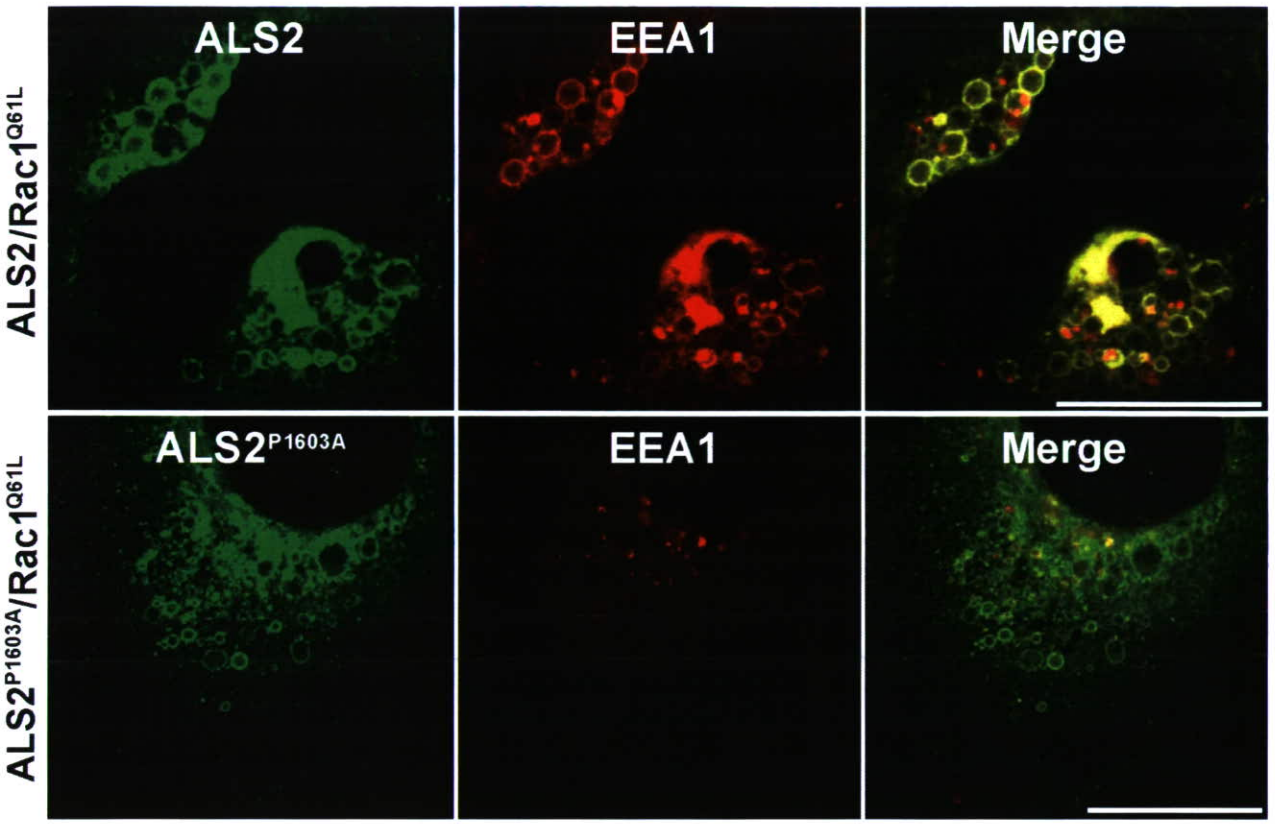
SUPPLEMENTARY FIGURE 1. EEA1 is less efficiently recruited to the Rab5GEF-defective ALS2 (ALS2P1603A)-localized macropinosomes. HeLa cells, transfected with either plasmid expressing FLAG-tagged WT ALS2 or plasmid expressing FLAG-tagged ALS2P1603A along with plasmid expressing 2XHA-tagged Rac1Q61L, were stained with anti-ALS2_RLD pAb (green) and anti-EEA1 mAb (red). Note that compared with the WT ALS2-localized macropinosomes, colocalization of ALS2 with EEA1 signals in the ALS2P1603A-localized macropinosomes is not prominent. Bars indicate 20 μm.

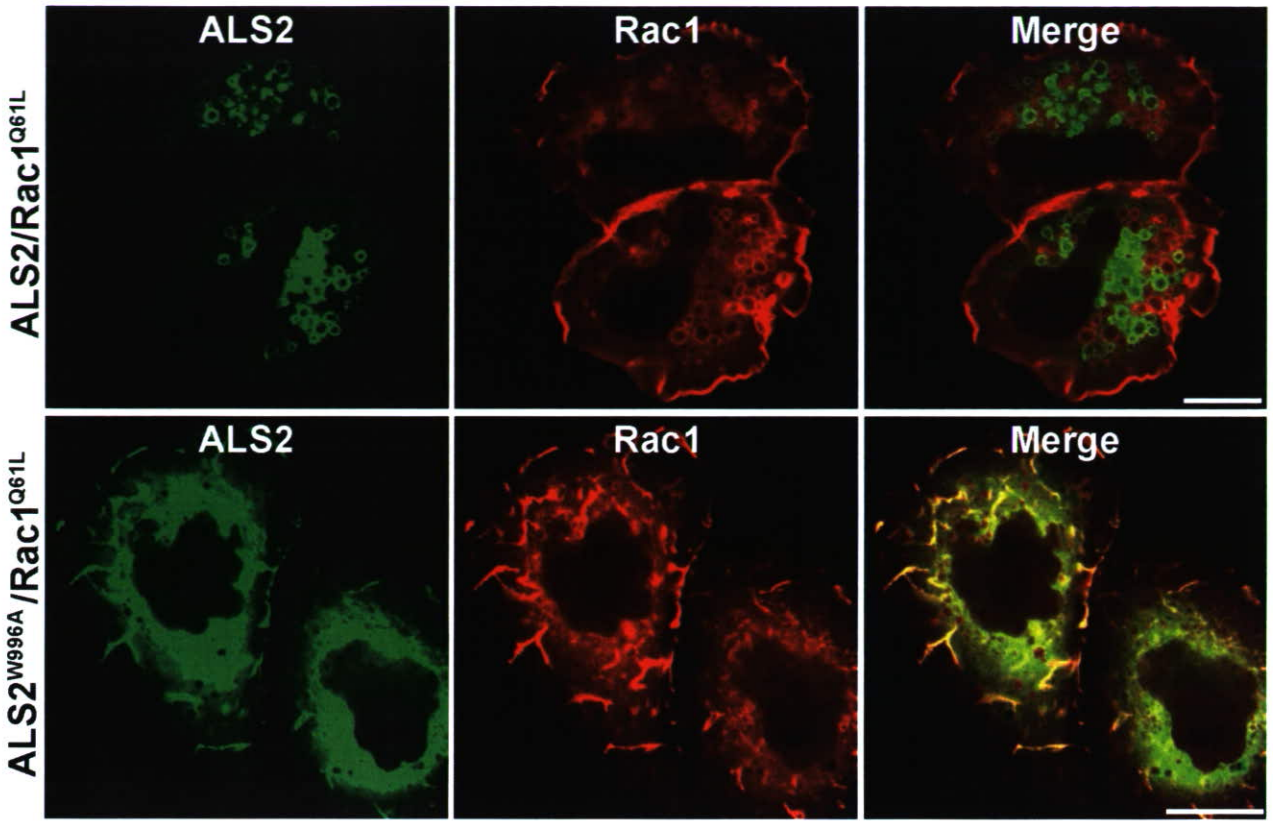
SUPPLEMENTARY FIGURE 2. The ALS2 mutant carrying a nonsynonymous mutation (W996A) in the PH domain (ALS2W996A) is redistributed to membrane ruffles, but not to intracellular macropinosomes upon Rac1 signaling. HeLa cells, transfected with either plasmid expressing FLAG-tagged WT ALS2 or plasmid expressing FLAG-tagged ALS2W996A along with plasmid expressing 2XHA-tagged Rac1Q61L, were stained with anti-ALS2_RLD pAb (green) and anti-HA mAb (red). Note that the ALS2W996A mutant is mainly localized to membrane ruffles, whereas WT ALS2 is exclusively localized to intracellular large macropinosomes upon Rac1 signaling. Bars indicate 20 μm.

SUPPLEMENTARY FIGURE 3. The ALS2-associated Rab5GEF activity provides a positive impact on the ALS2 redistribution to macropinosomes. A, The Rab5GEF-defective ALS2 mutant (ALS2P1603A) is redistributed to macropinosomes upon Rac1 signaling. HeLa cells were transfected with plasmid expressing FLAG-tagged WT ALS2 (top row) or FLAG-tagged ALS2P1603A (bottom row) along with plasmid expressing 2XHA-tagged Rac1Q61L. After 24 h, the cells were fixed and stained with anti-ALS2_RLD pAb (green) and phalloidin (red). B, The Rab5GEF-defective ALS2 mutant (ALS2P1603A) is not only relocalized to macropinosomes but also retained onto membrane ruffles. All experiment were conducted as in A except that the cells were fixed at relatively earlier time point (16 h after transfection). Note that although both the WT ALS2 and ALS2P1603A mutant are efficiently relocalized to macropinosomes upon Rac1 signaling, the ALS2P1603A mutant frequently exhibits the membrane ruffle localization. Bars indicate 20 μm.

SUPPLEMENTARY FIGURE 4. The ALS2 mutants carrying mutation(s) within the DH including ALS2E697A, ALS2T701A, and ALS2QD869-870AA are redistributed to macropinosomes upon Rac1 signaling (A), and ALS2T701A interacts with Rac1 in vitro (B). A, HeLa cells, transfected with expression plasmid either for FLAG-tagged ALS2E697A, ALS2T701A, or ALS2QD869-870AA along with expression plasmid for 2XHA-Rac1Q61L, were stained with anti-ALS2_RLD pAb (green). Bars indicate 20 μm. B, In vitro GST pull-down of the ALS2 mutant protein. All of the procedures were as in Fig. 1B except that FLAG-tagged ALS2T701A instead of FLAG-tagged WT ALS2 or ALS2_1-680 was used. The protein samples pulled down by GST or GST-fused GTPases indicated were immunoblotted with anti-ALS2_RLD pAb. ALS2T701A preferentially interacts with Rac1 and Rab5A as similarly as does WT ALS2 (Fig. 1).



Supplementary Fig. 1 Kunita et al.



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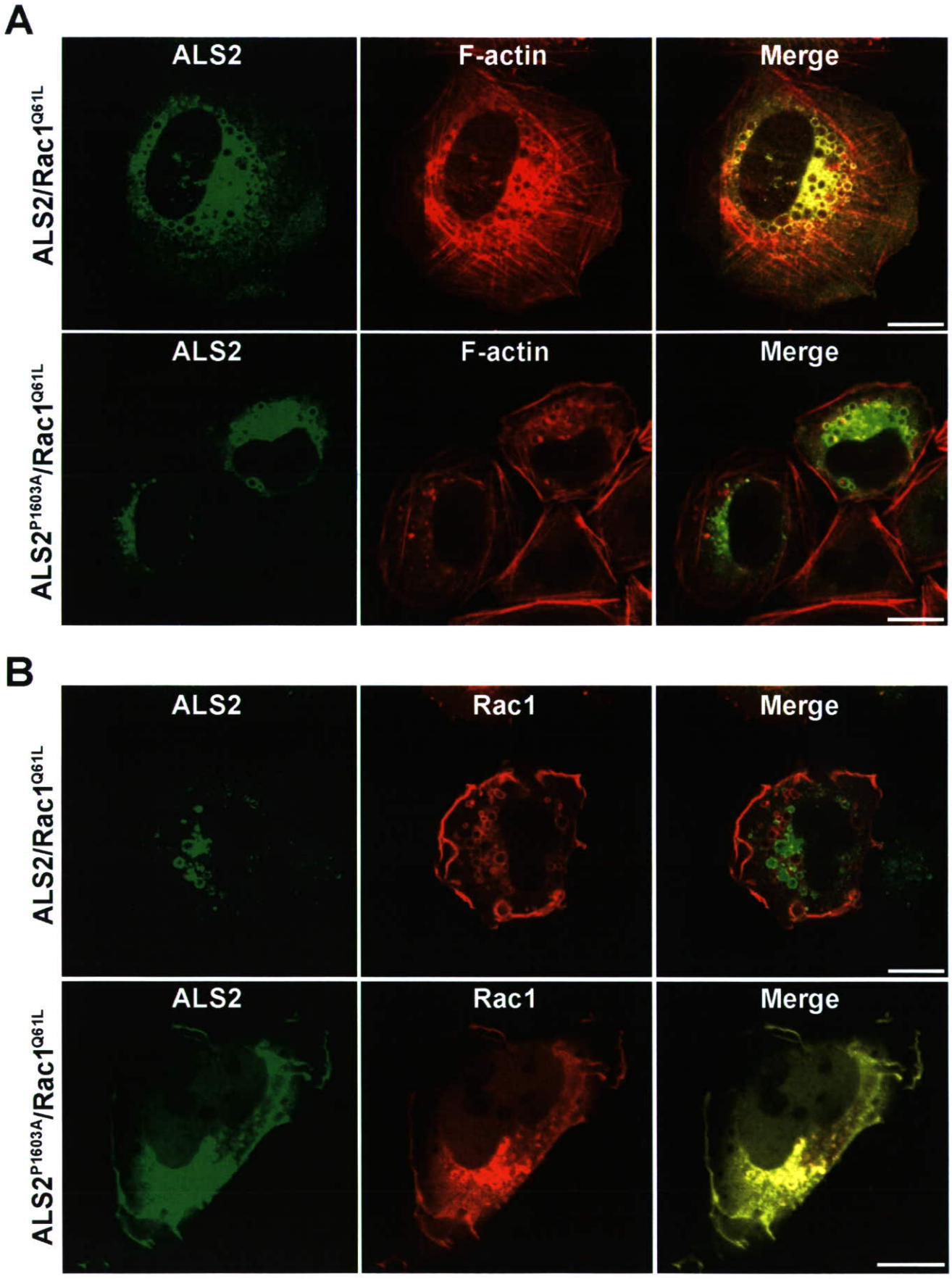
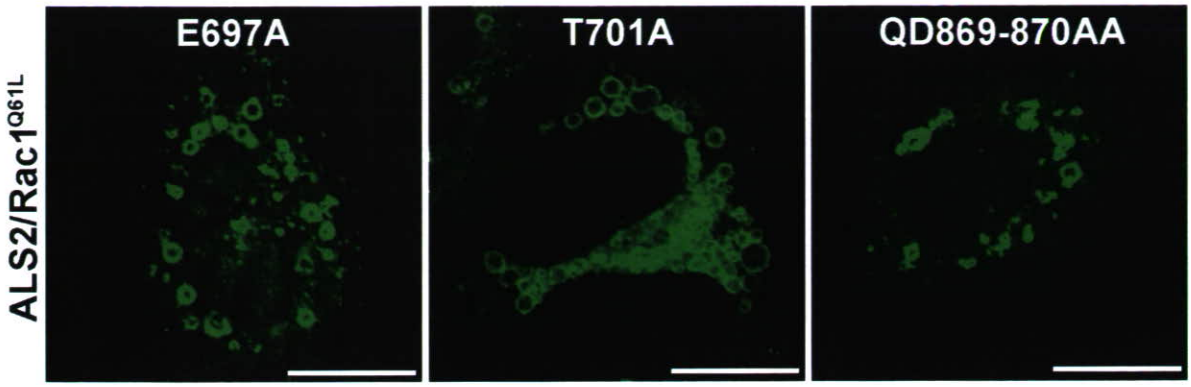


Supplementary Fig. 3 Kunita et al.

A



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Review

Molecular and cellular function of ALS2/alsin: Implication of membrane dynamics in neuronal development and degeneration

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Received 28 March 2007; received in revised form 18 April 2007; accepted 19 April 2007
Available online 4 May 2007

Abstract

ALS2 is a causative gene for a juvenile autosomal recessive form of motor neuron diseases (MNDs), including amyotrophic lateral sclerosis 2 (ALS2), juvenile primary lateral sclerosis, and infantile-onset ascending hereditary spastic paralysis. These disorders are characterized by ascending degeneration of the upper motor neurons with or without lower motor neuron involvement. Thus far, a total of 12 independent *ALS2* mutations, which include a small deletion, non-sense mutation, or missense mutation spreading widely across the entire coding sequence, are reported. They are predicted to result in either premature termination of translation or substitution of an evolutionarily conserved amino acid. Thus, a loss of functions in the *ALS2*-coded protein accounts for motor dysfunction and/or degeneration in the *ALS2*-linked MNDs. The *ALS2* gene encodes a novel 184 kDa protein of 1657 amino acids, ALS2 or alsin, comprising three predicted guanine nucleotide exchange factor (GEF) domains: the N-terminal RCC1-like domain, the central Dbl homology and pleckstrin homology (DH/PH) domains, and the C-terminal vacuolar protein sorting 9 (VPS9) domain. In addition, eight consecutive membrane occupation and recognition nexus (MORN) motifs are noted in the region between DH/PH and VPS9 domains. ALS2 activates Rab5 small GTPase and involves in endosome/membrane trafficking and fusions in the cells, and also promotes neurite outgrowth in neuronal cultures. Further, a neuroprotective role for ALS2 against cytotoxicity; i.e., the mutant Cu/Zn-superoxide dismutase 1 (SOD1)-mediated toxicity, oxidative stress, and excitotoxicity, has recently been implied. This review outlines current understandings of the molecular and cellular functions of ALS2 and its related proteins on safeguarding the integrity of motor neurons, and sheds light on the molecular pathogenesis of MNDs as well as other conditions of neurodegenerative diseases.

Keywords: Motor neuron disease; Amyotrophic lateral sclerosis; ALS2/alsin; Guanine nucleotide exchange factor (GEF); Small GTPase; Endosome dynamics

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0197-0186/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2007.04.010

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1. Introduction

Motor neuron diseases (MNDs) is a group of disorders characterized by the selective dysfunction and/or loss of either upper motor neurons (UMNs), or lower motor neurons (LMNs), or both, leading to relentlessly progressive weakness with variable degrees, muscle atrophy with eventual paralysis, and in many cases to death. One of the best characterized and known form of MNDs is amyotrophic lateral sclerosis (ALS), in which both UMN and LMN are affected (Boillée et al., 2006; Pasinelli and Brown, 2006), and thus the term of MND and ALS are sometimes interchangeable. However, it is generally accepted that MNDs also include other forms of disorders resulting from the dysfunction confined to UMN, such as primary lateral sclerosis (PLS) and spastic paraplegia (spastic gait; SPG), and those involved in the selective degeneration of LMNs, such as spinal muscular atrophy (SMA) (Fink, 2001; Verma and Bradley, 2001; Shaw, 2005; Chevalier-Larsen and Holzbaur, 2006; Gros-Louis et al., 2006; James and Talbot, 2006; Simpson and Al-Chalabi, 2006).

Most cases of MND/ALS are sporadic, and thus their causes are largely unknown. To delineate the molecular pathogenesis for such diseases, the identification of genes and their mutations that link to the familial forms of MND/ALS is essential. Indeed, recent advances in human genetics and genomics greatly facilitate the chromosomal mapping of disease loci, and the identification of the causative genes and mutations predisposing to many familial forms of MNDs (Gros-Louis et al., 2006; James and Talbot, 2006). The following molecular characterizations of the disease-causing and -related gene products, in conjunction with the generation of animal disease models, have large impacts on a wide range of disease studies to date. More than 30 causative genes underlying MND/ALS have been identified thus far (reviews in Boillée et al., 2006; Chevalier-Larsen and Holzbaur, 2006; Gros-Louis et al., 2006; James and Talbot, 2006; Pasinelli and Brown, 2006; Simpson and Al-Chalabi, 2006). One of the hallmarks for the MND/ALS studies was the identification of mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene as a cause for the autosomal dominant ALS with adult onset (ALS1) (Rosen et al., 1993). In this review, we focus on the second ALS-related gene, *ALS2*. The *ALS2* gene was originally identified by positional cloning, and the mutations in the *ALS2* gene account for a juvenile forms of autosomal recessive MND/ALS (Hadano et al., 2001a; Yang et al., 2001).

2. The *ALS2* gene

2.1. Mutation

ALS2 (OMIM 205100), also known as type 3 autosomal recessive ALS (RFALS type3), was originally reported in a large consanguineous Tunisian kindred and was characterized by a loss of UMNs and spasticity of limb and facial muscles accompanying distal amyotrophy of hands and feet (Ben-Hamida et al., 1990). The *ALS2* locus has been mapped to the 1.7 cM interval flanked by *D2S116* and *D2S2237* on chromosome 2q33 by linkage and haplotype analyses (Hentati et al., 1994; Hosler et al., 1998). The following physical cloning and mapping revealed that this interval spanned approximately 3 Mb of genomic DNA (Hosler et al., 1998; Hadano et al., 1999, 2001b), and allowed the generation of a transcript map of the *ALS2* critical region (Hadano et al., 2001a,b). In 2001, the *ALS2* (initially designated as *ALS2CR6*) gene was identified as a causative gene for ALS2 (Hadano et al., 2001a; Yang et al., 2001). At the same time, two additional *ALS2* mutations were found in patients with a rare juvenile recessive form of PLS (PLSJ; OMIM 606353) in both Kuwaiti and Saudi Arabian consanguineous families (Hadano et al., 2001a; Yang et al., 2001). Recently, although mutations or variations in the *ALS2* gene are not a common cause of sporadic or familial ALS (Al-Chalabi et al., 2003; Hand et al., 2003; Nagano et al., 2003), several independent homozygous *ALS2* mutations have been found in families segregating an infantile-onset ascending hereditary spastic paralysis (IAHSP; OMIM 607225) (Devon et al., 2003; Eymard-Pierre et al., 2002, 2006; Lesca et al., 2003; Panzeri et al., 2006), a single family of a recessive complicated hereditary spastic paraplegia (HSP) (Gros-Louis et al., 2003), and a single family of ALS2 (Kress et al., 2005). Thus far, a total of 12 independent *ALS2* mutations, which include the small deletions, non-sense mutations, or missense mutations spreading widely across the entire coding sequence, are reported (Table 1). These mutations are predicted to result in either premature termination of translation or substitution of an evolutionarily conserved amino acid. Since ALS2, PLSJ, and IAHSP/HSP are group of closely related recessive MNDs, and a loss of functions in the *ALS2* mutations may account for a number of recessive MNDs, it is likely that the *ALS2*-coded protein bears on maintaining the integrity of motor neurons.

2.2. Structure and expression

The *ALS2* gene comprises 33 introns and 34 exons and resides within 80.3 kb of genomic DNA on chromosome 2q33. Sequence of the *ALS2* transcript encompasses 6394 nucleotides (nt) with a single open reading frame (ORF: 4974 nucleotides long, 124–5097 nt), and is predicted to encode a 184 kDa protein consisting of 1657 amino acids (aa). A shorter transcript for *ALS2* (*ALS2_S*) that encompasses 2651 nt with a single 1191 nt ORF encoding 396 aa. The *ALS2_S* transcript is a product of alternative splicing at the 5′ donor site after exon 4, resulting in a premature stop codon after 25 amino acid residues in intron 4. Consistent with these findings, two transcripts of approximately

Table 1
Mutations in the *ALS2* gene in *ALS2*-linked motor neuron diseases

Mutations (Transcript or gene)[a]	Location	Type[b]	Mutant proteins[a]	Origin	Disease[c]	Phenotype[d]	References
c.138delA	Exon 3	fs	A47fsX4	Tunisian	ALS2	U/L	Hadano et al. (2001a) and Yang et al. (2001)
c.470G>A	Exon 4	ms	C157Y	Turkish	IAHSP	U	Eymard-Pierre et al. (2006)
c.553delA	Exon 4	fs	T185fsX5	Turkish	ALS2	U/L	Kress et al. (2005)
c.1007_1008delTA	Exon 4	fs	I336fsX5	Italian	IAHSP	U	Eymard-Pierre et al. (2002)
c.1425_1426delAG	Exon 5	fs	G476fsX71	Kuwaiti	PLSJ	U	Hadano et al. (2001a)
IVS5(c.1472)-1G>T(C.1472_1481delTTTCCCCCAG)	Intron 5	fs	V491fsX3	French	IAHSP	U	Eymard-Pierre et al. (2002)
c.1619G>A	Exon 6	ms	G540E	Italian	PLSJ	U	Panzeri et al. (2006)
c.1867_1868delCT	Exon 9	fs	L623fsX24	Saudi Arabian	PLSJ	U	Yang et al. (2001)
c.2537_2538delAT	Exon 13	fs	N846fsX13	Italian	IAHSP	U	Eymard-Pierre et al. (2002)
c.2992C>T	Exon 18	ns	R998X	Israeli	IAHSP	U	Devon et al. (2003)
c.3619delA	Exon 23	fs	M1207X	Algerian	IAHSP	U	Eymard-Pierre et al. (2002)
c.4721delT	Exon 32	fs	V1574fsX44	Pakistani	IAHSP	U	Gros-Louis et al. (2003)

[a] The description of the mutations are accorded by den Dunnen and Antonarakis (Hum Mut 15, 7–12, 2000).
[b] fs: frame shift, ms: missense, ns: nonsense.
[c] ALS2: amyotrophic lateral sclerosis 2 (OMIM 205100), PLSJ: juvenile primary lateral scelrosis (OMIM 606353), IAHSP: infantile-onset ascending hereditary spastic paralysis (OMIM 607225).
[d] U: upper motor neuron involvement, L: lower motor neuron involvement.

6.5 and 2.6 kb in various adult human tissues are detected by Northern blot analysis (Hadano et al., 2001a). Both transcripts are expressed ubiquitously with highest in the central nervous system (CNS), particularly in the cerebellum.

Murine ortholog for *ALS2*, officially designated as *Als2*, encompasses 6349 nt with a single ORF that is 4956 nt long (124–5079 nt), and is predicted to encode a 183 kDa protein consisting of 1651 aa. The entire ORF is well conserved between human and mouse (87% identity at DNA level). The *Als2* short variant (*Als2_S*) of a 2955 nt with a single 2787-nt ORF encoding 928 aa (~100 kDa), which is produced by alternative splicing at the 5′ donor site after exon 13, resulting in a premature stop codon after 74 amino acid residues in intron 13. Thus, the structure of the mouse *Als2_S* transcript is different from that of the human variant (Hadano et al., 2006). In adult mouse CNS, the *Als2* transcript is expressed to a variable degree in neuronal cells throughout the brain and spinal cord, particularly in neurons in the hippocampus, cerebellum, cerebral cortex, spinal gray matter (motor neurons), olfactory bulb, basal ganglia, and cranial nuclei (Hadano et al., 2001a) with most abundant in the granular layer of the cerebellum (Devon et al., 2005; Lein et al., 2007; Allen Brain Atlas, http://www.brain-map.org/welcome.do). During development, *Als2* expression is limited at an early embryonic stage (E9.5–E12.5), but is gradually increased in CNS after E14.5 and reaches adult levels at P7 (Devon et al., 2005), reflecting that *Als2* implicates in neuronal development.

3. The ALS2 protein (ALS2/alsin)

The human *ALS2* and mouse *Als2* genes encodes proteins of 1657 aa and 1651 aa, respectively, which are called ALS2 (Hadano et al., 2001a) or alsin (Yang et al., 2001). Analysis of the predicted amino acid sequences identified a high level of sequence similarity throughout the entire region of human and mouse ALS2 proteins (91% identity, 94% similarity). Database searches demonstrate the presence of orthologs in vertebrates as well as in fry (*D. melanogaster*; CG7158) and mosquito (*A. gambiae*), but not in nematode (*C. elegans*) and yeast (Devon et al., 2005). A number of interesting domains and motifs in the deduced ALS2 protein sequence is noted (Fig. 1). A region in the N-terminal half of ALS2 is highly homologous to regulator of chromosome condensation (RCC1) and retinitis pigmentosa GTPase regulator (RPGR) including its functional structural motif; a seven-bladed propeller (Hadano et al., 2001a; Topp et al., 2004). This domain is referred as to RCC1-like domain (RLD). RCC1 is a guanine nucleotide exchange factor (GEF) for the nuclear GTP binding protein Ran (Ras-related nuclear) (Dasso, 2001). A middle portion of ALS2 contains a tandem organization of a diffuse B cell lymphoma (Dbl) homology (DH) and pleckstrin homology (PH) domains, that is a hallmark of GEFs for Rho (Ras homologous member)-type GTPases (Rossman et al., 2005). The vacuolar protein sorting 9 (VPS9) domain, which has been found in a number of Rab5 (Ras-related in brain 5) GEFs (Zerial and McBride, 2001; Carney et al., 2006), is also found in the C-terminal region. In addition, a tandem array of eight membrane occupation and recognition nexus (MORN) motifs each consisting of 23 amino acids (Takeshima et al., 2000), which is implicated in the binding of plasma membranes, are also noted in the region between the DH/PH and VPS9 domains.

ALS2 is predominantly expressed in CNS with highest in the cerebellum, consistent with an expression pattern of the *ALS2*/*Als2* mRNA. Expression of ALS2 in non-CNS organs and tissues was generally low, except for in the testis (Hadano et al., 2006; Devon et al., 2006). A series of differential centrifugation experiments using brain tissues have revealed that ALS2 is enriched in P3 membrane fractions where a number of endosomal proteins, such as transferrin (Tf) receptor, early endosome auto-antigen 1 (EEA1), and synaptophysin, are co-fractionated, suggesting that ALS2 is predominantly distributed

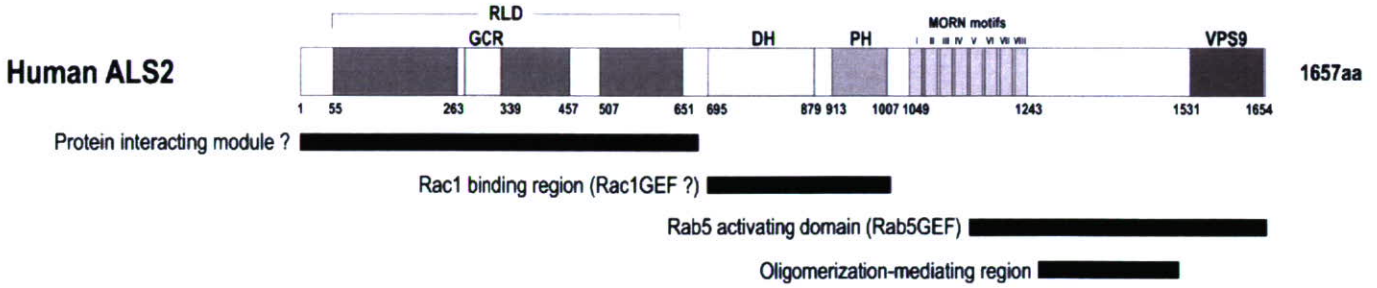

Fig. 1. Schematic representation of the human full-length ALS2 protein (ALS2) and its domains and motifs. ALS2 contains three predicted guanine nucleotide exchange factor (GEF) domains; i.e., RCC1-like domain (RLD), diffuse B cell lymphoma (Dbl) homology (DH) and pleckstrin homology (PH) domains, and vacuolar protein sorting 9 (VPS9) domain. In addition, eight consecutive membrane occupation and recognition nexus (MORN) motifs are noted in the region between DH/PH and VPS9 domains. GCR stands for glucocorticoid receptor homologous region. A number of the functionally assigned regions are also noted.

to endosomal membrane compartments (Yamanaka et al., 2003; Topp et al., 2004; Devon et al., 2005). Notably, a significant amount of ALS2 is also present both in P2 (mitochondrial/synaptosomal) and S3 (soluble) fractions (Suzuki-Utsunomiya et al., unpublished). Further, it has been shown that ALS2 is enriched in a centrosome preparations purified from human cortical brain (Millecamps et al., 2005).

Immunohistochemical analysis of ALS2 using anti-ALS2 antibodies combined with neuronal and glial markers have revealed that ALS2 is expressed in various neurons, but not glial cells in mice (Hadano et al., 2006), consistent with the mRNA *in situ* hybridization studies. ALS2 is highly expressed in the granular layers of the cerebellum (Devon et al., 2005; Hadano et al., 2006), and shows the colocalization with some, but not all calbindin immunopositive Purkinje cells (Hadano et al., 2006). High magnification studies have demonstrated that ALS2 is mainly distributed in a diffused manner, but several dot or patchy stainings are consistently observed in soma as well as in dendrite, suggesting that ALS2 localizes not only in the cytosol but also onto vesicular and/or membranous compartments in neurons (Hadano et al., 2006). However, as there are still inconsistencies in the results of the immunohistochemical ALS2 localization (Devon et al., 2005; Hadano et al., 2006), further careful assessments will be required.

It is noted that there is currently no evidences for the expression of the expected truncated protein products in lymphoblasts from patients with confirmed homozygous mutation in *ALS2* (Yamanaka et al., 2003) and short variants of the *ALS2/Als2* genes (Otomo et al., 2003; Yamanaka et al., 2003; Hadano et al., 2006). Interestingly, both the mutated ALS2 and a naturally truncated isoform of ALS2 (*ALS2_S*) proteins are rapidly degraded in human cultured cells (Yamanaka et al., 2003; Eymard-Pierre et al., 2006), suggesting that loss of function coupled with protein instability may, in part, account for the pathogenesis underlying the ALS2-linked MNDs.

4. Function of ALS2

4.1. Biochemical characteristics

The small GTPases act as binary switches by cycling between an inactive (GDP-bound) and an active (GTP-bound) state, and regulate a broad spectrum of cellular and molecular processes. GEFs are known to stimulate the exchange of GDP for GTP, thereby generating the active forms of the small GTPases (Vetter and Wittinghofer, 2001). For example, RCC1 (RanGEF) activates Ran GTPase, which implicates in nuclear transfer as well as chromatin condensation through the regulation of microtubule assembly (Dasso, 2001). The Rho subfamilies, which are activated by the DH/PH (Rossman et al., 2005) or DOCK (Côté and Vuori, 2002) -containing GEFs, are critical regulators of the organization of the actin cytoskeleton, various signaling cascades, and neuronal morphogenesis (Da Silva and Dotti, 2002; Etienne-Manneville and Hall, 2002; Luo, 2000; Snider et al., 2002; Van Aelst and Symons, 2002). Further, the Rab GTPases have emerged as key players for the vesicle budding, motility/trafficking, fusion, and signal transduction (Zerial and McBride, 2001; Miaczynska et al., 2004). In particular, the VPS9 domain-containing GEFs are shown to activate the Rab5 family of GTPases (Carney et al., 2006).

Since ALS2 contains multiple putative GEF domains; RLD, DH/PH, and VPS9, implying that ALS2 acts as a regulator/activator of multiple small GTPases (Hadano et al., 2001a; Yang et al., 2001). However, *in vitro* GDP dissociation and GTP binding assays (i.e., GEF assays) proved that ALS2 exhibits selective GEF activity on the members of Rab5 family (Rab5A, Rab5B, and Rab5C), but neither on other members of GTPases including Rab family (Rab3, Rab4, Rab7, Rab9, and Rab11), Arf family (ARF1 and ARF6), Rho family (Rac1, Cdc42, and RhoA), nor Ran (Otomo et al., 2003; Topp et al., 2004). Thus, ALS2 acts as a specific GEF for the Rab5 GTPase family *in vitro*. A series of the *in vitro* Rab5-GEF assays using the N- and C-terminally truncated ALS2 fragments reveals a region conferring the ALS2-associated Rab5GEF activity to the C-terminal half of ALS2 that contains the MORN-VPS9 domains. Lack of either the MORN motifs or VPS9 domain results in the loss of the Rab5-GEF activity (Otomo et al., 2003). Mutations in an evolutionarily conserved amino acid residue within the VPS9 domain; i.e., ALS2_P1603A and ALS2_L1617A, result in a marked decrease in the ALS2-associated Rab5-GEF activity (Otomo et al., 2003). Intriguingly, ALS2 forms a homophilic oligomer through two distinct C-terminal regions mapped within the interval between MORN motifs and VPS9 domain, which is crucial for the ALS2-associated Rab5 GEF

activity (Kunita et al., 2004). Moreover, the C-terminal region of ALS2 mediates the direct interaction between ALS2 and Rab5A (Otomo et al., 2003; Kunita et al., 2004; Hadano and Ikeda, 2005). Taken together, every element in the C-terminal ALS2 might be the determinant for the structural context of ALS2 that are indispensable for the ALS2-asociated Rab5 GEF activity (Fig. 1).

Notably, there are additional genes encoding members of the Rab5 family, including Rab21, Rab22a, Rab31 (Rab22b), and Rab22c, other than Rab5A, Rab5B, and Rab5C, in the human genome (Stenmark and Olkkonen, 2001). Recently, Rabex-5, the well-studied VPS9 domain-containing Rab5GEF, has been shown to activate not only Rab5 but also Rab21 and Rab22a (Delprato et al., 2004). Further, another VPS9 domain-containing proteins, Varp and Gapex-5, exhibits a predominant catalytic activity on Rab21 and Rab31, respectively (Zhang et al., 2006; Lodhi et al., 2007). These findings suggest that each member of the VPS9-domain containing Rab5GEFs has a distinct catalytic property on the Rab5 GTPase family. The VPS9 domain-containing ALS2 protein also behaves as a GEF for Rab22a and Rab31 but not for Rab21 (Hadano et al., unpublished).

ALS2 contains the DH/PH domain in the middle, which is a hallmark of GEFs for Rho GTPase family. Indeed, it has been shown that ALS2 specifically binds to Rac1 *in vitro* in a DH/PH-domain dependent manner, but neither to Rac2, Rac3, Cdc42, RhoA, RhoB, RhoC, nor RhoG (Topp et al., 2004; Kanekura et al., 2005; Kunita et al., 2007). Further, overexpression of ALS2 in cultured cells marginally enhances the level of active Rac1, implying a potency of ALS2 to activate Rac1 (Topp et al., 2004; Kanekura et al., 2005; Tudor et al., 2005). However, as earlier mentioned, ALS2 does not possess the GEF activity on Rac1 *in vitro* (Otomo et al., 2003; Topp et al., 2004). Thus, the ALS2-associated Rac1GEF activity is still matter of conjecture (Fig. 1). Most recently, we have shown that ALS2 turns to be activated by Rac1, and thus ALS2 is a Rac1 effector rather than Rac1GEF (Kunita et al., 2007).

The N-terminal RLD of ALS2 conserves a seven-bladed propeller sequence that is a distinguishing feature of RCC1 as a Ran GEF (Hadano et al., 2001a; Topp et al., 2004). However, no significant ALS2-associated Ran GEF activity was detected *in vitro* (Otomo et al., 2003). Since RLDs are identified in many other proteins of diverse functions, the RLD in ALS2 might function as an interface between protein–protein or protein–lipid bindings rather than GEF (Fig. 1). Indeed, the ALS2-RLD modulates the association of ALS2 molecules to the membrane compartments (Yamanaka et al., 2003; Kunita et al., 2007). Further, the N-terminal region containing the RLD directly interacts with the C-terminal portion of ALS2, modulating the ALS2 distribution and activity in the cells (Kunita et al., 2007).

4.2. Cellular biological characteristics

In non-neuronal cultured cells, such as HeLa and COS-7 cells, ectopically expressed ALS2 diffusedly distributes throughout cytoplasm with occasional localization to small vesicular structures in the perinuclear region and to the leading membrane edges of cellular peripheries (Otomo et al., 2003). Similar distribution profiles of endogenous ALS2 in NIH3T3 cells are observed (Topp et al., 2004). Vesicular ALS2 is partially colocalized with Rab5 and EEA1, markers for endosomes, indicating that ALS2 is the endosomal tribe (Otomo et al., 2003; Topp et al., 2004). Indeed, a loss of ALS2 results in the delayed fusion of epidermal growth factor (EGF)-positive endosomes in mouse embryonic fibroblasts (MEFs) (Hadano et al., 2006), supporting that endogenous ALS2 serves in Rab5-dependent endosome fusion (Fig. 2). Remarkably, ALS2 molecules on the leading membrane edges and ruffles significantly overlap with Rac1 (Topp et al., 2004). In a subset of cultured cells, some of ALS2 molecules are colocalized with the centrosomal markers, such as γ-tubulin and A kinase anchoring protein (AKAP-450) (Millecamps et al., 2005). It has also been reported that overexpression of ALS2 not only results in an abnormal endosomal phenotypes, but also in impairment of mitochondria trafficking and fragmentation of the Golgi apparatus (Millecamps et al., 2005). Thus, ALS2 is fairly committed in a wide range of membrane dynamics and cytoskeletal organizations in the cells.

A series of experiments using cultured cells expressing a truncated as well as missense ALS2 mutants has revealed that each ALS2 domain/region demonstrates specific subcellular tropism *in vivo* (Otomo et al., 2003; Yamanaka et al., 2003; Topp et al., 2004). The C-terminus of ALS2 carrying the MORN/VPS9 domains activates Rab5, and also mediates the endosomal localization for ALS2. On the other hand, the N-terminal RLD, in a context of full-length ALS2, acts suppressive in the membranous localization of ALS2 itself (Otomo et al., 2003), although the RLD-containing fragment itself associates with the membranous structures under certain conditions (Yamanaka et al., 2003; Kunita et al., 2007). Notably, the DH/PH domain constitutively promotes the MORN/VPS9 domain-mediated Rab5 activation and endosome fusions *in vivo* (Otomo et al., 2003). Further, the homo-oligomerization of ALS2 through its C-terminal regions is crucial for this ALS2-mediated endosome enlargement (Kunita et al., 2004). These findings suggest that the intracellular localization of ALS2 and its-associated Rab5GEF activity seem to be controlled by the internal domain(s) of ALS2, probably through the association with ALS2 itself and/or other protein or lipid molecules. Indeed, a pathogenic missense mutation in the RLD of ALS2 that results in the ALS2 mislocalization; i.e., loss of endosomal ALS2, has been reported (Panzeri et al., 2006). Thus, proper arrangement of the ALS2 subcellular localization may be crucial to exert the physiological ALS2 functions.

In neurons, endogenous and overexpressed ALS2 are found primarily on small vesicular/punctate structures both in cell bodies and in elaborated somato-dendritic neurites of embryonic cortical, hippocampal, and motor neuronal cultures (Otomo et al., 2003; Topp et al., 2004; Jacquier et al., 2006). Some cytoplasmic ALS2 stainings are also observed (Otomo et al., 2003; Topp et al., 2004). At a matured stage of cultured neurons, vesicular ALS2 is present in either dendrites, axons, or the cell bodies, with no apparent polarized localization (Topp et al., 2004). Co-localization studies with Rab5 and EEA1

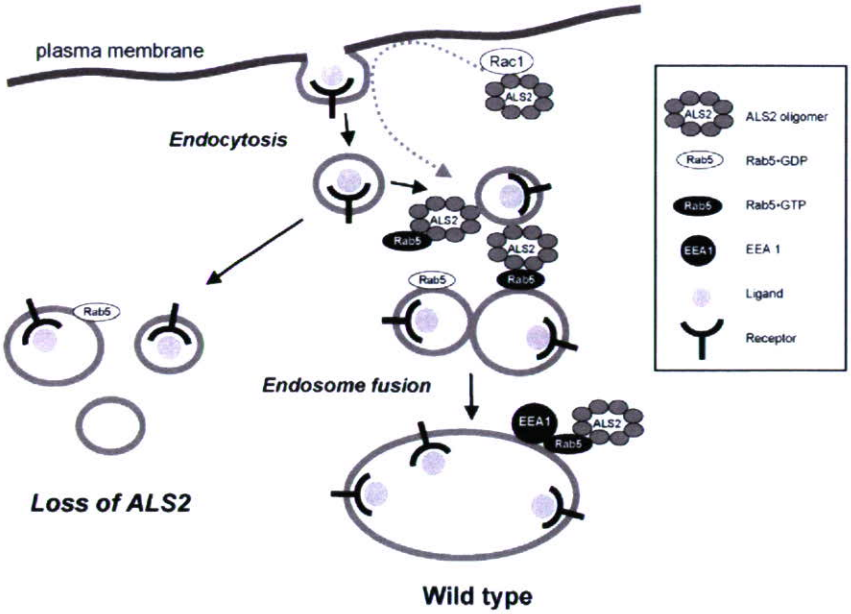

Fig. 2. Schematic representation of a model for the intracellular function of ALS2. ALS2 mediates Rab5-dependent endosome fusion in the cells. A particular molecular signal(s) (e.g., Rac1) activates and recruits the ALS2 oligomer to early endosomal compartments through Rac1-activated macropinocytosis (Kunita et al., 2007), wherein ALS2 binds to Rab5 GTPase. ALS2 activates Rab5 via its associated Rab5GEF activity. Activated Rab5 (Rab5-GTP) further facilitates the formation of protein complex comprising downstream effector molecules such as early endosome auto-antigen 1 (EEA1) and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (not shown), and promotes endosome fusion. A loss of ALS2 may perturb endosomal trafficking and fusion, thereby leading to dysfunction of intracellular molecular signaling; e.g., insulin-like growth factor 1 (IGF1) or brain-derived neurotrophic factor (BDNF) (see text), and ultimately resulting in dysfunction/degeneration of neuronal cells.

demonstrate that ALS2 is present in endosomal compartments in neurons (Otomo et al., 2003). On the other hands, at an immature stage, ALS2 is enriched on the tip of axon with a dense localization to Rac1/actin-positive lamellipodia and vesicles at growth cones (Tudor et al., 2005; Jacquier et al., 2006). Compelling evidences for the functional aspects of ALS2 in neuronal endosome dynamics have been obtained from two independent studies. The Rab5-dependent fusion activity is significantly reduced in the brain cytosol from the *Als2* knockout (KO) mice in comparison with wild-type (Devon et al., 2006). Further, siRNA-mediated knockdown of rat *Als2* in cultured motor neurons results in smaller-sized EEA1-positive endosomes (Jacquier et al., 2006). Thus, endogenous ALS2 certainly plays an important role in Rab5-dependent endosome fusion in neurons. On the other hand, studies using the ALS2 overexpression as well as *Als2* knockdown in conjunction with co-expression of the small GTPase have revealed that ALS2 stimulates neurite and axonal outgrowth in a Rac1-dependent manner (Tudor et al., 2005; Jacquier et al., 2006), suggesting that ALS2 enriched in growth cones may act as a modulator for neurite development. Collectively, ALS2 plays a role in membrane/vesicular trafficking and axonal outgrowth in neurons (Fig. 3), albeit the molecular linkages of these cellular events are still unclear.

4.3. ALS2 knockout mice

Als2 KO mice have been reported by five independent groups (Cai et al., 2005; Hadano et al., 2006; Devon et al., 2006; Yamanaka et al., 2006; Julien and Kriz, 2006). First two-lines of *Als2* KO mice were created by replacing the 39 bp of a *Bam*HI fragment in exon 3 with the *LacZ-NEO* cassette (Cai et al., 2005) and the stop codon followed by the *NEO* cassette (Hadano et al., 2006), respectively. Although the *Als2* gene in a targeted allele can be transcribed by its own promoter, the normal protein translation is terminated after first 14 amino acids, resulting the peptide lacks all the functional domains of ALS2. Third line was generated by using promoter trap gene targeting to replace exons 3 and 4 with SA-IRES-βgeo-pA cassette encoding bifunctional *LacZ*-neomycin fusion protein (Devon et al., 2006), and expected to produce fusion protein carrying only seven amino acid residues originated from ALS2 at the N-terminus. Forth line was produced by replacing exon 2 and part of exon 3 with the *NEO* cassette, resulting in a complete lack of transcription for the *Als2*-coded sequence (Julien and Kriz, 2006). Final line reported contain the *Als2*-null allele generated by replacing the 3′-half of exon 4 and intron 4 with the *NEO* cassette, which results in a truncated ALS2 peptide (174 aa) containing a portion of the N-terminal RLD (Yamanaka et al., 2006). Although there are slight differences in the gene targeting strategy, the translation of the full-length functional ALS2 protein is completely disrupted in all these mice (i.e., *Als2* KO mice) (Cai et al., 2005; Hadano et al., 2006; Devon et al., 2006; Yamanaka et al., 2006; Julien and Kriz, 2006).

Surprisingly, these studies combined demonstrate that absence of ALS2 does not develop *ALS2*-related disease phenotypes in mice; they show no obvious developmental and reproductive abnormalities with normal life-span. However, *Als2* KO mice exhibit age-dependent deficits in motor

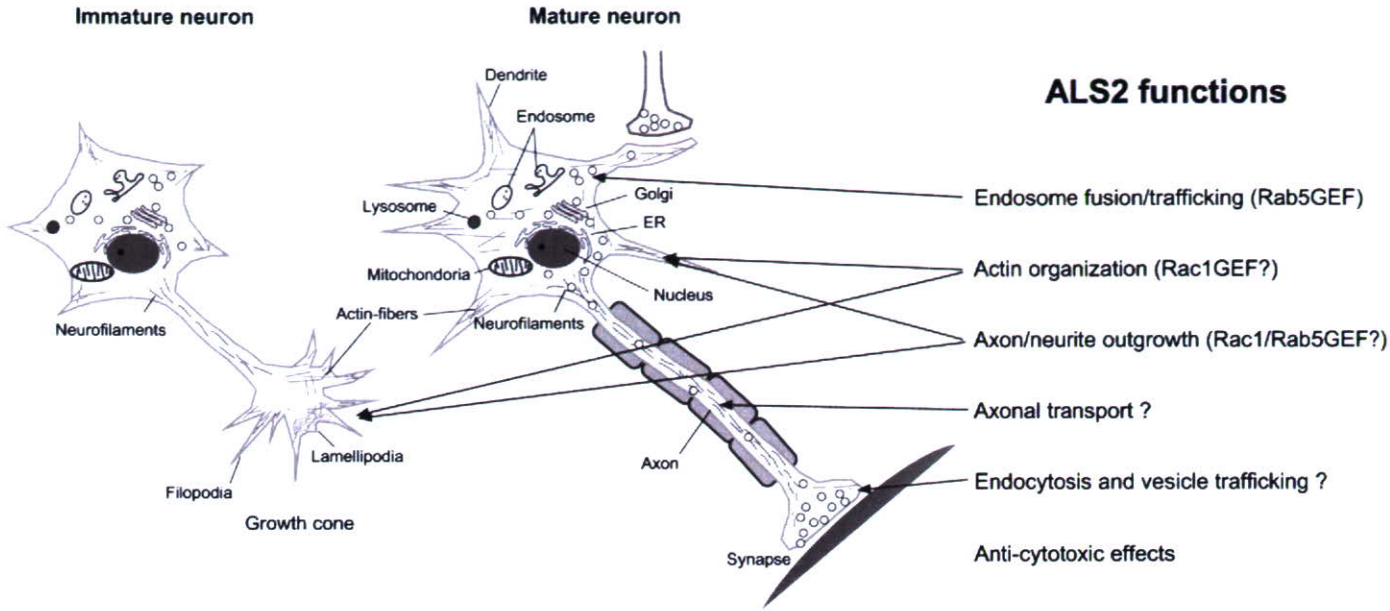

Fig. 3. Schematic representation of the proposed functions of ALS2 in developing and matured neurons. ALS2 plays a role in membrane/vesicular trafficking and axonal outgrowth in neurons. Further, a neuroprotective role for ALS2 against cytotoxicity; i.e., mutant SOD1-mediated toxicity, oxidative stress, and excitotoxicity, has recently been documented (see text).

coordination and motor learning, and aged *Als2* KO mice or primary cultured motor neurons derived from these mice are slightly vulnerable to oxidative stress (Cai et al., 2005). Additional findings include a significant decrease in the size of cortical neurons (Devon et al., 2006) and a progressive axonal degeneration in the lateral spinal cord (Yamanaka et al., 2006) or in corticospinal tract (Julien and Kriz, 2006). Further, *Als2* KO neurons shows a disturbance in endosomal transport of a subset of trophic receptors, such as insulin-like growth factor 1 (IGF1) and brain-derived neurotrophic factor (BDNF) (Devon et al., 2006). We have shown that *Als2* KO mice develop an age-dependent, slowly progressive loss of cerebellar Purkinje cells, disturbance of spinal motor neurons associated with astrocytosis and microglial activation, and evidences for the deficits in endosome dynamics (Hadano et al., 2006). Most recently, it has been reported that *Als2* KO mice exhibit lymphopenia and abnormal hematopoietic function, suggesting an involvement of immune system in the pathogenesis (Erie et al., 2007). Collectively, while loss of ALS2 does not produce severe phenotypes in mice, there are recognizable subclinical and cellular dysfunctions. Thus, it is anticipated that these animals should provide a useful means with which to investigate the ALS2 functions *in vivo* and the molecular pathogenesis underlying the ALS2-linked MNDs.

4.4. ALS2-interacting and its related proteins

The protein–protein interactions are one of the key molecular basis not only to the organizations of the structural protein complexes, such as cytoskeletal elements, but also to the regulation and coordination of an immense amount of molecular signalings within and/or between cells. Evidence is accumulating that ALS2 is a GEF for the Rab5 small GTPase family, and also acts as a regulator for the Rho-type GTPase Rac1. In fact, ALS2 specifically interacts with Rab5 and Rac1 (see above). Thus, ALS2 might involve in the regulation of Rab5- and Rac1-mediated signaling pathways, via their direct interactions, in the cells. To understand the pathogenesis of the ALS2-linked MNDs, the clarification of the ALS2-mediated molecular signaling both in normal and pathological conditions is essential. Here, we introduce recent advances in the identification and characterization of the ALS2-interacting proteins, which will provide a novel insight into the studies on the ALS2 functions and the pathogenesis.

4.4.1. SOD1: Cu/Zn-superoxide dismutase

Mutations in the *SOD1* gene account for the autosomal dominant ALS with adult onset (ALS1) (Rosen et al., 1993). The *SOD1* gene encodes Cu/Zn-superoxide dismutase (SOD1), and SOD1 mutants cause motor neuron degeneration through gain of toxic properties (Rakhit and Chakrabartty, 2006). Recently, it has been demonstrated that overexpression of ALS2 specifically inhibits SOD1 mutant-induced neurotoxocity in NSC34 cells (Kanekura et al., 2004). This protective effect is dependent on the DH/PH domain of ALS2, and most importantly, ALS2 directly binds to SOD1 mutant, but not to the wild-type SOD1 via its DH/PH domain (Kanekura et al., 2004). Further, such ALS2-mediated neuroprotective function against mutant SOD1-induced motor neuron death exerts through the activation of the Rac1/phosphatidylinositol 3-kinase (PI3K)/Akt3 prosurvival pathway, which is also dependent on the DH/PH domain of ALS2 (Kanekura et al., 2005; Matsuoka and Nishimoto, 2005). Thus, at least in a certain cell type, ALS2 antagonizes SOD1 mutant-induced neurotoxicity via their direct interaction and the activation of a particular anti-apoptotic signaling pathway. However, there are mutually contradictory aspects to their findings. Deficiency in ALS2 does not affect the pathogenesis of $SOD1^{G93A}$ mice on an

Als2-null background (Lin et al., 2007). Besides that, a missense mutation in ALS2 is also cytotoxic, and cell death induced by neurotoxic stimuli, such as *N*-methyl-D-aspartate (NMDA) and staurosporine, is significantly suppressed by expressing wild-type ALS2, which are both independent of the interaction with SOD1 (Panzeri et al., 2006). Rather the protection by ALS2 occurs through a signaling event impinging on a common cell-death pathway, in which mutant ALS2 decreases, while wild-type ALS2 increases the Bcl-xL:Bax ratio (Panzeri et al., 2006), albeit a direct molecular association yet to be demonstrated.

4.4.2. GRIP1: glutamate receptor interacting protein 1

Glutamate-mediated excitotoxicity, which is primarily mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-type glutamate receptors, involved in degeneration of spinal motor neurons in the sporadic ALS (Kawahara et al., 2004). AMPA receptor is a tetramer composing of a various combination of the AMPA-type glutamate receptor subunit 1–4 (GluR1–GluR4). GluR2 is of particular interest, because GluR2-containing AMPA receptors are calcium-impermeable and neurons lacking this type of receptors become more vulnerable to excitotoxicity (Kwak and Weiss, 2006). Glutamate receptor interacting protein 1 (GRIP1) is one of the regulators for GluR2 trafficking and the dynamics of GluR2-containing AMPA receptors (Hirai, 2001). Recently, GRIP1 has been identified as an ALS2-interacting protein, and shown to be associated through the N-terminal RLD of ALS2 (Lai et al., 2006). Importantly, loss of ALS2 results in a decrease of GluR2, and thus calcium-impermeable GluR2-containing AMPA receptors, at the synaptic/cell surface of *Als2* KO neurons, rendering neurons more susceptibility to glutamate receptor-mediated neurotoxicity (Lai et al., 2006). This suggests that ALS2 could modulate, through the interaction with GRIP1, AMPA receptor trafficking and contributes to a neuroprotective role against excitotoxicity.

4.4.3. ALS2CL: ALS2 C-terminal like

Recently, we and others have identified a novel ALS2 homolog, ALS2 C-terminal like (ALS2CL) highly homologous to the C-terminal half of ALS2, which is expressed in various tissues (Hadano et al., 2004; Devon et al., 2005). ALS2CL lacks the RLD, but contains the ALS2-like Rab5GEF domain; the MORN/VPS9 domain. ALS2CL exhibits a specific but a relatively weak Rab5-GEF activity with accompanying rather strong Rab5-binding properties (Hadano et al., 2004). Further, ALS2CL shows a different intracellular localization and has different effects on the Rab5-meidated endosomal morphology compared with those of ALS2. As aforementioned, ALS2 forms homo-oligomeric complex through its C-terminal regions (Kunita et al., 2004). Although a majority of ALS2CL is present as a rather dimeric form, it could interact with the ALS2-oligomer, resulting in the formation of the large ALS2/ALS2CL heteromeric complex *in vitro* (Suzuki-Utsunomiya et al., 2007). Importantly, in cultured cells, ALS2CL dominantly suppresses endosome enlargement induced by a constitutively active form of ALS2; ALS2_695-1657aa, ALS2 lacking the RLD, and results in an extensive perinuclear tubulomembranous phenotype co-localizing with microtubules, which are also dependent upon the ALS2CL-ALS2 interaction (Suzuki-Utsunomiya et al., 2007). Thus, ALS2CL is a novel ALS2-interacting protein that may contribute toward understandings of ALS2-mediated endosome dynamics in cells.

5. Conclusions

Since the discovery of the *ALS2* gene as a causative for a number of juvenile recessive MNDs in 2001 (Hadano et al., 2001a; Yang et al., 2001), extensive efforts toward the delineation of the ALS2 functions in normal as well as pathological conditions are being studied as described in this review. Although there are still many questions unanswered, it has become increasingly clear that ALS2 contributes to the small GTPase-mediated molecular as well as cellular events, and implicating in endosome dynamics, cytoskeletal organizations, anti-cytotoxicity, and neuronal development/maintenance (Figs. 2 and 3). Most recently, we have found that ALS2 is also involved in an unfamiliar endocytic mechanism, called macropinocytosis, and the following macropinosome trafficking and fusion (Kunita et al., 2007). Loss of the ALS2 functions due to the *ALS2* mutations ought to perturb such molecular and cellular mechanisms, thereby resulting in cellular dysfunction and ultimately in cell death. There are also increasing evidence accumulating that intracellular vesicle/membrane trafficking and endosome dynamics play crucial roles in the pathogenesis not only in MNDs other than ALS2/PLSJ/IAHSP (Evans et al., 2006; Kanekura et al., 2006; Mannan et al., 2006; Shirane and Nakayama, 2006; Goytain et al., 2007), but also in other conditions of neurodegenerative diseases, such as Alzheimer's disease (Grbovic et al., 2003), frontotemporal dementia (Skibinski et al., 2005), Parkinson's disease (Cooper et al., 2006), and Huntington's disease (Pal et al., 2006). Thus, membrane dynamics and its dysfunction may underlie the pathogenesis of a variety of neurodegenerative diseases, leading to a unifying comprehension of neuronal dysfunction. Nevertheless, it should also be emphasized that further studies, which could fill the gaps between understandings of molecular/cellular function and clinical manifestations, thereby uncovering the mechanisms of a selective neurodegeneration seen in all the neurodegenerative diseases, will be required to develop proper and effective remedies for these devastating diseases.

Acknowledgements

This work was funded by Japan Science and Technology Agency (JST) (to J-EI), the Ministry of Health, Labour and Welfare (to J-EI), a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (to SH), Japan Brain Foundation (to SH), and The Ichiro Kanehara Foundation (to SH). RK and KS-U are supported by a 2006 Tokai University School of Medicine Research Aid, and AO is supported by a Research Fellowship for Young Scientist from JSPS.

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doi:10.1093/brain/awm289 Brain (2008), 131, 229–239

CAG repeat size correlates to electrophysiological motor and sensory phenotypes in SBMA

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Spinal and bulbar muscular atrophy (SBMA) is an adult-onset, lower motor neuron disease caused by an aberrant elongation of a CAG repeat in the androgen receptor (AR) gene. The main symptoms are weakness and atrophy of bulbar, facial and limb muscles, but sensory disturbances are frequently found in SBMA patients. Motor symptoms have been attributed to the accumulation of mutant AR in the nucleus of lower motor neurons, which is more profound in patients with a longer CAG repeat. We examined nerve conduction properties including F-waves in a total of 106 patients with genetically confirmed SBMA (mean age at data collection = 53.8 years; range = 31–75 years) and 85 control subjects. Motor conduction velocities (MCV), compound muscle action potentials (CMAP), sensory conduction velocities (SCV) and sensory nerve action potentials (SNAP) were significantly decreased in all nerves examined in the SBMA patients compared with that in the normal controls, indicating that axonal degeneration is the primary process in both motor and sensory nerves. More profound abnormalities were observed in the nerves of the upper limbs than in those of the lower limbs. F-waves in the median nerve were absent in 30 of 106 cases (28.3%), but no cases of absent F-waves were observed in the tibial nerve. From an analysis of the relationship between CMAPs and SNAPs, patients were identified with different electrophysiological phenotypes: motor-dominant, sensory-dominant and non-dominant phenotypes. The CAG repeat size and the age at onset were significantly different among the patients with motor- and sensory-dominant phenotypes, indicating that a longer CAG repeat is more closely linked to the motor-dominant phenotype and a shorter CAG repeat is more closely linked to the sensory-dominant phenotype. Furthermore, when we classified the patients by CAG repeat size, CMAP values showed a tendency to be decreased in patients with a longer CAG repeat ($\geqslant$47), while SNAPs were significantly decreased in patients with a shorter CAG repeat ($<$47). In addition, we found that the frequency of aggregation in the sensory neuron cytoplasm tended to inversely correlate with the CAG repeat size in the autopsy study, supporting the view that the CAG repeat size differentially correlates with motor- and sensory-dominant phenotypes. In conclusion, our results suggest that there are unequivocal electrophysiological phenotypes influenced by CAG repeat size in SBMA.

Keywords: CAG repeat; spinal and bulbar muscular atrophy; electrophysiological phenotypes; motor-dominant; sensory-dominant

Abbreviations: CMAP = compound muscle action potential; MCV = motor conduction velocity; SBMA = spinal and bulbar muscular atrophy; SCV = sensory conduction velocity; SNAP = sensory nerve action potential

Advance Access publication December 4, 2007

Introduction

Spinal and bulbar muscular atrophy (SBMA) is a hereditary lower motor neuron disease affecting adult males (Kennedy *et al.*, 1968; Sobue *et al.*, 1989, 1993; Fischbeck *et al.*, 1997). The cause of SBMA is an aberrant elongation of a CAG repeat in the androgen receptor (AR) gene. Normally, 9–36 CAGs are observed in the AR gene in normal subjects, but 38–62 CAGs are observed in SBMA patients (La Spada *et al.*, 1991; Tanaka *et al.*, 1996; Andrew *et al.*, 1997). A similar gene mutation has been detected in Huntington's

disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA) and several types of spinocerebellar ataxia (Gatchel *et al.*, 2005). Since CAG is translated to glutamine, these disorders, including SBMA, are called polyglutamine diseases. In SBMA patients, there is an inverse correlation between the number of CAGs and the age at onset (Doyu *et al.*, 1992; Atsuta *et al.*, 2006). The histopathological hallmarks of this disease are an extensive loss of lower motor neurons in the spinal cord and brain stem, together with degeneration of the dorsal root ganglions (DRG) (Sobue *et al.*, 1989; Adachi *et al.*, 2005). Intranuclear accumulations of mutant AR protein in the residual motor neurons are another hallmark (Li *et al.*, 1998; Adachi *et al.*, 2005). The molecular basis for motor neuron degeneration is thought to be testosterone-dependent nuclear accumulation of the mutant AR, and androgen deprivation rescues neuronal dysfunction in animal models of SBMA (Katsuno *et al.*, 2002, 2003; Takeyama *et al.*, 2002; Chevalier-Larsen *et al.*, 2004). Androgen deprivation with a luteinizing hormone-releasing hormone (LHRH) analog also suppresses nuclear accumulation of mutant AR in the scrotal skin of SBMA patients (Banno *et al.*, 2006). Other candidates for potent therapeutics such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) or geranylgeranylacetone (GGA), enhancers of molecular chaperone expression and function, and a histone deacetylase (HDAC) inhibitor have also emerged from studies of animal models of SBMA (Minamiyama *et al.*, 2004; Katsuno *et al.*, 2005; Waza *et al.*, 2005).

The main symptoms of SBMA are weakness and atrophy of the bulbar, facial and limb muscles (Katsuno *et al.*, 2006). The onset of weakness is usually between 30 and 60 years of age. Postural tremor of the fingers is often observed prior to weakness. The symptoms are slowly progressive in SBMA, and the susceptibility for aspiration pneumonia increases as bulbar paralysis develops (Atsuta *et al.*, 2006). The most common cause of death is pneumonia. Many patients also have hypertension, hyperlipidemia, liver dysfunction and glucose intolerance. Serum creatine kinase is increased in the majority of patients.

In addition to motor symptoms, sensory impairment such as vibratory sensory disorder is often observed, and electrophysiological involvement has also been described in sensory nerves of SBMA patients (Harding *et al.*, 1982; Olney *et al.*, 1991; Li *et al.*, 1995; Guidetti *et al.*, 1996; Polo *et al.*, 1996; Ferrante *et al.*, 1997; Antonini *et al.*, 2000; Sperfeld *et al.*, 2002). In addition, sensory nerve axon loss, particularly of the central and peripheral rami of primary sensory neurons, has been documented to be profound (Harding *et al.*, 1982; Sobue *et al.*, 1989; Li *et al.*, 1995). Spinal dorsal column involvement and loss of axons in the sural nerve are common pathological features (Sobue *et al.*, 1989; Li *et al.*, 1995), and abnormalities in sensory nerve conduction and sensory evoked potentials are well known features of SBMA (Kachi *et al.*, 1992). Since the sensory symptoms are not generally severe in most patients, sensory nerve involvement has not been given much attention, particularly when compared to motor symptoms. However, the involvement of primary sensory neurons is one of the major phenotypic manifestations in SBMA (Sobue *et al.*, 1989).

The age at onset and the severity of motor symptoms are variable among SBMA patients (Kennedy *et al.*, 1968; Sperfeld *et al.*, 2002). One of the major factors determining clinical features is the CAG repeat size in the AR gene (Doyu *et al.*, 1992; Atsuta *et al.*, 2006). However, the age at onset and severity are also variable even among the patients with the same CAG repeat size (Doyu *et al.*, 1992; Atsuta *et al.*, 2006), indicating that some unknown genetic or environmental factors may influence the development of clinical heterogeneity (Atsuta *et al.*, 2006). In sensory impairments, there is also a variable degree of severity. Some patients show profound sensory symptoms and sensory nerve electrophysiological abnormalities, while other patients appear almost normal (Olney *et al.*, 1991; Li *et al.*, 1995; Guidetti *et al.*, 1996; Antonini *et al.*, 2000). In contrast to motor symptoms, the age at onset for sensory symptoms is rather difficult to determine, and the role of CAG repeat size in the severity of symptoms and the onset of sensory symptoms is unknown.

In order to clarify motor and sensory nerve involvement in SBMA, we examined nerve conduction properties including F-waves in 106 patients with genetically confirmed SBMA and 85 control subjects. We further analysed the influence of the CAG repeat size within the AR gene on the electrophysiological motor- and sensory-dominancy, as well as the histopathological background underlying the phenotypic diversity in nerve conduction of SBMA patients.

Subjects and Methods

Patients

A total of 106 male patients with the diagnosis of SBMA confirmed by genetic analysis and 85 male normal control subjects were included in this study. The data of SBMA patients were collected between May 2003 and May 2007. We analysed various electrophysiological examinations, motor function, sensory disturbance, disease duration and CAG repeat size in the AR gene in these patients. We defined the onset of disease as when the muscular weakness began, but not when tremor of the fingers appeared. As a functional assessment, we applied the Limb Norris score, Norris Bulbar score and ALS functional rating scale-revised (ALSFRS-R), which are aimed at motor function evaluations of patients with amyotrophic lateral sclerosis (ALS) (Norris *et al.*, 1974; The ALS CNTF Treatment Study (ACTS) Phase I-II Study Group, 1996).

All studies conformed to the ethics guideline for human genome/gene analysis research and the ethics guideline for epidemiological studies endorsed by the Japanese government. The ethics committee of Nagoya University Graduate School of Medicine approved the study, and all SBMA patients and normal subjects gave their written informed consent to the investigation.

Electrophysiological assessments

Motor and sensory nerve conduction studies were performed in the median, ulnar, tibial and sural nerves in 106 patients during their initial clinical assessment at Nagoya University Hospital using a standard method with surface electrodes for stimulation and recording as described previously (Sobue *et al.*, 1989; Kimura, 2001*a*, *b*; Koike *et al.*, 2003; Mori *et al.*, 2005). Motor conduction was investigated in the median, ulnar and tibial nerves, recording from the abductor pollicis brevis, abductor digiti minimi and abductor hallucis brevis, respectively. The following nerve segments were used for calculating motor conduction velocities (MCV): wrist to elbow for the median nerve, wrist to distally at the elbow for the ulnar nerve, and ankle to popliteal fossa for the tibial nerve. Sensory conduction was investigated in the median, ulnar and sural nerves, using antidromic recording from ring electrodes at the second and fifth digit for the median and ulnar nerves, respectively, and bar electrodes at the ankle for the sural nerve. Sensory conduction velocities (SCV) were calculated for the distal segment. Amplitudes of compound muscle action potentials (CMAP) and those of sensory nerve action potentials (SNAP) were measured from the baseline to the first negative peak. Control values were obtained in 56–85 age-matched normal volunteers (31–75 years) (Koike *et al.*, 2001; Mori *et al.*, 2005).

F-waves were also examined in the median and tibial nerves at the same time as the nerve conduction studies using a standard method as described previously (Kimura, 2001*c*). Sixteen consecutive supramaximal stimuli with frequency of 1 Hz were delivered to the median and tibial nerves, while recording from the same muscles as the normal nerve conduction studies. The following variables were estimated: occurrence, minimum latency and maximum F-wave conduction velocity (FWCV). FWCV was calculated using the formula $2D/(F-M-1)$, where D is the surface distance measured from the stimulus point to the C7 spinous process in the median nerves or to the T12 spinous process in the tibial nerves, F is the latency of the F-wave and M is the latency of the CMAP. Control values were obtained in 28–47 age-matched normal volunteers (31–75 years). All nerve conduction studies and F-wave studies were carried out on the right side of the body.

We defined the nerve conduction, CMAPs and SNAPs as abnormal, when these values were less than the mean −2 SD of normal controls on the examined nerves. We also expressed the CMAP and SNAP values as the percentage of the mean values of normal controls, when we need the standardized expression of the degree of CMAP and SNAP involvement as compared to normal controls.

Standard needle electromyography (EMG) was performed using concentric needle electrodes in 93 SBMA patients, with the muscles at rest and during weak and maximal efforts (Sobue *et al.*, 1993; Kimura, 2001*d*; Sone *et al.*, 2005).

Genetic analysis

Genomic DNA was extracted from peripheral blood of SBMA patients using conventional techniques (Tanaka *et al.*, 1996). PCR amplification of the CAG repeat in the AR gene was performed using a fluorescein-labelled forward primer (5′-TCC AGAATCTGTTCCAGAGCGTGC-3′) and a non-labelled reverse primer (5′-TGGCCTCGCTCAGGATGTCTTTAAG-3′). Detailed PCR conditions were described previously (Tanaka *et al.*, 1996, 1999). Aliquots of PCR products were combined with loading dye and separated by electrophoresis with an autoread sequencer SQ-5500 (Hitachi Electronics Engineering, Tokyo, Japan). The size of the CAG repeat was analysed on Fraglys software version 2.2 (Hitachi Electronics Engineering) by comparison to co-electrophoresed PCR standards with known repeat sizes. The CAG repeat size of the PCR standard was determined by direct sequence as described previously (Doyu *et al.*, 1992).

Immunohistochemistry for mutant AR in the sensory and motor neurons

For immunohistochemistry of primary sensory and spinal motor neurons, autopsy specimens of lumbar DRG and spinal cord from five genetically diagnosed SBMA patients (70.4 ± 11.0 years old) were used. The lumbar DRG and spinal cord were excised at autopsy and immediately fixed in 10% buffered formalin solution. The collection of tissues and their use for this study were approved by the Ethics Committee of Nagoya University Graduate School of Medicine. Lumbar DRG and spinal cord sections of 6 μm were deparaffinized, treated with 98% formic acid at room temperature for 3 min and with microwave oven heating for 10 min in 10 mM citrate buffer at pH 6.0, and incubated with an anti-polyglutamine antibody (1C2, 1 : 20 000; Chemicon, Temecula, CA). Subsequent staining procedures are performed using the Envision+ kit (Dako, Glostrup, Denmark).

For quantification of primary sensory neurons in which mutant AR accumulates, we prepared at least 100 transverse sections from the lumbar DRG, and performed 1C2 immunohistochemistry as described above. The frequency of 1C2-positive and -negative cells within the DRG was assessed by counting all the neurons with 1C2-positive cytoplasmic inclusions against total neuronal cells with obvious nuclei on every 10th section under the light microscope (BX51N-34, Olympas, Tokyo, Japan). The results were expressed as frequency of 1C2-positive cells in the 10 sections of the DRG. As for quantification of spinal motor neurons, the detailed procedure has been described previously (Adachi *et al.*, 2005). We have also examined five control autopsied specimens from patients died from non-neurological diseases, and found that there were no 1C2-positive cytoplasmic or nuclear staining.

Data analysis

Quantitative data was presented as means ± SD. Statistical comparisons were performed using the Student's *t*-test. Correlations among the parameters were analysed using Pearson's correlation coefficient. P values less than 0.05 and correlation coefficients (r) greater than 0.4 were considered to indicate significance. Calculations were performed using the statistical software package SPSS 14.0J (SPSS Japan Inc., Tokyo, Japan).

Results

Clinical and genetic backgrounds of SBMA patients

The clinical background of the SBMA patients is described in Table 1. All of the patients examined were of Japanese nationality. The duration from onset assessed from the first notice of motor impairment (Atsuta *et al.*, 2006) ranged from 1 to 32 years. There was no significant difference between the median CAG repeat size in the present study

Table 1 Clinical and genetic features of SBMA patients

Clinical and genetic features	Mean ± SD	Range	*n*
Age at examination (years)	53.8 ± 10.0	31–75	106
Duration from onset (years)	10.1 ± 6.8	1–32	106
Age at onset (years)	43.7 ± 10.4	25–68	106
CAG repeat size in AR gene (number)	47.8 ± 3.1	41–57	97[a]
Limb Norris score (normal score = 63)	53.9 ± 7.3	34–63	99
Norris Bulbar score (normal score = 39)	33.0 ± 4.3	20–39	99
ALSFRS-R (normal score = 48)	41.1 ± 4.3	22–48	99

[a]The abnormal elongation of the CAG repeat was confirmed by gene analysis using agarose gel electrophoresis without determining the repeat number in the remaining nine patients. AR = androgen receptor; ALSFRS-R = ALS functional rating scale-revised

and those reported previously in SBMA patients (La Spada *et al.*, 1991; Tanaka *et al.*, 1996; Andrew *et al.*, 1997).

All patients were ambulatory with or without aid, and none were bed-ridden. The mean Limb Norris score, Norris Bulbar score and ALSFRS-R also suggested that the ADL of patients in this study was not severely impaired. Vibratory sensation disturbance was detected in 78.2% of the SBMA patients. Touch and pain sensation abnormalities were found in 10.9 and 9.1% of the patients, respectively. Joint position sensation was intact in all of the patients examined.

In EMG, all the examined patients showed high amplitude potentials, reduced interference and polyphasic potentials, suggesting neurogenic changes in SBMA.

Nerve conduction and F-wave studies indicate CMAP and SNAP reduction as a profound feature of SBMA

MCV, CMAP, SCV and SNAP were significantly decreased in all the nerves examined in the SBMA patients when compared with those of the normal controls (Table 2). Sensory nerve activity could not be evoked in some cases, whereas activity in the motor nerves was elicited in all patients examined. The most profound finding in the nerve conduction studies was the reduction in the amplitude of the evoked potentials in both motor and sensory nerves. The mean values of CMAPs were reduced to 47–76%, and SNAPs were reduced to 31–47% of the normal mean values. The decrease in conduction velocity was relatively mild, but definitely present in both motor and sensory nerves. The conduction velocity was reduced to 94–96% in MCV and 87–91% in SCV of the normal mean values. The F-wave latencies were also mildly, but significantly prolonged in the median and tibial nerves of SBMA patients. The mean occurrence rate of F-waves in the median nerve was significantly less in SBMA patients, and they were absent in 30 cases (28.3%) (Table 2).

When we compared the CMAP and MCV values of the individual patients in the median, ulnar and tibial nerves, MCV was decreased only in the patients with a severely decreased CMAP (Supplementary Fig. 1). In addition, SCV reduction was observed only in the patients with severely decreased SNAP (Supplementary Fig. 1). These observations strongly suggest that the most profound impairment of the SBMA patients is a reduction of the amplitude of evoked potentials, possibly due to axonal loss (Sobue *et al.*, 1989; Li *et al.*, 1995).

As for the spatial distribution of electrophysiological involvements, the frequency of abnormal values of CMAP was most remarkable in the median nerve followed by the ulnar and tibial nerves (Table 3). The decrease in SNAP was also remarkable in the median and ulnar nerves when compared with those in the sural nerve (Table 3). The absence of F-waves was more frequent in the median nerve than in the tibial nerve (Table 3). These findings indicate that more significant abnormalities in nerve conduction and F-waves are observed in the nerves of the upper limbs than in those of the lower limbs.

Electrophysiologically defined motor and sensory phenotypes

When we analysed the relationship between the degree of motor and sensory nerve involvement by assessing the number of nerves showing abnormally reduced amplitudes (less than control mean − 2 SD) in the sensory (median, ulnar and sural nerves) and motor (median, ulnar and tibial nerves) nerves, we found that the patients could be distinguished by either a motor-dominant, sensory-dominant or non-dominant phenotype (Fig. 1A). It should be noted that there were patients showing only abnormally reduced SNAPs, while the CMAPs were well preserved (Fig. 1A). Alternatively, patients demonstrating CMAPs abnormalities with well preserved SNAPs were also seen (Fig. 1A).

When we analysed the relationship between CMAPs and SNAPs on a standardized scale of percentage of the mean values of normal controls in the median and ulnar nerves (Fig. 1B and C), we found that there were patients with different electrophysiological phenotypes. Some patients showed well preserved CMAPs, being 50% or more of the mean value in the controls, while showing profoundly reduced SNAPs of less than 50% of the mean value in the controls. In contrast, other patients showed well-preserved SNAPs and significantly reduced CMAPs (Fig. 1B and C). Finally, some patients showed a similar involvement of CMAPs and SNAPs. These observations suggest that a subset of SBMA patients shows predominantly motor impairments, while another subset shows predominantly sensory impairments.

The CAG repeat size correlates to electrophysiologically defined motor and sensory phenotypes

Since the CAG repeat size is a key factor dictating clinical presentation in polyglutamine diseases (Zoghbi *et al.*, 2000),