in KIF3Aβ are shown at the bottom of the panel.
dye Hoechst. We discovered that in both healthy and diseased samples and throughout the layers of the motor cortex KIF3A/KIF3Aβ immunoreactivity co-localized in a mixed population of cholinergic cells including pyramidal neurons and non-pyramidal cells (Fig. 5A, panels B1 and B2 and C1 and C2). In layer V in particular the Betz cells, giant upper motor neurons, and in layers III and V smaller pyramidal or non-pyramidal neurons were immunoreactive for KIF3A/KIF3Aβ (Fig. 5B). The co-localization of KIF3A/KIF3Aβ with the cholinergic marker ChAT was over 95% throughout the motor cortex layers.

These results revealed that KIF3Aβ is abundantly expressed in the motor cortex, including layer V upper motor neurons.

Expression levels of KIF1Bβ and KIF3Aβ mRNAs are not altered throughout disease progression in the motor cortex of SOD1 G93A mice, a model of FALS

Following the identification of KIAA0591/KIF1Bβ and KIF3Aβ as two motor proteins specifically down-regulated in sporadic cases of ALS in human, we were interested in testing whether these changes in expression levels would also be manifest in a widely used mouse model of SOD1-induced ALS, B6SJL-TgN(SOD1-G93A)1Gur. We first confirmed the existence of the KIF3Aβ isoform in mouse by cloning from healthy adult mouse motor cortex and sequencing a partial mouse cDNA spanning the sequences unique to the human KIF3Aβ ortholog (Table S1, AM231689). We then carried out real-time RT-PCR with oligonucleotides specific for mouse KIF3Aβ, analyzing the mutant mice against age-matched B6SJL-TgN(SOD1)2Gur controls. Three groups of animals were used: animals 60 days old, a stage when no disease symptoms can be detected, 90-day-old mice, at an age typically within the onset period of the first ALS-like symptoms, and in 126-day-old mice, corresponding to end-stage, close to death. In all three age groups and for both cDNAs the fold changes detected were practically unchanged in reference to the healthy, control transgenic strain and any small changes were not statistically significant ($p>0.05$) (Fig. 6), indicating that, throughout the progression of the disease, the molecular physiology of mutant SOD1-induced ALS is different from that of the human sporadic cases under study.

Genomic sequencing of the human KIF1B and KIF3A motor domain, and of SOD1 in SALS and healthy individuals

We next wanted to test whether the reduced levels of expression for the motors KIAA0591/KIF1Bβ and KIF3Aβ, in the particular individuals analyzed in our study, were a result of or were accompanied by mutations in the corresponding genes. This was pertinent given that the CMT 2A motor neuropathy has been associated with a specific mutation of the ATP-binding site in the KIF1Bβ motor domain (Zhao et al., 2001).

Genomic DNA from 24 sporadic ALS cases and six age-matched non-ALS individuals was subjected to nucleotide sequencing (Table 2B). The 24 ALS cases comprised the eleven patients also analyzed by real-time RT-PCR and an additional 13 SALS individuals from Cyprus (Table 2B).

Sequencing of the KIF1B gene motor domain revealed four known single nucleotide polymorphisms (SNPs) and a novel genotype in the 30 individuals examined (Table 4). The known SNPs were present in disease and control samples and corresponded to rs12402052 (in ten disease and one control samples), rs484609 (in twelve disease and two control samples), rs12141246 (in twelve disease and one control samples), and rs1339458 (in nine disease and two control samples). The first SNP is a synonymous substitution in exon 2 (A95), whereas the other three are intronic, not affecting the consensus splice or branch sites. The novel genotype was detected in three patients (two heterozygotes and one homozygote) and corresponded to a T/G intronic substitution, 110 bp upstream of the 3′ splice site of exon 8 (ss52050753, ss52085996; Table 4).

In the KIF3A gene, seven patients and one healthy individual carried the known intronic rs9784600 SNP (Table 4). In the SOD1 gene, we detected known intronic SNPs rs16988404 and rs2234694 in a number of patients, and a novel
Fig. 6. Quantitative results of real-time RT-PCR for FALS SOD1 G93A mice. Expression of mouse KIF1Bβ and KIF3Aβ was assessed in the motor cortex of the SOD1-G93A transgenic strain B6SJL-TgN(SOD1-G93A)1Gur FALS model and reference B6SJL-TgN(SOD1)2Gur strain (60, 90 and 126 days old). The quantitative results of real-time RT-PCR for FALS mice are expressed as a percentage of the normalized average control values per cDNA and indicate the absence of differential expression for both isoforms tested (p > 0.05). Error bars correspond to standard deviation of three independent experiments.

Discussion

This work sought to contribute to the understanding of the link between disruption in molecular motors, mediating axonal transport in motor neurons, and the pathophysiology of ALS. It was prompted by the finding that neurodegenerative diseases, such as CMT 2A, hereditary spastic paraplegia, and forms of motor neuron disease, are in some cases associated with mutations in specific motor protein genes (see Introduction). Furthermore, there are indications that axonal transport impairment may be involved in an even wider spectrum of human neurodegenerative diseases, including Huntington’s disease (Gunawardena et al., 2003) and early axonopathy in Alzheimer’s disease (Stokin et al., 2005).

We chose to conduct this analysis in the motor cortex, where upper motor neurons are located, rather than the spinal cord with lower motor neurons. Despite the general depletion of upper motor neurons in ALS (Kaufmann et al., 2004), most investigations of ALS focus on lower motor neurons, possibly owing to earlier clinical manifestations of defects in lower motor neurons and their easier accessibility. It is in line with this general trend that ALS-related defects of axonal transport are under-investigated in upper motor neurons, despite the reduction of corticospinal tract volume in ALS patients, indicating axonal degeneration of upper motor neurons (Wang et al., 2006). Naturally, signaling of upper motor neurons via their long axons in the corticospinal tract is required for lower motor neuron function (Purves et al., 2001) and subject to the same maintenance constraints acting on lower motor neurons. The differential effect of ALS pathology on upper and lower motor neurons is of high clinical relevance as it creates problems in the definition and diagnosis of ALS types (Ince et al., 2003), and in this respect a further characterization of ALS motor cortex is of general interest. Importantly, patients with SALS show alterations in the motor cortex, such as increased excitability and reduced inhibitory activity, which are not readily detectable in (SOD1-linked) FALS patients, thus stressing the general relevance of investigating upper motor neurons in SALS (Turner et al., 2005).

Investigating global motor protein expression in the human motor cortex of SALS patients, we first determined which kinesin-like/motor proteins (KLPs) are expressed in healthy motor cortex. The 15 KLP motors detected include KIF5A and KIF5C/KIAA531 (kinesin-1 family), KIF3A, KIF3Aβ, KIF3B and KIF3C (kinesin-2), KIF1A, KIF1Bβ, KIF1Bββ, KIAA591/KIF1Bββ and KIF1C (kinesin-3), C-terminal kinesin KIFC3 (kinesin-14), and also KLPs KIF13A2, KIF21A variant, and KIF21B variant (Table 1). Three of these motors, KIF3Aβ, KIF21A variant and KIF21B variant, are novel isoforms of the known KIF3A, KIF21A, and KIF21B proteins, respectively. We then compared, by quantitative analysis, combining real-time RT-PCR and statistical evaluation using three reference genes, the mRNA levels of all specifically detectable KLPs and of SOD1 in nine healthy individuals and eleven SALS patients. This analysis did not show a generalized disruption of the motor protein expression pattern, but instead revealed a specific down-regulation of motors KIAA591/KIF1Bββ (69% of healthy levels), KIF3Aβ (43% of healthy levels), and, interestingly, of SOD1 (71% of healthy levels). Although none of the fold-changes observed was dramatic as an average across samples, all were significant (p < 0.05) and reached as low as 19% of the control average in some patients. Importantly, quantitative analysis of protein expression for KIF3Aβ corroborated these findings by showing reproducible and statistically significant reduction of corresponding protein levels. We are confident that the down-regulation observed was not a generic effect, due to the loss of...
motor neurons in SALS motor cortex, because we showed that the expression of motor neuron markers such as KIF5C, an isoform particularly enriched or even unique in motor neurons (Kanai et al., 2000), remained unaffected (Table 3).

The two down-regulated motors, KIAA591/KIF1Bβ and KIF3Aβ, are very highly expressed in the central nervous system, including all four cortex areas of the cerebral cortex (motor, visual, temporal, and somatosensory) and the cerebellum, while their expression in other adult tissues in general is very low in comparison. In the motor cortex (area 4) the cellular localization of KIF3Aβ is completely overlapping with pyramidal and non-pyramidal cholinergic neurons and is marked in layers III and V, rich in pyramidal small and giant upper motor neurons. The presence of KIF3Aβ in cholinergic neurons, where it co-localizes with ChAT, in human is consistent with the localization of its homolog in cholinergic neurons in the central and peripheral nervous system of Drosophila, where it functions as a transporter of ChAT (Ray et al., 1999). We have not investigated whether co-localization in cholinergic presynaptic cells is a consistent feature throughout the CNS, but given the absence of cholinergic cell bodies in the cerebellum where KIF3Aβ is present, it is very likely that KIF3Aβ is not restricted to cholinergic systems.

The changes that were observed in SALS patients were not found in the FALS SOD1 G93A mouse model, neither presymptomatically nor at the onset of symptoms or at end-stage, the stage which would be equivalent to the post-mortem SALS cases examined in our study. This finding provides additional, indirect evidence that the observed quantitative changes in human SALS, specifically affecting molecular motors that are particularly abundant in motor neurons of the motor cortex (i.e. KIF3Aβ), are not a mere consequence of the destruction of motor neurons and their relative elimination from the motor cortex tissue since these conditions would apply also to day-126 transgenic SOD1 G93A mice. In conclusion, therefore, the down-regulation of motors KIAA591/KIF1Bβ and KIF3Aβ, that we observe in SALS samples, suggests that the qualitative characteristics of the axonal transport impairment in SALS and of the particular FALS mouse model differ. The lack of quantitative changes in KIF1Bβ expression in SOD1 G93A mice is consistent with a previous report analyzing KIF1Bβ levels in the spinal cord of early-disease-stage mice (Conforti et al., 2003). Similarly, work on the SOD1 G93R FALS strain reported KAP3 (a kinesin II-binding partner) up-regulation as an early event arising before axonal neurodegeneration, without change, however, of the other components of the kinesin II complex (Dupuis et al., 2000). This latter work also reported the up-regulation of the ubiquitously expressed KIF1A in mouse spinal cord, a finding that we could not confirm in the motor cortex of SALS patients.

Our finding of SOD1 down-regulation in SALS is intriguing as, at least in the specimens under study, it is not caused by genomic mutations in the protein ORF or splicing junctions as genomic sequencing has confirmed. While it is known that the reduction of SOD1 activity or half-life does not correlate well with ALS pathogenesis or disease phenotypes (Al-Chalabi and Leigh, 1998), it cannot be excluded as a contributing or aggravating factor in neuronal degeneration.

Motor proteins KIF1Bβ and the novel KIF3Aβ, that we show to be highly expressed in the motor cortex, are particularly interesting targets as candidates involved in other neurodegenerative diseases.

In human, KIF1B is expressed in two types of isoforms, KIF1Bα, originally reported as a mitochondria transporter (Nangaku et al., 1994) but also shown to interact with postsynaptic density and synaptic scaffold proteins (Mok et al., 2002), and at least three currently known KIF1Bβ-like forms, one of which, KIF1Bβ (NM015074), is a transporter of synaptic vesicle precursors and implicated in CMT 2A motor neuron disease (Zhao et al., 2001). In the motor cortex we have determined the expression of KIF1Bα, KIF1Bβ (NM015074), and KIAA0591/KIF1Bβ (AB017133) (Table 1). We have shown that KIAA0591/KIF1Bβ was specifically down-regulated in SALS patients. Although it is not clear whether the individual KIF1Bβ-like isoforms all share identical cellular cargoes, it is likely that they all serve as transporters of synaptic materials because of the high sequence similarity between them. KIAA0591/KIF1Bβ (AB017133) is an alternatively spliced form of KIF1Bβ (NM015074), with an additional 40-aa sequence within its ORF and a different extreme carboxy-terminus. The down-regulation of KIF1Bβ motors (or loss of function in the case of CMT 2A) and consequent impairment of the transport of synaptic material would debilitate synaptic function and hence axonal integrity.

The kinesin II complex is a multi-task motor formed by interaction of KIF3A with either KIF3B or KIF3C. It is involved in intraflagellar transport, renal cilogenesis, the establishment of the left–right axis in the embryogenesis of mammals (Nonaka et al.,

Table 4

<table>
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<th>Gene</th>
<th>SNP (dbSNP125, NCBI)</th>
<th>Proband Code</th>
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<tr>
<td>KIF1B</td>
<td>rs12402052 (C/G, A90 synonymous substitution)</td>
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<td>ss65406445 (T/C, 3′UTR)</td>
<td>D10, D13, D14, D19</td>
</tr>
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Summary of the sequencing results of the motor domain-encoding exons of KIF1B and KIF3A and of exons encoding the complete ORF of SOD1. Intronic mutations did not affect conserved splice or branch sites. Novel SNPs, deposited to the dbSNP125 of NCBI, are highlighted.
1998), signal transduction (Jimeno et al., 2002; Huangfu et al., 2003), transport of late endosomes and lysosomes (Banakis et al., 2004; Brown et al., 2005), and even mitosis (Haraguchi et al., 2006). Mutations in KIF3A have been implicated in situs inversus (Marszalek et al., 1999) and polycystic kidney disease (Lin et al., 2006). Critically, kinesin II is also important for neuronal development and function. Although KIF3C appears to be dispensable for normal neural development and behavior in mouse (Yang et al., 2001), kinesin II is critical, both for neurite building as a carrier of fodrin-associating vesicles (Takeda et al., 2000) and for the establishment of neuronal polarity via the targeting of PAR3 to the distal tip of the axon (Nishimura et al., 2004; Shi et al., 2004). In addition, in neurons KIF3A is detected within and probably transports tau mRNA-containing RNP complexes, locally translated in axons (Aronov et al., 2002). Importantly, reduction of kinesin II has been linked to neurodegeneration in mammals: kinesin II is a selective transporter of opsin and arrestin, but not α-transducin, in mammalian photoreceptors (Marszalek et al., 2000), and removal of KIF3A by photoreceptor-specific conditional mutagenesis causes a rapid photoreceptor cell degeneration (Marszalek et al., 2000; Pazour et al., 2002). In our work, it is of interest to note that all members of the kinesin II subfamily, KIF3A, KIF3B, and KIF3C, showed down-regulation in the motor cortex of SALS patients, although only the motor-neuron-enriched KIF3Aβ isoform is down-regulated in a statistically significant manner. The absence of genomic mutations in the motor-domain-encoding exons and splicing junctions in KIF3A, sequenced by us, indicates for the SALS population under study that this down-regulation is caused at the transcriptional level and possibly induced under conditions that are conducive to the culmination of the neurodegenerative pathogenesis. To what extent this contributes to axonal degeneration over time remains to be addressed. In mouse, heterozygous KIF3A animals did not show retinal degeneration (Jimeno et al., 2006), contrary to the dramatic effects observed with the complete removal of functional KIF3A (see above). However, the two paradigms and cell systems may not be directly comparable, and isoform-specific effects, such as the down-regulation of the motor neuronally enriched KIF3Aβ, may have a significant impact on the multi-factorial interplay that underlies SALS pathophysiology.

Indeed, the investigation of individual KLP isoforms, some of them as yet unidentified, may lead to a definite link between an impairment of axonal transport and motor neuron degeneration in ALS. The present study indicates KIAA0591/KIF1Bβ (AB017133) and KIF3Aβ as promising candidates for further functional in vivo assays and highlights the importance of the isoform specificity of analyses in the definition of ALS pathogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2007.02.005.

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