POSSIBLE INTERACTORS OF ALSIN AND EFFECTS OF ITS EXPRESSION ON FOUR MOTOR NEURON DISEASE-RELATED PROTEINS

by

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To my grandaunt

Emine Gökyer

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ABSTRACT

POSSIBLE INTERACTORS OF ALSIN AND EFFECTS OF ITS EXPRESSION ON FOUR MOTOR NEURON DISEASE-RELATED PROTEINS

Motor neuron disorders, characterized by selective motor neuron degeneration, consist of a large group of diseases with overlapping genes and common pathological mechanisms. Mutations in *ALS2*, associated with three types of motor neuron disorders with early age of onset, result in a loss of function of the protein, suggesting a crucial role of the protein product, alsin, in motor neuron integrity. However, the exact function of alsin is not known yet. This study aims to gain insights into alsin's function by investigating its relations both at mRNA and protein levels with proteins that are involved in different motor neuron disorders. Spastin (*Spg4*) and spartin (*Spg20*), selected as representative genes, are involved in upper motor neuron disorders, while heat shock protein B1 (*HspB1*) and heat shock protein B8 (*HspB8*) were selected due to their associations with lower motor neuron disorders. Since *ALS2* mutations result in a loss of function, an *Als2* knock-down stable cell line was generated. Q-RT PCR revealed statistically significant alterations in the expression levels of *Sgp20, Spg4, HspB1* and *HspB8* in this cell line, as compared to the control cell line. For the first time in literature, the inhibitory effect of *Als2* on *HspB1* expression under heat shock conditions implicated a possible role of alsin in heat shock response. At the protein level, it was observed that spartin and alsin were colocalized in the perinuclear region of differentiated neuronal N2a cells. Furthermore, immunoprecipitation studies revealed the co-precipitation of these proteins in the same protein complex, indicating a possible physical interaction. We hope that this thesis, the first study, showing relations of alsin with spartin and HspB1, will contribute to this unexplored field.

ÖZET

ALSİN PROTEİNİNİN OLASI ETKİLEŞİM PARTNERLERİ VE ALSİN EKSPRESYONUNUN MOTOR NÖRON HASTALIĞI İLE İLİŞKİLENDİRİLMİŞ DÖRT PROTEİNE ETKİSİ

Motor nöron hastalıkları (MNH), çeşitli patolojik mekanizmalar sonucu, motor nöronların özgün olarak dejenere olduğu hastalıklar grubudur. MNH'ye neden olan genlerden ALS2'deki mutasyonlar, proteinin işlevini kaybetmesi sonrası üç farklı MNH'nin erken-başlangıçlı tiplerine neden olduğundan, alsinin motor nöronlar için önemli görevleri bulunduğu tahmin edilmektedir. Çalışma çerçevesinde diğer MNH'lere yol açan bazı proteinlerle alsin proteininin mRNA ve protein düzeyindeki etkileşimleri incelenerek alsinin hücre içindeki işlevi hakkında daha detaylı bilgi edinilmesi amaçlanmıştır. Bu kapsamda, spastin (Spg4) ve spartin (Spg20) üst MNH'lerle**,** HspB1 ve HspB8 ise alt MNH'lerle ilişkili olarak alsinin olası etkileşim partnerleri olarak seçilmişlerdir. ALS2 mutasyonları, alsinin hücre içindeki işlevini kaybetmesine neden olduğu için, ilk etapta alsin geni stabil olarak susturulmuş hücre suşları oluşturulmuştur. Spg4, Spg20, HspB1 ve HspB8 genlerinin ekspresyon düzeyindeki değişimleri bu hücrelerde mRNA düzeyinde ölçüldüğünde istatistiksel olarak anlamlı sonuçlar elde edilmiştir. Bu sonuçlardan, ALS2 geni susturulmuş hücrelerin ısı şokuna verdiği cevaptaki azalma, günümüz literatüründe alsini ısı şokuyla ilişkilendiren ilk bulgu olması açısından önemlidir. Protein düzeyindeki deneyler sonucunda ise, alsin ve spartinin hücre çekirdeğine yakın bir bölgede birlikte bulundukları ve aynı protein kompleksi içinde çöktükleri gözlenmiştir. Bu sonuçlar alsin ve spartin proteinlerinin fiziksel olarak etkileşim içinde olabileceğine işaret etmektedir. Alsin ile spartin ve ısı şoku proteini B1 arasındaki etkileşimleri literatürde ilk defa gösteren bu tezin, henüz az araştırılmış bu alana katkı sağlayacağı umulmaktadır.

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O_2^-	Superoxide
^oC	Centigrade degree
V	Volt
v	Volume
w	Weight
µg	Microgram
µl	Microliter
Zn	Zinc

LIST OF ACRONYMS/ABBREVIATIONS

AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ALS2	Alsin
ANG	Angiogenin
APS	Amonium Persulfate
AR	Autosomal Recessive
ATXN2	Ataxin2
BSA	Bovine Serum Albumine
CYP7B1	Cytochrome P450, Family 7, Subfamily B, Polypeptide1
C9ORF72	Chromosome 9 Open Reading Frame 72
CMT	Charcot Marie Tooth
CNS	Central Nervous System
Cnt	Control
CO_2	Carbondioxide
Cu	Cupper
Cu^{+2}	Cupper Ion
DAO	D-amino acid oxidase
Dbl	Diffuse B Cell Lymphoma
DCTN1	Dynactin
DES	Desmin
DH	Diffuse B Cell Lymphoma Homologous Domain
DHMN	Distal Hereditary Motor Neuropathies
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EEA1	Early Endosome Antigen 1
EAAT2	Excitatory amino acid transporter 2
fALS	Familial Amyotrophic Lateral Sclerosis
FBS	Fetal Bovine Serum
FTD	Frontotemporal Dementia

FTLD	Frontotemporal Lobar Degeneration
FTLD-U	Frontotemporal Lobar Degeneration with Ubiquitinated Inclusions
FUS	Fused in Sarcoma
GDP	Guanosine Diphosphate
GEF	Guanine-nucleotide Exchange Factor
GRIP1	Glutamate Receptor Interacting Protein1
GTP	Guanosine Triphosphate
H_2O	Water
H_2O_2	Hydrogen Peroxide
HS	Horse Serum
HspB1	Heat Shock Protein B1
HspB8	Heat Shock Protein B8
HSP	Hereditary Spastic Paraplegia
IAHSP	Infantile-onset Ascending Hereditary Spastic Paraplegia
IF	Immunoflourescence
IgG	Immunoglobulin G
IP	Immunoprecipitation
JPLS	Juvenile Primary Lateral Sclerosis
kb	Kilobase
kDa	Kilodalton
KIF5A	Kinesin Heavy Chain Isoform 5A
L1CAM	L1 cell adhesion molecule
LB	Lysogeny Broth
LMN	Lower Motor Neuron
LMND	Lower Motor Neuron Disease
mA	Miliamper
MAPT	Microtubule associated protein tau
MEM	Modified Eagle Medium
MIT	Microtubule-interacting and Endosomal Trafficking
mg	Miligram
$MgCl_2$	Magnesium Chloride
Min	Minutes

ml	Mililiter
mm	Millimeter
MN	Motor neuron
MND	Motor Neuron Disorders
MORN	Membrane Occupation and Recognition Nexus
MT	Microtubule
mt	Mutant
N2a	Neuroblastoma 2A
NaCl	Sodium Chloride
ND	Neurodegenerative diseases
NEAA	Non-esential Amino acid
NF	Neurofilaments
ng	Nanogram
Nipa	Non-imprinted in Prader-Willi/Angelman syndrome region protein 1
OH^-	Hydroxyl radicals
OPTN	Optineurin
PD	Parkinson's disease
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pen/Strep	Penicillin/Streptomycin
PH	Pleckstrin Homology Domain
PLS	Primary Lateral Sclerosis
PLP1	Proteolipid Protein 1
pmole	Picomole
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
Rab5	Ras-related in Brain
Rac1	Ras-related C3 Botulinum toxin Substrate 1
Ran	Ras-related Nuclear GTPase
Ras	Rat Sarcoma Subfamily
RCC1	Regulator of Chromosome Condensation 1
Rho	Ras Homologous Member
Reep1	Receptor Accessory Protein 1

RIPA	Radio Immunoprecipitation Assay
RLD	RCC1-like Domain
ROS	Reactive Oxygen Species
rpm	Rotations per Minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RTN1	Reticulon
sALS	Sporadic Amyotrophic Lateral Sclerosis
SCA2	Spinocerebellar Ataxia Type-2
sHSP	Small Heat Shock Protein
SLC33A1	Solute Carrier Family 33 (acetyl-CoA transporter), Member 1
SMA	Spinal Muscular Atrophy
SMN1	Survival Motor Neuron 1
SnRNP	Small Nuclear Ribonucleoproteins
SBMA	Spinobulbar Muscular Atrophy
SDS	Sodium Dedocyl Sulfate
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
sec.	Seconds
SETX	Senataxin
shRNA	Short Hairpin RNA
SIGMAR	σ Non-opioid receptor 1
SOD1	Superoxide Dismutase1
SPG4	Spastin
SPG11	Spatacsin
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TDP-43	43 kDa TAR DNA binding protein
TSE	Transmissible Spongiform Encephalopathies
TEMED	Tetramethylethylenediamine
TLS	Translocated in Liposarcoma
UBQLN2	Ubiquitin-like Protein 2
UPS	Ubiquitin-Proteasome System
VABP	Vesicle-associated Membrane Protein-associated Protein B
VCP	Valosin-containing protein

VEGF	Vascular Endothelial Growth Factor
VPS9	Vacuolar Protein Sorting 9
WB	Western Blot
Wt	Wild-Type
ZFYVE27	zinc finger FYVE (Fab-1, YGL023, Vps27, and EEA1) domain containing 27

1. INTRODUCTION

Neurodegenerative diseases (NDs) include a large group of late-onset disorders, like Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FTD), polyglutamine diseases, transmissible spongiform encephalopathies (TSEs) and amyotrophic lateral sclerosis (ALS). Approximately 7 million people in the United States are suffering from these diseases (Lunn *et al*., 2011). Since aging is the main risk factor for NDs, this number may rise up to 13.2 million by 2050 due to increasing average life expectancy of the population with the advances in science and technology (Hebert *et al*., 2003). The situation is similar for the European countries. For instance, in the case of AD, it is estimated that in the next 50 years the number of patients will be 3 times larger than it was in the beginning of 2000s (Forman *et al*., 2004). Considering these facts, it is crucial to study the mechanisms underlying neurodegenerative diseases in order to find therapeutical approaches.

NDs are characterized by the selective degeneration of different types of neurons in distinct regions of the brain and the spinal cord. Although all of NDs have diverse clinical symptoms due to the region of the neuronal impairment, insoluble aggregate formation of various misfolded proteins is a common feature of these diseases (α-synuclein in PD, huntingtin in HD, amyloid-β in AD, SOD1 or TDP43 in ALS) (Soto, 2003; Double *et al*., 2010). Even though the exact pathological mechanisms are not very well known, some common cellular themes like ubiquitin-proteasome system (UPS) impairment, oxidative stress, mitochondrial dysfunction, axonal and endosomal transport deficits and neuroinflammatory events are emerging in the last decade (Jellinger, 2009).

According to the clinical symptoms, NDs can be categorized into 3 main groups in which only the cognition (eg. AD) or only the movement (eg. motor neuron disorders) or both the cognition and movement of the patients (eg. dementia with Lewy Bodies) are affected (Double *et al*., 2010).

1.1. Motor Neuron Disorders (MNDs)

In humans, voluntary movements are controlled by the upper motor neurons (UMNs) in the motor cortex and the lower motor neurons (LMNs) in the brainstem and the ventral horn of the spinal cord (Figure 1.1). By making direct or indirect synapses, UMNs and LMNs carry the signals from the motor cortex to the voluntary muscles (Baumer *et al.*, 2010).

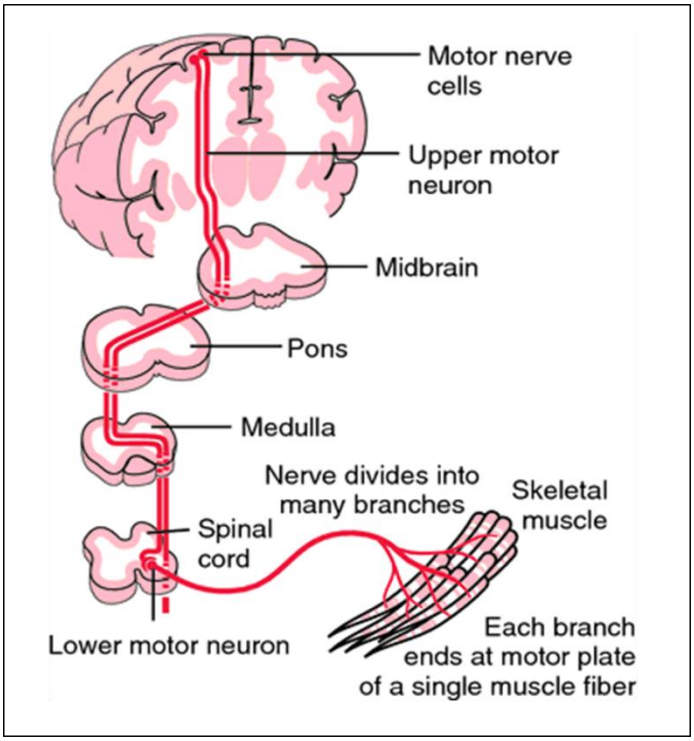

Figure 1.1. Upper and lower motor neurons (Damjanov, 2000).

Motor neurons (MNs) are highly differentiated and specialized cells with very long axons which can be more than 1 m long (nearly 99% of the total cell volume) in the human body (Blackstone *et al.*, 2010). Considering their extraordinary shape, intracellular transport and protective mechanisms against oxidative and heat stress conditions should be well functioning in MNs. Even a small impairment in one of these mechanisms may have deleterious effects on the neuron integrity leading to MN specific disorders.

Motor neuron disorders (MNDs) is a subgroup of NDs categorized with the selective degeneration of MNs leading to spasticity, muscle weakness, atrophy or paralysis (Sau *et al.*, 2011). According to the type of effected motor neuron, there are three categories of MNDs: those with LMN degeneration, those in which UMNs degenerate and those involving both of the UMN and LMN degeneration (Figure 1.2) (Figlewicz and Orrell, 2003).

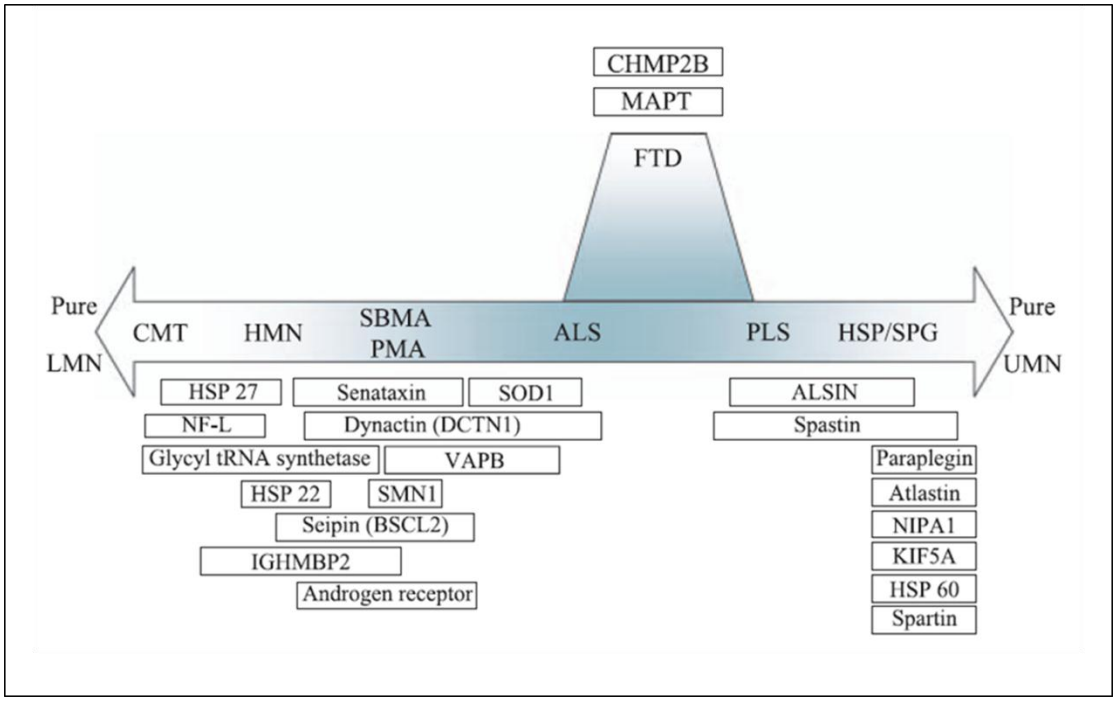

Figure 1.2. Motor Neuron Disorders (Andersen *et al*., 2007).

1.1.1. Upper Motor Neuron Disorders

Upper MNDs consist of two diseases which are Primary Lateral Sclerosis (PLS) and Hereditary Spastic Paraplegia (HSP). Due to selective UMN degeneration, lower limb spasticity and weakness are common clinical symptoms for this group of disorders (Gordon *et al*., 2009).

PLS is a rare MND which was first described by Jean-Martin Charcot in 1865 (Charcot, 1865). There are two types of this disease, a sporadic form (PLS type-1) and a juvenile form (JPLS). *ALS2* is the only gene associated with JPLS. Although selective UMN degeneration is a hallmark for PLS, after years from the diagnosis, there are patients who exhibit LMN degeneration too, which indicates that there may be some pathological overlaps with a mixed type MND, ALS (Rouleau *et al.*, 2009).

Hereditary spastic paraplegias (HSPs) are a heterogeneous group of disorders with autosomal dominant (AD), autosomal recessive (AR) and X-linked recessive inheritance. According to clinical symptoms, there are two forms of HSPs: pure (uncomplicated) form with spasticity in lower limbs only; and a complex (complicated) form in which many additional neurological features like deafness, optic atrophy or mental retardation can be observed in patients (Salinas *et al.*, 2008).

There are 45 loci and 20 genes which are associated with HSP (Table 1.1). Causative genes can be divided into 4 groups considering their functions. The proteins which are encoded by the first group of genes, are directly or indirectly involved in axonal or endosomal trafficking. Among these, the mutations in the *SPG4* (encoding the protein spastin) are the most common (nearly 40% of AD cases) causative factors. This may be the result of microtubule severing activity of the spastin which is crucial for the transport of cargoes and microtubule integrity during cytokinesis and axonal branching (Salinas *et al*., 2007). In addition to HSP, mutated *SPG4* also causes a juvenile form of ALS suggesting a common mechanism (Meyer *et al*., 2005). The protein products of the genes in the second group are related to mitochondria. Mutated forms of REEP1, HSP60 and paraplegin cause abnormalities in the mitochondrial respiratory chain and oxidative stress response and also disrupt the protein quality control processes in mitochondria (Salinas *et al.*, 2008). The third group consists of *PLP1*, *SPG35* and *L1CAM*. They encode the proteins which are necessary for the myelination process of the neurons in the central nervous system (CNS). However, the relationship between the UMN degeneration and myelination is not known in detail (Blackstone *et al*., 2010).

The roles of the remaining protein products are very diverse. For example while the KIAA0415 protein which is responsible for autosomal dominant HSP is involved in DNA repair, CYPB1 has implications in cholesterol metabolism. This diversity shows how much complex and multifaceted the pathological mechanisms for MNDs can be.

Table 1.1. The causative genes of HSP and their functions (Blackstone *et al*., 2011).

Gene Symbol	Protein name	Cell biological function
Membrane traffic and axonal transport		
SPG3A	Atlastin	ER morphogenesis/BMP signalling
SPG4 (SPAST)	Spastin	ER morphogenesis/Endosomal trafficking/ BMP signaling/Cytokinesis/Cytoskeletal regulation
SPG6 (NIPA1)	NIPA1	Endosomal trafficking/ BMP signalling
SPG8 (KIAA0196)	Strumpellin	Endosomal morphogenesis/Cytoskeletal regulation
SPG10	KIF5A	Microtubule-based motor proteins
SPG11	Spatacsin	Membrane traffic?
SPG15(ZFYVE26)	Spastizin	Membrane traffic?/Cytokinesis
SPG17 (BSCL2)	Seipin	ER membrane protein/Lipid droplet biogenesis
SPG20	Spartin	Endosomal trafficking/BMP signalling Lipid droplet biogenesis/Mitochondrial functions?
SPG21	Maspardin	Endosomal trafficking
Mitochondrial		
SPG31	REEP1	Mitochondrial chaperone?
SPG13 (HSPD1)	HSP60	Mitochondrial chaperone
SPG7	Paraplegin	Mitochondrial protease
Myelination		
SPG2 (PLP1)	PLP	Myelin protein
SPG35	Fatty acid 2-hydroxylase	Hydroxylation of myelination lipids
SPG1 (LICAM)	L1CAM	Cell adhesion and signalling
Miscellaneous		
SPG5 (CYP7B1)	CYP7B1	Cholesterol mechanism
SPG39	Neurotrophy target esterase	Phospholipid homeostasis/Target of organophosphates
SPG42	SLC33A1	Acetyl-CoA transporter
SPG48(KIAA0415)	KIAA0415	DNA repair

1.1.2. Lower Motor Neuron Disorders

Spinobulbar muscular atrophy (SBMA or Kennedy's disease) and spinal muscular atrophy (SMA) are typical lower MNDs (Figlewicz and Orrell, 2003). In addition to SBMA and SMA, distal hereditary motor neuropathies (dHMN) and Charcot Marie Tooth (CMT) disease can be also added to this group of disorders due to the distal motor neuron weakness (James and Talbot, 2006).

SBMA is an X-linked polyglutamine disorder; its clinical features are weakness, cramps, fasciculations and waste of the limb and face muscles (Poletti, 2004). Expanded CAG triplet repeats in the androgen receptor (AR) gene are associated with SBMA (La Spada *et al.,* 1991). These repeats cause conformational changes in the protein structure leading to aggregate formation. Mutated protein also sequesters several chaperones and the components of the ubiquitin-proteasome system (UPS) into these aggregates. Although the exact pathological mechanism is not known, it has been proposed that impairments in the protein clearance system of the cells due to intracellular aggregation formation, may lead to neurodegeneration in motor neurons (Williams and Paulson, 2008; Rusmini *et al*., 2010).

SMA is a fatal, autosomal recessive disorder, severely affecting infants and children. According to age of onset and motor functions, SMA is subdivided into four groups. Type-1 with an onset of 6 months, is lethal in two years. Patients with SMA type-2 show the first symptoms between 7 and 18 months and are not be able to walk independently. Type-3 and Type-4 patients with an onset age older than 18 months and 18 years, respectively, have milder symptoms of the disease (D'Amico *et al.*, 2011). All of these types of SMA are associated with the survival motorneuron 1 gene *(SMN1*) (Lefebvre *et al.*, 1995). SMN1 is a ubiquitously expressed protein with the highest levels in spinal cord (Battaglia *et al*., 1997). It functions in snRNP (small nuclear ribonucleoproteins) biogenesis, splicing machinery and axonal transport in motor neurons (Rossoll and Bassel 2009; Setola *et al.*, 2007). Because all of the mutations lead to loss of function mechanism, SMN1 might have a crucial role in MN integrity.

CMT, also known as hereditary motor and sensory neuropathy, is a heterogenous group of disorders affecting both the lower motor neurons and the sensory peripheral nerves. CMT patients have sensory abnormalities like loss of pain or temperature sensation, in addition to LMN disease symptoms (Szigeti and Lupski, 2009). Although there are many genes identified in CMT pathology with different functions, the result of the mutated protein products is impaired axonal transport (Pareyson and Marchesi, 2009). DHMNs are one of the subtypes of CMT without a sensory neuropathy. Unlike CMT, causative genes for nearly 80% of the cases are undefined. Among the known 20%, there are many overlapping genetic factors with CMT2 (*HSPB1, HSPB8, BSCL2, GARS, TRPV4*); and a juvenile form of ALS (*SETX*) and HSP (*BSCL2*) (Rossor *et al.*, 2011).

1.1.3. Mixed Type Motor Neuron Disorders: ALS

Amyotrophic Lateral Sclerosis (ALS) is a rapidly progressive, adult-onset motor neuron disorder, characterized by the selective degeneration of the upper motor neurons in the motor cortex and the lower motor neurons in the brain stem and ventral horn of the spinal cord. Degeneration of the motor neurons causes progressive muscle atrophy (Figure 1.3), weakness, fasciculations and spasticity. Fatal event is the respiratory muscle wasting generally occurring after 2-5 years from the onset of the disease (Rowland and Schneider, 2001).

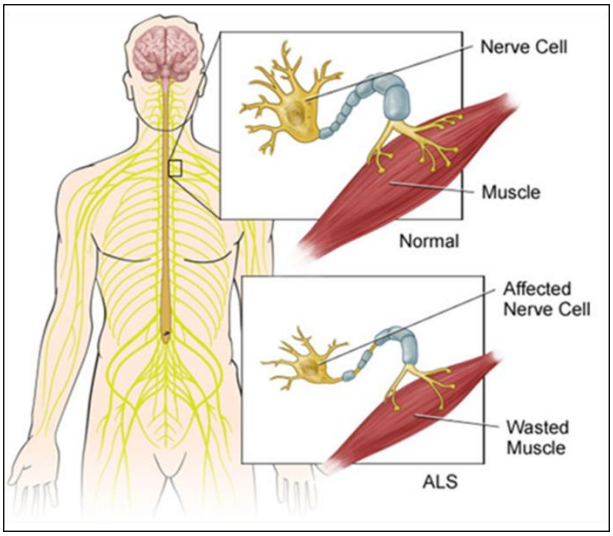

Figure 1.3. Muscle atrophy in ALS (yalemedicalgroup.org).

The symptoms of ALS were first reported in 1848 by a French neurologist François-Amilcar Aran in a 43-year-old woman who died after 2 years from the diagnosis (Aran, 1848). In 1869, another French neurobiologist and physician Jean-Martin Charcot described the disease as Amyotrophic Lateral Sclerosis (ALS) (Charcot *et al.*, 1869). In the memory of the famous baseball player Lou Gehrig, ALS is also known as Lou Gehrig's disease in the United States.

The incidence of ALS is 1-2 per year, and prevalence of ALS is approximately 2-4 per 100,000. Typical age of onset is 55 to 60 years, and the risk of getting ALS decreases rapidly after the age of 70. Although the number of male sALS patients were reported to be larger than females in past, the male/female ratio is getting closer to 1 according to the recent studies in Europe (Sathasivam, 2010; Logroscino *et al*., 2011).

1.1.3.1 Genetics of ALS. ALS can be inherited in autosomal dominant, autosomal recessive or X-linked manner. Only 5-10% of ALS cases have a familial history (fALS), the remaining 90-95% of cases are sporadic (sALS); interesting is that fALS and sALS are clinically indistinguishable. There are 19 genes associated with ALS recently (Table 1.2) (Ticozzi *et al*., 2011).

The genetics of ALS was not very well known, until a novel gene called superoxide dismutase 1 (*SOD1*) was discovered in 1993 (Rosen *et al*., 1993). *SOD1* is the major gene in ALS pathology causing 15-20% of fALS and 1-2% of the total ALS cases (Kabashi *et al.*, 2007). The protein product SOD1 which is a stable metal bound homodimer, inhibits the production of reactive oxygen species (ROS) by converting harmful superoxide radicals to molecular oxygen and hydrogen peroxide (Beckman *et al*., 1990). There are 166 reported SOD1 mutations (147 missense, 19 nonsense and deletion) leading to a gain of toxic function mechanisms (Andersen and Chalabi, 2011).

Another major progress in ALS genetics was achieved recently with the identification of 2 DNA/RNA binding proteins, residing in ubiquitinated protein aggregates of ALS and FTLD-U (frontotemporal lobar degeneration with ubiquitinated inclusions) patients. These were TDP-43 (43 kDa TAR-DNA binding protein) and FUS (fused in sarcoma) or TLS (translocated in liposarcoma) (Neumann *et al*., 2006; Arai *et al*.,

2006; Kwiatkowski *et al*, 2009; Vance *et al*., 2009). TDP43 and FUS together account for 4-5% of the fALS and 1-2% of sALS cases. The discovery of these proteins was very tantalizing because a new scenario, RNA metabolism, was emerged in pathological mechanisms for ALS (Tourenne and Cleveland, 2009).

The last two years were also extremely exciting regarding the new discoveries in ALS genetics. In 2010, Elden *et al*. showed that the intermediate-length polyQ expansions (between 23 to 33) in the ataxin 2 (*ATXN2*), gene which is when mutated associated with Spinocerebellar Ataxia Type-2 (SCA2), is a risk factor for ALS. This was the first link between the repeat expansions and ALS and was soon followed with the milestone discovery of hexanucleotide repeat expansions in a novel C9ORF22 gene in ALS and FTD patients (Renton *et al.*, 2011; Hernendaz *et al*., 2011).

Again in 2011, a new gene, *UBQLN2*, was identified as a causative factor in ALS with an X-linked dominant pattern of inheritance (Deng *et al.*, 2011). In the postmortem spinal cord tissues of patients with UBQLN2 mutations, UBQLN2-positive skein like inclusions were shown, containing proteins like TDP43, FUS, p62 and ubiquilin. UBQLN2 inclusions were also present in all sporadic ALS (sALS), familial ALS (fALS) and ALS-dementia patients without *UBQLN2* mutations; this suggests a common pathological mechanism for all types of ALS (Daoud and Rouleau, 2011).

Table 1.2. Genes associated with ALS (Ferraiuolo *et al.*, 2011).

Genetic Subtype	Chrm. Locus	Gene	Onset and Inheritance	Reference
Oxidative Stress				
ALS1	21q22	Superoxide dismutase 1 (SOD1)	Adult/AD	Rosen *et al.*, 1993
RNA Processing				
ALS4	9q34	Senataxin (SETX)	Juv./AD	Chen *et al.*, 2004
ALS6	16p11.2	Fused in sarcoma (FUS)	Adult/AD	Kwiatkowski *et al.*, 2009
ALS9	14q11.2	Angiogenin (ANG)	Adult/AD	Greenway *et al.*, 2006
ALS10	1p36.2	TAR DNA Binding protein (TARDBP)	Adult/AD	Sreedharan *et al.*, 2008
Endosomal Trafficking and Cell Signaling				
ALS2	2q33	Alsin (ALS2)	Juv./AR	Yang *et al.*, 2001
ALS11	6q21	Polyphosphoinositide phosphatase	Adult/AD	Chow *et al.*, 2009
ALS8	20q13.3	Vesicle-associated membrane protein-associated protein B (VAPB)	Adult/AD	Nishimura *et al.*, 2004
ALS12	10p13	Optineurin (OPTN)	Adult/AD/	Maruyama *et al.*, 2010
Glutamate Excitotoxicity				
ND	12q24	D-amino acid oxidase (DAO)	Adult/AD	Mitchell *et al.*, 2010
Ubiquitin/ protein degradation				
NDx	9p13-p12	Valosin-containing protein (VCP)	Adult/AD	Johnson *et al.*, 2010
ALSX	Xp11	Ubiquilin 2 (UBQLN2)	Adult/X-linked	Deng *et al.*, 2011
Cytoskeleton				
ALS-PD	17q21	Microtubule associated protein tau (MAPT)	Adult/AD	Hutton *et al.*, 1998
Axonal Transport				
ALS	2p13	Dynactin (DCTN1)	Adult/AD	Puls I *et al.*, 2003
Other genes				
ALS5	15q15-q21	Spatacsin (SPG11)	Juv./AR	Orlacchio *et al.*, 2010
ALS-FTD	9p13.3	σ Non-opioid receptor 1 (SIGMAR1)	Adult/AD Juv/AR	Luty *et al.*, 2010 Al-Saif *et al.*, 2011
ALS-FTD	9q21-q22	Chromosome 9 open reading frame (C9ORF72)	Adult/AD	Hosler *et al.*, 2000 Renton *et al.*, 2011 J-Hernandez *et al.*, 2011
Unknown genes				
ALS3	18q21	Unkown	Adult/AD	Hand *et al.*, 2002
ALS7	20p13	Unkown	Adult/AD	Sapp *et al.*, 2003

1.1.3.2. Molecular mechanisms in ALS pathology. Several mechanisms have been proposed to explain the selective motor neuron degeneration in ALS (Figure 1.4). Most of these mechanisms are based on SOD1-linked pathology, because its discovery was the primary milestone in ALS pathogenesis. However with respect to recent findings of the last two years, RNA metabolisms and UPS impairments would be also important factors in ALS pathogenesis.

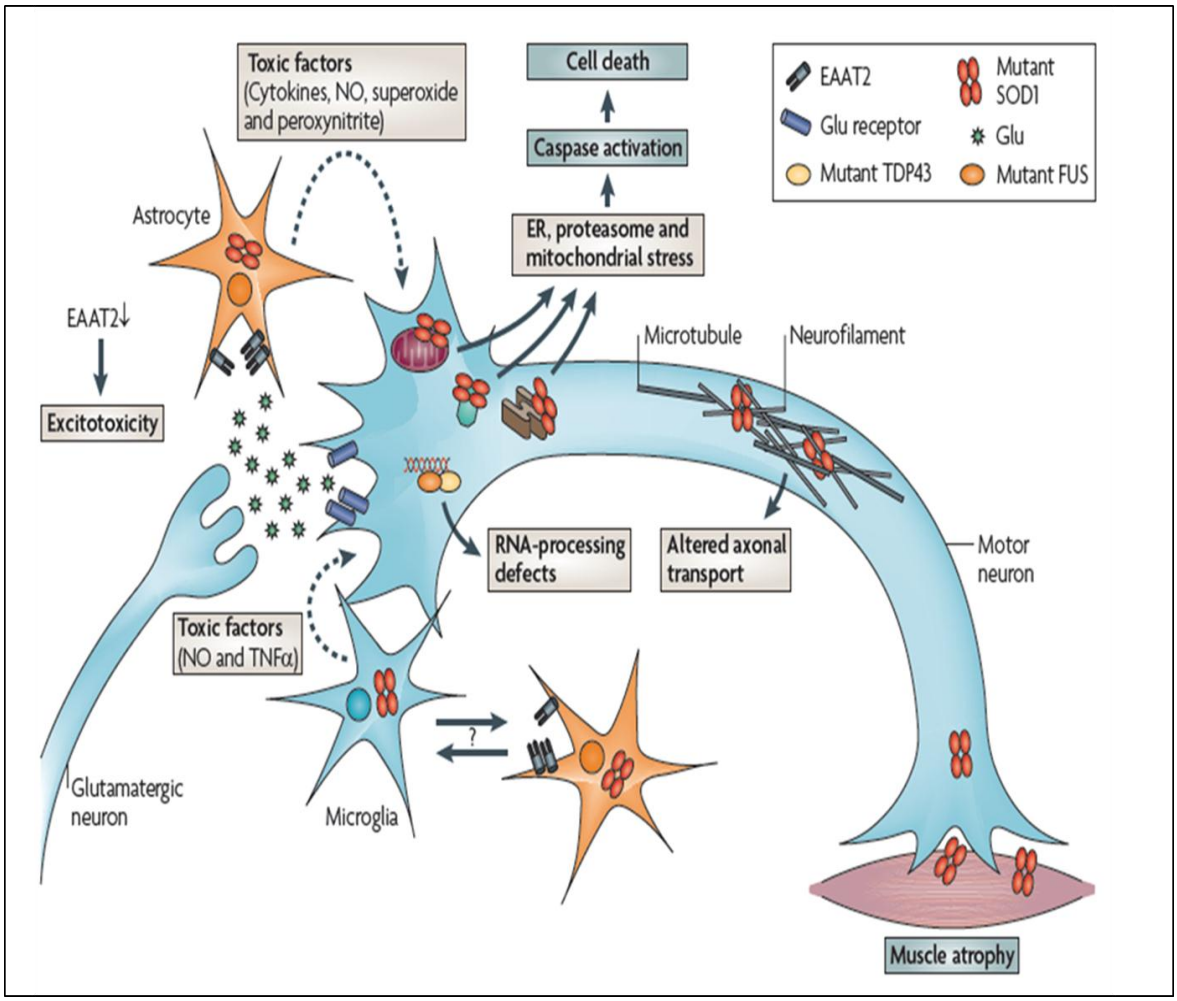

Figure 1.4. Proposed mechanisms for MN degeneration in ALS (Dion *et al.*, 2009).

8 mechanisms which were proposed for ALS pathology, are explained below:

- Oxidative Stress: Reactive oxygen species (ROS) are bi-products of the aerobic metabolism with harmful effects on proteins, lipids and also nucleic acids. Malfunctions in the ROS balancing system of the cell result in oxidative stress which will lead to motor neuron neurodegeneration (Barber *et al*., 2010). For instance, evidence of oxidative damage has been found in postmortem tissues of sALS and SOD1-related fALS patients (Abe *et al*., 1995; Beal *et al*., 1997). In addition, oxidative damage is also observed in both, cell culture and mouse models of ALS (Cookson *et al*., 2002; Poon *et al*., 2005). Although it has been initially proposed that the mutations will inhibit the dismutase activity of SOD1, leading to increasing ROS levels, observation of some properly functioning mutated SOD1 molecules eliminated this hypothesis. Thus, for SOD1-linked pathology in ALS is rather a gain of toxic function (GOF), than a loss of function (LOF). The mechanism of GOF is still elusive (Barber *et al*., 2010).

- Protein Aggregates: The presence of disease-specific protein aggregations are a hallmark for each neurodegenerative disease. In ALS, aggregations including SOD1, TDP43, FUS, VCP and UBQLN2 are observed in patients and even in some animal models. In addition, some other essential proteins like chaperons, members of UPS or even anti-apoptotic proteins can also be recruited to aggregations via interaction with the mutant proteins. It is still unknown, if these inclusions are the reason or consequence of the disease. But they have negative effects, like intracellular toxicity or impairment of the axonal transport on motor neurons, subsequently leading to cell death (Pasinelli and Brown, 2006; Ferraiuolo *et al.*, 2011).

- Mitochondrial Dysfunction: After Gurney *et al*., observed structurally altered mitochondria in SOD1 mouse models, ALS became a member of mitochondriopathies (Gurney *et al*., 1994). In mitochondrial dysfunction, mutant SOD1 may have two effects: (i) by accumulating in the outer surface of the mitochondria, SOD1 may inhibit the transportation of some proteins from cytosol to the mitochondrial matrix or (ii) by interacting with mitochondrial proteins with roles in energy metabolism and Ca^{2+} buffering, it may inactivate some of them, which also will lead to mitochondrial dysfunctioning (Liu *et al.*, 2004).

- Glutamate Excitotoxicity: Glutamate is the most common neurotransmitter in the CNS. During neurotransmission, if the excessive glutamate is not taken from the synaptic cleft immediately, the glutamate receptors will be over activated. This will lead to disruption in Ca^{2+} homeostasis inside the cell and can trigger apoptosis (Ilieva *et al.*, 2009). Excitatory amino acid transporter 2 (EAAT2) is one of the glutamate re-uptake molecules, transporting the glutamate from the synapses into the astrocytes. For most of the sALS cases and mouse models, the function of EAAT2 is lower than the control cases (Rothstein *et al.*, 1992; Fray *et al.*, 1998). This reduction can be explained by the negative effect of oxidative stress on EAAT2 due to SOD1 mutations or splicing defects which are observed in some sALS patients (Trotti *et al.*, 1999; Banner *et al.*, 2002).

- Altered Axonal Transport: Efficient transportation of cargoes, like proteins, RNA, mitochondria, ribosomes, endosomes and synaptic vesicles is very important for the motor neuron integrity. However, because of their extremely long axons, MNs are very fragile and thus sensitive to axonal transport defects (Sau *et al*., 2011). Among the molecular motors of axonal transport, dynactin is the only protein which is associated with ALS. A single base pair change on the gene disrupts the microtubule (MT) binding domain of the protein leading to loss of affinity on binding MTs (Puls *et al.*, 2003). Observations of retarding transportation of cargoes in G93A and G73R SOD1 mice, made SOD1 involve in axonal transport pathology, however how SOD1 impairs the axonal transport is still not known (Zhang *et al.*, 1997; Wiliamson and Cleveland, 1999).

- Dysregulated Endosomal Trafficking: In addition to axonal transport, endosomal trafficking is also a very crucial cellular mechanism for motor neurons. Via endocytosis, extracellular communication signals, like hormones or growth factors are taken inside the cell and transported to the relevant organelles. In addition, during the synaptic transmission, the production of the synaptic vesicles also depends on endocytosis. Alsin is the first endocytic protein associated with the juvenile form of ALS. The mutated form of alsin, which is unfunctional, leads to abnormalities, like enlarged endosomes disrupting endosome trafficking (Yang *et al.*, 2002).

- Role of non-neuronal cells: Although ALS is a result of selective degeneration of motor neurons, mouse models expressing mutant SOD1 only in motor neurons failed to represent the disease phenotype properly. This suggests that there is a complex, non-cell autonomous disease mechanism. To support this idea, Clement *et al.* generated chimeric mice expressing mutant human SOD1. As a result, they found out that healthy non-neuronal cells reduced the degeneration of mutant SOD1 expressing motor neurons and extend the life span of mutant mice. Reciprocally, the mutant non-neuronal cells had toxic effects on wild type motor neurons, but they could not trigger the degeneration by themselves (Clement *et al.*, 2003). Later on, with more detailed studies, the effects of astroglial and microglial cells were defined. When the expression of mutant SOD1 in both astrocytes and microglia is suppressed, the disease progression slows down in SOD1 mutant mice (Yamanaka *et al.*, 2008; Wang *et al.*, 2009). There are also some other promising studies about the effects of T lymphocytes and Schwann cells on motor neuron degeneration (Ilieva *et al.*, 2009).

- Dysregulated Transcription and RNA Processing: The discovery of two RNA/DNA binding proteins, TDP43 and FUS, brought a new point of view to pathologic mechanisms leading to ALS. TDP43-positive inclusions are found in SOD1-negative forms of fALS, FTLD-U cases and also in some other NDs (AD and PD). However, FUS-positive inclusions are observed in ALS (due to FUS mutations), FTLD-U and some polyglutamine disorders (HD and SCA). The pathologic mechanisms rising from these inclusions are still not understood but there are two major hypotheses. According to the loss of function hypotheses, because the normal nuclear localizations of both proteins are disrupted, their proper functioning on RNA metabolism will be impaired. However, a gain of function can also be postulated considering the toxic effects of cytoplasmic aggregations in many cellular processes (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009; Colombrita *et al.*, 2011). In addition to *TDP43* and *FUS*, two other genes (*SETX* and *ANG*), implicated in RNA processing, are also defined as causative factors for ALS, but since they are not as common as TDP43 and FUS, there are not many functional studies on the toxic effects of SETX and ANG (Blitterswijk and Landers, 2010).

1.2. Alsin

ALS2 is a causative gene for one of the juvenile forms of ALS, infantile-onset ascending HSP and a juvenile form of PLS (Yang *et al.*, 2001; Hadano *et al.*, 2001; Pierre *et al.*, 2002; Devon *et al.*, 2003). It resides in chromosome 2q33 and has 33 introns and 34 exons. There are two transcripts for *ALS2*, a long form (6394 nt) and a short form (2657 nt), which are produced by alternative splicing. Until today 19 different mutations in 17 families (4 novel mutations in 2 different families) have been defined in *ALS2* (Table 1.3). These mutations are predicted to cause a premature stop codon in the protein leading to the production of a truncated version of alsin, which is nonfunctional (Hadano *et al.*, 2010).

ALS2 encodes the ubiquitously expressed alsin, with the highest expression levels in the central nervous system. Although the 3-D structure of alsin is not defined yet, based upon sequence homology, it has been proposed that it is composed of three putative guanine nucleotide exchange factor (GEF) domains and 8 MORN (membrane occupation and recognition nexus) motifs (Figure 1.5). Although the exact function of alsin is not known, with its diverse GEF domains, it is categorized as a regulator and activator of small GTPases (Hadano *et al.*, 2001).

Figure 1.5. Primary structure of alsin (Topp *et al.*, 2004).

Table 1.3. Mutations reported in *ALS2*.

Name	Type	Ex./Int.	Origin	Mutation type	Disease	Age of onset	Mutated Protein	Reference
261delA	Del.	Ex.3	Tunusian	Frameshift	JALS	3-10 years	Ala46fsx50	Hadano *et al*., 2001; Yang *et al*., 2001
1548delAG	Del.	Ex.5	Kuwaiti	Frameshift	JALS	14 months	Thr475ThrfsX72	Hadano *et al*., 2001
1867-1868delCT	Del.	Ex.9	Saudi	Frameshift	JPLS	1-2 years	Leu623ValfsX624	Yang *et al*., 2001
3742delA	Del.	Ex.22	Algerian	Nonsense	IAHSP	1 year	Met1207Xfs1	Eymard-Pierre *et al*., 2002
1471delGTTTCCCCCA	Del.	Ex.6	French	Frameshift	IAHSP	18 months	Val491GlyfsX493	Eymard-Pierre *et al*., 2002
2660delAT	Del.	Ex.13	Italian	Frameshift	IAHSP	18 months	Ile336ThrfsX5	Eymard-Pierre *et al*., 2002
1130delAT	Del.	Ex.4	Italian	Frameshift	IAHSP	16 months	Asn846IlefsX13	Eymard-Pierre *et al*., 2002
C3115T	Subs.	Ex.18	Buchari-Jewish	Nonsense	IAHSP	1 year	Arg998X	Devon *et al*., 2003
4844delT	Del.	Ex.32	Pakistani	Nonsense	IAHSP	18 months	Val1574fsX44	Gros-Louis *et al*.,2003
553delA	Del.	Ex.4	Turkish	Nonsense	JALS	22 months	Thr185LeufsX5	Kress *et al*., 2005
G669A	Subs.	Ex.4	Turkish	Missense	IAHSP	1 year	Cys157Tyr	Eymard-Pierre *et al*., 2006
1619GA	Subs.	Ex.6	Italian	Missense	JPLS	2 years	Gly540Glu	Panzeri *et al*., 2006
1825_1826CAGTG	Ins.	NA	Hungarian	Nonsense	IAHSP	10 months	Glu609fsX9	Sztriha *et al*., 2008
3529GT	Subs.	NA	Hungarian	Nonsense	IAHSP	5 years	Gly1177X	Sztriha *et al*., 2008
2143CT	Subs.	Ex.10	Dutch	Nonsense	IAHSP	2 years	Gln715X	Verschuuren-Bemelmans *et al*., 2008
IVS9–2AT	Subs.	Intr.9	German	Frameshift	IAHSP	18 months	NA	Herzfeld *et al*., 2009
3565delG	Del.	Ex.22	Japanese	Frameshift	JALS	13 months	V1189WfsX19	Shirakawa *et al*., 2009
IVS22-5GC	Subs.	Intr.22	Japanese	Frameshift	JALS	3 years	G1172EfsX29	Shirakawa *et al*., 2009

The N-terminus of the protein harbors a region called RLD (regulator of chromatin condensation (RCC1) like domain), containing seven RCC1-like repeats in a seven bladed beta-propeller structure (Soares *et al.*, 2009). RLD provides surface for protein-protein interactions, like for glutamate receptor interacting protein1 (GRIP1) with the C-terminus of alsin (Kunita *et al.*, 2007; Hadano *et al.*, 2007). It is also homologous to the characteristic domain of RCC1 super family proteins. Although RCC1 is a hallmark domain of GEFs for small GTPase Ran (Ras-related nuclear) family, alsin does not have GEF activity on Ran proteins in vitro (Otomo *et al.*, 2003).

At the center of the gene, there is DH/PH (B-cell lymphoma (Dbl) homology and pleckstrin homology) domain which is a characteristic region of GEFs for Rho (Ras-homologous member) GTPases. As in the case of Ran, alsin does not have a GTPase activity for Rho proteins. However for one member of this family, the Ras-related C3 botulinum toxin substrate (Rac1), alsin is an effector and interaction partner. Rac1 is an important GTPase for neuron growth and regulates the actin cytoskeleton (Nikolic, 2002). On the other hand, with the observations of alsin's localization in growth cones of primary-cultured hippocampal neurons from mice and negative effects of alsin knock down in neuronal outgrowth of primary cultured rat spinal neurons, alsin is implicated in axonal outgrowth (Otomo *et al.*, 2008). The observation of Rac1's inhibitory effect against growth defects due to alsin KD in spinal motor neurons, suggests that neuron growth is controlled by alsin/Rac1 signalling (Jacquier *et al.*, 2006).

At the C-terminus of alsin, there is the VPS9 (Vacuolar protein sorting 9) domain, which has GEF activity for Rab5 GTPase. Unlike the other regions, it has been shown that with this region alsin acts as a Rab5-specific GEF (Otomo *et al.*, 2003). Because Rab5 is a key regulator of endocytosis, endosome fusion and trafficking, alsin may have indirect effects on these cellular mechanisms. For instance, in the case of micropinocytosis (one of endocytosis types) it has been shown that by the activation of Rac1, alsin is transported to the micropinosomes and with its GEF activity it activates Rab5 (Figure 1.6). As mentioned before, Rab5 is an essential element for intracellular trafficking and without alsin's activation, some Rab5-dependent endosomal abnormalities will occur (Kunita *et al.*, 2007).

Alsin's role in endocytosis is also supported with the observations of altered endosome trafficking, leading to motor behavioral abnormalities in alsin knock-out mouse models and knock-down mouse cell lines (Hadano *et al.*, 2006; Devon *et al.*, 2006; Jacquier *et al.*, 2006). In addition to GEF activity for Rab5, the DH/PH domain of alsin has also a neuroprotective effect against mutant SOD1 toxicity. With this domain, alsin specifically binds to mutant SOD1 and reduces cell death in NSC34 cell lines (Kanekura *et al.*, 2004).

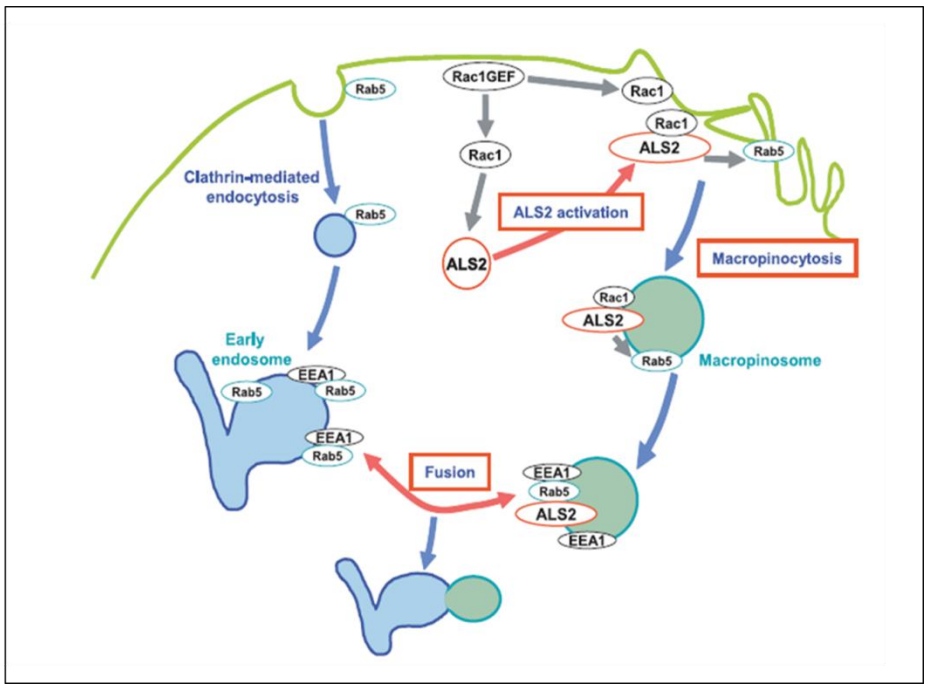

Figure 1.6. Proposed role for alsin in the endocytic pathway (Kunita *et al.*, 2007).

In addition to GEF domains, there are MORN motifs between DH/PH and VPS9, which are crucial parts of membrane binding proteins (Takeshima *et al.*, 2000). In the case of alsin, this domain is necessary for the activation of Rab5, and provides the physical interaction of alsin with micropinosomes or other endocytic vesicles (Hadano *et al.*, 2007).

1.3. Spastin

SPG4 is the first causative factor identified for the autosomal dominantly inherited HSP, accounting for nearly 40% of HSP cases (Hazan *et al.*, 1999). It is also associated with the juvenile form of ALS when it has a missense mutation (Meyer *et al.*, 2005). As in the case of alsin, defined mutations in the *SPG4* lead to loss of function of the protein product, called spastin. Spastin which is composed of 616 amino acids, belongs to the AAA (ATPases associated with various cellular activities) protein family. It contains two major domains: the MIT (microtubule-interacting and endosomal trafficking) domain at the N-terminus and the AAA domain at the C-terminus. In addition, there are one trans membrane and two nuclear signal (localization and export) domains at the N-terminus of the protein. Although the exact function of spastin is not known, its domains are informative (Salinas *et al*., 2007).

According to the primary protein structure, spastin belongs to the subfamily-7 of the AAA proteins. The members of this group have functions in microtubule severing, endosomal morphology and trafficking. Spastin, as the last identified group member, also has similar functions. Errico *et al*., showed that spastin can bind to microtubules (MTs) in vitro via its MIT domain which is activated by the ATPase activity of the AAA domain. In addition, overexpression of spastin in COS-7 and HeLa cells destabilizes MTs, which suggests an MT-regulatory role for spastin (Errico *et al*., 2005). Further evidence for direct functions came from a study on a Drosophila homologue of spastin (D-spastin). By using a technique called taxol-stabilization (stabilization of MTs with the chemical taxol) MTs were attached in a glass surface and exposed to purified D-spastin with or without ATP. Observation of the presence of disrupted MTs with the exposure of D-spastin and ATP proved that spastin has an ATP dependent severing activity. Besides, when they repeated the same experiment with the HSP mutant forms of D-spastin, depending on the mutation site, the efficiency of severing gets lower or even totally lost (Mecak and Vale, 2005). In another study with the human spastin, supporting results are obtained by Salinas *et al.* With a similar experimental design, they also discovered that spastin has bundling activity on MTs, independent from ATP hydrolysis (Salinas *et al.*, 2005). According to the studies on the regions of the interaction between spastin and MTs, spastin was found to be a highly dynamic protein. It is enriched in branching regions of the growth cones of immortalized

motor neurons, and during cell division of HeLa cells, its localization is altered due to the cell cycle phases (Errico *et al.*, 2004). Considering the importance of MT integrity on processes like neuronal plasticity, neurite growth, axonal transport and cell division; with its severing and bundling activities spastin may be the most important protein in HSP pathology (Figure 1.7).

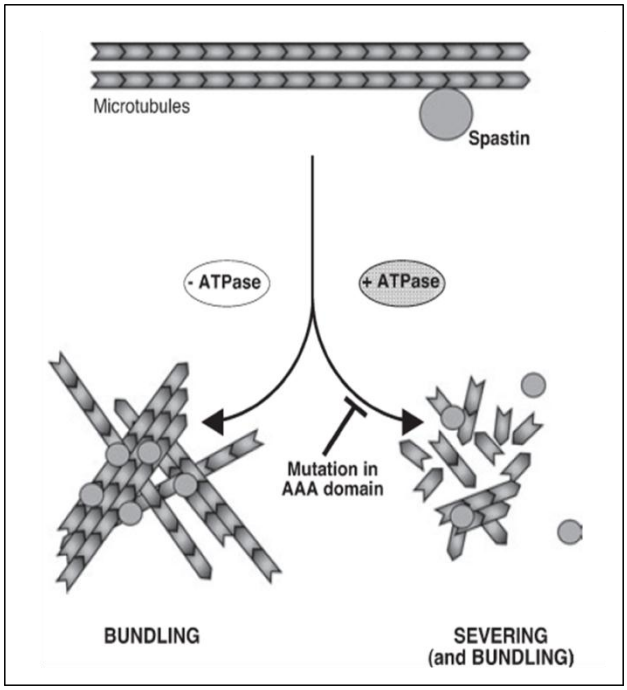

Figure 1.7. Severing and bundling activity of spastin.

MIT domain is a common region for several proteins with well-established functions in endosomal trafficking. Although a direct role for spastin in endosomal dynamics is not described yet, identification of interactions via its MIT domain with endosomal proteins like CHMP1B (chromatin modifying binding protein 1), ZFYVE27 (zinc finger FYVE (Fab-1, YGL023, Vps27, and EEA1) domain containing 27), atlastin and reticulon (RTN1) are promising. Among these proteins, the genes encoding for ZFYVE27, atlastin and REEP1 are mutated in some HSP cases, which suggests that they may have roles in a common pathological mechanism (Reid *et al*., 2005; Mannan *et al*., 2006a; Mannan *et al*., 2006b; Sanderson *et al*., 2006).

1.4. Spartin

Spg20 is the causative gene for Troyer Syndrome (TRS), an autosomal recessive and complicated form of HSP. So far, two mutations were identified in the *SPG20*. The first mutation is a homozygous single-nucleotide deletion (1110delA), described in Old Order Amish families (Pantel *et al*., 2002) and the second is a homozygous 2 bp deletion (364_365delAT) reported in an Omani kindred. Premature stop codons arising as a result of these mutations lead to a loss of function (Manzini *et al*., 2010).

Spg20 encodes for a ubiquitously expressed, 666-amino acid protein, named spartin, which has two isoforms, both in human and mouse. Spartin is composed of two conserved domains. In the C-terminus there is a plant-related senescence (PRS) domain, sharing a strong homology with some plant-specific gene sequences, that are being expressed under stress conditions. In the N-terminus, similar to spastin, spartin harbors an MIT domain (Cicarelli *et al.*, 2003).

As mentioned before, the MIT domain is a characteristic region of endosomal proteins. Although having an MIT domain, the exact function of spartin in endocytosis is not defined yet. However, many indirect implications were reported. Firstly, cytoplasmic spartin was also detected in a membrane fraction of total protein isolates, which indicates a possible role as a membrane-associated protein Then, EPS15 (epidermal growth factor receptor substrate 15), a major protein in endocytosis of EGFR (epidermal growth factor receptor), was defined as an interaction partner of spartin (Bakowska *et al.*, 2005). Based on this interaction, Bakowska *et al.* examined the effects of spartin on EGFR metabolism. In accordance with previous studies, as a result of the activated EGF in HeLa cells, cytoplasmic spartin gets transported to the cell membrane and binds to EGF-positive endosomes. Besides, in both spartin KD and OE conditions, EGFR uptake gets reduced. In the light of these results, with EPS15, spartin is a major actor in EGF/EGFR metabolism, which should be regulated properly for normal cell growth and divison (Bakowska *et al.*, 2007).

In addition to endosomal and membrane trafficking processes, spartin is also related to mitochondria physiology. The first link was established with the immunolocalization studies of Lu *et al*. showing spartin-mitochondria co-localization in neuronal and HeLa cells (Lu et al., 2006). This co-localization, despite the fact that spartin does not have a mitochondrial target sequence, was a question mark. However, the answer came recently from another study showing spartin's interaction with one of the mitochondrial phospholipids, cardiolipin, in human neuronal cells (Joshi *et al.*, 2011). This interaction depends on the PRS domain of the protein in the C-terminus, and in the case of the 1110 delA mutation (which resides in the PRS), spartin loses its mitochondrial localization. Considering that 1110A delA is one of two mutations defined in spartin-linked HSP, it can be suggested that the effects of spartin absence on mitochondria should be devastating (Lu *et al*., 2006). With this point of view, Joshi *et al*., investigated the mitochondrial membrane potential changes in spartin KD cells and cells derived from spartin KO mouse. Although the results were insignificant, the membranes of both cell types were all depolarized. In addition to that, the amount of Ca^{2+} uptake was lower in spartin depleted cells than in wild-type cells. As mentioned before, mitochondrial dysfunction is one of the pathological mechanisms in the ALS and also in other NDs. With the observations of depolarized mitochondria membrane and disrupted Ca^{2+} homeostasis, it can be hypothesed that loss of spartin will lead to mitohondrial dysfunction, ultimately resulting in cell death (Joshi et al., 2011).

1.5. Small Heat Shock Proteins

Small heat shock proteins (sHSP) are highly conserved, 12-43 kDa sized molecular chaperones with a common C-terminus called the α-crystallin domain. Under stress conditions, sHSPs get over-expressed and provide an isolated environment for aggregation-prone unfolded proteins to get properly folded (Sun *et al.*, 2005). In addition, sHSPs are also involved in apoptotic signaling and maintenance of cytoskeleton integrity. Most sHSPs are expressed ubiquitously and are related to many diseases, including some NDs, cancer, myopathy associated to mutations in DES gene and cataract. Considering the observations in ND patients' tissues, like abnormal expression levels of sHSPs and misfolded protein aggregations, the function of sHSPs seems vital for neurons (Perng and Quinlan, 2004; Wilhelmus *et al*., 2006).

HspB1 (Hsp27) is the first sHSP identified, when mutated, it causes dHMN and CMT2 (Evgrafov *et al.*, 2004). HspB1 was also shown to be related to AD, PD and multiple sclerosis, after the discovery of its presence in aggregations. By binding to apoptosis signaling proteins, HspB1 can reduce caspase activities in neuronal cells (Benn *et al.*, 2002 and Akbar *et al.*, 2003). In addition to its neuroprotective roles, the mutated forms of *HspB1* can lead to neurotoxicity. For example, mutant *HspB1*-expressing neuronal N2a cells are less viable than the cells expressing the wild-type protein (Evgrafov et al., 2004). Another study also discovered that HspB1-deficient cells are more sensitive to amyloid β-oligomer-mediated cell death (Kudva *et al.*, 1999).

HspB8 is the second sHSP associated with dHMN and CMT2 (Irobi *et al*., 2004). According to recent studies, HspB8 has motor neuron specific functions. For instance, although mutated HpsB8 causes degeneration in cultured motor neurons, it does not affect any cortical neurons or glial cells, used in the same experiment (Irobi *et al.*, 2010). In addition, transfection of cultured SOD1-mutant motor neurons with wild type HspB8 reduces the toxicity by increasing the mSOD1 solubility and decreasing the amount of aggregations (Crippa *et al.*, 2010).

2. PURPOSE

Motor neuron diseases are a heterogeneous group of disorders, caused by several genes with diverse pathological mechanisms. ALS is the most complex motor neuron disorder with the involvement of both of upper and lower motor neurons. *ALS2*, one of the causative genes for a juvenile form of ALS, and the main gene of interest in this study, is also involved in two upper motor neuron disorders: infantile-onset ascending HSP and the juvenile form of PLS. Early age of onset is the common feature of all three disorders. This suggests, that alsin should have a crucial role in motor neuron integrity, because its loss of function apparently causes an earlier damage to the neurons than most other mutated proteins involved in MNDs. In spite of its importance, the exact function of alsin is not known very well yet. This study aims to gain insights into alsin's function by investigating:

- the effects of alsin knock down conditions on the mRNA levels of four genes (*Spg4*, *Spg20*, *Hsp22* and *Hsp27*) that are also implicated in other MNDs.
- the interactions of alsin with the protein products of the above genes.

3. MATERIALS

3.1. Cell Lines

3.1.1. Neuroblastoma 2A (N2A)

N2a cells were obtained from ATCC Company's Cell Biology Collection, USA.

3.2. Buffers and Solutions

3.2.1. Cell Culture

Table 3.1. Cell culture materials.

Material	Company
0.5 % Trypsin-EDTA 1X	GibcoBRL, USA
Dimethyl sulfoxide (DMSO)	AppliChem, Germany
Fetal Bovine Serum (FBS)	GibcoBRL, USA
GlutaMAX I (Glu) 100X	GibcoBRL, USA
Horse Serum (HS)	GibcoBRL, USA
MEM Non-essential amino acid (NEAA) 100X	GibcoBRL, USA
Hygromycin	Sigma, USA
Opti-MEM I	GibcoBRL, USA

Table 3.1. Cell culture materials (continued).

MEM Non-essential amino acid (NEAA) 100X	GibcoBRL, USA
Hygromycin	Sigma, USA
Opti-MEM I	GibcoBRL, USA
Penicillin/Streptomycin (Pen/Strep)	GibcoBRL, USA
Minimum Essential Medium (MEM)	GibcoBRL, USA
Phosphate Buffered Saline (PBS)	GibcoBRL, USA
Retinoic Acid	Sigma, USA
RNaseZAP	Sigma, USA

3.2.2. Transformation and Transfection

Table 3.2. Transformation and transfection materials.

Material	**Company**
FuGENE®HD Transfection Reagent	Roche, Germany
LB Agar: 1 lt LB medium 15g Agar	 Sigma, USA Sigma, USA

Table 3.2. Transformation and transfection materials (continued).

Kanamycin	Sigma, USA
LB medium (1 Lt): 10g Tryptone 5g Yeast Extract 5g NaCl	 Sigma, USA Sigma, USA Sigma, USA

3.2.3. Protein Isolation

Table 3.3. Protein isolation materials.

Material	**Company**
RIPA Buffer	Santa Cruz, USA
Complete protease inhibitor cocktail tablets	Roche, Germany

3.2.4. Western Blot (WB)

Table 3.4. Western blot materials.

Material	**Company**
Acrylamide:bisacrylamide: 29.2 g/100 ml acrylamide 0.8 /100 ml N`N'-methylene-bis acrylamide	 Sigma, USA Sigma, USA

Table 3.4. Western blot materials (continued).

Ammonium Persulfate: 10 % APS (w/v) in dH2O	Sigma, USA
Blotto, NonFat Dry Milk	Santa Cruz Biotechnologies, USA
Chemiluminescence: 99.28 % ddH_2O 0.22 % Coumeric Acid 0.5 % Luminol 10 % Tris-HCl pH 8.5	 Sigma, USA Sigma, USA Sigma, USA
Hydrogen peroxide (H_2O_2)	Sigma Aldrich, Germany
Coomassie Brilliant Blue	Santa Cruz, USA
Developer	Kodak, USA
Fixer	Kodak, USA
PageRuler™ Plus Prestained Protein Ladder	Fermentas, USA
Resolving Gel (8%): 2.7ml Acryalmide 30% 1.25ml Tris- HCl 3M pH 8.8 100µl SDS 10% 5,9ml H_2O 75µl APS 10% 7,5µl TEMED	 Sigma, USA Sigma, USA Sigma, USA Sigma, USA Sigma, USA
BSA (2 mg/ml) Bio-Rad Quick Start	Bio-Rad, USA
Bradford dye reagent 1X	Bio-Rad, USA

Table 3.4. Western blot materials (continued).

Running Buffer: 25mM Tris-HCl, pH 8.3 192mM Glycine 0,1 % SDS	 Sigma, USA Sigma, USA Merck, Germany
Sample Buffer: 50 mM Tris 50 % β-mercaptoethanol 10 % Glycerol	 Sigma, USA Sigma, USA Sigma, USA
Stacking Gel (4%): 0,53ml Acryalmide 30% 1ml Tris- HCl 0,5M pH 6.8 40µl SDS 10% 2,39ml H2O 40µl APS 10% 4µl TEMED	 Sigma, USA Sigma, USA Merck, Germany Sigma, USA Sigma, USA Sigma, USA
TBS-TWEEN: 160mM NaCl 20mM Tris 0.05 % TWEEN	 Sigma, USA Sigma, USA Sigma, USA
Transfer Buffer: 25mM Tris-Glycine 20 % Methanol	 Sigma, USA Sigma, USA
TWEEN	CalbioChem, Canada

3.2.5. Immunofluorescence (IF)

Table 3.5. Immunofluorescence materials.

Nuclear dye DAPI	Santa Cruz Biotech., USA
Paraformaldehyde:	CalbioChem, Canada
Triton-X-100	CalbioChem, Canada

3.2.6. Immunoprecipitation (IP)

Table 3.6. Immunoprecipitation materials.

Protein G PLUS-Agarose	Santa Cruz, USA

3.3. Fine Chemicals

3.3.1. shRNA and Vectors

The shRNA sequence was designed by İzzet Enünlü in our laboratory and it was fused into a pSilencer™ 2.1-U6 hygro vector by using the online tool of Ambion Company (Figure 3.1).

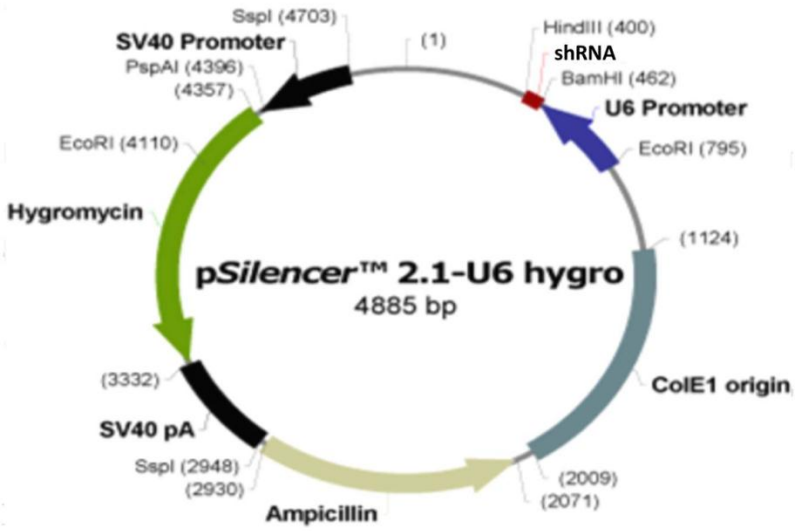

Figure 3.1. Map of the pSilencer™ 2.1-U6 hygro vector (www.ambion.com).

3.3.2. Primers

Primers used in this study were adopted from Harvard Primer Bank (Table 3.7).

Table 3.7. Primer sequences of the genes involved in the study.

SPG4	**Forward :** 5’ CGCATCGACGAGGAAGAGAAAGCA 3’
	Reverse : 5’ ACCTTGGCCCGTAACTATAACAGCGA 3’
SPG20a	**Forward:** 5’ AGCGGCGCTCAGAGATGGAGA 3’
	Reverse: 5’ TCCTTCTGCCCTAACTCGTCCGT 3’
SPG20b	**Forward :** 5’ GCAGCAAGGATGCCCGCCATAAA 3’
	Reverse : 5’ GTGGGCCACTTTCTCGCTCCATTCA 3’
HSPB1	**Forward :** 5’ ATCCCCTGAGGGCACACTTA 3’
	Reverse : 5’ CCAGACTGTTCAGACTTCCCAG 3’
HSPB8	**Forward :** 5’ AACCCTAAGGTCTGGCATGGT 3’
	Reverse : 5’ GACACACACTTTCCAAGGCTC 3’
Alsin	**Forward :** 5' TCCAGTTCTTGCTATGAGTCTCT 3'
	Reverse : 5' GGAATCCGTCATTTTCCCAGG 3'
β-Actin	**Forward :** 5' GGCTGTATTCCCCTCCATCG 3'
	Reverse : 5' CCAGTTGGTAACAATGCCATGT 3'

3.3.3. Antibodies

Table 3.8. Antibodies used in this study.

Antibody	Source	Company	Dilution Used	Usage
Alsin	Goat	Novus	1:250	IF
Anti-ALS2	Rabbit	Sigma Aldrich	1:50	IP
HspB1	Mouse	Santa Cruz	1:250/1:1000	IF/WB
HspB8	Mouse	Santa Cruz	1:250	IF
Spartin	Rabbit	Santa Cruz	1:250/1:1000	IF/WB
Spastin	Mouse	Santa Cruz	1:250	IF
OctA-Probe (D-8)	Rabbit	Santa Cruz	1:50	IP
Anti-rabbit lgG Red	Chicken	Santa Cruz	1:1000	IF
Anti-mouse IgG FITC	Bovine	Santa Cruz	1:1000	IF
Anti-goat IgG FITC	Donkey	Santa Cruz	1:1000	IF
Anti-mouse IgG Peroxidase (Fab specific) Peroxidase	Goat	Sigma Aldrich	1:3000	WB
Anti-mouse IgG (Whole molecule) Peroxidase	Rabbit	Sigma Aldrich	1:3000	WB
Anti-rabbit IgG (Whole molecule) Peroxidase	Goat	Sigma Aldrich	1:3000	WB

3.4. Kits

Table 3.9. Kits used in this study.

Procedure	Kit	Company
q-RT PCR	QuantiTect SYBR Green RT-PCR Kit	Qiagen, USA
RNA isolation	High Pure RNA Isolation Kit	Roche, Germany
Plasmid mini prep	High Pure Plasmid Isolation Kit	Roche, Germany

3.5. Equipment

Table 3.10. Equipment used in this study.

Equipment	Models
Autoclave	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Cover Slips	22x22 mm, ISOLAB Laborgeräte, Germany
Falcon Tubes	EasyOpen 50 ml Centrifuge Tubes, JET BIOFIL, USA
Films	Kodak, USA
Gel loading tips	Invitrogen, USA
Heat Blocks	BBA1, Grant Boekel, UK

Table 3.10. Equipment used in this study (continued).

Balances	Model VA124, Gec Avery, UK Model CC081, Gec Avery, UK TE612, Sartorious, Germany
Capillaries	LightCycler®Capillaries (20µl), Roche, Germany
Centrifuges	Allegra X22-R, Beckman Coulter, USA Centrifuge 1-15, Sigma, USA Centrifuge 2-16K, Sigma, USA
Deep Freezers	2021D (-20^{0}C), Arçelik, Turkey Sanyo (-70^{0}C), Sanyo, Japan
Electrophoretic Equipments	Minicell Primo E320, Thermo, USA Wide Sub Cell GT BIO-RAD, USA Mini-PROTEAN 3 Cell, BIO-RAD, USA Mini Trans-Blot, BIO-RAD, USA
Kodak, X-ray Processor	Kodak, USA
Magnetic Stirrer	Chiltern Hotplate Magnetic Stirrer, HS31, UK Hotplates MR3001, Heidolph, Germany
Microscope	DMI4000 B, Leica Microsystems, Germany
Microscope slides	Thermo Scientific, USA
Eppendorf Tubes	1.5 ml Boil-Proof Microtubes, Axygen, USA 0.2 ml and 0,5 ml Thin Wall Flat Cap PCR tubes, Axygen, USA
Balances	Model VA124, Gec Avery, UK Model CC081, Gec Avery, UK TE612, Sartorious, Germany

Table 3.10. Equipment used in this study (continued).

Flasks	Cell Culture Flask, 50 ml, 690160, GREINER BIO-ONE, Germany Cell Culture Flask, 250 ml, 658170, GREINER BIO-ONE, Germany
Incubator	Forma Series II Water Jacketed CO_2 Incubator Thermo Scientific, USA Hybex Microsample Incubator, SciGene,USA
Nitrocellulose Membrane	Nitrocellulose Pure Transfer Membrane , Santa Cruz Biotechnologies, USA
Real-Time PCR Systems	LightCycler 2.0 System, Roche, Germany
Refrigerator	4250T, Arçelik, Turkey
Reservoir	Corning Incorporated Costar, USA
pH Meter	PB-11, Sartorious, Germany
Pipettes	Pipetus, Hirschmann, Laborgeräte,Germany Pipetman, Gilson, USA 5-10-25 ml Serological Pipettes, GREINER BIO-ONE, Germany
Plates	6-well Tissue Culture Plates, BIOFIL, USA 12-well Cell Culture Plates, GREINER BIO-ONE, Germany

Table 3.10. Equipment used in this thesis (continued).

Hood	IP44/I, Wesemann, German Class II Biohazard Safety Cabinet, ESCO, USA
Power Supplies	Constant Power Supply, Pharmacia,Sweden EC250 Thermo Scientific, USA ECPS 3000/150 Model 200, BRL, USA Powerpac 1000, BIO-RAD, USA
Shaker	Duomax 1030 Platform Shaker, Heidolph, Germany Reax 2 Overhead Shaker, Heidolph, Germany
Spectrophotometer	NanoDrop ND-1000, Thermo, USA
Electrophoresis Tank	Mini Protean 3 Cell, BIO-RAD, USA
Thermocyclers	C312, Techne, UK iCycler, BIO-RAD, USA Techgene, Progene, UK Touchgene Gradient, Progene, UK
Tips	1000µl, 200µl, 100µl, 10µl ,Universal Fit Filter Tips, Axygen, USA
Vortex	Fisons WhirliMixer, UK Reax Top, Heidolph, Germany
Water Bath	Gemo DT104, TEST Laboratuvar Cihazları, Turkey
Water Purification	Millipore, USA

4. METHODS

4.1. Maintenance and Storage of the Cells

N2A cells were maintained in MEM, supplemented with 10% FBS, 1% Glu, 1% NEAA and 1% P/S. They were grown in 25 cm^2 cell culture flasks in a 5% CO_2-humidified incubator at 37°C. After achieving 100% cell confluency, the cells were washed with PBS once and trypsinized for 4 minutes. Next, dissociated cells were transferred from flasks to 15 ml eppendorf tubes and centrifuged for 4 minutes at 1500 rpm. The cell pellet was resuspended in 4 ml complete MEM and 1 ml of the cell suspension was transferred to new cell culture flasks.

For the cell storage, cells were grown in 75 cm^2 flasks. After centrifugation, the cells were resuspended in 10 ml freezing medium (25% FBS, 1% Pen/Strep, 1% GlutaMAX, 1% NEAA) and divided into 10 cryo-tubes with the addition of 10% DMSO in a drop wise manner. Next, the cells were incubated at -20°C for 2 hours, and they were kept in -80°C freezer for long term storage.

4.2. Generating *Als2* KD Stable Cell Line

4.2.1. Bacterial Transformation and Mini Prep

In order to generate an *Als2* KD stable cell line, the pSilencer™ 2.1-U6 hygro vector was used. The vector contains the shRNA sequence of the DH/PH domain of the *Als2* transcript. To eliminate possible effects of the vector on the cells, a second cell line (*Als2* control cell line) was generated by using a vector without the silencing sequence.

As a first step, *E.coli* DH5-alpha cells were transformed with the relevant vectors by using the following transformation procedure:

- 1 ng of silencing vector and 1 ng of control vector were added on 200 µl of DH5-alpha cells.

- The cells were incubated on ice for 30 minutes, heat-shocked for 1 minute at 42°C and kept on ice for 5 minutes.

After transformation, the cells were spread on LB agar plates with 100 mg/ml Kanamycin and incubated overnight at 37°C. Next day, one of the colonies was picked and grown in 5 ml LB with overnight incubation at 37°C on a shaker. In the final step the vectors were isolated from the cells by using the Roche High Pure Plasmid Isolation Kit and they were ready for transfection.

4.2.2. Transfection

The transfection mixture was prepared by mixing the FuGENE-HD transfection reagent and the isolated vectors in a 4:1 ratio (V/W) by incubation for 15 minutes at room temperature (RT). The N2a cells were grown for transfection in 6-well plate until they reached at least 90% cell confluency. During incubation, the cell medium (complete MEM) was replaced with Opti-MEM, a medium which provides an ideal protein-free environment for efficient transfection. After the incubation, the transfection mixture was added to the cells in a drop wise manner. For the next 24 hours, the transfected cells were grown in the incubator at the same conditions as the wild type ones. To ensure proper cell growth, Opti-MEM was replaced with complete MEM on the next day. As it can be seen from Figure 3.1, both of the control and silencing vectors, have a hygromycin resistance gene. In order to eliminate the cells without vectors, the growth medium was enriched for 10 days with three mg/µl hygromycin. With three day intervals, the medium was removed and refreshed. As a result of hygromycin application, the cells without vector are expected to die whereas the *ALS2* KD stable cell line should be established. In order to validate the *ALS2* KD level, q-RT PCR experiments were performed.

4.3. Total RNA Isolation

Before q-RT PCR, total RNA isolation was carried out by using High Pure RNA Isolation Kit from Roche. In order to inhibit RNase activity, each material, including pipettes, tips etc. were wiped with RNase inhibitor RNase-ZAP®. After achieving 100% cell confluency in 25 cm^2 flasks, the old medium of the cells was removed and they were washed with 1 ml ice-cold PBS. By help of a cell scraper, cells were dissociated from the flask and they were transferred to a 1.5 ml Eppendorf tube in 1 ml PBS, and centrifuged for 5 minutes at 1500 rpm with a standard tabletop centrifuge. Before the lysis step, the supernatant was removed and the cells were resuspended in 200 µl PBS. Then, 400 µl of lysis-binding buffer was added on the cell suspension and the mixture was vortexed for 15 seconds. The obtained cell lysate was transferred to a high pure filter-collection tube assembly. In order to prevent DNA contamination, cell lysis was incubated for 15 minutes at 15-25°C in a mixture composed of 10 µl of DNase I and 90 µl of DNase I incubation buffer. Then, sequential centrifugation steps were performed in order to remove DNA remnants. After each centrifugation, the buffers which were collected in the collection tubes were discarded. In the first and second step 500 µl of wash buffers I and II were added into tubes, respectively and the cell lysate was centrifuged for 15 seconds at 8000 x g. In the third step, 200 µl of wash buffer II was added and the suspension was centrifuged for 1 minute at 13000 x g to remove remaining salts from the washing buffers. In the last step, 50 µl of elution buffer was added to the silica membrane inside the column and the RNA isolate was stored at -80°C for future experiments.

4.4. Quantitative-Real Time PCR (q-RT PCR)

Q-RT PCR experiments were carried out by the using Qiagen QuantiTect SYBR Green RT-PCR Kit in Roche LightCycler 2.0. As in the case of total RNA isolation, all materials, used during q-RT PCR experiments; wiped with RNase inhibitor RNase-ZAP®.

For each PCR tube;

- 2µg of total RNA isolate,
- 20 pmol/µl forward and reverse primers (Table 3.7),
- 30 µl of 2X SYBR PCR master mix,
- 0.6 µl of QT-mix

was mixed and the total volume was completed to 60 µl with RNAse free water.

Then the prepared PCR mixture was divided into 3 separate capillary tubes, and the reactions were started with appropriate PCR conditions (Table 4.1). Sybr PCR master mix contains a dye (SYBR Green I), which emits fluorescence light, when it intercalates into the DNA double helix. During the PCR reaction, according to the amount of the fluorescent signal, the amount of RNA can be measured quantitatively.

Table 4.1. Reverse transcription and q-RT PCR conditions.

Reaction	**Temperature**	**Time**	
Reverse transcription	50°C	20 minutes	
Initial denaturation	95°C	15 minutes	
Denaturation	95°C	15 seconds	45 cycles
Annealing	60°C	20 seconds	
Extension	72°C	30 seconds	
Melting	65°C	15 seconds	
Cooling	40°C	30 seconds	

The results were analyzed with absolute quantification and normalized with β-actin levels. Besides, each experiment was repeated 3 times and the *Student's T-Test* was applied in order to measure significance level statistically.

4.5. Protein Isolation

The cells were grown in 75 cm^2 cell culture flasks until obtaining 100% cell confluency. After removing the growth medium from the flasks, the cells were rinsed with ice cold PBS and harvested by using a cell scraper in 5 ml PBS. Next, the cell suspension was centrifuged at 1500 rpm for 5 minutes and the pellet was resuspended in RIPA lysis

buffer, containing protease inhibitors. In order to break the cell and nuclear membranes, the cells were incubated with a cell and lysis buffer mixture on a rotating shaker for 15 minutes at RT. In the last step, after centrifugation at 18000 x g for 15 minutes at 4°C, the supernatant was transferred in a new Eppendorf tube and the proteins were stored at -80°C for later analyses.

4.6. Bradford Assay

Bradford analyses were performed by using BSA (2 mg/ml) and Bradford dye reagent (1X from Bio Rad Quick Start Reagents) in order to determine the concentration of proteins before loading them on SDS poly-acrylamide gels. For each standard measurement, the stock BSA solution was serial-diluted, from 0 mg/ml to 2 mg/ml with the lysis buffer. After the incubation of protein samples and the diluted BSA with Bradford dye reagent for 30 minutes at 37°C, the measurements were taken on the nanospectrometer, using the BSA Bradford assay program.

4.7. Immunoprecipitation

In immunoprecipitation studies, 10 µl of alsin antibody was added in 500 µl of protein isolate representing IP sample. In the case of negative control sample, 10 µl of oct-A-probe, an artificial antibody, was used. Then, the mixture was incubated for 1 hour on ice to provide antibody-protein binding. For the precipitation step, the protein-antibody mixture was incubated overnight with protein G PLUS-Agarose at 4°C on a rotating shaker. Next, the tubes were centrifuged at 500 g for one min at 4°C with a standard table top centrifuge. To get rid of the unbound proteins, the supernatant was discarded and the pellet was resuspended with 500 µl of lysis buffer. This step was repeated for 3 times. Finally, the pellet was resuspended in 40 µl of sample buffer (2x) and the IP product was kept at -80°C for Western Blot analysis.

4.8. Western Blot Analysis

4.8.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE experiments were carried out by using the Bio-Rad Mini-PROTEAN 3 cell system. The polyacrylamide gel was prepared by pouring 3 different layers between 0.75 mm glass cassettes. Concentration of the first layer (approximately 0.5 cm in height) from the bottom was 12%, and it was used as a plug to prevent leakage of the second layer during polymerization. Considering molecular sizes of the relevant proteins and the distance they will run along the gel, 12% SDS-polyacrylamide gel (approximately 4 cm in height) was prepared as a middle layer which is called the resolving gel. At the top, there was so-called stacking gel (approximately 2 cm in height) with a concentration, of 4% which was used to provide space and time for the proteins to get into the gel before starting to run properly. Before the polymerization of the stacking gel, the combs were placed between the glass cassettes.

During the polymerization of the SDS-polyacrylamide gel, the total protein isolates taken out of -80°C, were thawed on ice to prevent protease activation. Then they were mixed with 2X sample buffer with 1:1 ratio and incubated at 90°C for 8 minutes for denaturation. Next, 20 µl of each sample and 5 µl protein ladder were loaded onto the gel. In the first 20 minutes, samples and the ladder were run at 80 V in the running buffer, then the voltage was raised to 120 V for the next 1.5 hours. The gel was stained with Coomassie Brilliant Blue for 20 minutes and rinsed with a destaining solution until visualizing the protein bands on the gel.

4.8.2. Electro-blotting

In order to transfer the proteins from acrylamide gel to the nitrocellulose membrane, the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell system was used. First, the membrane and 4 whatman papers were cut according to the size of the acrylamide gel. Next, on the black side of the cassette a fiber pad, two whatman papers, the gel, nitrocellulose membrane, 2 whatman papers and another fiber pad was placed on top of each other. The cassette was locked, attached to the transfer apparatus and placed inside

the transfer chamber. Then, this assembly with an ice block nearby, was put in the transfer tank. The transfer of the proteins from the gel to the membrane was achieved at 120 mA for 1 hour via the transfer buffer, on a magnetic stirrer.

4.8.3. Hybridization with Primary and Secondary Antibodies

After the electro-blot step, the membrane was blocked via incubation with 5% (w/v) milk powder in TBST (TBS containing Tween) at RT for 1 hour. Appropriate primary antibody dilutions, prepared in 2% milk powder in TBST, applied on the membrane at 4°C for overnight (Table 3.8). Next day, excess primary antibodies were rinsed away with a washing step with TBST for 3 times with 10 minute intervals, and the membrane was incubated with secondary antibodies which were also prepared in 2% milk powder in TBST with appropriate dilutions for 1 hour at RT (Table 3.8). Before the detection step, unbound secondary antibodies were also washed away from the surface of the membrane by using TBST for 3 times with 10 minute intervals.

4.8.4. Detection of the Blot

Visualizing of the protein bands on detection films is the last step in Western Blot experiments. 10 ml of chemiluminescence solution was mixed with 2.5 µl of H_2O_2 in order to make a fluorescent mixture, which can bind to the secondary antibodies. This mixture was applied on the membrane which was placed under a transparent cover. Kodak detection films were placed on the cover and incubated for 3 minutes. Then films were soaked in developer solution (for 10-15 seconds), water and fixer solution, respectively, and air-dried.

4.9. Immunofluorescence

Immunofluorescence studies were carried out by using N2a cells which were grown on 22x22 mm cover slides inside the 6-well plates. In order to obtain neurite-like extensions, the cells were grown in differentitation medium (1% FBS, 1% Pen/Strep, 1% GlutaMAX, 1% NEAA), enriched with 3 µl of retinoic acid for 3 days. After generating differentiated cells, the growth medium was removed and the cells were washed with 1 ml PBS. For fixation, the cells were incubated with 4% paraformaldehyde for 30 minutes at RT, and the cell membrane was permealized with 0,4% of Triton-X-100 for 20 minutes at RT. Then, the cells were washed with PBS for 3 times. Next, the cell membrane was blocked by using 50% HS (horse serum) for 1 hour at RT. During this incubation time, appropriate primary antibody dilutions were prepared in PBS and the cells were incubated overnight with the antibodies at 4°C (Table 3.8). The next day, primary antibodies were washed away with PBS for 3 times, and secondary antibodies with the appropriate dilutions were applied on cells for 1 hour at RT in a dark environment (Table 3.8). In the final step, the mounting solution containing DAPI nuclear dye, was dropped on a clean slide which was covered by the cover slide with the cells. To prevent drying, slides were sealed with transparent nail polisher.

5. RESULTS

ALS2, a crucial gene for motor neuron integrity, is the gene of interest in this study. Despite of its importance, the exact function of *ALS2* is not known very well. Since *ALS2* mutations result in a loss of function of the protein product, an *Als2* KD stable cell line was generated by using the RNA interference technique in N2a cells. In order to clarify the function of *ALS2*, four genes implicated in other MNDs, *Spg20*, *Spg4*, *HspB1* and *HspB8*, were selected and possible relations both at mRNA and protein levels were investigated.

5.1. Quantitative-Real Time PCR Analyses

In q-RT PCR analyses, two experimental groups, *Als2* KD and *Als2* control stable cell lines, were used. As mentioned earlier in the Methods section, *Als2* KD stable cell line was generated by the transfection of N2a cells with pSilencer™ 2.1-U6 hygro with an *Als2* silencing sequence, and the *Als2* control stable cell line was generated with the same vector without the silencing sequence. The aims of the q-RT PCR experiments were to measure the knock-down efficiency of RNA interference and observe alterations at the mRNA levels of the four genes selected, *Spg20, Spg4, HspB1* and *HspB8,* in *Als2* KD background. The results were normalized with reference gene *β-actin* levels and the *Student's t-test* was applied to confirm the statistical significance.

5.1.1. Alterations at the *Als2* mRNA levels in *Als2* Knock-Down Cell Line

Prior to examining the changes in the expression levels of *Spg20, Spg4, HspB1* and *HspB8,* the knock-down efficiency of *Als2* at mRNA level was determined. Q-RT PCR analyses revealed that there is a 73.40% decrease at *Als2* mRNA level of *Als2* KD cell line as compared to *Als2* control cell line (Figure 5.1). The p-value smaller than 0.0001, showed that the result was statistically significant.

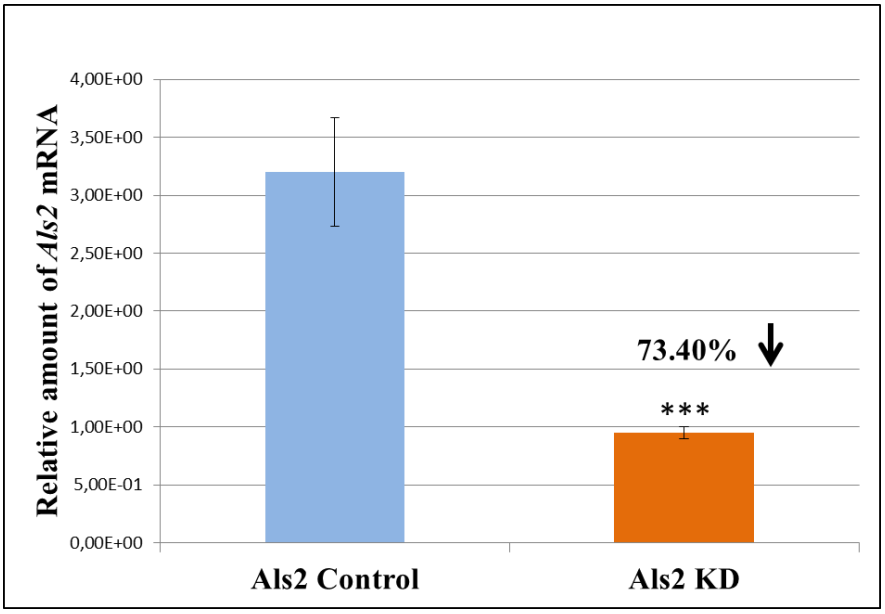

Figure 5.1. Knock-down level of *Als2* mRNA: 73.40% decrease (*** $p < 0.0001$).

5.1.2. Alterations at the mRNA Levels of *Spg4* (Spastin)

After validation of *Als2* knock-down level, the *SPG4* mRNA levels were measured. An 18% decrease in *SPG4* mRNA level in *Als2* KD background with a p-value smaller than 0.0001 was observed (Figure 5.2).

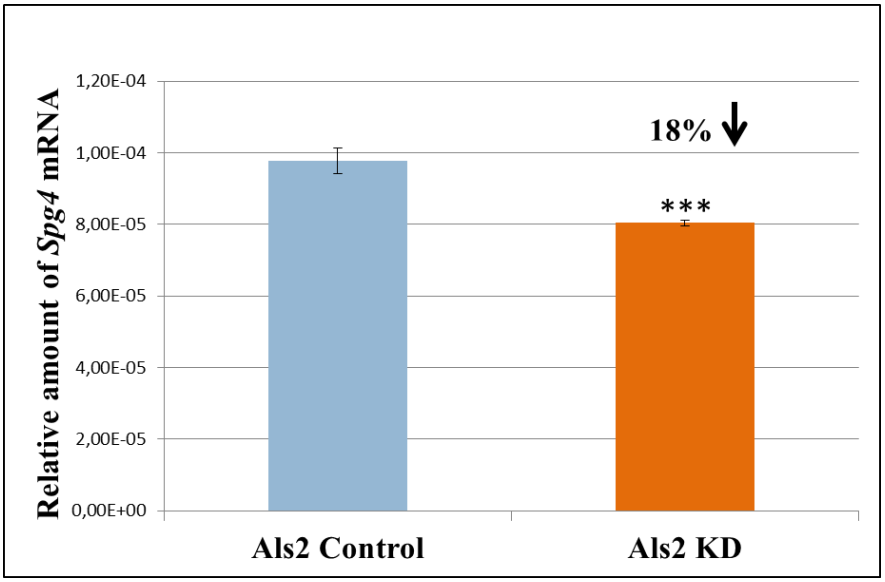

Figure 5.2. Alterations at *SPG4* mRNA levels: 18% decrease (*** $p < 0.0001$).

5.1.3. Alterations at the mRNA Levels of *Spg 20* (Spartin)

There are two transcripts originating from *Spg20*. The first isoform (isoform-a) is composed of 4013 bp, while the second (isoform-b) has 3626 bp, with 1 exon spliced. Since the exact functions of these isoforms are not clarified, 2 different primer sets were designed, considering possible functional differences. In *Als2* KD background, the *Spg20* isoform-a mRNA level was found to be increased by 48% (Figure 5.3a). As opposed to this result, the Spg20 isoform-b exhibited 32% decrease (Figure 5.3b). The p-values were smaller than 0.0001 in both cases showing statistical significance.

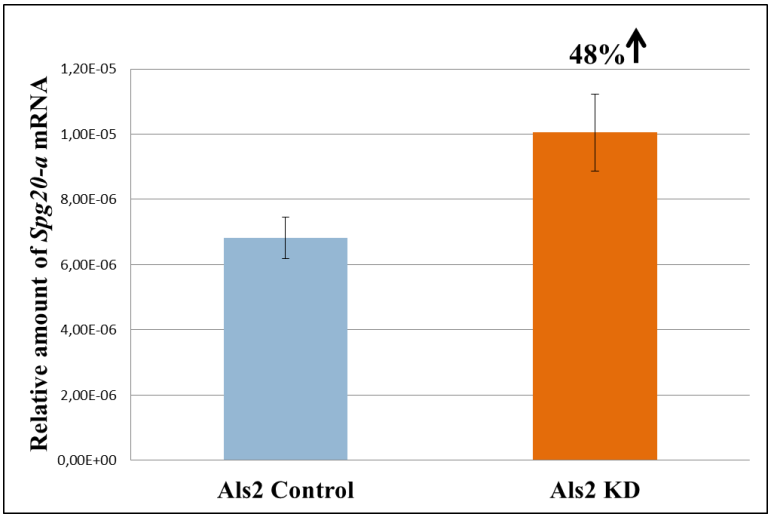

Figure 5.3a. Alterations at *SPG20* isoform-a mRNA levels: 48% increase (*** $p < 0.0001$).

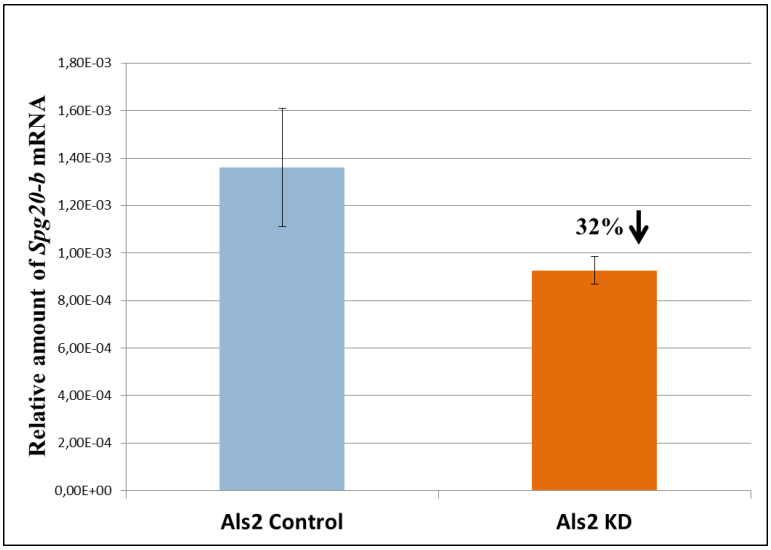

Figure 5.3b. Alterations at *SPG20* isoform-b mRNA levels: 32% decrease (*** $p<0.0001$).

5.1.4. Alterations at the mRNA Levels of *HspB1*

The third gene of interest in this study was the heat shock protein B1 (*HspB1*). Heat shock proteins are overexpressed under stress conditions in order to protect cells from misfolded protein aggregations and apoptosis. Considering this fact, to start with, it was aimed to observe the effect of alsin knock-down stress on *HspB1* expression. 66.42 % decrease was measured in the *Als2* KD cell line as compared to *Als2* control cell line (Figure 5.4a). In the second step, regarding *HspB1*'s role in heat shock response, both cell lines were heat-shocked at 42°C with 30-, 45- and 60-minute time intervals. At the end of the 30-minute heat-shock, surprisingly a 28.62% increase was observed, while 45.58 % and 43.97% decreases were detected at 45- and 60-minute heat-shocks, respectively (Figure 5.4b). The p-values for each result were smaller than 0.0001, showing statistical significance.

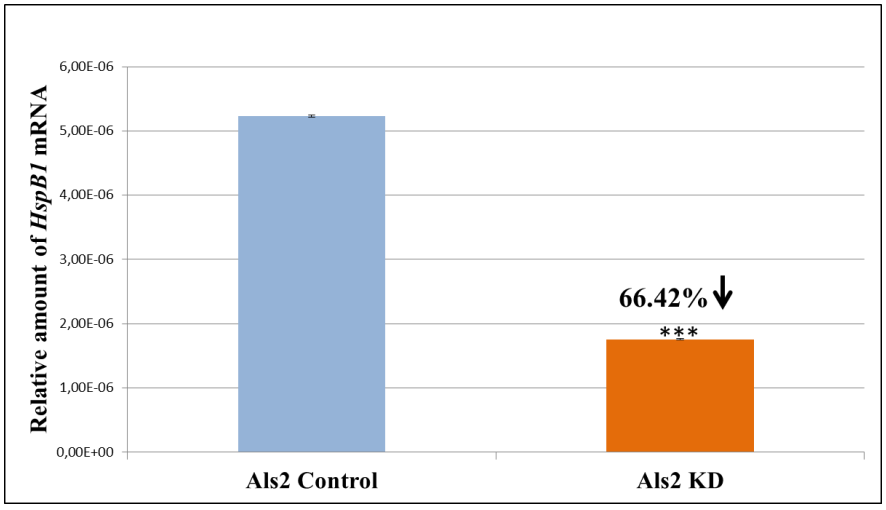

Figure 5.4a. Alterations at *HspB1* mRNA level: 66.42% decrease (without heat-shock) (*** $p < 0.0001$).

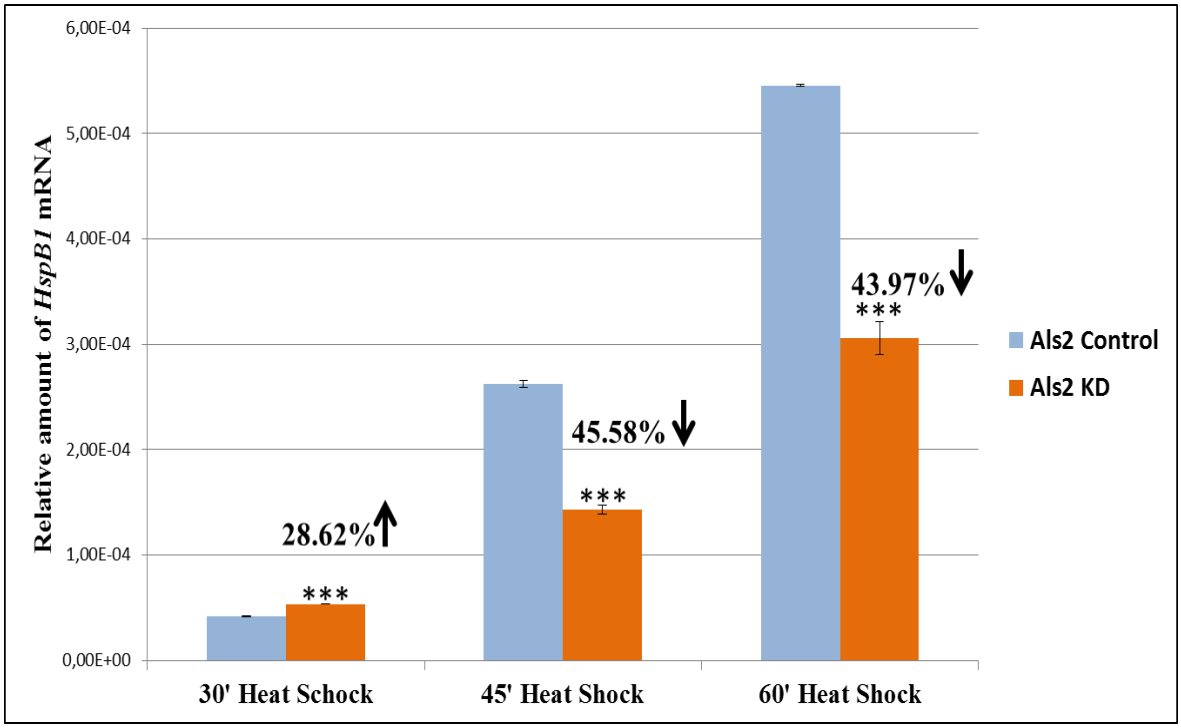

Figure 5.4b. Alterations at *HspB1* mRNA levels: 28.62% increase for 30' heat-shock; 45.58% and 43.97% decreases for 45' and 60' heat-shocks, respectively (*** $p < 0.0001$).

5.1.5. Alterations at the mRNA Levels of *HspB8*

Without heat-shock conditions, *HspB8* mRNA level of *Als2* KD cells was shown to be increased by 43.86% as compared to *Als2* control cell line (Figure 5.5a). In the case of heat-shock conditions, the results were 23.96%, 51.67% and 4.27% increases for 30-, 45- and 60-minute heat shocks, respectively (Figure 5.5b). The p-values were significant for each result.

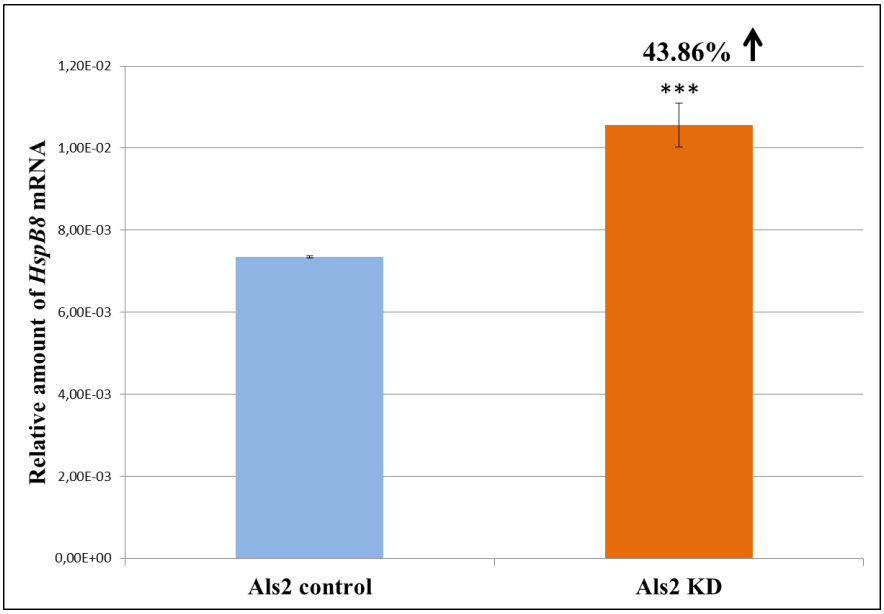

Figure 5.5a. Alterations at *HspB8* mRNA level: 43.86% increase (without heat shock) (*** $p < 0.0001$).

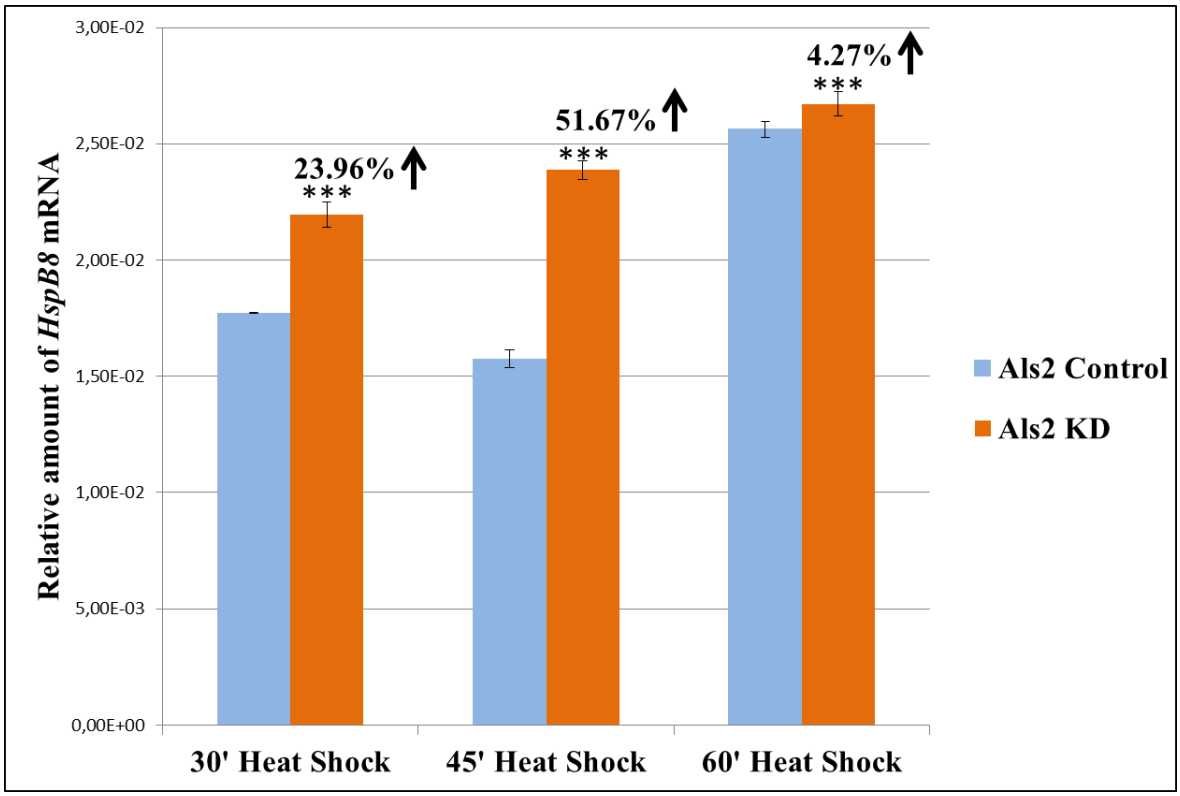

Figure 5.5b. Alterations at *HspB8* mRNA level: 23.96%; 51.67% and 4.27% increase in 30', 45' and 60' heat shock conditions, respectively (*** $p < 0.0001$).

The overall q-RT PCR results revealed that there were statistically significant changes at the mRNA levels of all the genes of interest in *Als2* KD cell lines, as compared to *Als2* control cells (Table 5.1.).

Table 5.1. The alterations in expression profiles of the genes investigated in this study.

	Alterations in mRNA levels in *Als2* KD background
*Als2 (*alsin*)*	73.40% ↑
Spg4 (spastin)	18% ↑
Spg20 (spartin) isoform-a	48% ↑
Spg20 (spartin) isoform-b	32% ↓
HspB1 without heat shock	66.42% ↓
HspB1 with 30’ heat shock	28.62% ↑
HspB1 with 45’ heat shock	45.58% ↓
HspB1 with 60’ heat shock	43.97% ↓
HspB8 without heat shock	43.86% ↑
HspB8 with 30’ heat shock	23.96% ↑
HspB8 with 45’ heat shock	51.67% ↑
HspB8 with 60’ heat shock	4.27% ↑

5.2. Comparison of protein levels of *Spg20* in *Als2* KD and *Als2* control cell lines

In order to correlate alterations at the mRNA levels to protein levels, Western Blot analyses were performed by using four different experimental groups, containing protein lysates isolated from *Als2* KD and *Als2* control cell lines. Spartin isoform-a and b were observed at 85 and 75 kDa on detection films, respectively. To ensure loading uniformity of the protein samples, membranes were also blotted with β-actin antibodies, and at 42 kDa, distinct bands for β-actin were observed (Figure 5.6).

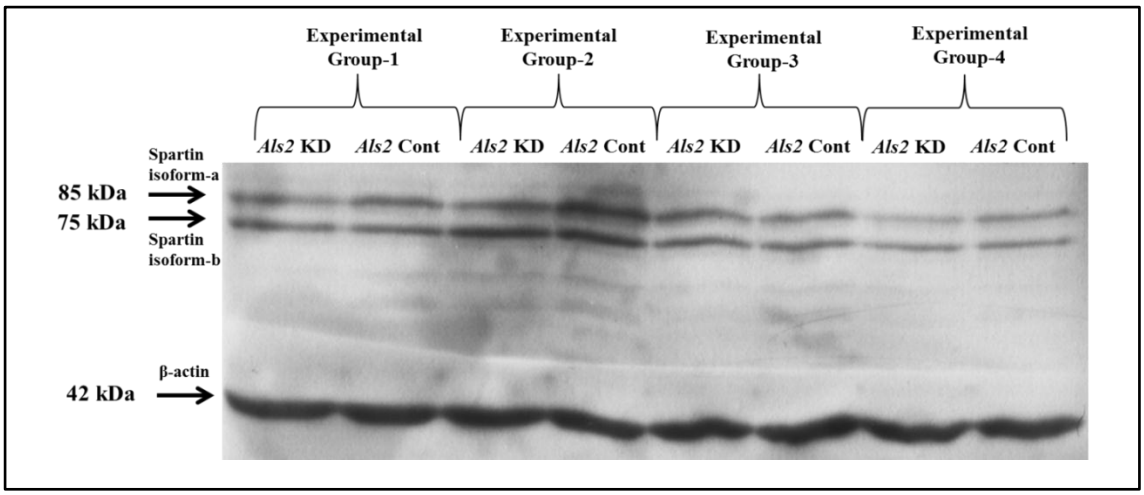

Figure 5.6. Spartin isoforms and β-actin were detected in four different protein lysates derived from *Als2* cont and *Als2* KD cell lines.

In order to compare protein levels, the intensities of the bands on the detection film were measured by using an internet-based program (Image J). Each isoform band in *Als2* KD and *Als2* control cell lines were normalized with β-actin and they were compared with each other. There was 11% decrease at spartin isoform-a protein level and 3% increase at spartin isoform-b. This result suggests that there may not be a correlation between alterations of protein and mRNA levels for spartin (Figure 5.7).

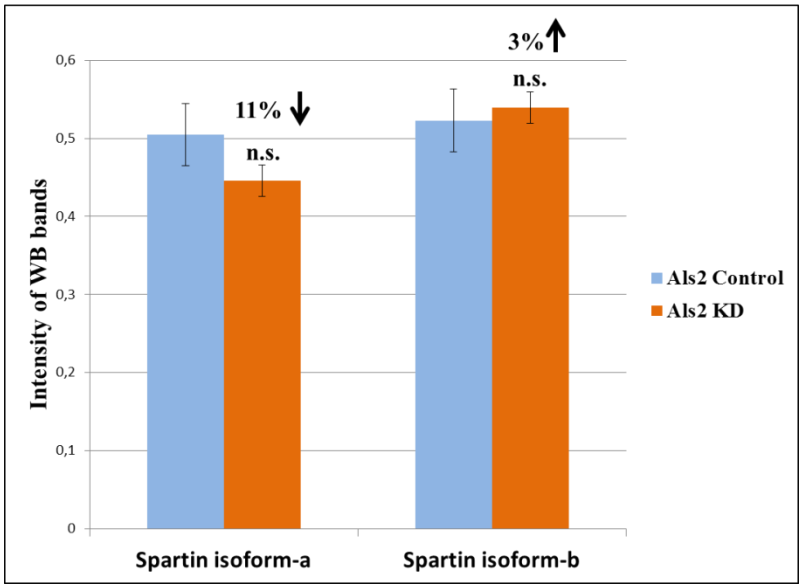

Figure 5.7. Spartin protein levels in *Als2* KD and control cell lines (n.s.: not significant).

5.3. Immunofluorescence Analyses

Since the results in literature are very controversial, regarding overexpression studies of alsin's and spartin's cellular distributions, immunofluorescence studies were carried out to clarify the localizations of these endogenous proteins. Before cell fixation, wild type N2a cells were differentiated by serum deprivation and retinoic acid application for 3 days. Endogenous alsin, stained with an FITC (green fluorescent dye), showed a general diffused pattern in the cell body and neurite-like extensions, there were strong signals deriving from the peri-nuclear region of the cell. Furthermore, endogenous spartin, stained with a rhodamine-red fluorescent dye, displayed a similar pattern to alsin in the cytoplasm with the same high intensity in the peri-nuclear region. The weak signals in the nucleus imply that spartin's expression was too low in the nucleus. When two staining pictures were merged, a yellow-coloured punctuated pattern was observed in the peri-nuclear region of the cells; this indicates that spartin and alsin were partially co-localized in differentiated N2a cells (Figure 5.7).

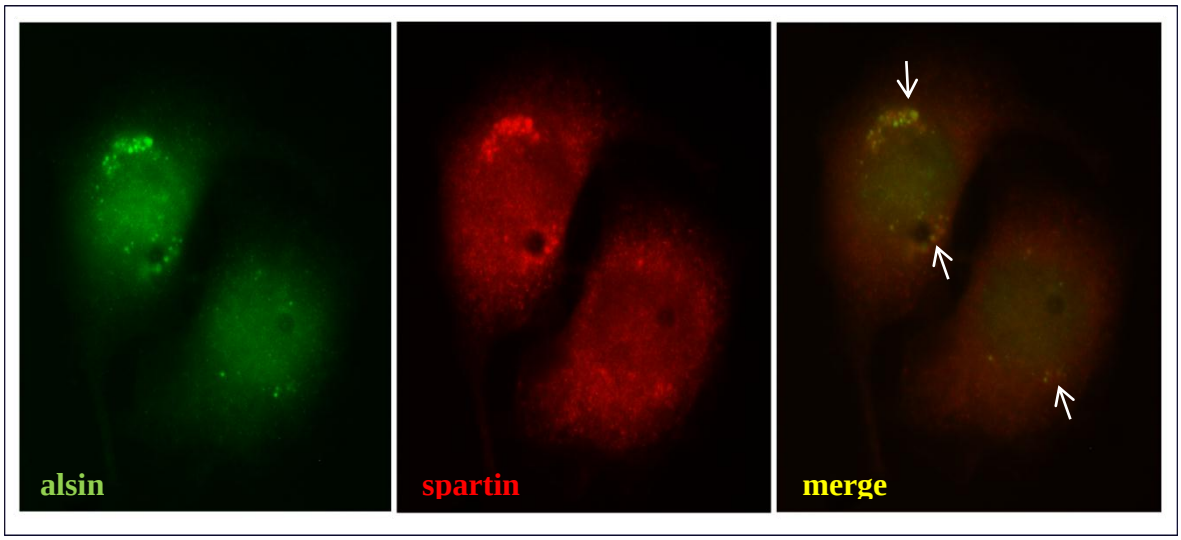

Figure 5.8. Endogenous alsin and spartin were found to be co-localized in the peri-nuclear region of differentiated wild type N2a cells.

5.4. Immunoprecipitation Analyses

The observation of alsin's and spartin's co-localization in N2a cells implies that these two proteins may be present in the same protein complex. In order to confirm their co-existence, immunoprecipitation analyses were performed by using protein lysates isolated from wild type N2a cells. There were 3 experimental groups in the case of IP studies. The first one is the positive control group containing total protein lysate. In this group, two distinct bands at 75 and 85 kDa, representing both spartin isoforms, were observed after Western Blot analyses (Figure 5.9a). The second experimental group (negative control) was the protein lysate which was precipitated with an unrelated antibody and G protein beads. After blotting with anti-spartin antibody, no bands were observed, representing spartin; there was only a band related to the heavy chain of the antibody on the detection film (Figure 5.9b). The third group was the protein lysate precipitated with an alsin antibody and the G protein beads. When the spartin antibody was applied on the Western Blot membrane, a distinct band for the isoform-a was detected in addition to the heavy chain of the spartin antibody (Figure 5.9c). These results implied that alsin and spartin isoform-a were residing at the same protein complex.

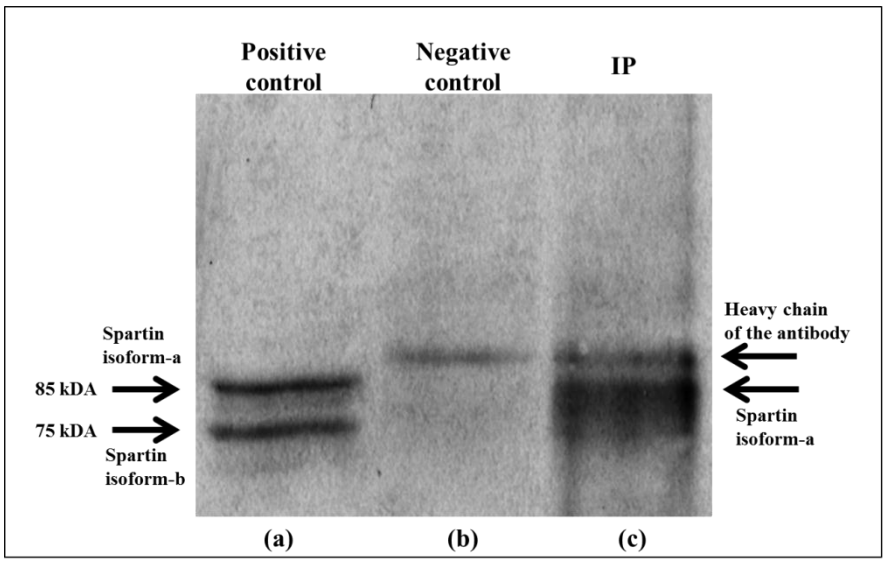

Figure 5.9. Western blot analyses of total protein lysate and immunoprecipitates of wild type N2a cells (a) two spartin isoforms (a and b) at 85 and 75 kDA, respectively. (b) heavy chain of the spartin antibody (c) heavy chain of the spartin antibody and spartin isoform-a at 85kDA.

5.5. Protein studies for alsin, HspB1, HspB8 and spastin

In the framework of this study, the protein levels of alsin, HspB1, HspB8 and spastin were also investigated. In the case of alsin, the protein could not be detected with Western Blot experiments due to its low expression levels. For HspB1 and HspB8, four different groups of protein lysates (*Als2* control, *Als2* KD, heat-shocked *Als2* control and heat-shocked *Als2* KD) were loaded on the SDS-PAGE gels to confirm the mRNA changes at protein levels. However, ladder-like band patterns were observed on detection films, suggesting unspecific binding of the antibodies to unrelated proteins on the membranes. The same unspecific band pattern was also observed in the case of spastin (Figure 5.10).

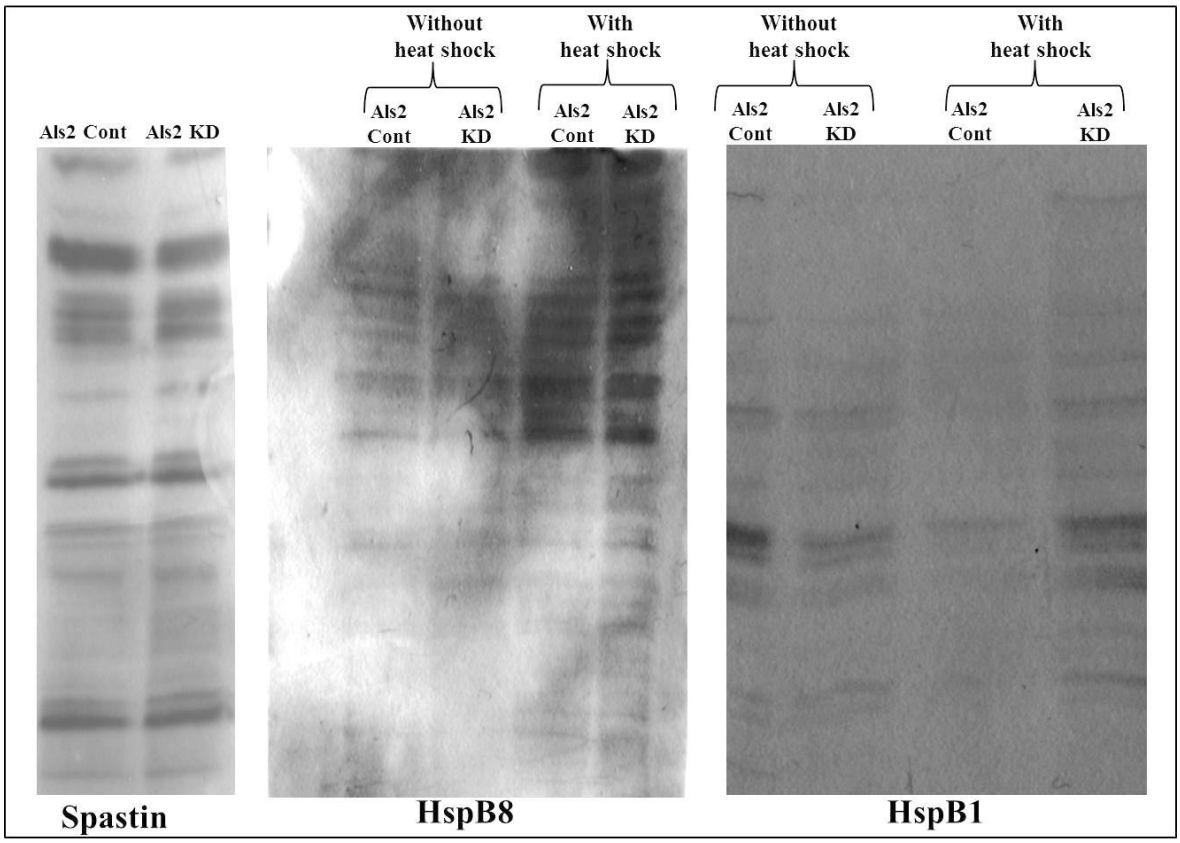

Figure 5.10. Western Blot analyses for spastin, HspB8 and HspB1.

6. DISCUSSION

Neurodegenerative diseases (NDs) are becoming more and more common and a serious health burden with the populations' advanced life spans all around the world. Considering possible negative effects of the rising number of patients on societies in future, studying neurodegenerative processes emerged as a very important topic in the last decade (Hebert *et al*., 2003). In NDs, specific neurons in specific regions of the brain and/or the spinal cord are affected. According to the region affected, the disease may affect cognition, movement or both. Motor neuron disorders (MNDs) are a sub-group of NDs characterized by selective degeneration of motor neurons resulting in movement abnormalities. In the last 16 years, many genes were identified as causative factors for MNDs (Double *et al*., 2010). Although some of these genes are related with only one type of MND, some of them are linked to more than one type, which makes the boundaries between MNDs less clear (Figure 6.1).

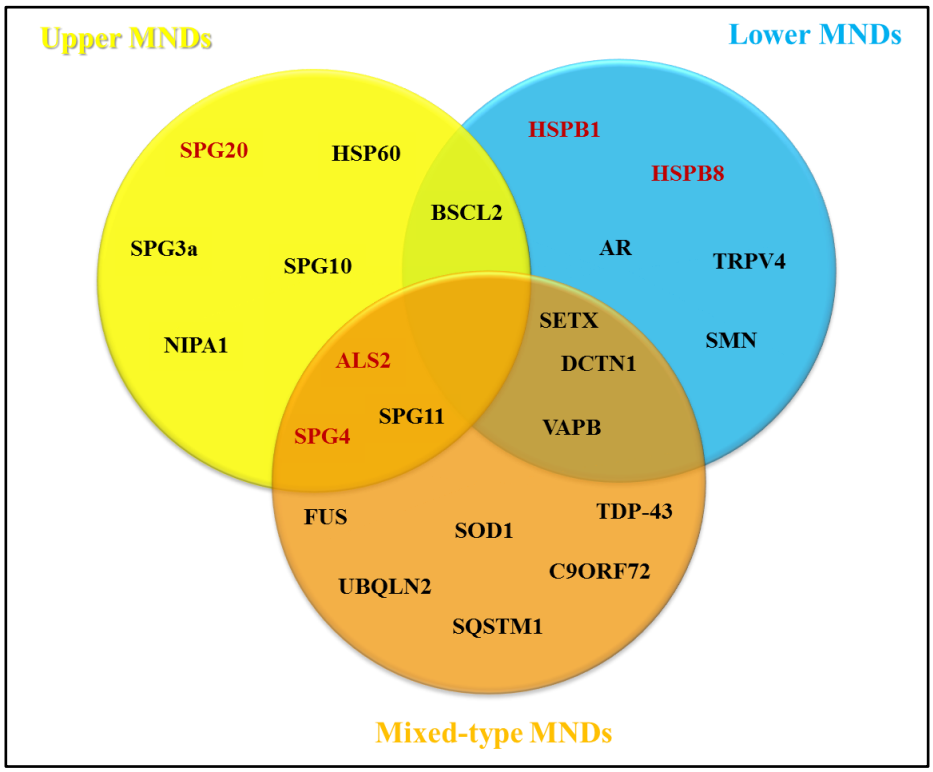

Figure 6.1. Schematic representation of the genes associated with MNDs (The genes investigated in this study are shown in red).

ALS2 is one of the genes which is found to be associated with more than one type of MND. Mutations in *ALS2* result in an unstable and non-functional protein product, either leading to degeneration of upper motor neurons, causing Primary Lateral Sclerosis and Hereditary Spastic Paraplegia, or degeneration of upper and lower motor neurons giving rise to Amyotrophic Lateral Sclerosis. The remarkable issue about *ALS2* mutations is that although MNDs generally have late-onset characteristics, in the case of mutated *ALS2*, the disease symptoms start at very early ages, leading to juvenile or infantile forms of these diseases (Hadano *et al*., 2010). This situation indicates that the protein product of *ALS2*, alsin, has a major role in motor neuron integrity, in which motor neurons cannot compensate the absence of alsin and degenerate in a very short period of time.

Alsin, with its three putative RCC1-, Rho- and VPS9- GEF domains, is regarded as an activator and regulator of small GTPases. Since the identification of alsin as a Rab5 activator and a Rac1 effector, it is implicated in endosomal trafficking and axonal growth via the interactions with the above two proteins. However, the exact functions of alsin in these mechanisms are not very well known (Hadano *et al*., 2007). Considering limited information about alsin's assumed essential role on motor neuron integrity, the aim of this study was to gain detailed information about the functions of alsin by investigating its relations both at mRNA and protein levels with HspB1, HspB8, spartin and spastin. As it can be seen in Figure 6.1, all three types of MNDs are connected to each other via overlapping genes. This situation suggests that there may be some direct or indirect relations between those proteins which can affect each other's functions both in healthy or disease conditions. In this sense, *SPG20* and *SPG4* were chosen to be studied in this thesis, considering their upper MND associations. HspB1 and HspB8 were selected, on the other hand, due to their lower MND implications.

In order to mimic alsin-linked abnormalities in N2a cells, generation of an *ALS2* KD stable cell line was the first step in this study. With q-RT PCR experiments, a 73.40% significant decrease in *ALS2* mRNA level was observed (Figure 5.1). At protein level, Western Blot experiments were performed to confirm this significant reduction by using four different antibodies deriving from different origins. However, specific bands for alsin were not observed. In literature, it has been shown that in a total detergent-soluble fraction of mouse brain lysate, the percentage of alsin protein quantity was 0.0003% (Yamanaka *et*

al., 2003). Compared to mouse brain lysate, the amount of alsin in N2a cell lysates, that were used in the experiments of this study, is much lower, thus the absence of the bands can be explained by endogenous alsin's low expression levels. In order to overcome this problem in future, different protein isolation procedures, which would further enrich protein concentrations, can be used. Another approach could be the generation of alsin-overexpressing cell lines. A major additional problem is the non-functional antibodies. Since there is a lack of commercially available alsin-specific antibodies, production of an *ALS2* antibody will help to solve the problem.

6.1. *Als2* and HSP genes: *Spg20* and *Spg4*

In this part of the study, the relations between alsin and two genes that are associated with HSP were investigated. *SPG4* (spastin) and *SPG20* (spartin) harbor an MIT domain which is a characteristic region of endosomal proteins (Cicarelli *et al*., 2003). Considering alsin's implications in endosomal trafficking, spartin and spastin may be related to alsin with roles in a common endocytic pathway.

In the first step, changes at expression levels of the above two genes were measured in *Als2* KD conditions and significant alterations were observed (Figure 5.2, Figure 5.3a and Figure 5.3b). In the second step, in order to confirm those changes at protein levels, Western Blot analyses were performed. In the case of spastin, due to unspecific antibody binding, a distinct band for the protein could not be detected (Figure 5.10). Hence, the experiments should be repeated by using new antibody sets. For spartin, four different protein lysate sets were used and distinct bands at 75 and 85 kDa, representing isoforms of the protein were observed (Figure 5.6). After quantification of band intensities using an internet-based program, no correlation was observed between changes at mRNA and protein levels. Although there was a 48% increase at spartin isoform-a mRNA level, there was a 11% decrease at protein level. A similar result was obtained in isoform-b with a 32% decrease at mRNA level and a 3% increase at protein level. The reason for this discrepancy may lie in the limitation of the Western Blot system, the resolution power of which does not measure the changes observed (corresponding to 50% decrease at mRNA level).

In order to investigate the relationship between spartin and alsin at protein level, IF studies were performed and a similar pattern of staining was observed for both alsin and spartin. They were all diffused in the cytoplasm and co-localized in peri-nuclear region of the differentiated N2a cells (Figure 5.8). Since there was no isoform-specific antibody for spartin, isoform-specific localization could not be obtained. In addition to IF analysis, to check if these two proteins are residing in the same protein complex or not, immunoprecipitation experiments were performed. Those studies revealed that alsin and spartin isoform-a precipitated together in the same protein complex. This result suggests a possible physical interaction between alsin and spartin isoform-a. Since both proteins are implicated in endosomal trafficking, have membrane binding features and can be recruited to endosomes, it is plausible to examine direct interactions by using yeast-two-hybrid systems in future experiments. There are also supportive functional studies on alsin and spartin showing that there may be a direct link between them. For instance, both proteins have roles in endocytosis of EGFR (epidermal growth factor receptor). In addition to spartin's interaction with EPS15, which is a major protein in EGF uptake, in spartin knock-down conditions, rate of EGFR internalization was found to be decreased significantly (Bakowska *et al*., 2007). In the case of alsin, the cultured fibroblasts derived from *Als2* knock-out mice displayed significant delay in trafficking and fusion of EGF-positive endosomes and vesicles (Hadano *et al*., 2006). Considering EGF's importance in cell growth and differentitation, spartin and alsin may have related roles, other than in MND pathology, via effecting EGF metabolism.

6.2. Heat Shock Response and Alsin

Small heat shock proteins are molecular chaperones protecting cells from stress. In cellular stress conditions, with increased heat-shock protein expression levels, HSPs assist proper folding of denatured proteins and prevent aggregations (Irobi *et al*., 2010). In the framework of this thesis, the changes at mRNA levels of *HspB1* and *HspB8* by creating stress conditions were analyzed in an N2a cell line. While knocking down of ALS2 was the first stress factor applied, additional stress factors, e.g. 30-, 45- and 60-minute heat shocks, were applied on *Als2* KD stable cell lines and the changes were measured.

In the case of *HspB1*, the expression of the gene decreased >50%, when *Als2* was knock downed in N2a cells (Figure 5.4a). This result was not in accordance with an upregulation of the small heat-shock proteins under stress conditions. After heat-shock applications using different time intervals, there was a rising pattern of expression as the heat-shock time increased in the control and *Als2* KD cell lines independently. However, when the two experimental groups were compared with each other, except the 30 minute heat-shock, heat-shock response was nearly 50% lower in *Als2* KD stable cell line, similar to the expression pattern seen in Figure 5.4a (Figure 5.4b). These results suggest that knocking down *Als2* may have an inhibitory effect on the heat shock response of *HspB1* at mRNA level. The lack of heat-shock response may cause abnormalities in protein folding mechanisms which would lead to protein aggregations. Another important point is the role of *HspB1* against apoptosis. As mentioned earlier, *HspB1* can bind to apoptotic signalling proteins and reduce caspase activites (Akbar *et al*., 2003). In the light of this study's results, it can be suggested that in *Als2* KD conditions, the cells were more prone to enter apoptosis under stress conditions.

HspB8, the second heat shock gene investigated, displayed different expression patterns in q-RT PCR experiments. After knocking down *Als2*, unlike *HspB1*, there was a 50% increase at *HspB8* mRNA level (Figure 5.5a). In the *Als2* KD stable cell line, the responses were in a rising-manner, as heat-shock time increases (orange bars in Figure 5.5b). On the other hand, in the control cell line, the data was inconsistent, showing that the level of the expression after 45 minute heat-shock, was lower than the expression after 30 minute heat-shock (blue bars in Figure 5.5b) This may be the result of the big response when the cell was first exposed to heat-shock, decreasing its expression after 30 minutes As the heat-shock time extended, the response became inadequate and the cell increased again the expression dramatically. When the results of both experimental groups were compared, it was observed that for each heat-shock condition, the amount of heat-shock response in *Als2* KD group was higher than the control group. These results show that knock-down conditions have enhancing effects on expression levels of *HspB8*. Another interesting point is the difference between *HspB8* expression levels of *Als2* KD and the control, which is very small (4.27%) after 60 minute heat-shock compared to 30 and 45 minutes. There are two explanations for this result: (i) The cell may reach its maximum heat-shock response, independent from the knock-down stress after 60 minutes,

or (ii) as it is the case for *HspB1*, alsin knock-down has also an inhibitory effect on *HspB8*. This becomes obvious as the time of heat-shock extends beyond 60 minutes. In order to check this, q-RT PCR should be performed, after heat-shocks longer than 60 minutes.

Although significant changes were observed at mRNA levels, without confirming alterations at protein levels, it is risky to conclude that there is a direct link between heat shock response and alsin expression. In this respect, by using two different protein lysates (with or without heat-shocks) derived from *Als2* KD and *Als2* control cell lines, Western Blot experiments were performed. However, distinct protein bands for HspB1 and HspB8 could not be observed. Due to unspecific antibody binding, there were too many protein bands on the detection films (Figure 5.10). By using different antibody dilutions and blocking buffer concentrations, the problem was trouble-shooted, but this was not successful either. Hence, Western Blot analyses should be repeated by using new antibody sets in the future.

7. CONCLUSION

The aim of this study was to gain insights into the functions of alsin, a protein which is associated with MNDs, by investigating its relations with other MND-related proteins. This is the first study in the literature implicating alsin in heat-shock response. With the inhibitory effect of alsin on heat-shock response of *HspB1* at mRNA level, considering HspB1's pro-apoptotic function, cells with alsin mutations will be more prone to enter apoptosis under stress conditions. In order to get a clear idea about alsin's role in heat-shock, further studies at protein level should be performed.

Another important finding is the identification of a possible interaction between alsin and spartin. In addition to significant changes at mRNA levels of spartin isoforms in *Als2* KD background, IF and IP studies support the presence of an interaction between alsin and spartin. In this sense, yeast-two-hybrid experiments should be performed in future, which may help to clarify endocytic abnormalities in alsin- and spartin- linked motor neuron degeneration.

MNDs are affecting millions of people all around the world, with increasing numbers of patients every year. Each MND-associated gene/protein can be regarded as one piece of a jigsaw puzzle pointing to the mechanisms of motor neuron degeneration. We hope that this study, along with future functional research, will contribute to decoding the unknowns, finding and connecting the right pieces. Only a complete understanding of the whole mechanism, underlying motor neuron degeneration, will pave the ways for long-awaited therapies.

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