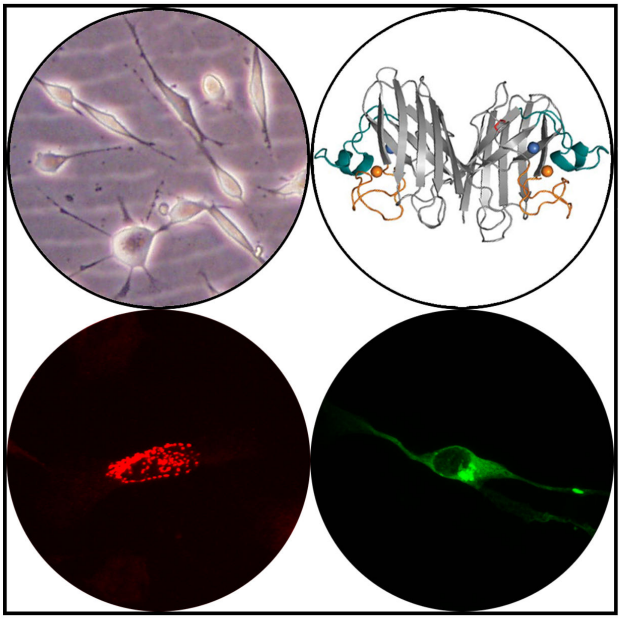
Amyotrophic Lateral Sclerosis:
Mammalian Cell Models, Copper-Zinc Superoxide Dismutase and Biological Characteristics



Catarina Heitor Gomes

Dissertation presented to obtain the
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Supervisor: Dr. Júlia Costa.

President of the jury: Dr. Luís Paulo Rebelo.

Members of the jury: Dr. Mª João Saraiva; Dr. Cláudio Gomes; Dr. Carlos Farinha; Dr. Mamede de Carvalho; Dr. Maria Helena Santos.

Cover: *By author*: NSC-34 cells used as mammalian cell model in this Thesis, visualized by inverted microscopy. Crystal structure of dimeric human Copper-Zinc Superoxide Dismutase (adapted from Valentine et al., 2005). Two of the pathological characteristics of Amyotrophic Lateral Sclerosis mimicked in NSC-34 cells upon mutant SOD1 overexpression: mutant SOD1 aggregation (green) detected by fluorescence microscopy and Golgi apparatus fragmentation (red) detected by immunofluorescence microscopy using an antibody anti-GM130, a *cis*-Golgi marker.

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Ph.D. Thesis

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is the most severe and common adult onset disorder that affects motor neurons in the spinal cord, brainstem and cortex, resulting in progressive weakness and death from respiratory failure within two to five years of symptoms onset.

The majority of the ALS cases are sporadic, but 5-10% of cases are familial (FALS), nevertheless they are clinically and pathologically very similar, which suggests they share common pathogenic mechanisms. The discovery of the copper-zinc superoxide dismutase (SOD1) mutated gene 15 years ago, that accounts for 20% of the FALS cases, allowed the creation of cellular and animal models of ALS, and numerous studies have been performed to investigate the toxicity of the mutant enzyme. These models also allow the design and testing of therapeutic strategies. It is accepted that SOD1-linked FALS arises through a toxic gain-of-function, and several mechanisms have been proposed. These include protein misfolding and toxicity from intracellular aggregates, disruption of the neurofilament network and intracellular trafficking along neurofilaments, and involvement of non-neuronal cells in the vicinity of motor neurons.

One major goal of this Thesis has been to establish cellular models of FALS through overexpression of mutant $SOD1^{G93A}$ in model cells of motor neurons, NSC-34 cells, to mimic pathological characteristics associated with the disease. The importance of cellular models to study pathological mechanisms associated with the disease was emphasized.

In Chapter 2, we have started by constructing and characterizing stable NSC-34 cell lines overexpressing human mutant SOD1 with the substitution of a glycine by an alanine at position 93 ($SOD1^{G93A}$). Mutant expressing cells were shown to have decreased proliferation and differentiation. One of the pathological characteristics of ALS patients is the

fragmentation of the Golgi apparatus. We have analyzed the Golgi morphology by immunofluorescence microscopy using an antibody anti-GM130, a *cis*-Golgi marker. A higher percentage of cells overexpressing mutant SOD1 showed Golgi fragmentation when compared with cells overexpressing wild-type SOD1. This was found not to be due to apoptosis as no caspase-3 activation, loss of mitochondrial transmembrane potential or nuclear fragmentation were detected by Western blot, fluorescence activated cell sorting (FACS) analysis or fluorescence microscopy, respectively. Therefore, NSC-34/hSOD1^{G93A} cells constituted a model of FALS with respect to Golgi fragmentation.

In Chapter 3, primary cultures of spinal cord cells from transgenic SOD1^{G93A} rat embryos E14 were established and characterized. Two types of cultures were studied, spinal cord sections or dissociated spinal cords. Both cultures were found to contain neurons, including motor neurons, and astrocytes as evaluated using marker antibodies by immunofluorescence microscopy. The morphology of the *trans*-Golgi Network and the *cis*-Golgi apparatus from wild-type and transgenic cells were found to be similar when detected with antibodies anti-TGN38, anti-GM130 or anti-GRASP65 by immunofluorescence microscopy. These cell cultures were found to resemble the spinal cord environment and, thus, constituted a good system to study the different cell types involved in the ALS pathology.

In Chapter 4, NSC-34 cells were transiently transfected with mutant SOD1^{G93A} fused to the enhanced green fluorescent protein, which formed aggregates as found in tissues from FALS patients. These aggregates were detected with an anti-ubiquitin antibody. Another feature was that endogenous TAR DNA-binding protein of 43 kDa was found predominantly in the nucleus but not in the aggregates, similarly to that reported for patients with mutant SOD1 associated FALS but contrary to patients with sporadic ALS. Small molecules, such as trehalose, have been used to decrease protein aggregation in models of various neurodegenerative

diseases due to their properties as chemical chaperones. Here, the percentage of cells containing mutant SOD1 aggregates and the amount of detergent insoluble protein were also found to be decreased upon exposure to trehalose at millimolar concentrations.

In Chapter 4, mutant SOD1 overexpression was also found to cause decreased levels of three concomitantly overexpressed glycoproteins - two secretory, β-trace protein and erythropoietin, and one from the plasma membrane, L1, irrespective of the presence of mutant SOD1 aggregates. Furthermore, there was no intracellular accumulation of these proteins nor alterations in their *N*-glycosylation, which indicated that Golgi fragmentation did not cause detectable changes in glycoprotein trafficking nor glycosylation. As it is known that mutant SOD1 triggers endoplasmic reticulum stress, which in turn reduces global protein synthesis the decreased levels of concomitantly overexpressed glycoproteins maybe the consequence of endoplasmic reticulum stress.

Previously in the literature, cytosolic SOD1 protein was found extracellularly. In Chapter 5, wild-type and mutant SOD1 were found to be secreted in association with membrane vesicles from NSC-34 cells. The separation of those vesicles by sucrose density gradient showed that SOD1 co-localized with the exosomal marker CD9. Therefore, SOD1 secretion occurred via exosomes. Furthermore, exosomes produced by NSC-34 cells were found to interact with the same cells, which may constitute a way to transfer toxicity between cells.

In conclusion, the work described in this Thesis provides evidence that cellular models overexpressing mutant SOD1 are important tools for the study of pathogenic mechanisms in FALS such as, fragmentation of the Golgi apparatus, mutant SOD1 aggregation and SOD1 secretion. They can also be used as tools to test possible therapeutic compounds in preclinical assays.

Resumo

A Esclerose Lateral Amiotrófica (ELA) é a doença neurológica mais grave e comum do adulto que afecta os neurónios motores na espinal medula, tronco cerebral e cérebro, a qual resulta em fraqueza progressiva. A morte por insuficiência respiratória ocorre no prazo de dois a cinco anos após o início dos sintomas.

A maioria dos casos de ELA são esporádicos e 5-10% são hereditários, no entanto, ambos são clínica e patologicamente muito semelhantes, o que sugere que partilham mecanismos fisio-patológicos comuns. A descoberta de mutações no gene da cobre-zinco superóxido dismutase (SOD1) há 15 anos atrás, as quais correspondem a 20% dos casos familiares, permitiu o desenvolvimento de modelos celulares e animais de ELA, e têm sido realizados inúmeros estudos para investigar a toxicidade do enzima mutado. Estes modelos permitem também o desenho e o teste de estratégias terapêuticas. Actualmente, sabe-se que as mutações na SOD1 relacionadas com a forma familiar de ELA, advêm de um ganho de função tóxica, e têm sido propostos vários mecanismos. Estes incluem desnaturação de proteína e toxicidade de agregados intracelulares, alterações dos neurofilamentos e do tráfego intracelular ao longo destes, e envolvimento de células não-neuronais nas proximidades dos neurónios motores.

Um dos principais objectivos desta Tese foi a constituição de modelos celulares da forma familiar de ELA através da sobre-expressão da SOD1 humana mutante, na qual a glicina, na posição 93, foi substituída por uma alanina ($SOD1^{G93A}$), em células modelo de neurónios motores, células NSC-34, para mimetizar as características patológicas associadas à doença. Enfatizou-se a importância dos modelos celulares no estudo dos mecanismos patológicos associados à doença.

No capítulo 2, começou-se por construir e caracterizar linhas celulares estáveis de NSC-34 por sobre-expressão da forma humana mutada de $SOD1^{G93A}$. As células que expressavam a forma mutante de SOD1 revelaram o decréscimo das capacidades de proliferação e diferenciação. Uma das características patológicas dos pacientes com ELA é a fragmentação do complexo de Golgi. Analisou-se nas células construídas a morfologia do complexo de Golgi por microscopia de imunofluorescência utilizando o anticorpo anti-GM130, que marca o *cis*-Golgi. Observou-se que uma maior percentagem de células que sobre-expressavam SOD1 mutante apresentava fragmentação do complexo de Golgi, quando comparado com as células que sobre-expressavam a forma *wild-type* da mesma proteína. Esta fragmentação não era consequência de apoptose, uma vez que não se verificou activação da caspase-3, perda de potencial transmembranar mitocondrial ou fragmentação nuclear por *Western blot*, *fluorescence activated cell sorting* (FACS) ou por microscopia de fluorescência, respectivamente. Por conseguinte, as células NSC-34/$hSOD1^{G93A}$ constituem um modelo da forma hereditária de ELA no que diz respeito à fragmentação do complexo de Golgi.

No capítulo 3, foram estabelecidas e caracterizadas culturas primárias de células de espinal medula de embriões E14 de ratos transgénicos $SOD1^{G93A}$. Estudaram-se dois tipos de culturas, secções de espinal medula ou células da espinal medula dissociadas. Ambas as culturas possuíam neurónios, incluindo neurónios motores, e astrócitos, identificados com anticorpos marcadores por microscopia de imunofluorescência. A morfologia do *trans*-Golgi reticular e do *cis*-Golgi de células provenientes do animal *wild-type* ou transgénico revelou-se semelhante quando detectada com anticorpos anti-TGN38, anti-GM130 ou anti-GRASP65 por microscopia de imunofluorescência. Estas culturas de células assemelham-se ao ambiente da espinal medula e, assim,

constituem um bom sistema para estudar os diferentes tipos de células envolvidas na patologia de ELA.

No capítulo 4, células NSC-34 foram transientemente transfectadas com o vector codificante da $SOD1^{G93A}$ mutante acoplada à proteína fluorescente verde que formou agregados semelhantes aos encontrados em tecidos de pacientes com forma familiar de ELA. Estes agregados foram detectados com um anticorpo anti-ubiquitina. A proteína endógena de 43 kDa, *TAR DNA-binding protein* TDP-43, foi encontrada predominantemente no núcleo, e não nos agregados, de forma semelhante ao descrito nos pacientes com SOD1 mutante associada à forma hereditária de ELA mas, contrário ao descrito para pacientes com ELA esporádica. Pequenas moléculas, como a trealose, têm sido usadas para diminuir a agregação de proteína em modelos de doenças neurodegenerativas, devido às suas propriedades como *chaperone* químico. Aqui, a percentagem de células que possuíam agregados de SOD1 mutante e a quantidade de proteína insolúvel em detergente foram reduzidas na presença de trealose em concentrações na ordem de milimolar.

No capítulo 4, observou-se ainda que a sobre-expressão de SOD1 mutante provocava a diminuição dos níveis de três glicoproteínas concomitantemente sobre-expressas - duas de secreção, proteína *β-trace* e eritropoietina, e uma de membrana plasmática, L1, independentemente da presença de agregados de SOD1 mutante. Além disso, não houve acumulação intracelular destas proteínas nem alterações na sua *N*-glicosilação, o que indicou que a fragmentação do complexo de Golgi não provocou alterações detectáveis no tráfego de glicoproteínas nem na sua glicosilação. Uma vez que se sabe que SOD1 mutante provoca *stress* do retículo endoplasmático que, por sua vez, reduz a síntese proteica global, a diminuição dos níveis das glicoproteínas concomitantemente sobre-expressas pode ser consequência do *stress* do retículo endoplasmático.

Anteriormente, a proteína citosólica SOD1 foi encontrada extracelularmente. No Capítulo 5, descreveu-se que a SOD1 *wild-type* e mutante eram secretadas em associação com vesículas membranares em células NSC-34. O fraccionamento dessas vesículas por gradiente de densidade de sacarose mostrou que a SOD1 co-localizava com o marcador exosomal, CD9. Por conseguinte, a secreção de SOD1 ocorreu via exossomas. Além disso, os exossomas produzidos pelas células NSC-34 interagiram com as mesmas células, pelo que podem constituir uma forma de transferência de toxicidade entre células.

Em conclusão, o trabalho descrito nesta Tese fornece evidência de que os modelos celulares, através da sobre-expressão de SOD1 mutante, são ferramentas importantes no estudo dos mecanismos patogénicos associados à forma familiar de ELA, tais como, a fragmentação do complexo de Golgi, agregação e secreção de SOD1 mutante. Estes modelos podem também ser usados como ferramentas para testar possíveis compostos terapêuticos em ensaios pré-clínicos.

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Outline of the Thesis

The work described in this Thesis is concerned with the study of mammalian cell models of Amyotrophic Lateral Sclerosis (ALS) by overexpression of mutant $SOD1^{G93A}$. ALS is a devastating neurodegenerative disease that results from the death of motor neurons in the brainstem, cortex and spinal cord, leading to progressive muscular atrophy and paralysis, and for which no effective therapy has been found yet. Since the discovery of mutations in the gene coding for Cu,Zn superoxide dismutase (SOD1) 15 years ago, that account for 2% of all ALS cases, several mechanisms to explain the death of motor neurons have been proposed but more studies are needed to fully understand this disease.

This dissertation starts with a general introduction to ALS, mainly epidemiological, clinical and neuropathological aspects of the disease are presented. Particular relevance is given to the genetics of familial ALS, especially to SOD1 mutations. This is followed by an overview on the cellular pathogenic mechanisms in ALS, with emphasis to protein instability and SOD1 aggregation, SOD1 structure, folding and stability, mechanisms of mutant SOD1 degradation, morphology of Golgi apparatus and intracellular trafficking, apoptosis and the role of non-neuronal cells in ALS. Finally, a summary of clinical trials for ALS treatment is presented.

Chapter 2 describes the establishment of a cell model of the ALS disease by overexpression of mutant $SOD1^{G93A}$ in NSC-34 cells where Golgi apparatus disruption occurs independently from apoptosis.

In Chapter 3, two types of primary cultures of spinal cord, sections or dissociated cells, from rat embryo E14 from transgenic $SOD1^{G93A}$ animals are established and characterized by immunofluorescence microscopy.

Chapter 4 describes mutant SOD1 aggregation in NSC-34 cells, the levels of co-expressed secretory and membrane glycoproteins, as well as the effect of trehalose, a chemical chaperone, in preventing mutant SOD1 aggregation.

In Chapter 5, NSC-34 cells overexpressing mutant $SOD1^{G93A}$ are used to study the secretion of cytosolic SOD1 via exosomes. These membrane vesicles could explain cell-to-cell transfer of mutant toxicity as they can interact with NSC-34 cells.

Chapter 6 consists of a general discussion, where main conclusions and future perspectives of the work are presented.

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ABBREVIATIONS

Abbreviation	Full form
AD	**A**utosomal **D**ominant
ALS	**A**myotrophic **L**ateral **S**clerosis
ANG	**ANG**iogenin
AR	**A**utosomal **R**ecessive
BDNF	**B**rain-**D**erived **N**eurotrophic **F**actor
BSA	**B**ovine **S**erum **A**lbumin
CFSE	**C**arboxy**F**luorescein diacetate **S**uccinimidyl **E**ster
CHO	**C**hinese **H**amster **O**vary
CNS	**C**entral **N**ervous **S**ystem
CNTF	**C**iliary **N**euro**T**rophic **F**actor
COX-2	**C**yclo**OX**ygenase **2**
CSF	**C**erebro**S**pinal **F**luid
DAPI	4',6-**D**i**A**midino-2-**P**heny**l**Indole
DiOC$_6$(3)	3,3'-**Di**hexyl**O**xa**C**arbocyne iodide
DOC	sodium **D**e**O**xy**C**holate
EAAT2	astroglial **E**xcitatory **A**mino **A**cid **T**ransporter **2**
ECL	**E**nhanced **C**hemi**L**uminescent
EDTA	**E**thylene**D**iamine**T**etra-**A**cetic acid
EE	**E**arly **E**ndosome
EGFP	**E**nhanced **G**reen-**F**luorescent **P**rotein
Endo H	**Endo**glycosidase **H**
EPO	**E**rythro**PO**ietin
ER	**E**ndoplasmic **R**eticulum
FACS	**F**luorescence **A**ctivated **C**ell **S**orting
FALS	**F**amilial **A**myotrophic **L**ateral **S**clerosis
FBS	**F**oetal **B**ovine **S**erum
FCS	**F**oetal **C**alf **S**erum

Abbreviation	Full form
FDA	**F**ood and **D**rug **A**dministration
FIG4	**F**actor-**I**nduced **G**ene 4 protein
FITC	**F**luorescein **I**so**T**hio**C**yanate
FTD	**F**ronto**T**emporal **D**ementia
FUS	**FU**sed in **S**arcoma
GDNF	**G**lial cell line-**D**erived **N**eurotrophic **F**actor
GFAP	**G**lial **F**ibrillary **A**cidic **P**rotein
GFP	**G**reen-**F**luorescent **P**rotein
Glc	**Gl**u**c**ose
GM130	*cis*-**G**olgi **M**atrix protein of **130** kDa
GRASP55	**G**olgi**R**e**A**ssembly **S**tacking **P**otein of **55** kDa
GRASP65	**G**olgi**R**e**A**ssembly **S**tacking **P**otein of **65** kDa
GTP	**G**uanoside-5'-**T**ri**P**hosphate
HEK	**H**uman **E**mbryonic **K**idney
HMM	**H**igh **M**olecular **M**ass
HRP	**H**orse**R**adish **P**eroxidase
hSOD1	**h**uman copper-zinc **S**uper**O**xide **D**ismutase **1**
Hsp	**H**eat-**s**hock **p**rotein
IGF-1	**I**nsulin-like **G**rowth **F**actor-**1**
IgG	**I**mmuno**g**lobulin **G**
Inher	**Inher**itance
LC	**L**ight **C**hain
LE	**L**ate **E**ndosome
Lyso	**Lyso**some
MAPT	**M**icrotubule-**A**ssociated **P**rotein **T**au
MCP-1	**M**onocyte-**C**hemoattractant **P**rotein-**1**
MN	**M**otor **N**euron

Abbreviation	Full form
mSOD1	**m**ouse copper-zinc **S**uper**O**xide **D**ismutase **1**
MVB	**M**ulti**V**esicular **B**ody
NB	**N**euro**B**asal
NF	**N**euro**F**ilament
NF-L	**N**euro**F**ilament-**L**ight chain
NGFR	**N**erve **G**rowth **F**actor **R**eceptor
NO	**N**itric **O**xide
PAGE	**P**oly**A**crylamide **G**el **E**lectrophoresis
PBS	**P**hosphate **B**uffered **S**aline
PCR	**P**olymerase **C**hain **R**eaction
PDL	**P**oly-**D**-**L**ysine
PI	**P**ropidium **I**odide
PNGase F	**P**eptide:***N***-**G**lycosid**ase F**
p115	**p**rotein of **115** kDa
PVDF	**P**oly**V**inyledene **D**i**F**luoride
rSOD1	**R**at copper-zinc **S**uper**O**xide **D**ismutase **1**
RT	**R**oom **T**emperature
SALS	**S**poradic **A**myotrophic **L**ateral **S**clerosis
SC	**S**pinal **C**ord
SD	**S**prague **D**awley
SDS	**S**odium **D**odecyl **S**ulphate
SETX	**SE**na**T**a**X**in
SNARE	**S**oluble ***N***-ethylmaleimide-sensitive factor **A**ttachment protein **RE**ceptor
SOD1	Copper-Zinc **S**uper**O**xide **D**ismutase **1**
STS	**ST**auro**S**porine
Suc	**Suc**rose

Abbreviation	Full form
TARDBP	**TAR D**NA-**B**inding **P**rotein
TBS	**T**ris **B**uffer **S**aline
TDP-43	**T**ar **D**NA-binding **P**rotein-**43**
TgN	**T**rans**g**e**N**ic
TGN	***Trans***-**G**olgi **N**etwork
TLS	**T**ranslated in **L**ipo**S**arcoma
TNFα	**T**umor **N**ecrosis **F**actor **α**
Tre	**Tre**halose
Tris	**Tris**(hydroxymethyl)aminomethane
TRITC	**T**etramethyl **R**hodamine **I**so-**T**hio**C**yanate
TUDCA	**T**auro**U**rso**D**eoxy**C**holic **A**cid
TX-100	**T**riton **X**-**100**
VAPB	**V**esicle-**A**ssociated membrane **P**rotein **B**
VEGF	**V**ascular **E**ndothelium **G**rowth **F**actor
VSVG	**V**esicular **S**tomatitis **V**irus **G** protein
WB	**W**estern **B**lot
wt	**w**ild-**t**ype
β-TP	**β**–**T**race **P**rotein

AMINO ACID NOMENCLATURE

Abbreviations		Amino acid name
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartate (Aspartic Acid)
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamate (Glutamic Acid)
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

Chapter 1

General introduction

1. General introduction

1.1. Amyotrophic Lateral Sclerosis

The most common adult onset disorder of motor neuron (MN) is Amyotrophic Lateral Sclerosis (ALS). This neurodegenerative pathology was first described by the French neurobiologist and physician Jean-Martin Charcot in 1874 (Charcot, 1874), being initially known as Charcot's sclerosis (Pasinelli and Brown, 2006).

ALS is a fatal neurodegenerative disease that results from selective dysfunction and death of upper and lower MN in the spinal cord (SC), brainstem and cortex, which leads to generalized weakness and muscular atrophy, and death occurs due to respiratory failure within 2-5 years (Bruijn et al., 2004). The aetiology is likely to be multifactorial, as it involves complex interactions between genes and environmental factors that initiate the disease and lead to MN cell death (Majoor-Krakauer et al., 2003). Despite more than a century of research, there is currently no cure and the available therapy, the Riluzole, prolongs survival by only a few months (Majoor-Krakauer et al., 2003).

Approximately 90-95% of ALS cases are sporadic (SALS), whereas 5-10% of cases have a family history, familial ALS (FALS). The majority of familial cases are clinically and pathologically very similar to sporadic cases, leading to the hypothesis that they share common pathogenic mechanisms (Goodall and Morrison, 2006).

1.1.1. Epidemiological and clinical aspects

ALS is a relatively rare neurodegenerative disease with an annual incidence rate (number of new cases per year) ranging from 0.4 to 2.4 per 100,000 individuals in developed countries (Mayeux, 2003), with a prevalence (total number of cases of disease) of 4-6 per 100,000

individuals. ALS is an age-dependent disorder in which incidence and mortality rates are higher with increasing age. The average age at onset of ALS is 63 years but it varies from ages 37 to 88 (Mayeux, 2003).

There are four well-studied geographic areas with 50-100 times higher prevalence of ALS (Wijesekera and Leigh, 2009): in the Chamorro population of the Western pacific islands of Guam and Rota, the Japanese Kii peninsula, the Western coast of Irian Jaya in Indonesia and the isolated tribe at Anguru on Groote Eylandt in the Gulf of Carpentaria in North Australia (Majoor-Krakauer et al., 2003). In these endemic areas in the South Pacific, ALS accounts for about one in ten deaths. For the Chamorro population of Guam a connection between ALS with dementia and Parkinsonism has been established in some cases, which is recognized as a clinical subtype of ALS, the Guamanian ALS/parkinsonism dementia complex.

The present lack of a biomarker makes it impossible to diagnose ALS in a pre-clinical stage before irreversible cell death occurs (Eisen, 2009). The El Escorial Criteria, developed in the late 1980s, form the basis of the clinical diagnosis in the absence of *post-mortem* examination (Mayeux, 2003).

The initial features of this disease can include muscle twitching, cramping or stiffness; muscle weakness affecting an arm or a leg; slurred and nasal speech; or difficulty chewing or swallowing (Mayeux, 2003).

1.1.2. Neuropathological features of ALS

The pathological hallmarks of ALS are the degeneration and loss of MN evident in cortex, brainstem and SC with the presence of intraneuronal inclusions in degenerating neurons (Figure 1A-C) and glia (Strong et al., 2005; Lobsiger and Cleveland, 2007).

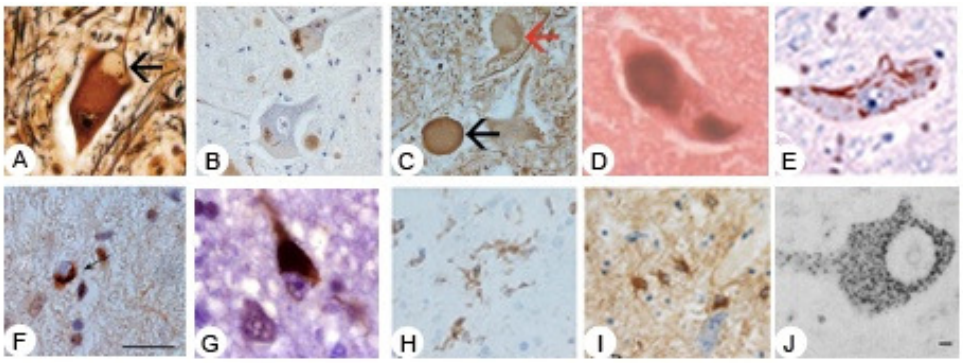

Figure 1: Pathological features described for ALS. (A) Intensely argyrophilic, punctuate aggregates corresponding to the Bunina bodies are apparent (arrow) (magnification 40x). (B) Ubiquitin-immunoreactive threads and aggregates are observed as early markers of aberrant protein aggregation in otherwise healthy appearing MN. (C) Axonal spheroids are intensely stained using a monoclonal antibody recognizing highly phosphorylated neurofilament-heavy chain (black arrow), whereas perikarial neurofilament aggregates are more amorphous and heterogeneous (red arrow) (magnification 40x). (D) Hyaline inclusion from the transverse section of the SC from FALS stained with anti-SOD1 (magnification 225x) (E) Abnormal cytoplasmic staining of TDP-43 in lower MN of a SALS patient. (F) Glial inclusion displaying abnormal cytoplasmic immunoreactivity for TDP-43 in the SC from a SALS patient (bar: 20 µm). (G) FUS/TLS immunohistochemistry in a surviving MN from the SC of an ALS patient with the FUS/TLS^{R521H} mutation: nuclear FUS/TLS is trapped in the cytoplasm. (H, I) Glial pathology. Both microglial (H, human leukocyte antigen DR, HLA-DR, immunoreactivity) and astrocytic (I, glial fibrillary acidic protein immunoreactivity) abnormalities are evident in ALS SC. (H) In the region of ventral horn, both phagocytic and activated ramified appearance of microglia is evident. (I) Astrocytic activation in the ALS SC (magnification 40x). (J) Fragmentation of Golgi apparatus in a patient with SALS. Immunostaining of SC MN showing a fragmented Golgi apparatus represented by small immunostained elements of the organelle in perikaryon and dendrites. Immunostaining was against MG160, a conserved sialoglycoprotein of the medial cisternae of the Golgi apparatus (bar: 10 µm). Adapted from (Mourelatos et al., 1993; Niwa et al., 2002; Strong et al., 2005; Lagier-Tourenne and Cleveland, 2009; Maekawa et al., 2009).

The number of lower MN can be reduced by up to 50% at autopsy. The remaining neurons are atrophic and contain intraneuronal aggregates of the following types: bunina bodies, ubiquitinated inclusions and hyaline conglomerate inclusions (Goodall and Morrison, 2006; Wijesekera and Leigh, 2009). Bunina bodies are small round, single or branched eosinophilic (they bind eosin that is acidic), and they have not been widely described in other diseases (Figure 1A). They stain positive for cystatin and transferrin and are present in 70-100% of cases. Ubiquitinated inclusions can be divided according to morphology in “skein-like” (filamentous) or “Lewy body-like” (round and compact) inclusions (Figure 1B). They are detected in up to 95% of ALS cases. Hyaline conglomerate inclusions have the appearance of glass after staining with eosin or haematoxylin, they are argyrophilic, they contain phosphorylated and non-phosphorylated neurofilaments (Figure 1C), in some FALS cases they stain for SOD1 (Figure 1D) and they are less specific for the disease (Wood et al., 2003; Wijesekera and Leigh, 2009).

Recently, TDP-43 has been identified as the major protein in ubiquitinated inclusions of neurons (Figure 1E) and glial cells (Figure 1F).

This year a mutant form of the DNA/RNA-binding protein FUS/TLS has been found to be associated with MN degeneration and ALS. The mutant protein forms large globular and elongated aggregates in the cytoplasm in SC MN and dystrophic neurites (Figure 1G) (Kwiatkowski et al., 2009; Vance et al., 2009).

Other pathological hallmarks in ALS are activation of microglia (microgliosis, Figure 1H) and proliferation of astrocytes (astrogliosis, Figure 1I) (Strong et al., 2005; Lobsiger and Cleveland, 2007).

Another consistent neuropathological feature of upper and lower MN in ALS is the atrophy and fragmentation of neuronal Golgi apparatus (Figure 1J) observed for the three clinical subtypes of ALS: SALS, FALS (Mourelatos et al., 1990; Fujita et al., 2000) and Guamanian

ALS/parkinsonism dementia complex (Mourelatos et al., 1994). The fragmentation consists of dispersion of the normal network of large and irregular elements of the Golgi apparatus into numerous shortened disconnected cisternae (Gonatas et al., 2006). Importantly, lesions of the Golgi apparatus were detected in spinal MN of presymptomatic transgenic $SOD1^{G93A}$ mice, suggesting that the Golgi apparatus is targeted early in disease progression. Golgi apparatus fragmentation may lead to functional impairment of intracellular protein trafficking essential for secretion and axonal transport (Mourelatos et al., 1996). Transfection of CHO cells with mutant SOD1 induced Golgi apparatus dispersion and dysfunction of the secretory pathway (Stieber et al., 2004).

1.2. Genetics of familial and sporadic ALS

ALS has been considered a sporadic disorder, but in 10% of patients the disease is familial (FALS), transmitted as either an autosomal dominant or recessive disease. In 1991, the first ALS gene (*ALS1*) for an autosomal-dominant form of FALS, was mapped to chromosome 21q. Two years later the cytosolic copper-zinc superoxide dismutase (SOD1) gene was shown to be the *ALS1* gene that accounts for 20% of the FALS cases, and only 1-2% of all patients with ALS (Gros-Louis et al., 2006), but 2-7% of apparently sporadic patients have also SOD1 mutations. To date, molecular genetic analysis allowed the identification of several genetic loci and genes (Table 1) as well as genetic polymorphisms, as risk factors involved in ALS (Majoor-Krakauer et al., 2003; Valdmanis et al., 2009).

Environmental risk factors, including behavioral and occupational exposures, such as smoking, heavy exercise or trauma, exposure to pesticides, lead or solvents, chemicals used in agricultural work, diet high in glutamate content, common environmental exposure in a particular region, as in Guam (section 1.1.1.) and exposure to neurotoxins have been

investigated in ALS patients, but no significant factor has been consistently related to ALS (Gros-Louis et al., 2006; Valdmanis and Rouleau, 2008).

1.2.1. FALS

1.2.1.1. SOD1

The SOD1 gene spans 11 kilobases of genomic DNA, comprises five exons and four introns and encodes a highly conserved 153-amino acid long protein of 16 kDa (Vucic and Kiernan, 2009).

Table 1: Genetics of FALS. Loci identified for the inherited form of ALS are presented.

Classification[a]	Locus	Gene	Inher	Onset	References
ALS1	21q22	*SOD1*	AD/AR	Adult/ Juvenile	(Siddique et al.,1991; Rosen 1993)
ALS2	2q33	*Alsin*	AR	Juvenile	(Hentati et al., 1994; Hadano et al., 2001; Yang et al., 2001)
ALS3	18q21	Unknown	AD	Adult	(Hand et al., 2002)
ALS4	9q34	*SETX*	AD	Juvenile	(Chance et al., 1998; Chen et al., 2004)
ALS5	15q15	Unknown	AR	Juvenile	(Hentati et al., 1998)
ALS6	16q12	*FUS/TLS*	AD	Adult	(Kwiatkowski et al., 2009; Vance et al., 2009)
ALS7	20ptel	Unknown	AD	Adult	(Sapp et al., 2003)
ALS8	20q13.3	*VAPB*	AD	Adult	(Nishimura et al., 2004a; Nishimura et al., 2004b)
ALS9	14q11.2	*ANG*	AD	Adult	(Greenway et al., 2004; Greenway et al., 2006)
ALS10	1p36	*TARDBP*	AD	Adult	(Yokoseki et al.,2008; Sreedharan et al., 2008)
ALS11	6q21	*FIG4*	AD	Adult	(Chow et al., 2009)
ALS-FTD	9q21-q22	Unknown	AD	Adult	(Hosler et al., 2000)
ALS with Parkinsonism and dementia	17q21.1	*MAPT*	AD	Adult	(Hutton et al., 1998)

[a]Abbreviations are: AD, autosomal dominant; ANG, angiogenin; ALS, amyotrophic lateral sclerosis; AR, autosomal recessive; FIG4, factor-induced gene 4 protein; FTD, frontotemporal dementia; FUS, fused in sarcoma; Inher., inheritance; MAPT, microtubule-associated protein Tau; SETX, Senataxin; SOD1, superoxide dismutase 1; TARDBP, TAR DNA binding protein; TLS, translated in liposarcoma; VAPB, vesicle-associated membrane protein B.

To date, one hundred and forty one mutations spanning the entire SOD1 polypeptide chain have been identified (http://alsod.iop.kcl.ac.uk/Als/reports/report Summary.aspx), including one hundred and fifteen amino acid substitutions, three insertions and four deletions (Simpson and Al-Chalabi, 2006; Vucic and Kiernan, 2009). The mutations that are either nonsense or deletion mutations, either introduce novel nucleotides or remove existing ones, resulting in alteration of the polypeptide length. All SOD1 mutations described to date are associated with autosomal-dominant late onset ALS (typical ALS), except for two mutations, the substitution of an aspartate by an alanine at position 90 or by a glutamine at position 96 (D90A or D96N, respectively), which can both cause dominant and recessive ALS (Bruijn et al., 2004; Valdmanis and Rouleau, 2008). The most common SOD1 mutations are D90A, followed by A4V and I113T. The A4V gives raise to the most aggressive form of ALS with reduced survival time after onset (Cudkowicz et al., 1997; Vucic and Kiernan, 2009).

Clinical characteristics, penetrance, age of onset, disease progression and survival, vary greatly between and among different mutations (Majoor-Krakauer et al., 2003; Gros-Louis et al., 2006) suggesting that environmental and genetic factors are modifying the phenotype (Simpson and Al-Chalabi, 2006). For example, some SOD1 mutations are associated with survivals longer than 17 years (G37R, G41R, E100K, H46R), others with survivals around 10 years (G93C), or with survivals of 5-6 years (E100G, G85R), some are associated with shorter survivals such as 1-3 years (L37V, H43R, G93A), or 1 year (A4V) and some have highly variable survival times (I113T, D90A) (Valentine et al., 2005).

It is still not understood at present the mechanisms underlying the toxicity of each SOD1 mutant to MN, causing ALS, but it seems to result from a gain-of-toxic properties (Pasinelli and Brown, 2006). It is noteworthy

that SOD1 knockout mice did not develop MN disease supporting this notion.

1.2.1.2. Other mutations

The first description of a rare juvenile-onset recessive form of FALS (*ALS2*) appeared in 1994, and was linked to chromosome 2 (Hentati et al., 1994). Later, the *ALS2* gene was identified primarily in families of Arabic origin (Tunisian and Kuwaiti families), displaying a juvenile onset (age 3 to 23 years old) with a relatively long survival (15 to 20 years). The *ALS2* gene comprises 34 exons that encode for Alsin, a 1,657-amino acid protein with three motifs homologous to guanine-nucleotide exchange factors that include several cell-signaling motifs. To date, there are ten reported mutations in the *ALS2* gene, eight of which are deleterious mutations, one is a nonsense mutation and one is a splice site mutation. They all lead to premature termination of the transcript and a truncated Alsin protein suggesting a loss of the guanine-nucleotide exchange factor function that results in a deficit in the intracellular trafficking (Hadano et al., 2001; Vucic and Kiernan, 2009).

A rare autosomal dominant form of ALS with juvenile onset (mean age at onset 17 years) and slow progression has been localized to a locus on chromosome 9q34 (*ALS4*). Different missense mutations in the *senataxin* gene (*SETX*) have been identified (Chen et al., 2004). The encoded protein is 2,667-amino acid long and contains a C-terminal domain found in the superfamily 1 of DNA/RNA helicases. It is conceivable that the abnormal SETX protein impairs the capacity of neurons to maintain DNA and produce error-free mature mRNA, thereby resulting in neurodegeneration.

One additional loci for adult onset autosomal-dominant form of FALS (*ALS6*) was assigned to chromosome 16q12.1-16q21 (Abalkhail et al., 2003; Sapp et al., 2003). Recently, genomic sequencing revealed a

sequence variant in exon 15 of the fused in sarcoma/translated in liposarcoma (*FUS* also known as *TLS*) gene (Kwiatkowski et al., 2009; Vance et al., 2009). So far 16 mutations in the 15 exons of the *FUS* gene have been described in patients with FALS, but none in patients with SALS (Valdmanis et al., 2009). It is estimated that FUS/TLS mutations are detected in about 4% of FALS cases. FUS/TLS is a 526-amino acid long protein ubiquitously expressed and normally located in the nucleus, that is involved in DNA repair and the regulation of transcription, RNA splicing, and export to the cytoplasm. The FUS/TLS mutations described led to cytoplasmic retention and apparent aggregation of FUS/TLS in ALS patients (Figure 1G) (Lagier-Tourenne and Cleveland, 2009; Van Damme and Robberecht, 2009).

A locus mapped at chromosome 20q13.33, causative of an autosomal-dominant late-onset atypical ALS (*ALS8*), was identified in a large Caucasian-Brazilian family. Further work led to the identification of a missense P56S mutation in the vesicle associated membrane protein/synaptobrevin associated protein B gene (*VAPB*) (Nishimura et al., 2004b). *VAPB* has six exons that encode for a ubiquitously expressed 33 kDa homodimer protein named VAMP-B or VAP33. VAMP-B is a membrane protein found at the endoplasmic reticulum (ER). The P56S causes VAMP-B aggregation and Golgi apparatus fragmentation (Teuling et al., 2007). These findings suggest that ALS may be caused by dysfunction in the intracellular membrane trafficking and secretion or malfunction of VAPB in mediating unfolded protein response (Suzuki et al., 2009; Vucic and Kiernan, 2009).

In a group of typical-onset patients with ALS from Ireland and Scotland, candidate-based gene sequencing resulted in the detection of seven amino acid changes in the angiogenin *(ANG)* gene at the *ALS9* locus (Greenway et al., 2006). The angiogenin is a 123-amino acid long protein that is expressed in MN. It is induced under hypoxic conditions to elicit

angiogenesis (like vascular endothelium growth factor, discussed in section 1.3.7.) and it is a potent inducer of neovascularisation *in vivo*, needed for cell proliferation. Several of the missense changes identified reside within highly conserved residues in the catalytic domains of the angiogenin and were shown to reduce the wild-type activity of the protein (Valdmanis et al., 2009).

ALS10 is a recently described typical form of ALS linked to the *TARDBP* gene on chromosome 1p36.22, in which mutations in both sporadic and familial ALS cases, as well as in frontotemporal dementia (FTD) were found. This gene codes for the 43-kDa TAR DNA-binding protein (TDP-43), which binds RNA and DNA, thereby regulating the transcription and splicing of nuclear material (Sreedharan et al., 2008; Wijesekera and Leigh, 2009). Other reported functions of TDP-43 include cell cycle regulation and apoptosis, mRNA transport and regulation of local translation at synapses (Geser et al., 2009). To date 30 mutations have been identified and the overall *TARDBP* mutation frequency in FALS is 2 to 5% (Van Damme and Robberecht, 2009; Wijesekera and Leigh, 2009). With the exception of the Y374X truncation mutation, all other *TARDBP* mutations are missense mutations mostly affecting highly conserved and protein-protein interacting amino residues in the C-terminal region (Neumann, 2009). TDP-43 is a 414-amino acid protein ubiquitously expressed, present in neuronal and glial nuclei (more than 90%). Mutant TDP-43 has been found in ubiquitin-positive cytoplasmic aggregates present in neurons and glial cells (Wang et al., 2008a). Biochemical analysis revealed a characteristic pathologic profile due to N-terminal truncation, abnormal hyperphosphorylation and ubiquitination (Neumann, 2009). Hence, it is plausible that neural toxicity is mediated by disruption of key nuclear functions (Neumann et al., 2007; Vucic and Kiernan, 2009).

1.2.2. SALS

Several studies have identified gene polymorphisms as risk factors for the more common SALS, which may influence the onset and progression of ALS (Beleza-Meireles and Al-Chalabi, 2009). Some of the protein products of the genetic polymorphisms reported include: neurofilament heavy/light chain subunit (intermediate filaments expressed in adult neurons) (Xu et al., 1993; Figlewicz et al., 1994); glutamate excitatory amino acid transporter 2, EAAT2 (Rothstein et al., 1995) and glutamate receptor, AMPA (Carriedo et al., 1996); ciliary neurotrophic factor (Orrell et al., 1995); and, vascular endothelium growth factor (angiogenesis factor) (Lambrechts et al., 2003).

1.3. Understanding cellular pathogenic mechanisms in ALS

As familial and sporadic ALS are clinically and pathologically similar, understanding the pathophysiological processes in FALS may provide a greater understanding of the mechanisms underlying neurodegeneration in SALS (Vucic and Kiernan, 2009). Indeed, the identification of pathogenic alleles of SOD1 has led to the generation of cellular and transgenic rodent models for the study of ALS. The exact pathophysiological mechanisms underlying neurodegeneration remain elusive, but they seem to be multifactorial (Figure 2), with evidence emerging of a complex interaction between genetic factors and molecular pathways.

The combination of genetic, pathological and biochemical studies of both human ALS tissue obtained *post-mortem* and from biopsies, and animal models, led to the identification of intrinsic pathogenic characteristics of ALS MN, including: formation of protein aggregates, proteasome dysfunction, ER stress pathways, increased sensitivity to cell death signals, glial cell pathology, neuroinflammation, altered mitochondrial function, impaired energy production, alterations in calcium homeostasis,

oxidative stress-mediated damage, glutamate excitotoxicity, cytoskeletal abnormalities and impaired axonal transport, growth factor deficiency and aberrant RNA metabolism (Rothstein, 2009).

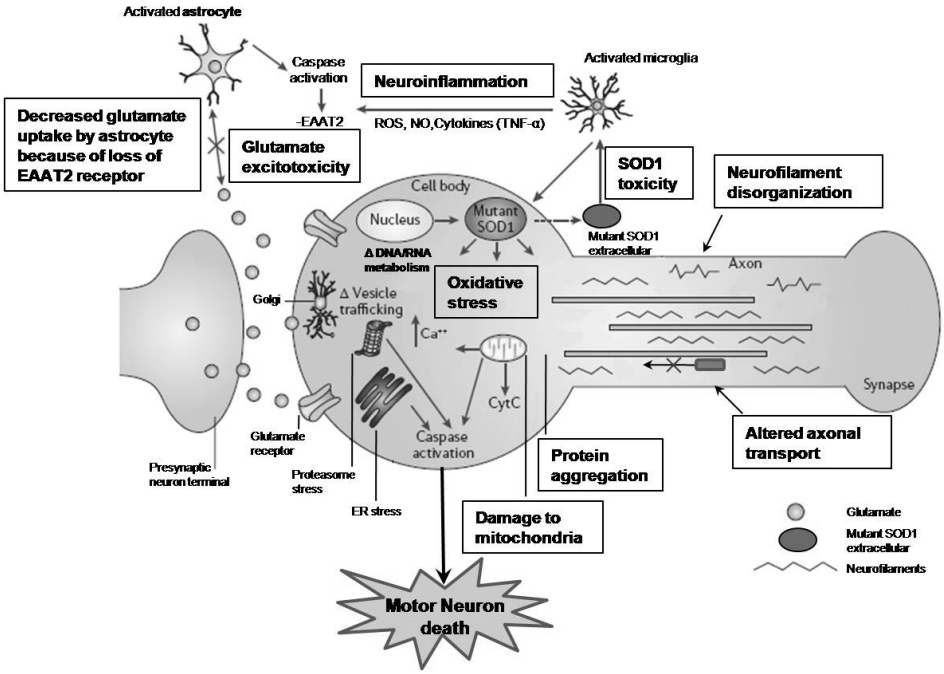

Figure 2: Schematic representation of proposed mechanisms that could converge in ALS and cause MN degeneration. Adapted from Pasinelli and Brown (2006).

1.3.1. Protein instability and SOD1 aggregation

Several lines of evidence point out that the toxicity of SOD1 mutants causing ALS arise from the acquired gain of a toxic function, resulting from the propensity of SOD1 mutants to form large aggregates. In transgenic rodents with mutant SOD1-mediated ALS and in some human ALS cases, aggregates that are immunoreactive for SOD1 are detected in MN and within the astrocytes surrounding them (Vucic and Kiernan, 2009). In these transgenic mice, these aggregates become evident by the time of disease

onset and increase in abundance with disease (Johnston et al., 2000; Wang et al., 2002).

Aggregates found in SALS and FALS patients, as well as mouse models, may also contain ubiquitin (Ince et al., 1998; Watanabe et al., 2001), a protein adduct which typically targets proteins for disposal via the proteasome. Misaccumulation of ubiquitinated, misfolded proteins might adversely affect the proteasome machinery and impair normal protein degradation.

In vitro studies have shown that mutant proteins oligomerize over time to form small pore-like structures that are similar to some forms of β–amyloid proteins (Matsumoto et al., 2006; Nordlund and Oliveberg, 2006). In fact, fibrillar SOD1 species generated *in vitro* from wild-type and mutant SOD1 bind the amyloid markers Congo Red and Thioflavin-T, suggesting the presence of amyloid structure. Micrometer-scale SOD1 aggregates in transgenic mice have also stained positive with amyloid indicators such as Thioflavin S, suggesting the presence of amyloid structures (Shaw and Valentine, 2007; Chattopadhyay and Valentine, 2009).

As in other neurodegenerative disorders, it remains unclear whether protein aggregates directly cause cellular toxicity and have a key role in pathogenesis, or aggregates are innocent by-products of the neurodegeneration process, or their formation may actually be a beneficial process by being part of a defence mechanism to reduce intracellular concentrations of toxic proteins (Cleveland and Rothstein, 2001; Wijesekera and Leigh, 2009). It has been proposed that they could deregulate organelle function including Golgi apparatus, ER and mitochondria, or cause axonal transport defects possibly linked to aberrant accumulations of intermediate filaments, overwhelming the capacity of the protein folding chaperones and/or of ubiquitin proteasome system to degrade important cellular regulatory factors (Vucic and Kiernan, 2009). Additionally, aggregates may impair protein degradation and recycling, not

only of mutant SOD1 but also of other proteins, and sequestering proteins that are essential for cellular processes, such as heat-shock proteins (Hsp) (Julien and Kriz, 2006; Pasinelli and Brown, 2006). Indeed, mutant SOD1 directly interacts with Hsp70, Hsp40, Hsp27 and αβ-crystallin (Shinder et al., 2001; Wang et al., 2003).

It is currently understood that mutant SOD1 aggregation is a multistep pathway of sequential protein monomerisation, demetallation and oligomerisation, but the precise toxic biochemical species of SOD1, whether monomeric, multimeric, soluble, insoluble or disulfide reduced, remain elusive (Khare et al., 2004; Turner and Talbot, 2008a).

1.3.2. SOD1 structure, folding and stability

The SOD1 protein is a homodimer of two subunits of 16 kDa, in which each monomer consists primarily of an eight-stranded antiparallel Greek-key β-barrel, with two large loops, the so-called "electrostatic" loop and the "metal binding" loop (Figure 3). The electrostatic loop has a number of charged residues. The metal binding loop contains many of the residues necessary for binding of the metals (Valentine et al., 2005; Rakhit and Chakrabartty, 2006). Each subunit can form an intramolecular disulfide bond between cysteines 57 and 146, which is unusual for an intracellular protein, as it is present in the reducing environment of the cytoplasm. It can coordinate one catalytic Cu^{2+} ion and one Zn^{2+} ion in its active site. The catalytic copper is bound by four histidines. The zinc ion is thought to play a stabilizing structural role and act as a positive charge sink. The Cu^{2+} and Zn^{2+} ions are coordinated in close proximity and share a unique bridging histidine side chain (Rakhit and Chakrabartty, 2006; Shaw and Valentine, 2007). The dimer is held together primarily by numerous main-chain to main-chain hydrogen bonds, water-mediated hydrogen bonds, and hydrophobic contacts (Valentine et al., 2005). Dimerization of SOD1

reduces the solvent accessible surface area, greatly increasing its stability (Rakhit and Chakrabartty, 2006).

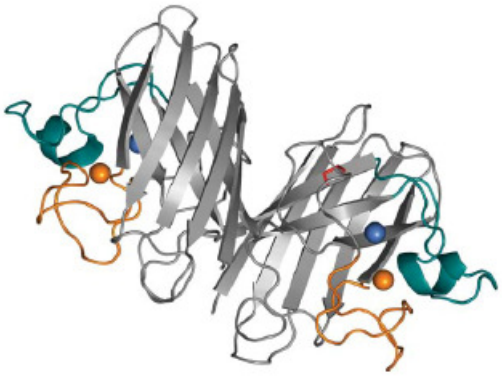

Figure 3: **Crystal structure of metal bound dimeric human SOD1**. Copper and zinc ions are shown as blue and orange spheres, respectively. The zinc loop is depicted in orange and the electrostatic loop in teal. The intrasubunit disulfide bond is shown in red. Adapted from Valentine et al. (2005).

The main function of the SOD1 enzyme involves free radical scavenging whereby, through cyclical reduction and oxidation (dismutation) of the copper ion, catalyses the conversion of the superoxide anion (O_2^-), a toxic by-product of mitochondrial oxidative phosphorylation, to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), which in turn is reduced to water by glutathione peroxidase and catalase, thus preventing oxidative damage.

This ubiquitously expressed metalloenzyme is highly expressed constituting 0.5-1% of soluble protein in the brain and SC. It is mainly located within the cytosol, with a smaller fraction at the intermembrane space of mitochondria (Furukawa and O'Halloran, 2006).

SOD1 is a highly stable protein in its fully metallated (holo), disulfide-oxidized form and remains folded at temperatures near the boiling point of water, 85-95ºC (depending on buffer). In addition, it retains a

dimeric quaternary structure in 1% sodium dodecyl sulphate (SDS) and enzymatic activity after 1 h in 4% SDS, 8 M urea or 6 M guanidine-HCl. Additionally, it has also been shown to be resistant to proteolytic digestion (Valentine et al., 2005; Chattopadhyay and Valentine, 2009).

There are minimal changes in the crystal structure of fully metallated mutant SOD1, nevertheless there are popular hypotheses to explain effects of mutations in SOD1, which include decreased thermostability of holo or metal-deficient (apo) SOD1, increased hydrophobicity and aggregation propensity, loss of metals, aberrant chemistry and increased susceptibility to posttranslational modifications, including augmented vulnerability to disulfide reduction (Hart, 2006; Chattopadhyayand Valentine, 2009). Mutant SOD1 has been shown to have decreased half-life *in vivo*, but to different degrees depending on the mutation. The faster degradation of the mutants seems to involve the ubiquitin-proteasome system (Valentine et al., 2005).

SOD1 enzymatic activity varies greatly depending on which mutation is present and does not correlate with disease severity, with some mutant forms maintaining full activity, some being inactive (H46R) and others near normal (G93A) (Bruijn et al., 2004). Nevertheless, it was shown that loss of SOD1 stability, faster and higher aggregation propensities relate to faster death of ALS patients, revealing that protein instability and aggregation propensity are synergistic risk factors (Wang et al., 2008b; Prudencio et al., 2009a).

Understanding how charge, hydrophobicity, local pH, conformational destabilization and disordered regions of the polypeptide each contribute to SOD1 aggregation can provide multiple approaches to stop SOD1 aggregation and to treat or prevent these familial forms of ALS (Shaw and Valentine, 2007).

1.3.3. Mechanisms of mutant SOD1 degradation

In every eukaryotic cell, there are two main systems for the degradation of cytoplasmic proteins: the ubiquitin–proteasome system and autophagy. The ubiquitin-proteasome system is the main intracellular proteolytic system that accounts for most of the selective intracellular protein degradation and mainly degrades short-lived damaged and misfolded proteins (Cheroni et al., 2009). Autophagy is less selective and is involved in degradation of long-lived proteins in addition to organelles, such as mitochondria (Pasquali et al., 2009).

Alterations in the functionality of the ubiquitin-proteasome system have been proposed to be responsible for the accumulation of potentially harmful ubiquitinated proteins in ALS (Cheroni et al., 2009). Expression of mutant SOD1 leads to proteasomal inhibition that may result in accumulation of mutant SOD1 and motor neuronal death (Urushitani et al., 2002; Cheroni et al., 2005). A decrease in constitutive proteasome subunits during disease progression in the SC of transgenic mice expressing the FALS $SOD1^{G93A}$ was found (Cheroni et al., 2009). However, it is likely that additional degradation pathways such as autophagy participate in SOD1 turnover (Kabuta et al., 2006).

Autophagy is an ubiquitous dynamic process that involves the formation of double membrane structures, which fuse with lysosomes where their content is degraded (Klionsky and Emr, 2000). Autophagic degradation is implicated in the removal of aggregated proteins associated with pathological conditions such as Alzheimer's, Parkinson's, Huntington's and prion diseases (Pasquali et al., 2009). *In vitro*, autophagy inhibition induced mutant SOD1-mediated cell death, and increased SOD1 levels in detergent-soluble and insoluble fractions (Kabuta et al., 2006). Additionally, *in vivo*, it was shown that autophagy was activated in SC of $SOD1^{G93A}$ mice (Morimoto et al., 2007; Li et al., 2008). Accumulating data indicate that ER

stress is also a potent trigger of autophagy that can counterbalance this stress (Hoyer-Hansen and Jaattela, 2007).

Endoplasmic reticulum stress is triggered by the accumulation of misfolded proteins within the ER lumen (Nishitoh et al., 2008), it can activate two adaptive pathways: the conserved ER to nucleus signaling pathway called the unfolded protein response, and ER-associated protein degradation. The latter eliminates misfolded or unassembled proteins from the ER; its targets are selected by a quality control system within the ER lumen and are ultimately destroyed by the cytoplasmic ubiquitin-proteasome system. Studies showed that mutant SOD1 aggregated in association with the ER (Tobisawa et al., 2003), induced ER stress but specifically impaired ER-associated protein degradation (Nishitoh et al., 2008). Additionally, an ER stress inhibitor delayed formation of insoluble aggregates of the mutant SOD1 and suppressed cell death (Oh et al., 2008). Up-regulation of the full spectrum of unfolded protein response markers in lumbar cord tissue from human SALS patients was also demonstrated. Furthermore, increased levels of protein disulphide isomerase were found in the cerebrospinal fluid (CSF) of these patients (Atkin et al., 2008). Unfolded protein response, which is known to reduce global protein synthesis and to induce the synthesis of chaperones (Harding et al., 2002), is part of a pathophysiological process, contributing to neuronal death in both familial and sporadic disease (Atkin et al., 2008).

1.3.4. Morphology of Golgi apparatus and intracellular protein trafficking

The Golgi apparatus is a cytoplasmic organelle involved in the transport, processing, and targeting of proteins synthesized in the rough ER and destined for the secretory pathway (Figure 4) (Nakagomi et al., 2008). The Golgi apparatus also serves as site for the post-translational modification of proteins and lipids, mostly glycosylation. In most higher eukaryotic cells, the Golgi apparatus consists of multiple flattened, parallel,

interconnected cisternae and vesicles that carry molecular “cargo” from one cisterna to the next by the coordinated fission of vesicles from the lateral edge of one cisterna and fusion to the next cisterna. It is organized around the microtubule-organizing center in the perinuclear region (Gonatas et al., 2006).

The structural and functional integrity of the Golgi apparatus is maintained by different systems of proteins and structures that include microtubules and microtubule-associated proteins, the actin-associated cytoskeleton, the Golgi matrix proteins and proteins that ensure the targeting and fusion of transport vesicles to the correct compartment, such as GTP-binding proteins and SNAREs (Gonatas et al., 2006).

Originally, the Golgi apparatus was thought to be a static organelle, but it is actually a highly dynamic structure. The steady-state structure of the Golgi stacks is partly a consequence of the balance of anterograde and retrograde transport through the Golgi (Hicks and Machamer, 2005).

As the Golgi apparatus is involved in numerous important functions quality control of proteins in the Golgi apparatus and ER must be stringent to ensure appropriate cellular function. It is reasoned that fragmentation of the Golgi might have detrimental effects and lead to dysfunction of the cytoplasmic machinery in neurons as well as in non-neuronal cells (Nakagomi et al., 2008). Indeed, the fragmentation of the neuronal Golgi apparatus has been reported in ALS (Fujita and Okamoto, 2005), as well as in Alzheimer’s disease (Stieber et al., 1996), Parkinson’s disease (Gosavi et al., 2002), spinocerebellar ataxia type 2 (Huynh et al., 2003), in Creutzfeldt-Jacob disease (Sakurai et al., 2000), among others (Fan et al., 2008). Furthermore, α-synuclein expression blocked ER-to-Golgi vesicular trafficking (Cooper et al., 2006), similarly, misfolded growth hormone caused Golgi apparatus fragmentation and disrupted the traffic of two secretory proteins (Graves et al., 2001).

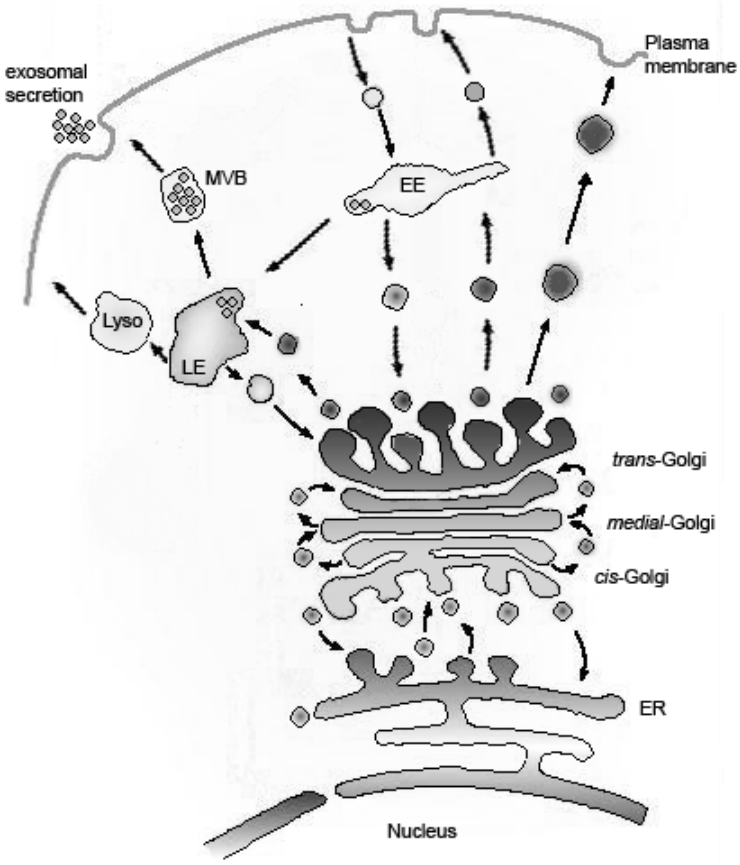

Figure 4: Schematic representation of the secretory and endocytic pathways. In the secretory pathway newly synthesized proteins are exported from the ER to the *cis*-Golgi. Material is transported along the Golgi apparatus until the *trans*-Golgi network. The cargo targeted to endosomal compartments is transported to the early endosome (EE) and to the late endosome (LE), while membrane components and secretory cargo are transported to the plasma membrane. Molecules are internalized into EE, transported into the LE and subsequently packed into lysosomes (Lyso) for degradation. Multivesicular bodies (MVBs) derived from the LE can fuse with the plasma membrane, releasing the internal vesicles as exosomes. Adapted from Simons and Raposo (2009) and Keller et al. (2006).

1.3.5. Apoptosis in ALS

Evidence has suggested that the ultimate mechanism of neuronal damage in human ALS occurs through programmed cell death, resembling apoptosis (Sathasivam and Shaw, 2005a; Pasinelli and Brown, 2006). Apoptosis is an energy-dependent process characterised by cell shrinkage, compaction and contraction of cytoplasm organelles, membrane blebbing, nuclear chromatin condensation and fragmentation (Goodall and Morrison, 2006). Apoptosis can be triggered by three mechanisms: activation of cell surface receptors of the tumour necrosis family, such as Fas; cytochrome *c* release from the mitochondria; and stress to the ER. Apoptosis processes are tightly regulated by a series of proteins including caspases, the Bcl-2 family of pro- and anti-apoptotic proteins and inhibitors of apoptosis proteins (Wijesekera and Leigh, 2009). Caspases are cysteine proteases with aspartate specificity that can be divided into upstream effector (such as caspase-1, -8 and -9) and downstream-executioner caspases (such as caspase-3 and -7), the former activating the latter in the apoptotic pathway. Upon activation, the caspases cleave other intracellular targets including other caspases resulting in an amplified cell death cascade.

The first evidence of an apoptotic response in ALS was the increased activation of effector caspases-1 and -9 in the CSF (Ilzecka et al., 2001) and in the SC tissue (Li et al., 2000; Inoue et al., 2003) of ALS patients. Further, SC expression of caspase-1 gene was significantly increased in ALS cases (Anagnostou et al., 2008). Caspase activation is also very prominent within astrocytes of mutant SOD1 mice (Li et al., 2000; Pasinelli et al., 2000) raising the possibility that caspase-1 activation may contribute to an inflammatory pathway causing astrocytosis. It has been demonstrated that caspase-3 activation in glial cells proteolytically inactivates the astroglial glutamate excitatory amino acid transporter 2, EAAT2 (Boston-Howes et al., 2006), involved in excitotoxicity.

In different cellular and animal systems, overexpression of the mutant SOD1 protein either increases the predisposition of the cells to apoptosis or causes apoptosis by activating several caspases, suggesting that mutant SOD1 is a pro-apoptotic molecule initiating a slowly progressive cell death process that kills MN and the surrounding cells over time (Pasinelli and Brown, 2006). Moreover, inhibition of one or more caspases prolongs life (Li et al., 2000; Cleveland and Rothstein, 2001), and the increase in the expression of the anti-apoptotic factor Bcl-2 slows disease onset and increases survival (Kostic et al., 1997), of mutant mouse models.

Changes in the structure of the Golgi apparatus may trigger and propagate apoptosis (Nakagomi et al., 2008) as it is known that pro-apoptotic caspase-2 is localized to the Golgi apparatus (Hicks and Machamer, 2005). As referred previously, Golgi apparatus fragmentation is an early event in ALS (Mourelatos et al., 1996) and therefore may be a cause of apoptosis in ALS.

1.3.6. Non-neuronal cells affect motor neurons

There is a controversy on the contribution of MN and other non-neuronal cells to the ALS pathology. Some authors have shown that neuron-specific expression of mutant SOD1 is sufficient to induce ALS in transgenic mice (Jaarsma et al., 2008; Wang et al., 2008c), proposing a model in which mutant SOD1 triggers neuronal degeneration, which in turn facilitates aggregate formation and functional deficits in surrounding glia. In contrast, others reported that expression of mutant SOD1 in MN (Pramatarova et al., 2001; Lino et al., 2002) or astrocytes (Gong et al., 2000) in isolation is insufficient to induce neurodegeneration. Analysis of chimeric mice with mixed populations of cells expressing either endogenous or transgenic mutant SOD1 support the non-cell autonomous MN death in ALS. In fact, MN expressing transgenic G93A or G37R SOD1 failed to degenerate if they were adjacent to large numbers of supporting

cells without the mutant protein. Reciprocally, normal MN (not expressing mutant SOD1) surrounded by mutant-expressing non-neuronal cells, such as astrocytes or microglia, showed reduced survival, indicating that the mutant-expressing cells had transferred damage to them, causing neurotoxicity (Clement et al., 2003; Di Giorgio et al., 2007; Nagai et al., 2007; Di Giorgio et al., 2008; Marchetto et al., 2008; Yamanaka et al., 2008). This evidence contributes to the idea that the death of the MN depends, at least in part, on a contribution from surrounding glia and possibly other cell types.

Even if it is not yet clear whether inflammation plays a crucial role in MN degeneration, evidence in humans indicates that microglial and astroglial activation is one of the earliest microscopic manifestations widespread in ALS patients (see Figure 1H, I) (Henkel et al., 2004; Turner et al., 2004a). Microglia consists of the resident immune cells of the central nervous system (CNS), that resembles peripheral tissue macrophages, and it is the primary mediator of neuroinflammation. Microglia exist in a “resting” state characterized by a small cell body and fine, ramified processes and minimal expression of surface antigens. They can be activated upon an injury, exerting their toxic effect on neurons and macroglia (astrocytes and oligodendrocytes) through the release of cytotoxic and inflammatory substances (oxygen radicals, NO, glutamate, proinflammatory cytokines such as interleukins, COX-2, TNFα, MCP-1 and prostaglandins) (Bruijn et al., 2004), causing them to down-regulate expression of neurotrophic factors and release additional neuroinflammatory mediators that further activate microglia, thus creating a potentially lethal cycle. The expression of mutant $SOD1^{G93A}$ resulted in activated and neurotoxic microglia (Beers et al., 2006; Xiao et al., 2007; Liu et al., 2009). Further, lowering mutant SOD1 expression within microglia significantly extended the survival of transgenic $SOD1^{G37R}$ mice, by slowing disease progression after onset (Boillee et al.,

2006a). However, in transgenic mice $SOD1^{G93A}$, it had no effect on MN degeneration (Gowing et al., 2008).

Recent findings demonstrated that chromogranins (soluble acidic glycophosphoprotein components of secretory large dense-core vesicles in neurons and endocrine cells) can interact selectively with mutant forms of SOD1, acting as chaperone-like proteins to promote secretion of these molecules. Extracellular mutant SOD1 and chromogranin A can trigger microglial activation and death of MN in culture, emphasizing the likely crosstalk between MN, microglial cells and potentially other non-neuronal cells that may cooperate to drive disease progression (Urushitani et al., 2006).

There is growing evidence showing the existence of an intercellular mode of communication by the transfer of molecules from one cell to another via exosomes. Exosomes are defined as membranous vesicles in which the same topological orientation as at the plasma membrane is maintained, with a diameter of 30-100 nm that float on sucrose gradient to a density that ranges from 1.13 to 1.19 g/ml, their unique protein and lipid composition enable their identification (Simons and Raposo 2009). Exosomes result from the fusion of multivesicular bodies with the plasma membrane and the subsequent release of their cargo (Figure 4) (Keller et al., 2006). Many cell types release exosomes including hematopoietic cells, dendritic cells, tumor cells, neurons, astrocytes and microglia (Vella et al., 2008). In Alzheimer's disease β–amyloid peptides are released in association with exosomes, suggesting a role in pathogenesis (Rajendran et al., 2006). Similarly, abnormal scrapie prion protein is released in association with exosomes which are infectious to other cells (Fevrier et al., 2004). This evidence contributes to the idea that exosomes can allow the propagation of pathogenic proteins.

1.3.7. Other mechanisms

Numerous studies have focused on the role of the mitochondrion in the pathogenesis of ALS. This is based on reports of morphological (vacuolated and dilated mitochondria with disorganized cristae and membranes and paracrystalline inclusions) and biochemical (defects in the respiratory chain complexes I and IV and elevated levels of mitochondrial calcium) mitochondrial abnormalities in human, mouse and cellular models of ALS (Pasinelli and Brown, 2006; Muyderman et al., 2009). Although SOD1 is generally considered to be a cytosolic protein, it has been established that there is a pool of enzymatically active SOD1 in the mitochondrial intermembrane space, where it could form pores and aggregates, interact with proteins affecting the protein translocation machinery or with Bcl-2 rendering it non-functional providing a direct molecular link between mutant SOD1, mitochondrial mediated cell death, and apoptosis (Vucic and Kiernan, 2009).

Another mechanism proposed for ALS pathogenesis is oxidative stress, which increases with age, so fits in with the middle-life onset of this disease (Carter et al., 2009). Oxidative stress is the result of unregulated production of reactive oxygen species such as hydrogen peroxide, peroxynitrite, superoxide and hydroxyl radicals. Several studies have found evidence of increased oxidative damage in ALS *post-mortem* tissue, as well as in SOD1 mice (Abe et al., 1995). The discovery of mutations in SOD1, an anti-oxidant enzyme, causative of ALS has supported this hypothesis. Therefore, it was hypothesized that toxicity was caused by aberrant copper chemistry performed by the mutant protein, yielding nitration of tyrosine residues on proteins and production of hydroxyl radicals, and ultimately cell injury and death (Tu et al., 1997). Another hypothesis of aberrant chemistry was proposed, where SOD1 mutants would run the catalytic cycle backwards, converting oxygen to superoxide that in turn could be converted

to peroxynitrite by combination with nitric oxide, promoting intracellular damage and protein nitration (Barber et al., 2006).

Another hypothesis is that glutamate-induced excitoxicity plays a role in the aetiology of both sporadic and SOD1 mutant caused ALS. Glutamate is the major excitatory neurotransmitter in the CNS, it is released from presynaptic terminals and activates specific receptors on the dendrites of the postsynaptic neurons, by diffusing across the synaptic cleft. Excitotoxicity occurs when excessive glutamate-induced stimulation of post-synaptic glutamate receptors takes place, triggering increased intracellular calcium influxes leading to neuronal degeneration (Van Den Bosch et al., 2006). Increase in glutamate levels in the CSF and abnormalities in glutamate transport have been found in patients with ALS (Rothstein, 2009). In 60% of SALS patients and in transgenic mutant SOD1 rodent models a pronounced loss of the main astroglial excitatory amino acid transporter 2, EAAT2, and activity have been observed in the affected areas (Rothstein et al., 1995; Howland et al., 2002). Finally, the major argument favoring that glutamate excitotoxicity plays a role in ALS pathogenesis is that Riluzole, an anti-glutamate agent prolongs life in human trials and is the only available treatment for ALS at present (discussed in section 1.4.).

A hallmark of ALS pathology is the aberrant accumulation of intracellular aggregates staining for cytoskeleton neurofilament proteins in the cell body and proximal axon (section 1.1.2.). Neurofilament proteins are the major type of intermediate filaments expressed in neurons and play an important role in stabilization of neurites and in the determination of axonal caliber. Neurofilaments are formed by the co-assembly of three subunits: NF-light (61 kDa), NF-medium (90 kDa) and NF-high (110 kDa). NF-high gene deletions have been found in 1% of SALS cases and neurofilament mutations have also been identified in FALS (Vucic and Kiernan, 2009). Axonal proteins, such as neurofilaments, are synthesized in the cell body

and need to be transported in an anterograde manner along axon and dendrites to reach synapses, whereas other components, such as trophic factors need to be transported in a retrograde manner from the synaptic regions back to the cell body (Pasinelli and Brown, 2006). Studies in SOD1 ALS mouse models have revealed that both slow and fast anterograde axonal transport are slowed months prior to disease onset and is concomitant to the accumulation of neurofilaments, which may physically compromise the transport system (Wijesekera and Leigh, 2009). Furthermore, mutant SOD1 has been shown to interact with the dynein-dynactin complex possibly causing its dysfunction (Ligon et al., 2005).

Astrocytes and microglia are important sources of neurotrophic factors. As damage of these cells occurs in ALS, deregulation of trophic support has been hypothesised to be involved in MN degeneration. Indeed, decreased levels of ciliary neurotrophic factor, brain-derived neurotrophic factor, the glial cell line derived neurotrophic factor and the insulin-like growth factor-1 have been observed in ALS patients *post-mortem* and *in vitro* models (Wijesekera and Leigh, 2009). Further evidence for the participation of growth factors in the pathology of ALS came from the discovery that deletion of the hypoxia response element from mouse with consequent decrease in vascular endothelial growth factor expression, leads to muscle weakness, atrophy, and death resembling neurodegeneration with ALS symptoms. Vascular endothelial growth factor is an important angiogenesis factor and its receptors have been discovered on neurons and astrocytes (Sathasivam, 2008).

1.4. ALS treatment

ALS is a complex disease with multiple causes, making the discovery of effective pharmacologic therapies challenging and at present clinical trials have failed to identify any truly effective therapies. In fact, since 1995, Riluzole (Rilutek®; Sanofi-Aventis) is currently the only Food and Drug Administration (FDA)-approved compound for ALS. It is thought to act in part by inhibiting glutamate release and the post-synaptic glutamate receptor cascade, and hence reducing glutamate excitotoxicity (Doble, 1996; Wijesekera and Leigh, 2009).

Ongoing trials (Table 2) are exploring the value of antiglutamatergic agents, as well as antioxidants, mitochondrial enhancers, neurotrophic factors, neuroinflammation protectors and anti-apoptotic drugs as modifiers of disease (Andrews, 2009).

Gene therapy holds promise for ALS, as it has the potential to deliver treatments to damaged MN and overcome the difficulty of crossing the blood–brain barrier. Many strategies are under investigation, including the delivery of genes encoding neurotrophic factors, anti-apoptotic drugs and antioxidants using a range of viral vectors administered by direct injection into the affected areas of the CNS, or remotely via intramuscular injection and retrograde transport to MN cell bodies (Goodall and Morrison, 2006).

An alternative gene therapy approach is to “knock-down” genes that might be causing toxicity, such as mutant SOD1. Successful studies of increased survival of SOD1 ALS mouse models have been demonstrated using small interfering RNAs targeted to mutant SOD1 (Miller et al., 2005; Raoul et al., 2005; Yokota et al., 2007), and antisense oligonucleotides (Turner et al., 2003a), remarkably, mutant SOD1 silencing with lentiviruses almost doubled the longevity of mice expressing mutant $SOD1^{G93A}$ (Ralph et al., 2005).

Recently, stem cell therapy has also been considered for the treatment of ALS. The most effective stem cell approaches could be those focused on glial cells, to restore healthy astrocytes with normal glutamate transport activity, or used for trophic and neuroprotective factors delivery to existing MN (Nirmalananthan and Greensmith, 2005; Mitchell and Borasio, 2007). The feasibility and safety of implantation of autologous mesenchymal stem cells into the SC has been tested in ALS. No major adverse events arose, with some clinical improvement in only some of the ALS patients analyzed (Mazzini et al., 2008).

Due to emerging evidence for the existence of secretory pathways for SOD1 mutants linked to ALS and for neurotoxicity of extracellular mutant SOD1, benefits of active immunization in the G93A and G37R transgenic mouse models with recombinant SOD1 mutant protein and disease specific epitope peptides derived from recombinant SOD1 mutant protein as immunogens have been demonstrated. The development of vaccines to remove aggregated abnormal SOD1 as a possible treatment in ALS, needs to be evaluated in models of SALS, and eventually tested in humans with ALS (Urushitani et al., 2007; Brooks, 2009).

In view of the complexity of the disease, a combination of different therapies acting in synergy, to target different processes may be the most effective (Bruijn et al., 2004; Brooks, 2009).

Table 2: Human clinical trials for ALS therapy. Adapted from Andrews (2009).

Compound	Putative target	Results/Progress	References
Previously trialed			
Vitamin E, *N*-acetyl-L-cysteine, L-methionine	Oxidative stress	No benefit	Orrell et al., 2005; Orrell et al., 2008
BDNF	Neurotrophic factor	Negative	Nirmalananthan and Greensmith, 2005
IGF-1	Neurotrophic factor	No benefit	Sorenson et al., 2008
CNTF	Neurotrophic factor	Negative	Miller et al., 1996a; Bongianni et al.,2004
Xaliproden	Neurotrophic factor	Negative	Meininger et al., 2004
Branched-chain amino acids	Glutamate metabolism	No benefit	Parton et al., 2008
Riluzole	Anti-glutamate	Prolonged survival	Bensimon et al., 1994
Gabapentin	Anti-glutamate	Negative	Miller et al., 2001
Topiramate	Anti-glutamate	Negative	Cudkowicz et al., 2003
Lamotrigine	Anti-glutamate	Negative	Ryberg et al., 2003
Nimodipine	Calcium regulation/ Excitotoxicity	Negative	Miller et al., 1996b
Verapamil	Calcium regulation/ Excitotoxicity	Negative	Miller et al., 1996c
Dextromethorphan	Excitotoxicity	No benefit	Gredal et al., 1997
Celecoxib	Anti-inflammatory	Negative	Cudkowicz et al., 2006
Creatine	Cellular energy deregulation	Negative	Rosenfeld et al., 2008
Pentoxifylline	Anti-inflammatory	No benefit	Meininger et al., 2006
Minocycline	Neuroinflammation	Negative	Gordon et al., 2007
TCH346	Anti-apoptotic	No benefit	Miller et al., 2007
Glatiramer acetate	Immune modulation	No benefit	Meininger et al., 2009
Interferon beta 1A	Immune modulation	Negative	Beghi et al., 2000
Thalidomide	Neuroinflammation	No benefit	Stommel et al., 2009
Tamoxifen	Anti-inflammatory/ Excitotoxicity	Prolonged survival	Brooks, 2009
Co-enzyme Q10	Mitochondrial enhancer/ Antioxidant	No benefit	Andrews, 2009
Sodium valproate	Histone deacetylase inhibition	No benefit	Andrews, 2009
Sodium phenylbutyrate	Histone deacetylase inhibition	Safe and tolerable	Cudkowicz et al., 2009
Minocycline/Creatine	Neuroinflammation/ Cellular energy deregulation	No benefit	Gordon et al., 2008; Andrews, 2009
Celecoxib/Creatine	Anti-inflammatory/ Cellular energy deregulation	Slowed deterioration	Gordon et al., 2008; Andrews, 2009
Ongoing and upcoming clinical trials			
Arimoclomol	Hsp induction	Phase I/II/III	Kieran et al., 2004; Cudkowicz et al., 2008
Ceftriaxone	Glutamate toxicity	Phase III	Rothstein et al., 2005
Talampanel	Glutamate toxicity	Phase II	*
Lithium	Neuroprotection/ Autophagy inducer	Delayed progression/ Phase II/III	Fornai et al., 2008
Memantine	Excitotoxicity	Phase II	Joo et al., 2007
GSK1223249	Nerve growth	Phase I/II	*, GlaxoSmithKline
Pioglitazone	Anti-inflammatory	Phase II	Schütz et al., 2005
Cistanche total glycosides	Anti-apoptotic	Phase II	*
E0302 (mecobalamin)	Excitotoxicity	Phase II/III	Kaji et al., 1998
TRO19622	Mitochondrial function	Phase II/III	Bordet et al., 2007
TUDCA	Antioxidant/Antiapoptotic/ Neuroprotective	Phase II	*
Tretinoin/Pioglitazone	Neuroprotective/ Anti-inflammatory	Phase I/II	*
VEGF	Neurotrophic	Phase I/II	*
AEOL-10150	Antioxidant	Phase I	Orrell, 2006
KNS-760704	Mitochondrial enhancer	Phase II	Gribkoff and Bozik, 2008; *
Growth hormone (Somatropin)	Neurotrophic	Phase II	*
MCI-186 (edaravone)	Antioxidant	Phase III	Takahashi 2009; *
ONO-2506	Anti-glutamate/ Anti-inflammatory	Phase III	Andrews 2009

*) www.clinicaltrials.gov

1.5. Aims of this Thesis work

As referred in the previous sections mutant SOD1 is known to play an important role in ALS pathogenic mechanisms. The major goal of the work presented in this Thesis is to study cellular features caused by the overexpression of mutant SOD1 in experimental *in vitro* cell models of ALS, which may contribute to the understanding of pathological characteristics of the disease.

The first objective is to investigate consequences for the morphology of the Golgi apparatus of the overexpression of $SOD1^{G93A}$ in the MN cell line NSC-34, and the relationship with apoptosis.

The second objective consists of the study of primary cultures obtained from SC of embryos from the transgenic rat model $SOD1^{G93A}$ and the characterization of the Golgi morphology from the MN.

The third objective is based on the fact that protein aggregation is one of the hallmarks of the disease. The fusion protein $SOD1^{G93A}$-enhanced green fluorescent protein will be overexpressed in the NSC-34 cell line to obtain aggregates. Consequences for the levels of the glycoproteins β-trace protein and erythropoietin, and for the trafficking of the cell adhesion molecule L1 to the plasma membrane will be investigated. Additionally, the effect of chemical chaperones on mutant protein aggregation will be studied.

The fourth objective relates with the role of the cytosolic protein SOD1 in the extracellular environment, which is believed to be the cause of non-cell autonomous spreading of toxicity. The mechanism of SOD1 secretion in association with secretory vesicles, the exosomes, by the NSC-34 cell line, and their interaction with other MN, will be investigated.

Chapter 2

Establishment of a cell model of ALS disease: Golgi apparatus disruption occurs independently from apoptosis

Work presented in this chapter corresponds to the following article:

Gomes, C., Palma, A.S., Almeida, R., Regalla, M., McCluskey, L.F., Trojanowski, J.Q. and Costa, J. (2008) Establishment of a cell model of ALS disease: Golgi apparatus disruption occurs independently from apoptosis. Biotechnol Lett **30**(4): 603-10.

2. Establishment of a cell model of ALS disease: Golgi apparatus disruption occurs independently from apoptosis

2.1. Abstract

The Golgi apparatus appears disrupted in MN of ALS. Here, mouse motor neuron-like NSC-34 cell lines stably expressing human superoxide dismutase 1 (hSOD1)^{wt} and mutant hSOD1^{G93A}, as an ALS cell model, were constructed. The number of cells with disrupted Golgi apparatus increased from 14 to 34%. Furthermore, NSC-34/hSOD1^{G93A} cells showed lower levels of proliferation and differentiation. Golgi apparatus disruption was not caused by apoptosis as determined by several techniques, including caspase-3 activation. Therefore, NSC-34/hSOD1^{G93A} cells are a suitable cell model to study Golgi apparatus dysfunction in ALS.

2.2. Introduction

Amyotrophic lateral sclerosis is a fatal neurodegenerative disease that results from selective dysfunction and death of upper and lower MN in the SC, brainstem and cortex, which leads to generalized weakness, muscular atrophy and death (Bruijn et al., 2004). Approximately 90-95% of ALS cases are sporadic, whereas 5-10% of cases have familial ALS, 20% of which presenting mutations in the protein Cu,Zn superoxide dismutase (hSOD1).

At cellular level, neuronal Golgi apparatus becomes disrupted or disorganized to form numerous round, smaller, oval and uniform vesicles in ALS MN. Golgi apparatus disruption was observed in patients with FALS with hSOD1 mutations and in transgenic mice expressing $hSOD1^{G93A}$, and in SALS. However, the molecular mechanism underlying Golgi apparatus disruption in ALS is not known at present (Gonatas et al., 2006).

Several authors have shown evidence of apoptotic features associated with MN degeneration in ALS models as well as in human tissues of ALS patients (Sathasivam and Shaw, 2005a). Apoptotic cells also show fragmented Golgi apparatus due to cleavage of Golgi apparatus matrix proteins such as GRASP65, Golgin-160 or p115 (Hicks and Machamer, 2005).

The NSC-34 cell line is a mouse neural hybrid cell between neuroblastoma and SC cells that has several characteristics of MN including generation of action potentials, expression of neurofilament proteins, acetylcholine synthesis, storage and release (Cashman et al., 1992).

In the present work, we have found increased Golgi apparatus disruption in non-apoptotic NSC-34 cells expressing recombinant $hSOD1^{G93A}$, therefore mimicking a pathological characteristic of the disease.

2.3. Materials and methods

2.3.1. NSC-34 cell culture.

NSC-34 cells were grown in Dulbecco´s modified Eagle´s medium-high glucose (Gibco), supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 0.1 mg/ml streptomycin (P/S, Invitrogen). Transfection of NSC-34 cells with pCI-neo expression vector (NSC-34^{v}) and pCI-neo expression vector containing human wild-type, hSOD1^{wt} (NSC-34/hSOD1^{wt} cells) and mutant hSOD1^{G93A} (NSC-34/hSOD1^{G93A} cells) was performed using the calcium phosphate method, and selection was with 0.5 mg/ml geneticin sulphate. Cells were kept in a humidified incubator at 37ºC under 5% CO_2. Cells were grown on plastic surfaces with or without coating with 10 µg/ml poly-D-lysine (PDL, Sigma) and 0.26 mg/ml Matrigel (BD Biosciences).

2.3.2. Protein analysis.

Cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 5 mM EDTA, 2% (w/v) SDS, 1% (w/v) Nonidet P-40, with a protease inhibitor cocktail Complete (PI, Roche) for 10 min on ice. Protein samples after ethanol precipitation were applied onto 12 or 15% (v/v) acrylamide SDS-PAGE gels. For Western blot analysis proteins were electrotransferred to polyvinyledene difluoride (PVDF) membranes and further processed as previously described (Morais and Costa, 2003). Detection was by the ECL Plus method (GE Healthcare).

2.3.3. Antibodies.

Primary antibodies used for Western blot were: polyclonal anti-SOD1 (dilution 1:15,000, Santa Cruz); monoclonal anti-GM130 (dilution 1:200, BD Biosciences) and polyclonal anti-cleaved caspase-3 (dilution 1:1,000,

Asp175, Cell Signalling Technology). As secondary antibodies anti-rabbit-HRP (1:4,000) and anti-mouse-HRP (1:3,000) were used.

2.3.4. Immunofluorescence microscopy.

For immunofluorescence microscopy cells were grown until 80% confluence in 24-well-plates with glass coverslips precoated with PDL/Matrigel. Immunofluorescence was as previously described (Brito et al., 2007). Coverslips were examined with a Leica DMRB microscope coupled to a COHU high performance CCD camera and Leica QFISH software, or with a Leica Confocal (SP2+AOBS) microscope. The secondary antibody was anti-mouse immunoglobulin G conjugated to Alexa Fluor 594 (1:500).

2.3.5. FACS analysis of cell viability and mitochondrial transmembrane potential.

Cells were detached with trypsin-EDTA (Gibco) and stained with 20 nM 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$, Molecular Probes] and 1 µg/ml propidium iodide (PI, Sigma) and analysed by flow cytometry with a two-laser FACS cytometer (CyFlow space, Partec), as described before (Vieira et al., 2002). Twenty five thousand events were acquired and histograms were analyzed using Partec FloMax.

Cell viability was determined with PI (excitation 488 nm with the blue solid state laser; detection >620 nm) and mitochondrial transmembrane potential with $DiOC_6(3)$ (excitation 488 nm with the blue solid state laser; detection at 530±20 nm).

2.4. Results

2.4.1. NSC-34/hSOD1^{G93A} cells show decreased proliferation and differentiation

To study the effect of hSOD1^{G93A} on Golgi apparatus morphology NSC-34 cells were transfected with vectors pCI-neo coding for hSOD1^{wt} and hSOD1^{G93A} and selected with 0.5 mg/ml geneticin sulphate. Expression of hSOD1 was confirmed by Western blot analysis (Figure 5A).

The newly constructed cell lines had similar growth rates during the initial 2 days in culture. However, on the 5th day of culture the NSC-34/hSOD1^{G93A} cell number was only 60% of that observed for NSC-34/hSOD1^{wt} (Figure 5B). Since cell viabilities were similar for the three cell lines and the highest number of cells released to the supernatant was 13%, the results suggested that transfection of NSC-34 cells with hSOD1^{G93A} caused decreased proliferation rates.

NSC-34 cells differentiate *in vitro* at low cell concentrations and exhibit multiple neurite-like processes (Cashman et al., 1992). Cells were grown on a plastic surface with or without coating with PDL/Matrigel, which is known to promote neuron adhesion and differentiation (Figure 5C). The percentage of differentiated NSC-34/hSOD1^{G93A} cells was significantly lower (17%) than NSC-34/hSOD1^{wt} cells (35%; $P<0.05$ by Student's *t*-test), on the PDL/Matrigel surface (Figure 5D). Therefore, mutant hSOD1 caused reduced cell differentiation.

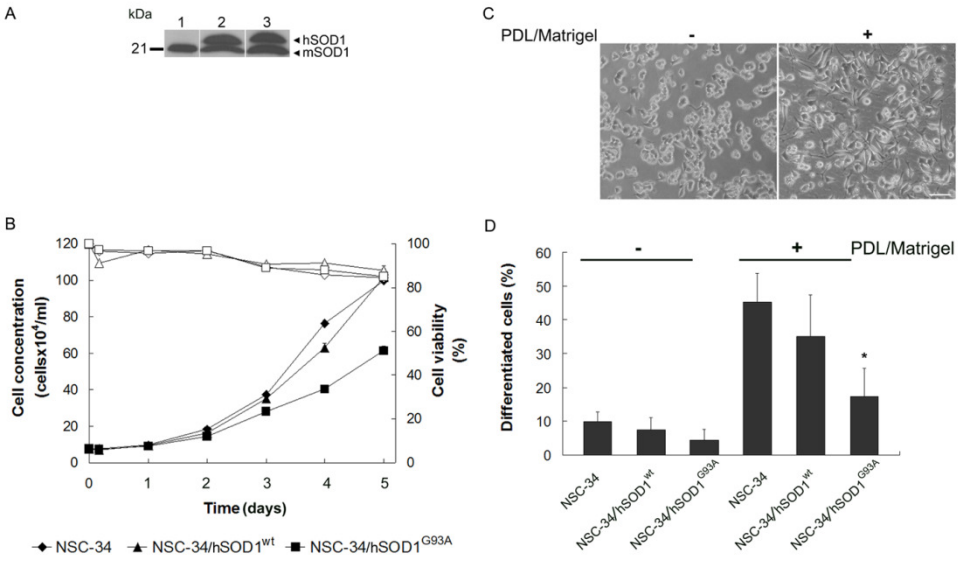

Figure 5: Construction and characterization of stable NSC-34 cell lines expressing recombinant hSOD1^{wt} and hSOD1^{G93A}. (A) Western blot analysis of hSOD1^{wt} and hSOD1^{G93A} from NSC-34 (lane 1), NSC-34/hSOD1^{wt} (lane 2) and NSC-34/hSOD1^{G93A} (lane 3) cells. Murine SOD1 (mSOD1) migrates faster than hSOD1. Primary antibody was polyclonal rabbit anti-SOD1. Detection was performed by the ECL method. (B) Growth curve and viability of NSC-34, NSC-34/hSOD1^{wt} and NSC-34/hSOD1^{G93A} cells. Results are the average of twelve measurements. Closed symbols - cell concentration; open symbols - cell viability. (C) Visualization of NSC-34 cells by inverted microscopy. Bar: 50 µm. (D) Percentage of differentiated cells. Cell counting were the average of duplicates from three independent experiments, a total of 28,895 cells being counted. Differentiation of NSC-34/hSOD1^{G93A} cells on PDL/Matrigel was significantly lower than that observed for NSC-34/hSOD1^{wt} (*P<0.05 by Student's t-test).

2.4.2. NSC-34/hSOD1^{G93A} cells have increased levels of Golgi apparatus disruption

Golgi apparatus was detected by immunofluorescence microscopy, using the anti-Golgi matrix protein GM130 antibody that marks the *cis*-Golgi. Under basal conditions the Golgi apparatus was detected as a compact organelle at the periphery of the nucleus for the NSC-34/hSOD1^{wt} cells, whereas it appeared as round and small vesicles dispersed throughout the cytoplasm for a subpopulation of NSC-34/hSOD1^{G93A} cells (Figure 6A). The percentage of cells with disrupted Golgi apparatus increased from 14 to 34%, for the mutant, and it was significant with $P<0.001$ using the Student's *t*-test (Figure 6C).

As the Golgi apparatus is known to be fragmented under apoptotic conditions, apoptosis was induced in the cells with staurosporine (STS), which is a broad spectrum kinase inhibitor. In the presence of STS, Golgi apparatus was disrupted, however, it exhibited a morphology distinct from that observed for the NSC-34/hSOD1^{G93A} cells, where the Golgi apparatus was disrupted into punctated structures of smaller dimensions (Figure 6A).

Nuclei appeared intact after staining with DAPI in the cells with disrupted Golgi apparatus (Figure 6B), which indicated that these cells had not undergone advanced apoptotic events.

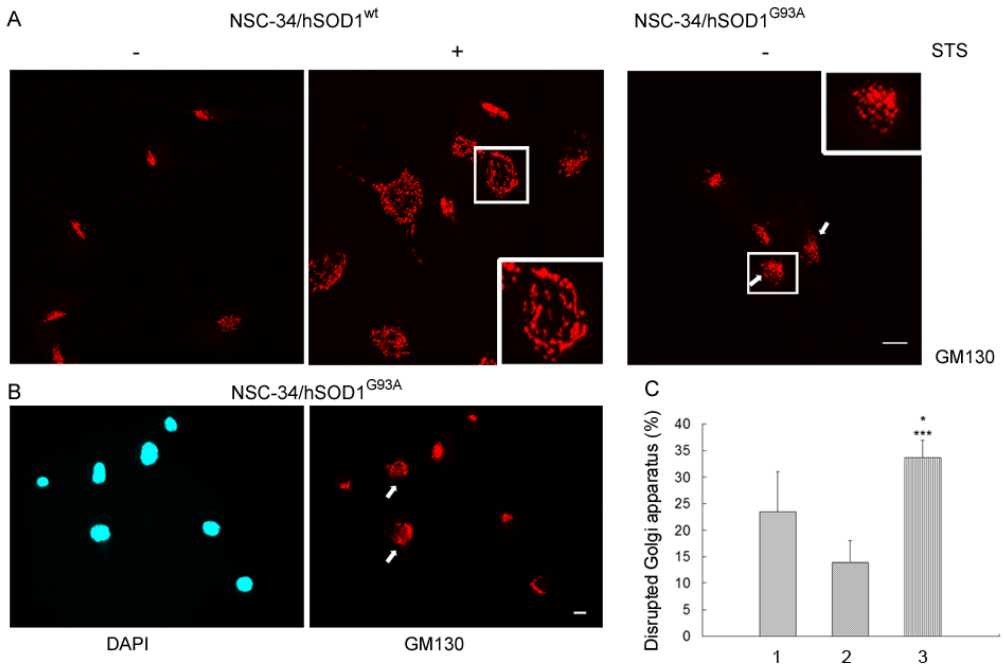

Figure 6: Immunofluorescence microscopy monitoring of Golgi apparatus disruption in NSC-34/hSOD1^{G93A} cells. (A) Golgi apparatus from NSC-34/hSOD1^{wt}, under basal conditions and after exposure overnight to 0.1 μM STS, and NSC-34/hSOD1^{G93A} cells. Primary antibody was monoclonal anti-GM130. Arrows indicate disrupted Golgi. Bar: 10 μm. The inset was two-fold magnified. (B) Double staining of nuclei (DAPI, blue) and Golgi apparatus (red, as in A). (C) Percentage of cells with disrupted Golgi apparatus. The quantification of the number of cells with disrupted Golgi apparatus was performed blind in seven different coverslips for each cell line, from three independent experiments in a total number of 2,949 cells. NSC-34/hSOD1^{G93A} (3) cells showed a significant increase in percentage of disrupted Golgi apparatus when compared with NSC-34/hSOD1^{wt} (2) cells (***P<0.001 by Student's t-test). There was also a significant difference between NSC-34^{v} (1) and NSC-34/hSOD1^{G93A} Golgi apparatus disruption (*P<0.05 by Student's t-test).

2.4.3. Golgi apparatus disruption was not due to apoptosis or Golgi apparatus protein degradation

As nucleus fragmentation is a late event in apoptosis, NSC-34/SOD1^{G93A} cells were analysed for earlier occurring apoptotic events such as mitochondrial membrane permeability to PI, mitochondrial transmembrane potential and caspase-3 cleavage.

PI is a membrane impermeable dye that is excluded by viable cells with an intact membrane, but that enters dead cells and binds DNA. $DiOC_6(3)$, detects mitochondrial transmembrane potential ($\Delta\Psi m$) and, consequently, stains viable cells. The combination of these two markers allowed us to divide the cells into four different populations: Q4 - PI impermeable and $DiOC_6(3)$ positive cells [$PI^-/DiOC_6(3)^+$] – live cells; Q3 - PI impermeable and $DiOC_6(3)$ negative cells [$PI^-/DiOC_6(3)^-$] – early apoptotic cells; Q1 - PI permeable and $DiOC_6(3)$ negative cells [$PI^+/DiOC_6(3)^-$] – apoptotic cells and Q2 - PI permeable and $DiOC_6(3)$ positive cells [$PI^+/DiOC_6(3)^+$] – non apoptotic dead cells (Figure 7A).

Under basal conditions we observed that all the cell lines had similar low levels of early apoptotic (Q3) and apoptotic cells (Q1) (5-8%). When NSC-34/hSOD1^{G93A} cells were treated with 0.1 μM STS, higher levels of apoptotic cells were found for the NSC-34/hSOD1^{G93A} (45%) than for the NSC-34/hSOD1^{wt} (31%) cells (Figure 7B). These results suggested that NSC-34/hSOD1^{G93A} cells were more vulnerable to apoptotic stimuli when compared with the controls.

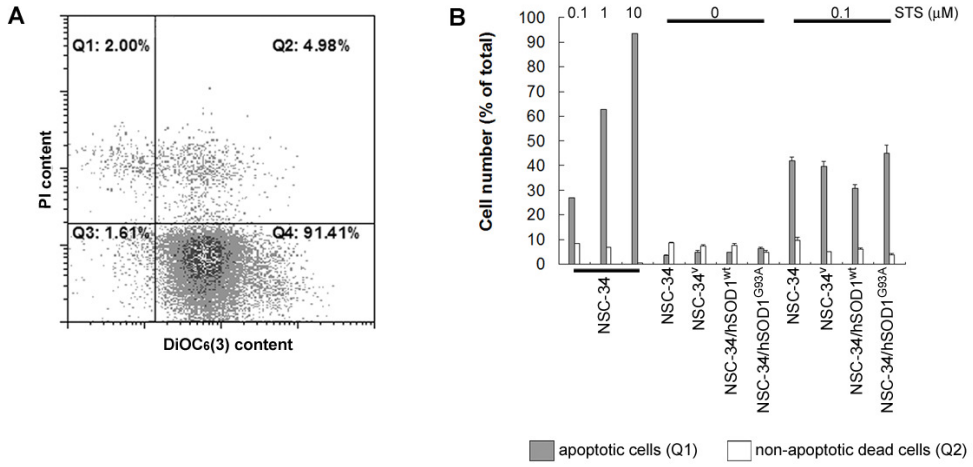

Figure 7: Biparametric $DiOC_6(3)$-PI flow cytometry analysis of NSC34/hSOD^{wt} and NSC-34/hSOD1^{G93A} cells. (A) PI and $DiOC_6(3)$ staining of NSC34/hSOD^{wt} cells. (B) Percentage of apoptotic and non-apoptotic cells in the absence or presence of STS.

Caspase-3, an effector caspase of the apoptotic cascade, which is activated upon proteolytic processing to 19 and 17 kDa products, was also analysed. Under basal conditions there was no caspase-3 cleavage for the NSC-34/SOD1^{G93A} cells (Figure 8). As a positive control of apoptosis, cells were FCS depleted, and the two cleavage products at 19 and 17 kDa were detected (Figure 8). Another positive control consisted of exposure to 0.1 μM STS. Also in this case caspase-3 activation was observed but only the 17 kDa cleavage product was detected.

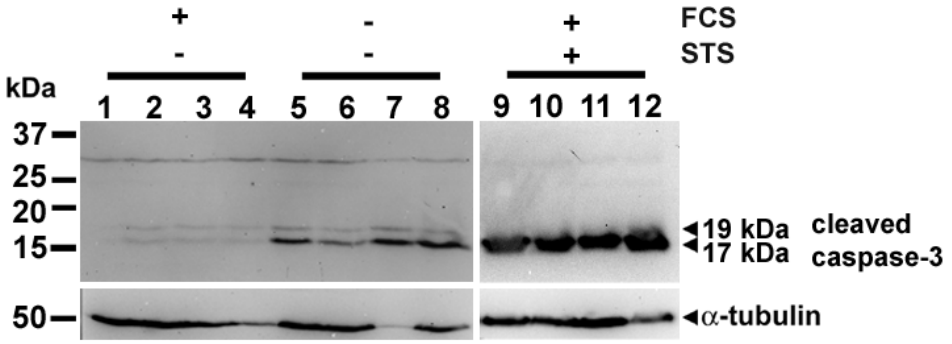

Figure 8: Western blot analysis of caspase-3 cleavage products. Protein was obtained from $2x10^5$ cells: NSC-34 (lanes 1, 5, 9), NSC-34^v (2, 6, 10), NSC-34/hSOD1^{wt} (3, 7, 11) and NSC-34/hSOD1^{G93A} (4, 8, 12). Cells were cultured in the presence of FCS in the control, and depleted of serum for 48h, or supplemented with 0.1 µM STS for 16h, under stress conditions. Primary antibody used was polyclonal rabbit anti-cleaved caspase-3. Detection was performed by the ECL method.

These results together showed that under basal conditions NSC-34/hSOD1^{G93A} cells did not present increased levels of apoptotic cells. Thus, Golgi apparatus disruption in mutant transfected cells occurred independently of apoptosis.

To investigate if Golgi apparatus disruption would result from proteolytic cleavage of proteins involved in maintaining the Golgi apparatus steady-state, GRASP55, p115 and GM130 were analysed by Western blot. However, GRASP55, p115 and GM130 levels were found at essentially the same levels for the different cell lines in basal conditions (results not shown). Therefore, Golgi apparatus disruption was not due to variations in these Golgi apparatus proteins. Alterations of other Golgi apparatus proteins cannot be ruled out.

2.5. Discussion

Observations from several authors have shown that Golgi apparatus appears disrupted in MN from ALS patients and from transgenic mouse models of the disease (Gonatas et al., 2006). Here, increased Golgi apparatus disruption for mutant NSC-34/hSOD1^{G93A} cells has been found, therefore mimicking a cellular feature of ALS and a pathological hallmark of the disease.

When stably transfected with mutant hSOD1^{G93A}, NSC-34 cells have been used as a cellular model of ALS for the study of other pathological characteristics of the disease (Kirby et al., 2005; Sathasivam et al., 2005b). However, this is the first study where Golgi apparatus disruption has been found in this cell model.

Secreted glycoproteins such as growth factors or plasma membrane glycoproteins including receptors or cell adhesion molecules cross the ER and Golgi apparatus where they are glycosylated and sorted to their final destination. These proteins are essential for neuron differentiation and adhesion. Therefore, it is possible that Golgi apparatus disruption from NSC-34/hSOD1^{G93A} cells could result in impaired trafficking and glycosylation of growth factors, receptors or cell adhesion molecules, similarly to that described by Stieber et al. (2004) for the CD4 receptor from CHO (chinese hamster ovary) cells transfected with SOD1 mutants. Such impairment could constitute the basis for the decreased cell differentiation and adhesion observed in this work. Others have also observed defective neurite outgrowth in cells expressing mutant SOD1^{G93A} (Lee et al., 2002). Accordingly, in a cellular model of another neurodegenerative disease, Parkinson's, impaired trafficking from the ER to the Golgi apparatus has been observed caused by overexpression of misfolded alfa-synuclein (Cooper et al., 2006).

Evidence indicated that ALS tissues and cells show apoptotic features (Sathasivam et al., 2005b). On the other hand, it is known that Golgi apparatus disruption occurs during apoptosis as result of GRASP65 and Golgin-160 cleavage by caspase-3 among other factors (Hicks and Machamer, 2005). In this work, several methodologies were used to investigate the apoptotic status of the mutant transfected cells and the results clearly showed that NSC-34/hSOD1^{G93A} cells were not apoptotic in basal conditions. Therefore, this cell model has the advantage of exhibiting Golgi apparatus disruption without apoptosis and will allow the study of the molecular basis of Golgi apparatus disruption in ALS independently from apoptosis. These could include alterations in the cytoskeleton and disruption of kinesin dependent trafficking of organelles similarly to that found in astrocytes from neurodegenerative tauopathy (Yoshiyama et al., 2003), or changes in Golgi apparatus proteins distinct from those analyzed here.

In summary, NSC-34/hSOD1^{G93A} cells showed increased Golgi apparatus disruption without caspase-3 activation. Therefore, they constitute an adequate model to study the mechanisms underlying Golgi apparatus disruption in ALS and to test therapeutic compounds for the disease.

2.6. Acknowledgments

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Chapter 3

Primary cultures of spinal cord cells from rat embryo: wild-type *vs.* transgenic SOD1^{G93A}

3. Primary cultures of spinal cord cells from rat embryo: wild-type *vs.* transgenic SOD1^{G93A}

3.1. Abstract

ALS is a neurodegenerative disease that results from degeneration and death of MN in brain and SC. Some ALS cases result from mutations in ubiquitously expressed SOD1. Changes in the morphology of Golgi apparatus, like fragmentation, have been observed in MN from mice and patients with ALS. Aggregates of protein that stain for SOD1 are described in patients and cell models of ALS.

In the present work, primary cultures of SC sections and SC dissociated cells, from E14 rat embryos from wild-type (wt) and transgenic (TgN) SOD1^{G93A} animals modelling ALS have been established and characterized by immunofluorescence microscopy. They were found to contain neurons, revealed by the neurofilament staining with anti-NF-L antibody, among the neurons, MN were present, and marked with the specific antibody nerve growth factor receptor (anti-NGFR). The cultures contained also the supporting cells of neurons, astrocytes, marked with glial fibrillary acidic protein (anti-GFAP) antibody. The morphology of *cis*-Golgi and *trans*-Golgi network in these SC derived cell cultures has been observed by immunofluorescence microscopy with the antibodies anti-GM130 and GRASP65, and anti-TGN38, respectively, during 31 days in culture, without differences between wt and TgN. SOD1 accumulation was monitored by immunofluorescence microscopy. Furthermore, high molecular mass forms of mutant SOD1^{G93A} in SC from adult rats were detected by Western blot.

3.2. Introduction

Mutations in superoxide dismutase 1 (SOD1), a Cu,Zn enzyme that catalyzes the conversion of superoxide anions to hydrogen peroxide, have been implicated in some cases of FALS. It was initially thought that the toxicity of different SOD1 mutants could result from decreased free-radical scavenging activity, but now it is believed to result from a gain of a new toxic property (Bruijn et al., 2004).

A number of factors contribute to the selective vulnerability of SOD1 within MN to oxidative modification: SOD1 is present at high concentration in MN (Pardo et al., 1995), and its normal enzymatic function exposes it to high levels of oxidative stress, predisposing it, compared to other proteins, to oxidative damage. It also has a long life-time in MN because of slow transport in motor axons that potentiates oxidative modification (Rakhit and Chakrabartty, 2006).

Understanding why MN are particularly vulnerable in ALS may be important for deciphering pathogenic mechanisms and finding treatments. The vast majority of neuronal cells are more susceptible to oxidative stress than other cellular populations because they are post mitotic, and damage is going to accumulate throughout life. MN in particular are highly specialized cells, and it seems likely that this specialization renders them vulnerable to injury. Significant features are likely to include the extreme size of MN, their high metabolic activity, their sensitivity to mitochondrial dysfunction, their elevated neurofilament content, their reduced capacity to buffer calcium and an impaired ability to activate the heat shock defense mechanism (Nirmalananthan and Greensmith, 2005; Boillee et al., 2006b; Goodall and Morrison, 2006).

One of the mechanisms proposed to explain the selective MN death linked to ALS is the formation of protein aggregates that are immunoreactive to SOD1. Whether these aggregates damage MN (or other

cells in the SC), and if so through what mechanism(s), it is still an unsolved puzzle. Several possible toxicities of the protein aggregates have been proposed including aberrant chemistry; loss of protein function through co-aggregation with the aggregates; depletion of protein folding chaperones; dysfunction of the proteasome overwhelmed with misfolded protein; and inhibition of specific organelle function, including mitochondria and peroxisomes, through mutant aggregation onto or within such organelles (Bruijn et al., 2004).

It is now clear that toxicity to MN from SOD1 mutant is non-cell autonomous that is, it requires mutant damage not just within MN but also to non-neuronal cells like surrounding glia and possibly other cell types (Bruijn et al., 2004). The cellular neighbourhood does matter. The essential nature of glial cells in supporting MN with trophic factors, and/or through the maintenance of glutamate homeostasis by an active uptake of excess glutamate through glutamate transporters, makes plausible a glial role in SOD1-mediated toxicity (Cleveland and Rothstein, 2001).

At cellular level, Mourelatos et al. (1990) have observed that the neuronal Golgi apparatus became disrupted or disorganized to form numerous round, smaller, oval and uniform vesicles in ALS MN. Golgi apparatus disruption was observed in patients with FALS with hSOD1 mutations (Fujita et al., 2000; Fujita et al., 2002; Stieber et al., 2004) and in transgenic mice expressing hSOD1^{G93A} (Mourelatos et al., 1996; Stieber et al., 2000). Golgi apparatus disruption was also reported to occur in SALS (referred in Stieber et al., 2004). However, the molecular mechanism underlying Golgi apparatus disruption in ALS is not known at present.

We have established a cellular model of ALS, from SC cells of embryos E14 wild-type and SOD1^{G93A} that were shown to contain neurons including MN and astrocytes, among other cells. The morphology of *cis*-Golgi and *trans*-Golgi network in these SC derived cell cultures has been observed during 31 days in culture, by immunofluorescence microscopy,

and no changes were observed. The accumulation of SOD1 was monitored by immunofluorescence microscopy. High molecular mass forms of mutant $SOD1^{G93A}$ in SC from adult rats were detected by Western blot.

These cultures provide a good system to study the different cell types that may be involved in the ALS pathology, as it has been emphasized that a crosstalk between MN, astrocytes, microglial cells and other non-neuronal cells may cooperate to drive disease progression.

3.3. Materials and methods

3.3.1. Animal Model.

The rats used were Sprague Dawley Tac:N:(SD) and TgN(SOD1G93A)L26H, model for ALS, purchased from Taconic (Howland et al., 2002). The ovulation of female rats was synchronized with luteinizing hormone-releasing hormone agonist (40 μg), pregnant mare serum gonadotropin (10 Iu) and human chorionic gonadotropin (10 Iu) (Rouleau et al., 1993). Females SD were mated with male SD and TgN rats, to obtain the wild-type and transgenic embryos, respectively. Tail biopsies of littermate were analyzed for the presence of the transgene, after digestion with 1.25 mg/ml proteinase K overnight at 56ºC, by polymerase chain reaction (PCR) using primers sodi3-f (5'-GTGGCATCAGCCCTAATCCA-3') and sodE4-r (5'-CACCAGTGTGCGGCCAATGA-3') specific to human SOD1. PCR conditions were: an initial denaturation step at 95ºC for 5 min, 35 cycles with 1 min of denaturation at 95ºC, 1 min of annealing at 60ºC, 2 min of elongation at 72ºC, and a final elongation for 5 min, at 72ºC. The PCR product for human SOD1 transgene is 200 base pair.

3.3.2. Primary cell culture from rat embryos SC.

Two types of primary cell cultures from rat embryos SC were used. The first consisted of SC sections and the second consisted of dissociated cells. They were prepared as described below.

The cultures were prepared from embryos E14. The pregnant rats were euthanised with CO_2, the ventral surface of the animal was swabbed with 70% ethanol, and embryos were removed. The SCs were dissected under a stereomicroscope, in Hanks' Balanced Salt Solution, cut into small pieces, and kept in Neurobasal (NB, Gibco) medium with 1% penicillin/streptomycin (Invitrogen) on ice.

The SC sections were cultured in 24-well-plates with glass coverslips pre-coated as described below, containing culture medium as follows: NB medium, supplemented with 10 ng/ml glial-derived neurotrophic factor (GDNF, Invitrogen), 10 ng/ml brain-derived neurotrophic factor (BDNF, Invitrogen), 1% (v/v) B-27 (Gibco), 1% (v/v) N-2 (Gibco) and 1% (v/v) muscle extract from chicken embryos as described by Henderson et al. (Henderson et al., 1983), for the initial 3 days in culture.

For the SC dissociated cell cultures the steps after the dissection of the SC were performed under sterile conditions. The tissue was let to settle, and excess NB was removed. Sterile phosphate buffered saline (PBS, 1ml/2 SC) was used to wash the tissue. After removal of excess PBS, 0.05% trypsin-EDTA (Gibco) was added (1ml/2 SC) and incubated for 15 min at 37ºC, agitating gently to allow trypsin to get to all tissues. The supernatant was removed. The pellet was extracted 3 times with 0.8 ml of NB medium, 0.1 ml of bovine serum albumin (BSA, 4% (w/v) in NB) and 0.1 ml (first time) and 20 μl (next times) of DNase I (1mg/l in NB medium), per 2 SC. The tissue was gently agitated and then passed through a fire-polished Pasteur pipette to dissociate the tissue, till turbidity was observed. The collected supernatants were pooled and the same volume of NB medium was added, and was layered gently onto a cushion of 4% (w/v) BSA (1ml/2

SC), centrifuged for 10 min at 300x*g* at room temperature (RT) (Camu and Henderson, 1992). The pellet was resuspended in 1 ml of NB medium. The cells were counted using 0.1% Trypan blue solution in PBS using a Fuchs-Rosenthal haemocytometer.

Cells (representing mixed neuron/glia) were seeded at a density of $2x10^5$, $1x10^6$ and $2x10^6$ cells/well into 24-well-plates with glass coverslips precoated as described below, containing culture medium as follows: NB medium, 10 ng/ml GDNF (Invitrogen), 1% (v/v) B-27 (Gibco), 1% (v/v) N-2 (Gibco), 2% (v/v) horse serum (Gibco), 0.5 mM L-glutamine (Gibco), 25 μM 2-mercaptoethanol (Gibco), 25 μM L-glutamate (Sigma) and 10 ng/ml ciliary neurotrophic factor (CNTF, PeproTech EC), for the first 3 days in culture (Arce et al., 1998; Comoletti et al., 2001). After this, glutamate was removed from the culture medium. And for both cell culture types (SC sections or dissociated) medium was changed twice a week, and 1 or 0.5 μM cytosine β-D-arabino-furanoside, 10 or 5 μM 5-fluoro-2'deoxyuridine and 10 or 5 μM uridine were added to reduce proliferation of non-neuronal cells (Pleasure et al., 1992; Filipeanu et al., 2002). Cells were grown in a humidified incubator at 37ºC in a 5% CO_2 atmosphere.

The 24-well-plate containing the glass coverslips were incubated with poly-L-ornithine, (1.5 μg/ml in sterile bdH_2O, Sigma), for 30 min to overnight at RT. After removing the solution and allow to dry in hood, laminin (3 μg/ml in NB medium 1:100 P/S, Sigma) was added and incubated for 2 hours to overnight at 37ºC in the CO_2 incubator. Laminin was removed and replaced immediately with NB medium with the supplements described.

3.3.3. Immunofluorescence microscopy.

For immunofluorescence microscopy cells were grown on glass coverslips. Cells were rinsed twice with PBS containing 0.5 mM $MgCl_2$. Cells were fixed with 4% (w/v) paraformaldehyde in PBS with or without 4% (w/v) of sucrose for 20 min, and permeabilized with 0.1% (w/v) Triton X-100 (TX-100) in PBS for 15 min. Fixed cells were blocked with 1% (w/v) BSA in PBS for 1 hour, incubated at RT for 2 and 1 h with primary and secondary antibodies, respectively. Antibodies were diluted in PBS containing 1% (w/v) BSA and washes were done with PBS. Coverslips were mounted in Airvol and examined with a Leica DMRB microscope. Images were acquired using a COHU high performance CCD camera coupled to the microscope and Leica QFISH software.

The antibodies used are described below. To characterize the cells present in the cultures the primary antibodies rabbit anti-neurofilament light chain (NF-L, 1:2,000), neuron marker, mouse anti-glial fibrillary acidic protein (GFAP, 1:1,000, Chemicon International), astrocyte marker, mouse anti-nerve growth factor receptor (NGFR, 1:10, Chemicon International) and goat anti-NGFR p75 (1:10, Santa Cruz Biotechnology, Inc), MN marker, were used.

To monitor the Golgi morphology in the cultures the antibodies rabbit anti-Golgi ReASsembly Protein (GRASP65, 1:250), mouse anti-Golgi Matrix protein (GM130, 1:500, BD Biosciences), that mark the *cis*-Golgi, and sheep anti-*trans*-Golgi Network (TGN38, 1:200) were used.

To detect SOD1 in these cultures the antibody rabbit anti-SOD1 (1:200, Santa Cruz Biotechnology, Inc) was used.

The secondary antibodies used were: 1:100 anti-rabbit IgG conjugated to FITC (for NF-L), 1:64 anti-mouse IgG conjugated to TRITC (for GFAP and NGFR), anti-rabbit IgG conjugated to Alexa Fluor 488 (for GRASP65), 1:500 anti-mouse IgG conjugated to Alexa Fluor 594 (for NGFR, GFAP and GM130), anti-sheep IgG conjugated to Alexa Fluor 488

(for TGN38), anti-goat IgG conjugated to Alexa Fluor 488 (for NGFR) and anti-rabbit IgG conjugated to Alexa Fluor 594 (for SOD1).

3.3.4. SOD1 analysis of SC from wild-type and transgenic adult rat model of ALS SOD1^{G93A}.

SC of wt and TgN adult rats were lysed on ice with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate (DOC), 1% (w/v) TX-100, 1% PI (Roche)) by increasing cycles of sonication. Protein extracts were centrifuged at 20,000x*g*, 10 min, 4ºC. The protein concentration of the supernatants was determined by the bicinchoninic acid method (Smith et al., 1985).

After ethanol precipitation protein samples were applied onto 12% (v/v) acrylamide SDS-PAGE gels.

3.3.5. Western blot analysis.

SOD1 protein was analysed by SDS-PAGE as described, followed by Western blot on PVDF membranes. The following primary antibody was used: polyclonal anti-SOD1 (dilution 1:15,000, Santa Cruz). As secondary antibody anti-rabbit-HRP (1:4,000) was used. Protein was detected by the ECL Plus method following supplier's protocol (Amersham Biosciences).

3.4. Results

3.4.1. SC derived cell cultures

Two types of SC derived cell cultures, SC sections and SC dissociated cells, from rat embryos E14 wild-type and SOD1^{G93A} were performed as described in Materials and methods. The two cultures were observed by inverted microscopy and characterized with specific antibodies for different cells.

The cells migrated from the SC sections. Initially only few cells could be observed, but after 3 days a monolayer was seen with different cells that started to contact with each other (Figure 9A, arrow).

The culture from dissociated cells after 7 days started to become organized in clusters rich in cells that connected with each other via neurofilament containing structures (Figure 9B, arrowhead and 11C). The connections between clusters in the TgN cultures appeared thicker than those observed in their wt counterparts (Figure 9B, TgN).

Both cultures contained several cell types. After one month in culture, the cells started to detach.

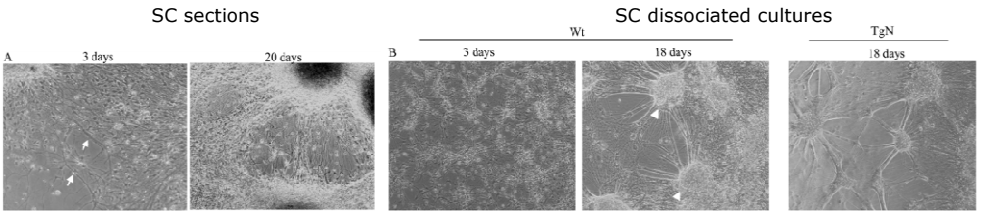

Figure 9: Monitoring the SC cultures by inverted microscopy. Arrows - contacts established between the cells in SC sections. Arrowheads – clusters of cells in SC dissociated cultures.

Since a mixed culture was performed the cells were marked with different antibodies to different cell types, such as NF-L that marked neurons, NGFR that marked MN and GFAP that marked astrocytes, by immunofluorescence microscopy (Figure 10), and the different cell types were distinguished.

The cultures of SC sections were found to be rich in neurofilaments belonging to neurons (Figure 10A), and among these MN were found (Figure 10B), these cultures contained also astrocytes (Figure 10C) that acted as support cells for MN.

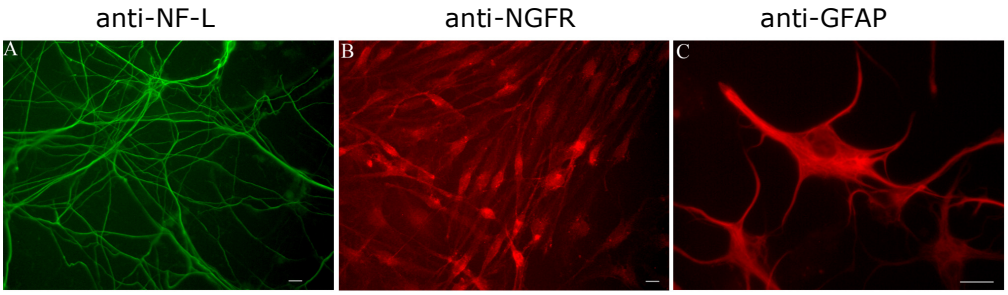

Figure 10: Identification of different cells in the cultures of SC sections by immunofluorescence microscopy. Antibodies used were anti-NF-L (A – 5 days), anti-NGFR (B – 7 days) and anti-GFAP (C – 8 days). Bar: 10 μm.

The effect of mitosis inhibitors in these mixed cultures was tested in order to diminish the cell proliferation, so that the visualization of MN was facilitated. Two concentrations of three mitosis inhibitors were tested: 1 or 0.5 μM cytosine β-D-arabino-furanoside (known to have antiproliferative effect), 10 or 5 μM 5-fluoro-2'deoxyuridine (known to reduce cell proliferation and the rate of DNA synthesis) and 10 or 5 μM uridine. We observed that for the lowest concentration of inhibitors tested, MN were kept healthy, and astrocytes were still alive but at a lower number. When cells were exposed to the highest concentration tested, astrocytes got shrank and ultimately died, and neurons were still present, but the neurofilaments stained with anti-NF-L were disrupted and were less abundant (data not shown).

The cultures of dissociated SC were then characterized by immunofluorescence microscopy. Different cell densities at the starting culture were tested: $2x10^5$, $1x10^6$ and $2x10^6$ cell/well. At the lowest density, cells did not grow properly since staining for neurons (NF-L) or for astrocytes (GFAP) was almost absent (data not shown). On the other hand, when cells were seeded at $2x10^6$ cell/well too many cells had grown and

detached easily from the coverslips. So, $1x10^6$ cell/well, were seeded for further monitoring.

Using immunofluorescence microscopy a mixed culture of neurons, marked with the antibody anti-NF-L, and astrocytes marked with the antibody anti-GFAP (Figure 11) was observed as previously described for SC sections. These cells had a characteristic organization, the astrocytes were seen mainly below the neurons, and the neurons were also found in great number in the clusters of cells and they contacted with neighbour cells via neurofilament containing structures.

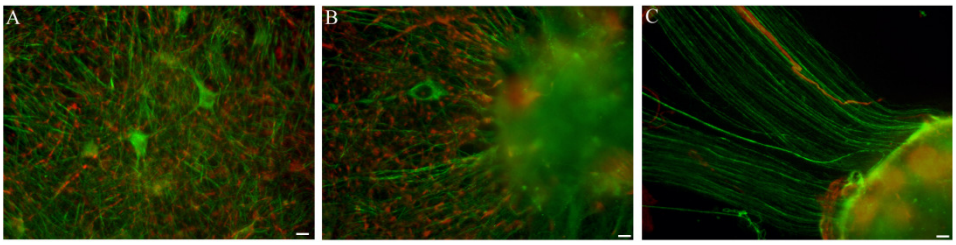

Figure 11: Monitoring of SC dissociated cultures by immunofluorescence microscopy. Antibodies used were anti-NF-L (green) and anti-GFAP (red). Bar: 10 μm.

3.4.2. Monitoring of MN Golgi morphology in the SC derived cell cultures

After the characterization of the cultures, the Golgi morphology was monitored by immunofluorescence microscopy.

MN Golgi morphology from SC sections was monitored with markers for *cis*-Golgi (GRASP65) and *trans*-Golgi Network (TGN38), the Golgi apparatus was not dispersed in the wt MN nor in the TgN ones (data not shown), different from what had been reported in ALS models *in vivo* and *in vitro* (Mourelatos et al., 1990; Fujita et al., 2000; Stieber et al., 2004). This could be explained due to the fact that these cultures were performed using embryos E14, so the changes in the Golgi apparatus morphology might not be observed at this stage.

The Golgi apparatus morphology from SC dissociated wild-type and transgenic cells was also monitored during 29 days by immunofluorescence microscopy. A similar staining of the *cis*-Golgi, marked with anti-GM130 antibody, of MN in wt and TgN cells (Figure 12) was observed.

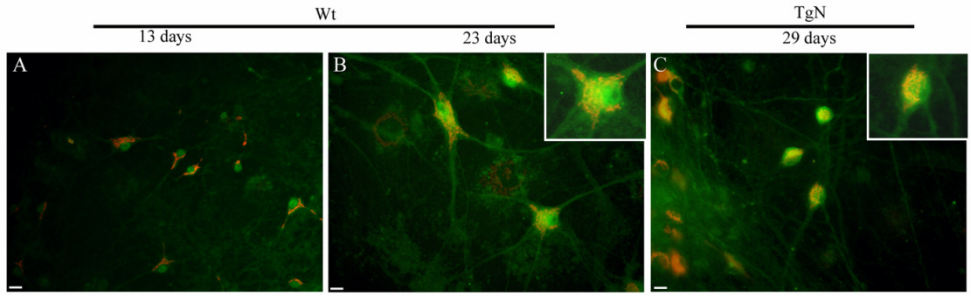

Figure 12: Monitoring of *cis*-Golgi in the SC dissociated cultures by immunofluorescence microscopy. Cells were stained with monoclonal anti-GM130 (red) and anti-NGFR (green). Bar: 10 μm.

3.4.3. Analysis of SOD1 from SC of wild-type and transgenic rat model of ALS (SOD1^{G93A})

One of the hallmarks of ALS pathology is the presence of prominent, intracellular cytoplasmic inclusions in MN and astrocytes that are highly immunoreactive for SOD1. Several possible toxicities of the protein aggregates have been proposed: aberrant chemistry, loss of protein function through coaggregation with the aggregates, depletion of chaperones, dysfunction of the proteasome and inhibition of mitochondria (Bruijn et al., 2004).

The presence of protein aggregates immunostained with SOD1 were investigated in SC dissociated cultures by immunofluorescence microscopy. The polyclonal antibody anti-SOD1 was used to detect the accumulation of SOD1 in time. The wt cells had low staining with the antibody while transgenic cells overexpressing human SOD1^{G93A} appeared strongly stained and accumulation of SOD1 was detected in the axons and dendrites of the neurons (Figure 13).

Several authors have described SOD1 insoluble aggregates in postnatal tissues from transgenic mouse models of ALS (Johnston et al., 2000; Turner et al., 2003b; Wang et al., 2003; Liu et al., 2004; Turner et al., 2004b), the presence of these protein aggregates in SC from adult rat model of ALS ($SOD1^{G93A}$) was then analysed.

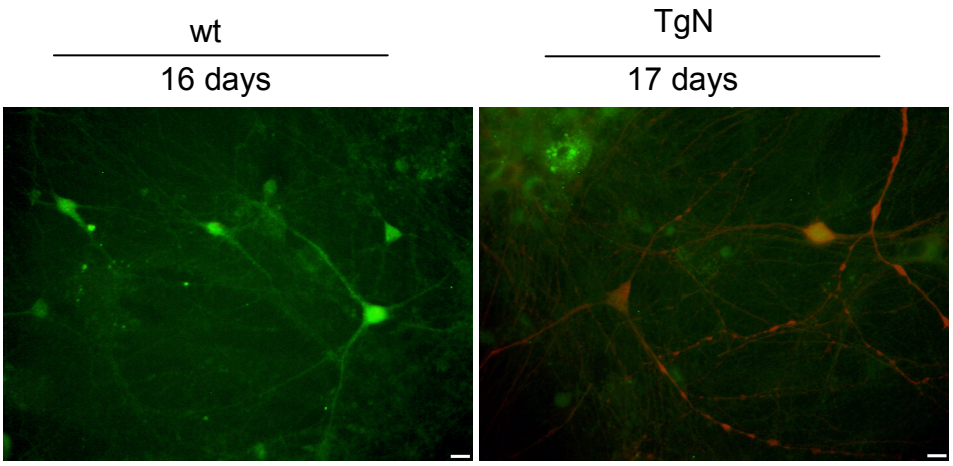

Figure 13: Immunofluorescence microscopy of SOD1 in the SC dissociated cell cultures. Cells were stained with polyclonal anti-SOD1 (red) and anti-NGFR (green). Bar: 10 μm.

Proteins of SC from adult wt (259 days old, Figure 14A) and TgN (127 days old, Figure 14B) rats were extracted by increasing cycles of sonication to promote solubilization of protein aggregates. In Figure 14A the endogenous rat SOD1 (rSOD1), and a small amount of the dimer and trimer of rSOD1 were detected. In Figure 14B the monomer and dimer of human $SOD1^{G93A}$ (hSOD1), and forms of SOD1 of high molecular mass (HMM) were observed. There was an increasing extraction of the HMM forms with increasing cycles of sonication, and with 10 cycles there was a maximum in the extraction of these forms. So, with increasing cycles of sonication HMM aggregates containing SOD1 were detected.

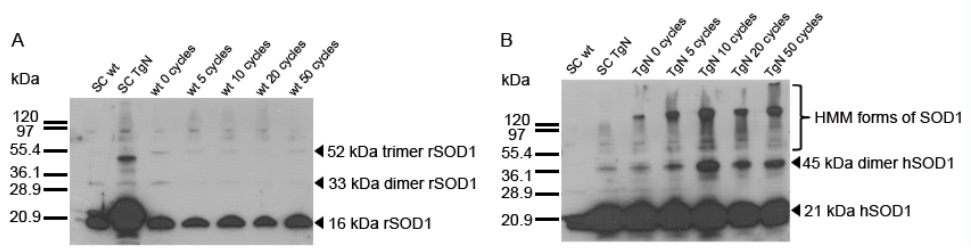

Figure 14: Western blot analysis of SOD1 in the SC from rat expressing wild-type rat or G93A human SOD1. (A) Cellular extract from SC of rat 259 days old, expressing endogenous wild-type SOD1 (B) Cellular extract from SC of rat 127 days old, expressing human $SOD1^{G93A}$. In each lane 20 μg of protein were loaded.

3.5. Discussion

We have established a cellular model of ALS, from SC cells of embryos E14 wild-type and $SOD1^{G93A}$ that were shown to contain neurons, including MN, and astrocytes among other cells.

Since the SC is composed of different cell types including neurons like MN, and glial cells like oligodendrocytes and astrocytes, the mixed cultures resembled the SC environment where the glial population removed harmful proteins and toxic products, and secreted neurotrophic factors, which helped neurons survive and grow. So, these cultures provided a good system to study the different cell types that may be involved in the ALS pathology.

SC cultures were used to study one of the features associated with the disease where there have been reports of fragmented Golgi apparatus in ALS models *in vivo* and *in vitro* (Mourelatos et al., 1990; Mourelatos et al., 1996; Fujita et al., 2000; Stieber et al., 2004; Gonatas et al., 2006). The Golgi was not dispersed in the wt MN nor in the TgN ones. In transgenic mice that express $SOD1^{G93A}$ Golgi fragmentation was observed months before the onset of paralysis (Mourelatos et al., 1996). Concomitantly, there is increasing evidence that Golgi apparatus fragmentation occurs before

any signs of apoptosis (Gonatas et al., 2006). In the cultures studied the lack of altered Golgi morphology could be explained due to the fact that these cultures were performed using embryos E14, so the typical alterations might not be observed at this stage.

The presence of abnormal protein aggregates or inclusions has been described in several neurodegenerative diseases including ALS, where prominent, intracellular, cytoplasmic inclusions in MN and astrocytes were found (Bruijn et al., 2004). In SC dissociated cultures accumulation of SOD1 was detected in the axons and dendrites of the neurons from cells overexpressing human $SOD1^{G93A}$.

As the aggregates are normally seen in postnatal tissues (Wang et al., 2003; Turner et al., 2004b), SC extracts from adult wt and TgN rats were analysed for the presence of these protein aggregates. Increasing cycles of sonication to promote solubilization of protein aggregates were used. There have been reports that sonication might cause itself protein aggregates (Stathopulos et al., 2004), but when increasing cycles of sonication were tested $SOD1^{wt}$ did not aggregate, as expected, while $SOD1^{G93A}$, after the same procedure revealed high molecular mass forms that had a maximum at 10 cycles and then reached a steady state, meaning that with 10 cycles there was a maximum in the extraction of these forms.

One of the proposed mechanisms accounting for the toxicity of the SOD1 aggregates is the loss or sequestration of essential components, such as molecular chaperones or extracellular trophic signals required for neuron proper function. Several proteins have now been identified to be coimmunoprecipitated with mutant SOD1: copper chaperone for SOD1 (CCS), neuronal glutamate transporters (Goodall and Morrison, 2006), Hsp70, Hsp40, Hsp25, $\alpha\beta$-crystallin (Shinder et al., 2001; Wang et al., 2003) and Bcl-2, anti-apoptotic protein (Pasinelli et al., 2004), but we can speculate that more proteins could be entrapped in these aggregates.

Further studies to characterize and explain the protective/harmful role of these aggregates are needed.

3.6. Acknowledgements

We gratefully acknowledge Dr. Dolores Bonaparte, Instituto Gulbenkian de Ciência, Oeiras, Portugal, who kindly helped with the animal model.

Chapter 4

Mutant SOD1 does not cause intracellular accumulation of secretory glycoproteins in a cell model of Amyotrophic Lateral Sclerosis

Work presented in this chapter corresponds to the following article:

Gomes, C., Escrevente, C. and Costa, J. (2009) Mutant SOD1 does not cause intracellular accumulation of secretory glycoproteins in a cell model of Amyotrophic Lateral Sclerosis. *Submitted*.

4. Mutant SOD1 does not cause intracellular accumulation of secretory glycoproteins in a cell model of Amyotrophic Lateral Sclerosis

4.1. Abstract

Protein inclusions rich in mutant SOD1 have been found in tissues from patients with FALS. Here, the mouse motor neuron-like NSC-34 cell line, transiently transfected with human $SOD1^{G93A}$ fused to the enhanced green fluorescent protein tag, exhibited mutant SOD1 aggregates contrary to the cells overexpressing wild-type SOD1, thus mimicking the pathology. The aggregates were immunoreactive for ubiquitin, but not for the TAR DNA binding protein TDP-43 that was found in the nucleus. These characteristics mimicked the pathology of mutant SOD1 associated FALS.

To investigate if the disruption of the Golgi apparatus previously reported would cause impairment of the trafficking of secretory or membrane glycoproteins, co-overexpression of mutant SOD1 with secretory (β-trace protein and erythropoietin), or plasma membrane (L1 cell adhesion molecule) glycoproteins was performed. Decreased levels of all glycoproteins were observed for mutant transfected cells, which were not due to their intracellular accumulation. This decrease was observed irrespective of the presence of aggregates. Furthermore, no major changes in the *N*-glycosylation of β-trace protein were observed.

Aggregate formation correlated with mutant SOD1 detergent insolubility, and both parameters were significantly decreased in the presence of millimolar concentrations of the chemical chaperone trehalose.

These cells may be used to study mechanisms of pathogenesis associated with ALS and to test potential therapeutic compounds.

4.2. Introduction

Amyotrophic lateral sclerosis is a fatal neurodegenerative disease that results from selective dysfunction and death of upper and lower MN in the SC, brainstem and cortex, which leads to generalized weakness, muscular atrophy and death (Bruijn et al., 2004). Approximately 90-95% of ALS cases are sporadic, whereas 5-10% of cases have familial ALS, 20% of which presenting dominant mutations in the cytosolic antioxidant protein SOD1, that is ubiquitously expressed.

In vivo, protein inclusions rich in mutant SOD1 have been found in tissues from FALS patients, mutant SOD1 animals and cellular models (Johnston et al., 2000; Bruijn et al., 2004; Kabashi and Durham, 2006). Several possible toxicity mechanisms of the protein aggregates have been proposed including aberrant chemistry; loss of protein function through co-aggregation with the aggregates; depletion of protein folding chaperones; dysfunction of the proteasome, and inhibition of specific organelle function, including mitochondria and peroxisomes, through mutant aggregation onto or within those organelles (Bruijn et al., 2004; Julien and Kriz, 2006). In sporadic and familial ALS patients the most widely seen inclusions immunostain for ubiquitin, and similarly in mutant SOD1 mice protein inclusions mainly immunoreactive for SOD1 and ubiquitin have been detected (Bruijn et al., 1998; Watanabe et al., 2001; Cheroni et al., 2005). Ubiquitinated inclusions might represent the cellular defence against misfolded and/or abnormally modified proteins.

Small molecules have been used to prevent protein aggregation that is common in neurodegenerative diseases, such as ALS. For instance, trehalose, has been used successfully to reduce aggregate formation in a transgenic mouse model of Huntington disease (Tanaka et al., 2004), to inhibit the formation of fibrillar aggregates of insulin (Arora et al., 2004), to reduce Aβ aggregation and cytotoxicity related to Alzheimer's disease (Liu

et al., 2005), to impair prion protein aggregation and to protect prion-infected cells against oxidative damage (Beranger et al., 2008), and in cell models of oculopharyngeal muscular dystrophy (Davies et al., 2006). Trehalose is a natural disaccharide used in freeze-dried products to prevent protein denaturation. It is also one of many small molecules accumulating to high intracellular concentrations that are able to influence protein misfolding/folding and aggregation via direct protein-small molecule interactions and has, thus, been termed a chemical chaperone (Davies et al., 2006).

The NSC-34 cell line is a mouse neural hybrid cell between neuroblastoma and SC cells that has several characteristics of MN including expression of neurofilament proteins, generation of action potentials, acetylcholine synthesis, storage and release (Cashman et al., 1992). In previous work, we have shown that the overexpression of mutant $SOD1^{G93A}$ in NSC-34 cells caused increased fragmentation of the Golgi apparatus (Chapter 2, Gomes et al., 2008). Studies from other groups have shown that overexpression of α-synuclein in yeast, which constitutes a cell model of Parkinson´s disease, led to a block in ER to Golgi vesicular trafficking (Cooper et al., 2006).

In this work, we have reported that overexpressing mutant $SOD1^{G93A}$ in NSC-34 cells caused decreased levels of two concomitantly overexpressed secretory glycoproteins - β-trace protein (β-TP) and erythropoietin (EPO) in the supernatant, and of the cell adhesion molecule L1 at the cell surface. This decrease was not due to their intracellular accumulation. In addition, trehalose was found to decrease mutant SOD1 aggregation and insolubility *in vivo*.

4.3. Materials and methods

4.3.1. NSC-34 cell culture.

NSC-34 cells were grown in Dulbecco´s modified Eagle´s medium-high glucose (DMEM; Gibco), supplemented with 10% foetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen).

NSC-34 cells were stably and transiently transfected with the vector pEGFP-N1 (Clontech) encoding human $SOD1^{wt}$ linked at the *C*-terminus to the enhanced green fluorescent protein tag (ph$SOD1^{wt}$-EGFP) and mutant $SOD1^{G93A}$ with the same tag (ph$SOD1^{G93A}$-EGFP) or vector encoding only the tag (pEGFP) (Turner et al., 2005), using Lipofectamine™ 2000 (Invitrogen). For transient co-transfections of approximately $1.6x10^5$ cells, 1 µg of each plasmid DNA was used. For stable co-transfections of approximately $8x10^5$ cells the calcium phosphate precipitation method (Sousa et al., 2003) and 5 µg of each plasmid DNA, were used. Selection of stably transfected cells started 48 h after transfection and was carried out for two weeks with 0.5 mg/ml geneticin sulphate.

NSC-34 cells stably transfected with wild-type SOD1 or mutant $SOD1^{G93A}$, as previously described (Chapter 2, Gomes et al., 2008), were transiently transfected with the vector pEGFP-N1 (Clontech) encoding vesicular stomatitis virus G plasma membrane protein tagged with green fluorescent protein, VSVG-GFP using Lipofectamine™ 2000 (Invitrogen) (Toomre et al., 1999). Ten µg of plasmid DNA were used to transfect approximately $8x10^5$ cells.

Cells were grown in a humidified incubator at 37ºC under 5% CO_2.

4.3.2. Detergent insolubility and Western blot analysis.

For the supernatant analysis cells transiently or stably transfected were grown for 24 h in complete medium, then the medium was replaced by medium without FBS for 16 and 24 h. The collected supernatants were

centrifuged at 500x*g* for 5 min at 4ºC to remove cells and then they were precipitated with ethanol for further analysis.

For protein analysis stably transfected NSC-34 cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 1%(w/v) DOC, 1% (w/v) TX-100, 1% protease inhibitors (PI, Roche)) for 10 min on ice. Cell lysates were cleared by centrifugation at 10,000x*g* for 10 min at 4ºC. After ethanol precipitation protein samples were applied onto acrylamide SDS-PAGE gels.

For the analysis of detergent insolubility of protein NSC-34 cells transiently transfected with hSOD1^{wt}-EGFP, hSOD1^{G93A}-EGFP or the corresponding empty vector, were lysed for 10 min on ice, with 50 mM Tris-HCl, pH 7.5, containing 1% PI, and different compositions of salt and detergent as follows: i) 150 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) DOC, 1% (w/v) TX-100; ii) 0.1% (w/v) SDS, 1% (w/v) DOC, 1% (w/v) TX-100; iii) 150 mM NaCl, 1% (w/v) DOC, 1% (w/v) TX-100; iv) 150 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) TX-100; v) 150 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) DOC; vi) 150 mM NaCl, 0.1% (w/v) Nonidet P-40, 0.1 or 1% (w/v) SDS. Buffer iv) was found to reveal SOD1^{G93A} insolubility. Lysates were clarified by centrifugation at 15,800x*g* for 10 min. Resulting supernatants (soluble fraction) were precipitated with 10% (w/v) trichloroacetic acid, and the pellets were washed with acetone and applied onto SDS-PAGE gels. The pellets resulting from a 15,800x*g* centrifugation (insoluble fraction) were solubilized in the same buffer and sonicated for 15 sec. After ethanol precipitation protein samples were applied onto SDS-PAGE gels.

Western blot was performed on PVDF membranes that were blocked 1 h with PBS, pH 7.2 or TBS (for L1), pH 7.4, 0.1% (w/v) Tween-20, 5 or 1% (w/v) powdered milk (for SOD1). Then, they were incubated with the following primary antibodies: monoclonal anti-L1 L1-11A (dilution 1:2) (kind gift from Prof. Peter Altevogt), polyclonal anti-β-TP (dilution 1:5,000), polyclonal anti-EPO (dilution 1:5,000) (kind gifts from Dr.

Harald Conradt), polyclonal anti-SOD1 (dilution 1:15,000, Santa Cruz) and monoclonal anti-α-tubulin (dilution 1:5,000, Sigma). As secondary antibodies anti-rabbit (1:3,000) and anti-mouse (1:4,000) coupled to HRP were used. Proteins were detected by the ECL Plus method following supplier's protocol (GE Healthcare) or Immobilon™ Western Chemiluminescent HRP Substrate method following the supplier's protocol (Millipore). All incubations were performed at RT.

4.3.3. Protein deglycosylation.

For glycosidase digestion, supernatants from NSC-34 cells doubly transfected with β-TP and empty vector, wild-type or mutant SOD1, were incubated with endoglycosidase H (Endo H) (New England Biolabs) or peptide:*N*-glycosidase F (PNGase F) (Roche) according to the supplier's protocol. Briefly, cell supernatants were denatured in glycoprotein denaturing solution, at 100°C for 10 min, and incubated with deglycosylation enzyme (1 U of PNGase F or 1,000 U of Endo H) in the corresponding buffer. Hydrolysis was carried out at 37°C overnight. The positive control, the human recombinant β-TP, was deglycosylated with PNGase F, as described, for 3 h at 37ºC. After ethanol precipitation protein samples were applied onto 15% (v/v) acrylamide SDS-PAGE gels.

4.3.4. Fluorescence microscopy.

NSC-34 cells transiently transfected with phSOD1^{wt}-EGFP, phSOD1^{G93A}-EGFP or pEGFP were grown until 80% confluency, for fluorescence microscopy, in 24-well-plates with glass coverslips precoated with PDL (10 μg/ml, Sigma) and Matrigel (0.26 mg/ml, BD Biosciences). Immunofluorescence was as previously described (Chapter 2, Gomes et al., 2008). Coverslips were examined with Leica Confocal (SP2+AOBS) microscope. Primary antibodies were mouse anti-ubiquitin (1:50, Santa

Cruz) and rabbit anti-TDP-43 (1:200, Proteintech Europe Ltd). The secondary antibodies used were 1:500 anti-mouse IgG conjugated to Alexa Fluor 594 and 1:500 anti-rabbit IgG conjugated to Alexa Fluor 594.

For inclusion quantification, aggregate-positive cells were counted as a percentage of total EGFP-positive cell transfectants. If an EGFP-positive cell had one or many aggregates, the aggregate score was one. If an EGFP-positive cell did not have any aggregate, the aggregate score was zero.

4.3.5. Statistical analysis.

The relative level of wild-type and mutant protein in the insoluble pellet fraction of cellular extract was quantified by densitometry analysis of immunoblots with ImageJ software version 1.41 from two independent experiments in duplicate. The ratio between mutant and wild-type protein without the presence of trehalose was set to 1.

For comparison of the groups exposed to trehalose with the control group without the presence of any sugar, analysis of variance using one-way ANOVA method followed by Dunnett's *post hoc* comparison test was used.

4.4. Results and discussion

4.4.1. hSOD1^{G93A}-EGFP aggregates do not change endogenous TDP-43 localization

In order to mimic the mutant SOD1 aggregates present in ALS (Bruijn et al., 2004), NSC-34 cells were transiently transfected with phSOD1^{wt}-EGFP or phSOD1^{G93A}-EGFP, as reported by Turner et al. (2005). The expression of wild-type and mutant hSOD1 was analysed by fluorescence microscopy. The cells expressing SOD1^{wt} and the corresponding empty vector showed a widespread and diffuse fluorescence

(Figure 15A), whereas in the cells transfected with hSOD1^{G93A}-EGFP large and prominent cytoplasmic protein aggregates were observed (Figure 15B and E).

It is well described that the aggregates of mutant SOD1 observed in patients and rodent models of ALS stain positively for ubiquitin (Bruijn et al., 1997; Bendotti and Carri, 2004). Accordingly, analysis by immunofluorescence microscopy with an anti-ubiquitin antibody showed that mutant SOD1 aggregates from NSC-34 cells also stained intensively for ubiquitin (Figure 15B, C and D).

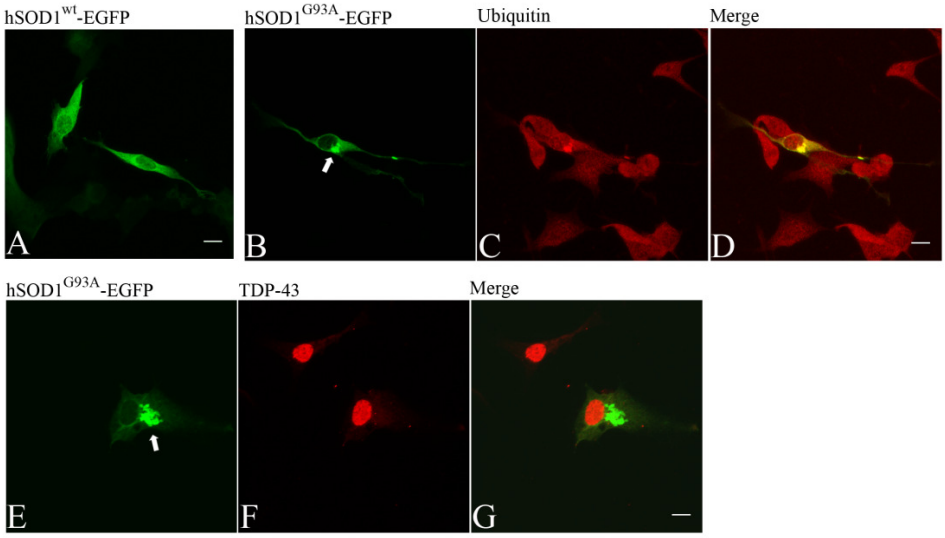

Figure 15: Immunofluorescence microscopy of mutant SOD1^{G93A}-EGFP aggregates in NSC-34 cells. NSC-34 cells transiently overexpressing hSOD1^{wt}-EGFP (A) or hSOD1^{G93A}-EGFP (B and E). Colocalization of mutant SOD1 aggregates with ubiquitin (C and D), and with TDP-43 (F and G). Arrows indicate mutant SOD1 aggregates. Bar: 10 µm.

Recently, aggregation of the TDP-43 protein has been widely described as a pathological hallmark of SALS and other neurodegenerative diseases, namely frontotemporal lateral dementia (Neumann et al., 2006).

In healthy conditions, TDP-43 is localized in the nucleus, whereas in the pathology phosphorylated protein and *C*-terminal fragments form aggregates found in the cytoplasm. The presence of TDP-43 aggregates in FALS cases is contradictory, with some groups describing the absence of TDP-43 aggregation in mutant SOD1 FALS pathology (Mackenzie et al., 2007), whereas others have reported their presence in patients (Robertson et al., 2007). Concerning animal models of FALS with SOD1 mutations, the evidence is also contradictory with some groups not finding TDP-43 pathology (Robertson et al., 2007; Turner et al., 2008b) contrary to others (Shan et al., 2009). Here we investigated if mutant SOD1 aggregation would be associated with endogenous TDP-43 aggregation in NSC-34 cell lines. TDP-43 was predominantly detected in the nucleus of the cells as in healthy conditions and no large aggregates were detected (Figure 15F). This shows the lack of TDP-43 pathology in a cell model of SOD1 FALS according to that previously described for patients (Mackenzie et al., 2007; Neumann, 2009).

4.4.2. Decreased levels of secretory and plasma membrane glycoproteins in NSC-34/hSOD1^{G93A} cells is not due to intracellular accumulation

Previously, we reported that a higher percentage of NSC-34/hSOD1^{G93A} cells had fragmented Golgi apparatus (Chapter 2, Gomes et al., 2008). To investigate the effects of mutant SOD1 aggregates on the levels of two secretory glycoproteins, β-TP (Hoffmann et al., 1994) and EPO (Nimtz et al., 1993), were transiently co-overexpressed with hSOD1^{wt}-EGFP or hSOD1^{G93A}-EGFP in NSC-34 cells. β-TP is a 24 kDa secretory glycoprotein, also known as prostaglandin D synthase, and is a major polypeptide constituent of human CSF (Hoffmann et al., 1993). EPO is an approximately 30 kDa secretory glycoprotein, which is a type I cytokine that

regulates human erythropoiesis. More recently EPO and its specific receptor have been found in human CNS and may have a neuroprotective role (Brines and Cerami, 2005). In ALS, despite several drawbacks it may be considered a potential therapeutic agent (Butsch and Cudkowicz, 2007).

For cells expressing β-TP/hSOD1^{G93A} a lower amount of β-TP was detected in the supernatant at 16 and 24 h when compared with the levels observed for the hSOD1^{wt} expressing cells (Figure 16A). This decrease was not due to intracellular accumulation of β-TP, as visualized in the cellular extract analysis. As loading control mouse SOD1 (mSOD1) was used, and the results indicated that all the lanes had comparable amounts of protein.

Similarly, the levels of EPO from the supernatants of EPO/hSOD1^{G93A} cells were lower when compared with the levels observed for the EPO/hSOD1^{wt} cells at 16 and 24 h (Figure 16B). Also in this case the decrease observed was not due to intracellular accumulation of EPO.

Since there was no intracellular accumulation of the two glycoproteins, the decreased levels observed in the supernatant could be due to impaired protein synthesis or to higher proteolytic degradation of the overexpressed protein in cells expressing mutant SOD1.

To investigate if the same reduction of β-TP levels in the supernatant would be observed in stably transfected cells, which did not exhibit SOD1 aggregation (data not shown), NSC-34 cells were double stably transfected with plasmids coding β-TP and hSOD1^{wt}-EGFP or hSOD1^{G93A}-EGFP and selected with 0.5 mg/ml geneticin sulphate. For this study, clones expressing similar amounts of wt and mutant SOD1 were selected. Similar to that observed for the transiently transfected cells, the stably transfected cells expressing mutant SOD1 had a lower amount of β-TP in the supernatant at 16 and 24 h (Figure 16C), which was not due to intracellular accumulation (data not shown).

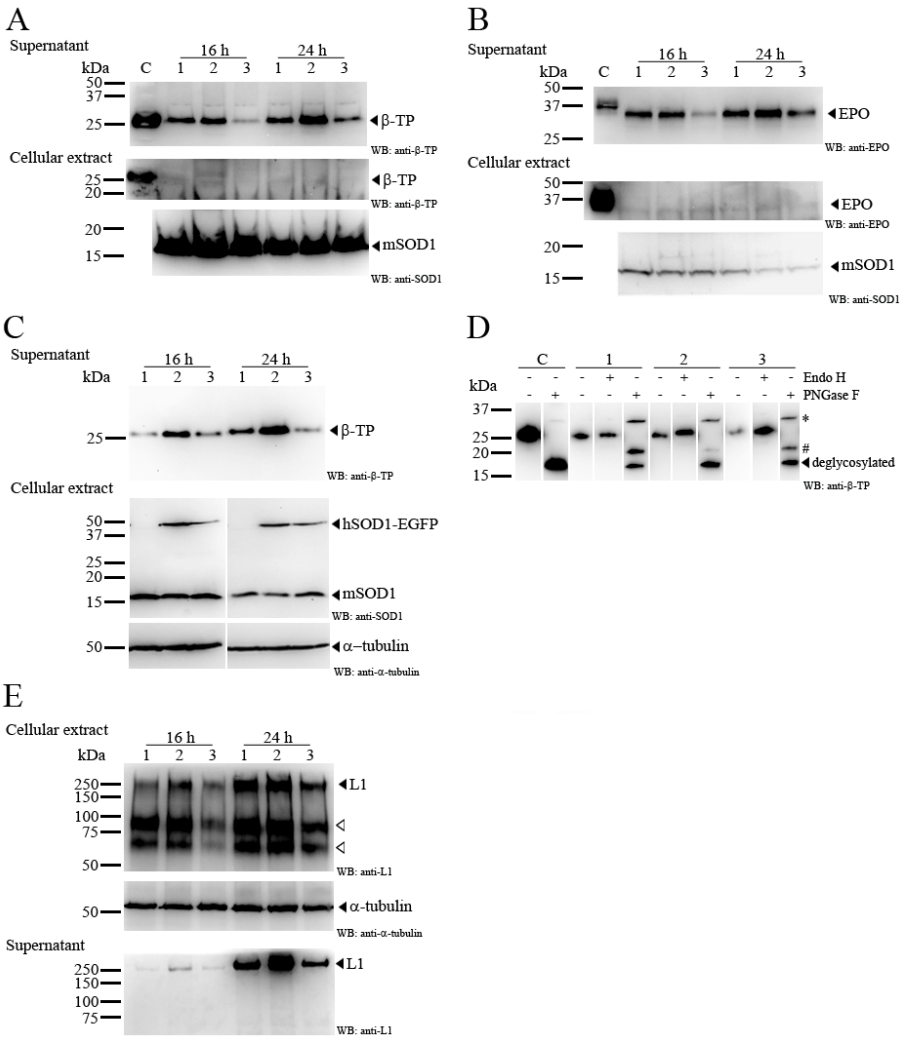

Figure 16: Western blot analysis of β-TP, EPO and L1 glycoproteins from the supernatants and cellular extracts of NSC-34 cells overexpressing mutant SOD1. (A) β-TP from the supernatants and the corresponding cell extracts (transient overexpression); C: 10 ng β–TP from human hemofiltrate. (B) EPO from the supernatants and the corresponding cell extracts (transient overexpression); C: 10 ng recombinant human EPO from CHO cells. (C) β-TP from the supernatants and the corresponding cell extracts (stable overexpression). (D) Deglycosylation with Endo H and PNGase F of β-TP obtained in (C). Arrowhead indicates deglycosylated β-TP. *Dimer of deglycosylated β-TP. #Mono-glycosylated form of β-TP. (E) L1 from cell extracts and shedded to the supernatant. Open arrowheads

indicate cellular proteolytic products of L1. 1- empty vector pEGFP; 2- hSOD1^{wt}-EGFP; 3- hSOD1^{G93A}-EGFP. These results are representative immunoblots from at least three different experiments.

Glycosylation is one of the most representative post-translational modifications, which occurs in the ER and Golgi apparatus concomitantly to the trafficking of glycoproteins. To investigate if there were changes in *N*-glycosylation, overexpressed β-TP, which has two potential *N*-glycosylation sites, was deglycosylated with endoglycosidase H (removes high-mannose and hybrid-type *N*-glycans through cleavage between the two *N*-acetylglucosamine residues of the chitobiose core) and peptide *N*-glycosidase F (removes high-mannose, hybrid- and complex-type *N*-glycans through cleavage between proximal *N*-acetylglucosamine and asparagine residues). β-TP was found to be resistant to Endo H and sensitive to PNGase F, which indicated that it contained complex-type *N*-glycans (Figure 16D). As the deglycosylation profile was similar for β-TP from wt or mutant SOD1 overexpressing cells, the results suggested that no major changes in *N*-glycosylation occurred in the secreted protein.

We also co-expressed the plasma membrane glycoprotein L1 with wt or mutant SOD1. L1 is a glycoprotein of 200-220 kDa that is predominantly localized on the plasma membrane, and is involved in the development of the nervous system (Maness and Schachner, 2007; Nishimune et al., 2005; Gouveia et al., 2008). L1 is proteolytically cleaved by the metalloprotease ADAM10 at the plasma membrane, and the soluble ectodomain is shedded to the supernatant (Escrevente et al., 2008). Similarly to the secretory proteins, a lower amount of L1 in the cellular extract and shedded to the supernatant was also observed in mutant transfected cells (Figure 16E).

One of the mechanisms of cellular proteolysis involves the conjugation of target proteins with ubiquitin and degradation by the

proteasome. Here, cellular L1 was not ubiquitinated, as observed that after L1 immunoprecipitation no ubiquitin was found associated with this protein by Western blot (data not shown). This result suggested that the decreased levels were not due to degradation by the proteasome.

Studies from other groups have shown that $SOD1^{G93A}$ overexpressed in NSC-34 cells induced ER stress and specifically impaired ER-associated degradation (Nishitoh et al., 2008). Unfolded glycoproteins that accumulate in the ER usually undergo the ER-associated degradation pathway that consists of several steps involving translocation to the cytosol, deglycosylation by a peptide:*N*-glycanase, poly-ubiquitination and degradation by the proteasome (Meusser et al., 2005). As the secretory and membrane glycoproteins tested here in transient or stable transfection did not accumulate in the ER, and their deglycosylated form was not detected in the cellular extracts, the results strongly suggested that the decreased levels observed were not due to accelerated degradation via this pathway. Instead, it is more probable that they were due to attenuated protein synthesis. Supporting this conclusion was the observation by several authors that ER stress at its initial stage causes a reduction of global protein synthesis (Harding et al., 2002). Therefore, it is plausible that mutant SOD1 that triggered ER stress in the NSC-34 cells (Nishitoh et al., 2008), subsequently caused reduced levels of synthesis of overexpressed β-TP, EPO and L1. As there is evidence that ER stress causes autophagy (Hoyer-Hansen and Jaattela, 2007) it is also possible that the decreased levels of these glycoproteins were due to increased autophagy. Further studies will be required to further elucidate this matter.

This effect was not due to the aggregates as it was also observed for cells stably overexpressing mutant SOD1, which did not exhibit visible aggregates. This agrees with the previous hypothesis that in neurodegenerative diseases intermediate size oligomers are the toxic species as proposed, for example, for mutant demetallated SOD1 in FALS

(Banci et al., 2008) or for α-synuclein in Parkinson's disease (Gosavi et al., 2002; Outeiro et al., 2008).

To further address the effects of the Golgi apparatus fragmentation (Chapter 2, Gomes et al., 2008) on protein trafficking and processing, the temperature sensitive mutant of vesicular stomatitis virus G plasma membrane protein tagged with GFP, VSVG-GFP, was transiently co-expressed (Gallione and Rose, 1985) in stable NSC-34/hSOD1^{G93A} cells (data not shown). This mutant has been used to dissect the dynamics of the secretory pathway, as its transport can be synchronized in a process dependent of the temperature. At the non-permissive temperature of 39-40ºC, the VSVG reversibly misfolds and accumulates in the ER, at 19.5ºC the exit from the *trans*-Golgi network is blocked and VSVG accumulates, but at 32ºC it folds correctly, exits the *trans*-Golgi network and it reaches the plasma membrane (Toomre et al., 1999). In NSC-34 cells the non-permissive temperature was found to be 42ºC, as VSVG retention in the ER was observed, as monitored by sensitivity to deglycosylation with Endo H. However, there were no detectable differences in the processing of VSVG between cells expressing mutant SOD1^{G93A} or wild-type protein.

4.4.3. Trehalose diminishes mutant SOD1 aggregates and detergent insolubility

To determine whether trehalose would reduce mutant protein aggregates observed in the NSC-34/hSOD1^{G93A} cells (Figure 15), these cells were incubated with 1 or 10 mM trehalose for 72 h. The decrease in the percentage of cells containing aggregated SOD1 was statistically significant in the presence of 10 mM trehalose (Figure 17A). When the cells were exposed to glucose or sucrose as controls, no effect was observed on SOD1 aggregation. This supports the idea that the reduction in the number of cells with aggregates of mutant SOD1 was a trehalose specific effect.

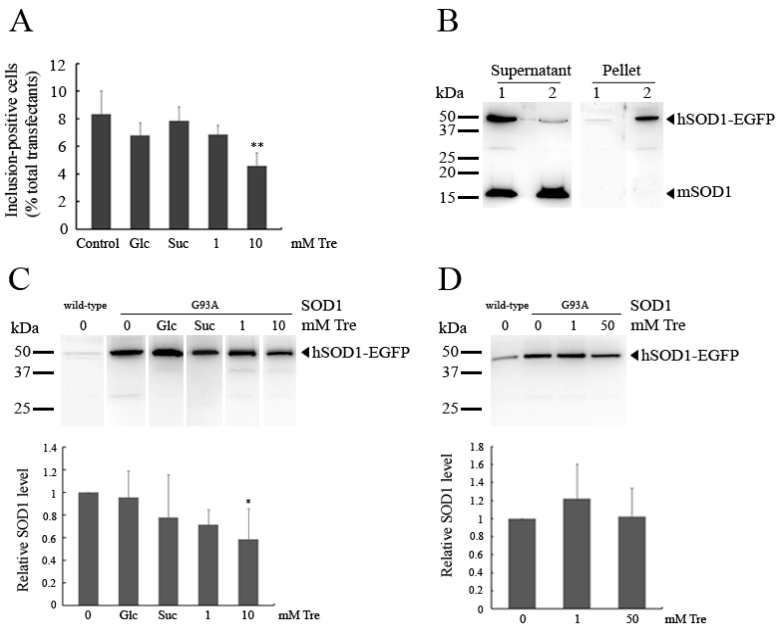

Figure 17: Effect of trehalose on SOD1^{G93A}-EGFP aggregation and insolubility in NSC-34 cells. (A) Ratio between aggregate-containing cells and total transfected cells. Cells were treated with trehalose (Tre), 50 µM glucose (Glc) or sucrose (Suc) for 72 h. (B) Western blot analysis of hSOD1 from supernatants and pellets obtained from cell lysates of NSC-34/hSOD1^{wt}-EGFP (1) and NSC-34/hSOD1^{G93A}-EGFP (2) cells. (C) Western blot analysis of hSOD1^{G93A} from the pellets of cell lysates obtained from cells previously treated with Tre, 50 µM Glc or sucrose Suc for 72 h. (D) Western blot analysis of hSOD1^{G93A} from the pellets of cell lysates incubated with Tre for 48 h. Results are from two different experiments in duplicate. The relative level of insoluble mutant SOD1^{G93A} *vs.* insoluble wtSOD1 was quantified by densitometry (for C and D). Data are presented as the means±S.D. *, p<0.05. **, p<0.01 (Graph Pad Prism4 software).

The effect of trehalose was also monitored using a different assay for protein aggregation: detergent insolubility that is usually found for aggregated proteins including SOD1^{G93A} (Turner et al., 2005). Here, different buffers (see Material and methods) were tested and it was found that the buffer 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 1%

(w/v) TX-100 and 1% PI, allowed the fractionation between soluble and insoluble SOD1^{G93A}. Mutant SOD1 was found to be enriched in the pellet fraction whereas wt SOD1 was predominantly found in the soluble fraction (Figure 17B). When the cells were incubated with 10 mM trehalose there was a statistically significant decrease in the amount of insoluble mutant SOD1 (Figure 17C). When the cells were exposed to either glucose or sucrose no effect on the mutant protein solubility was observed. This result supports the beneficial role of trehalose in decreasing protein detergent insolubility.

To investigate if trehalose could solubilize mutant SOD1 after the aggregates were formed, cellular extracts 24 and 48 h after transient transfection were incubated with 1 or 50 mM trehalose overnight, for 24 or 48 h. Cellular extracts incubated for 48 h with trehalose, 48 h after transfection, displayed a similar amount of insoluble mutant protein (Figure 17D). These results together indicated that trehalose prevented *de novo* formation of aggregates but did not promote the resolubilization of insoluble protein. This is in agreement with that previously reported for PrP^{Sc} in prion-infected cells (Beranger et al., 2008) and suggested for poly-glutamine mediated aggregation (Tanaka et al., 2004). Trehalose probably decreased mutant SOD1 insolubility due to its properties as a chemical chaperone (Davies et al., 2006).

As previously referred, the levels of β–TP were decreased in the supernatant of cells expressing β–TP/SOD1^{G93A}-EGFP, additionally trehalose diminished protein aggregates and insolubility in NSC-34/hSOD1^{G93A}–EGFP cells. In order to address if trehalose would be beneficial in restoring the levels of secreted β–TP in the cells expressing mutant SOD1^{G93A}-EGFP, stable NSC-34/β–TP/SOD1^{G93A}-EGFP cells were incubated with 10 or 50 mM trehalose for 24 h and the levels of secreted β–TP were analysed. However, upon trehalose exposure the levels of

secreted β–TP were not restored in cells expressing mutant SOD1 (data not shown).

Other small-molecular weight compounds, known as compatible solutes or osmolytes from thermophiles have an auxiliary function in improving protein stability *in vitro* (Santos and da Costa, 2002). Particularly, α-D-mannosylglycerate has been found to suppress Alzheimer's disease Aβ aggregation and neurotoxicity (Ryu et al., 2008). In order to evaluate the effect of compatible solutes from thermophiles, on the reduction of mutant SOD1 insolubility, the cells overexpressing $SOD1^{G93A}$-EGFP were incubated with 0.1, 1.0, 10 or 25 mM mannosylglycerate, with 0.1, 1, 10 or 50 mM mannosylglyceramide, with 0.1 or 1 mM diglycerol phosphate, with 0.1 or 1 mM mannosyl-lactate or with 0.1 or 1 mM di-myo-inositol-1,1'-phosphate for 48 h. No significant decrease in the amount of insoluble mutant SOD1 upon exposure to the different compounds was observed (data not shown).

To investigate if these compounds could aid in the solubilization of mutant SOD1 after the aggregates were formed, cellular extracts 48 h after transient transfection were incubated with 1 mM mannosylglycerate, mannosylglyceramide, diglycerol phosphate, mannosyl-lactate or di-myo-inositol-1,1'-phosphate overnight. No decrease in the amount of detergent insoluble mutant protein was found (data not shown), similarly to that observed with trehalose.

4.5. Conclusions

In this work, a lack of TDP-43 pathology in the cell model of SOD1 FALS was found, according to that previously described for patients. Mutant SOD1 overexpression caused decreased levels of secretory and plasma membrane glycoproteins, which was not due to intracellular

accumulation. It is possible that mutant SOD1 by triggering ER stress caused reduced synthesis of those glycoproteins. It was also found that trehalose decreased *de novo* formation of aggregates in the FALS cell model.

4.6. Acknowledgements

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Chapter 5

Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis

Work presented in this chapter corresponds to the following article:

Gomes, C., Keller, S., Altevogt, P., and Costa, J. (2007) Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis. Neurosci Lett **428**(1): 43-46.

5. Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis

5.1. Abstract

A familial form of the neurodegenerative disease ALS, is caused by dominant mutations in the cytosolic Cu,Zn superoxide dismutase (SOD1). There has been evidence for secretion of SOD1, by an unknown mechanism. In this work stable mouse motor neuron-like NSC-34 cells overexpressing hSOD1^{wt} (NSC-34/hSOD1^{wt}) and mutant hSOD1^{G93A} (NSC-34/hSOD1^{G93A}) have been used as an ALS cell model. SOD1 was found to be secreted in association with a membrane fraction that pelleted at 100,000x*g*. Sucrose density gradient separation of this fraction showed that wt and mutant SOD1 were found between 0.5 and 1.16 M sucrose and co-localized with the exosomal marker CD9. Therefore, SOD1 secretion occurred via exosomes. p115, a cytosolic and Golgi apparatus protein involved in vesicle tethering, was also found in exosomes, contrary to the ER protein calnexin. SOD1 secretion mediated by exosomes could explain cell-to-cell transfer of mutant toxicity.

5.2. Introduction

Amyotrophic lateral sclerosis is a fatal neurodegenerative disease that results from selective dysfunction and death of upper and lower MN in the SC, brainstem and cortex (Bruijn et al., 2004). In 20% of familial ALS patients population more than 100 mutations in the protein SOD1, that is ubiquitously expressed, have been identified.

There has been evidence for secretion of cytosolic SOD1, by an unknown mechanism (Mondola et al., 1996; Mondola et al., 1998; Mondola et al., 2003). As SOD1 is a cytoplasmic protein that lacks a signal peptide there must be an alternative mechanism for the extracellular export of this protein.

More recently, Turner et al. (2005), have shown that extracellular secretion of mutant SOD1 is impaired in NSC-34 cells modelling familial ALS. Urushitani et al. (2006) reported that SOD1 mutants linked to ALS can be secreted by interaction with chromagranins. The authors also showed that extracellular SOD1 mutants can trigger microgliosis and death of MN in culture, suggesting a pathogenic mechanism based on toxicity of secreted SOD1 mutant proteins (Urushitani et al., 2006). The importance of the MN environment has been emphasized, and it is now believed that toxicity to MN derives from damage developed within cell types beyond the MN. However, the mechanism by which the toxicity of mutant SOD1 may be transferred from one cell to another is still unclear (Clement et al., 2003).

Concomitant to these reports, there is an increasing body of evidence that different cell types, including neurons (Keller et al., 2006; Faure et al., 2006) are capable of producing and releasing microvesicles called exosomes. Exosomes are small lipid membrane microvesicles (30-100 nm diameter) that are formed by fusion of multivesicular bodies with plasma membrane and the subsequent release of their cargo. Exosomes are biologically active entities which are important for a variety of pathways,

and one of the biological functions of exosomal release is the secretion of membrane proteins meant to be discarded, or to be passed onto other cells (Keller et al., 2006; van Niel et al., 2006). In neurodegenerative diseases, such as Alzheimer's disease, β-amyloid peptides, which are intracellularly generated, were shown to be released to the extracellular space, in association with exosomes (Rajendran et al., 2006). Similarly, in prion disease, infectious prion protein mediates its intercellular transfer via exosomes, bypassing cell-cell contact in the dissemination of prions (Fevrier et al., 2005).

In the present work human and endogenous mouse SOD1 were found to be secreted from NSC-34 cells via exosomes. These microvesicles might constitute a way of cell-to-cell communication and transfer of mutant SOD1 toxicity.

5.3. Materials and methods

5.3.1. Protein analysis.

NSC-34/hSOD1^{wt} and NSC-34/hSOD1^{G93A} cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 1% (w/v) DOC, 1% (w/v) TX-100, 1% PI (Roche)) for 10 min on ice. Cell lysates were cleared by centrifugation at 10,000x*g* for 10 min at 4ºC. After ethanol precipitation protein samples were applied onto 15% (v/v) acrylamide SDS-PAGE gels.

5.3.2. Isolation of membrane vesicles.

For supernatant analysis, NSC-34/hSOD1^{wt} and NSC-34/hSOD1^{G93A} cells were grown for 4 days in complete medium. A day before exosome preparation, culture medium was replaced with serum-free medium. Culture supernatants were collected and sequentially centrifuged at 500x*g* for 10 min (to remove cells), 10,000x*g* for 20 min (to remove cellular debris) and

100,000x*g* for 2 h (to pellet membrane vesicles). Vesicles were directly solubilized in sample buffer, centrifuged at 150,000x*g* for 2 h or further processed for gradient centrifugation.

5.3.3. Sucrose density gradient fractionation.

Vesicles resuspended in 0.25 M sucrose were loaded on top of a step gradient comprising layers of 2, 1.3, 1.16, 0.8, 0.5 and 0.25 M sucrose. The gradients were centrifuged at 100,000x*g* for 2.5 h using a Beckman SW 41 Ti rotor. Twelve 1 ml fractions were collected from the top of the gradient and precipitated with chloroform/methanol (1:4 (v/v)). Samples were analysed by SDS-PAGE and Western blot as described below.

5.3.4. Carboxyfluorescein diacetate, succinimidyl ester labelling of exosomes.

NSC-34/hSOD1^{G93A} exosomes from 100,000x*g* centrifugation were incubated in 0.1% (w/v) BSA/PBS with 7.5 μM carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes), at 37ºC, for 30 min. Medium with 10% (v/v) FBS depleted of exosomes, by centrifugation overnight at 100,000x*g*, 12ºC, was then added and it was centrifuged overnight at 100,000x*g*, 12ºC.

5.3.5 Immunofluorescence microscopy of CFSE labeled exosomes.

Ten μg of CFSE labeled exosomes were added to the NSC-34 cells overnight and these were analyzed by immunofluorescence microscopy. Briefly, cells grown on coverslips were washed twice in 150 mM NaCl, 500 mM CH_3COOH, 10 min, 4ºC. Then, they were fixed with 4% (w/v) paraformaldehyde, permeabilized with 0.1% (w/v) TX-100 and blocked with 1% (w/v) BSA in PBS. Cells were probed with the monoclonal antibody anti-α-tubulin (1:2,000, Sigma) and with the polyclonal anti-caveolin-1 (1:50, Santa Cruz). Secondary antibodies used were: 1:500 anti-mouse IgG

conjugated to Alexa Fluor 594 and 1:500 anti-rabbit IgG conjugated to Alexa Fluor 594.

5.3.6. Western blot analysis.

Western blot was performed on PVDF membranes that were blocked 1 h with PBS, pH 7.2, 0.1% (w/v) Tween-20, 5% (w/v) milk. They were incubated with the following primary antibodies: polyclonal anti-SOD1 (dilution 1:10,000, Santa Cruz); anti-CD9 (dilution 1:1,000, Santa Cruz), anti-calnexin (dilution 1:15,000) (kind gift from Prof. Helenius) and anti-p115 (dilution 1:1,000, BD Biosciences). As secondary antibodies anti-rabbit (1:4,000) and anti-rat (1:5,000) coupled to horseradish peroxidase were used. Proteins were detected by the ECL Plus method following supplier's protocol (Amersham Biosciences). All incubations were performed at RT.

5.4. Results

To investigate if NSC-34/hSOD1^{wt} and NSC-34/hSOD1^{G93A} cells were capable of secreting wt and mutant hSOD1, cell culture supernatants were analysed by Western blot after sequential centrifugation at 500 and 10,000x*g* to remove dead cells and cell debris (Figure 18). Overexpressed hSOD1 (~22 kDa) as well as endogenous mouse SOD1 (mSOD1, 16 kDa) were detected. In addition, a degradation product was found in the cell debris fraction. Post-10,000x*g* supernatants were further centrifuged at 100,000x*g* to collect a fraction enriched in exosomes (Gutwein et al., 2005). There was hSOD1^{wt} and hSOD1^{G93A} present in this fraction, which indicated that the cells secreted SOD1 to the extracellular medium associated to membrane vesicles. It appeared that the ratio between hSOD1 and endogenous mSOD1 was lower for the mutant than for the wild-type form of hSOD1.

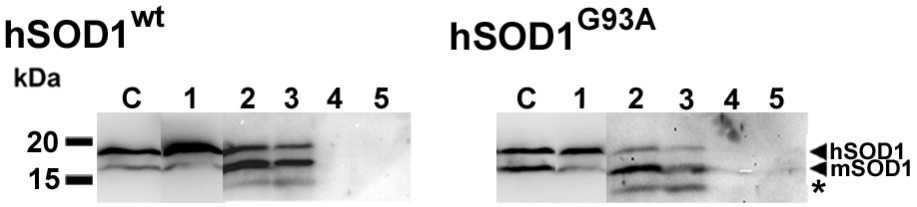

Figure 18: Western blot analysis of SOD1 protein from the supernatant of NSC-34/hSOD1^{wt} and NSC-34/hSOD1^{G93A} cells. C: cellular extract (2x10^5 cells). Cell supernatants were sequentially centrifuged at 500x*g*, 10,000x*g*, 100,000x*g* (2.5x10^7 cells) and 150,000x*g* (2.5x10^7 cells) and the corresponding pellets (lanes 1-4, respectively) analysed; 5: post-150,000x*g* supernatant (2x10^6 cells). hSOD1: human SOD1; mSOD1: mouse SOD1. *Degradation product. These results are representative immunoblots from at least three different experiments.

The secreted hSOD1 was totally present in the 100,000x*g* fraction, as there was no enzyme associated with the pellet of a subsequent centrifugation at 150,000x*g*, nor in the corresponding supernatant. As exosomes collected from the 100,000xg pellet may be contaminated with apoptotic blebs (Gutwein et al., 2005), this pellet was further fractionated in a sucrose density gradient that ranged between 0.25 and 2.00 M. CD9 was used as a marker protein to identify exosome-containing fractions (Keller et al., 2006), and revealed that exosomes were recovered in fractions between 0.50 and 1.16 M sucrose, corresponding to a density between 1.06 and 1.15 g/ml (Figure 19). The density of these vesicles is compatible with those described for exosomes purified from other cells, such as ovarian carcinoma cell lines (Gutwein et al., 2005), dendritic cells (Thery et al., 2001), cortical neurons (Faure et al., 2006), and N2a cells (Rajendran et al., 2006).

Endogenous mouse and human SOD1 were found in the exosomes as revealed for its presence in the fractions corresponding to exosomes by

comparison with the exosomal marker CD9 (Figure 19, lanes 4-7). A peak corresponding to a molarity of 1.16 M was observed for both mouse and human SOD1 (Figure 19, lane 6). These data demonstrated that the medium from NSC-34 cells had exosomes containing SOD1.

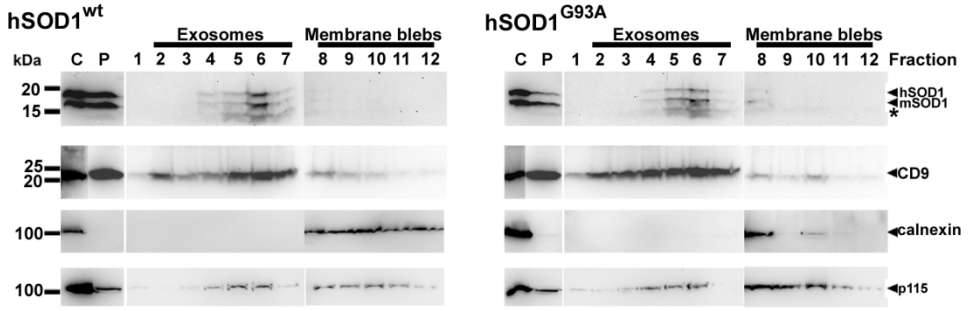

Figure 19: Western blot analysis of SOD1 protein after sucrose gradient fractionation. Media from NSC-34/hSOD1^{wt} and NSC-34/hSOD1^{G93A} cells. C: cellular extract (2x10^5 cells); P: 100,000x*g* pellet (2.5x10^7 cells) (corresponding to exosomes and membrane blebs); 1–12: fractions resulting from gradient sucrose fractionation (1.25x10^8 cells). hSOD1: human SOD1; mSOD1: mouse SOD1. *Degradation product. These results are representative immunoblots from at least three different experiments.

As negative control, calnexin, an ER resident protein, was used because it is known not to be included in exosomes, as exosomes have an endosomal origin and typically do not contain proteins of the nucleus, mitochondria, or ER, but all exosomal proteins are typically found in the cell cytosol or at the plasma membrane (Keller et al., 2006). Indeed calnexin was not found in the exosome-containing fractions. However, it was associated with apoptotic membrane blebs. p115 a cytosolic Golgi apparatus vesicle tethering protein involved in the fusion of transport vesicles with acceptor compartments, which allows maintenance of Golgi apparatus architecture (Seemann et al., 2000), was also found associated with exosomes and apoptotic membrane blebs (Figure 19).

In order to evaluate if the NSC-34 cells would interact with and internalize the produced exosomes, NSC-34 cells were exposed to CFSE labeled exosomes (Keller et al., 2009) produced from NSC-34/hSOD1^{wt} or NSC-34/hSOD1^{G3A} cells. CFSE passively diffuses into cells. It is colorless and nonfluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well retained (Bronner-Fraser, 1985). Upon overnight exposure of the cells to 10 µg exosomes, a major localization of the exosomes at the membrane of the cells was observed (Figure 20A). In addition, some colocalization with the intracellular markers α-tubulin and caveolin-1 was also detected, which showed that part of the exosomes were internalized by the cells (Figure 20B).

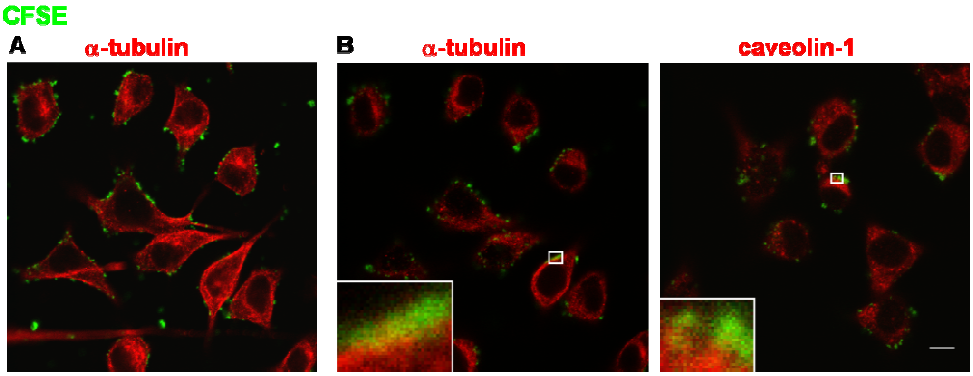

Figure 20: NSC-34 cells exposed to labeled exosomes analyzed by confocal microscopy. (A) Membrane distribution of the exosomes at the surface of NSC-34 cells. (B) Colocalization of exosomes with α-tubulin and caveolin-1, in NSC-34 cells. Single optical sections with 10 fold magnification insets. Bar: 10 µm.

5.5. Discussion

A familial form of the neurodegenerative disease ALS is caused by dominant mutations in the ubiquitously expressed cytosolic SOD1. In the present work, we have shown that wild-type and mutant SOD1 were present in the supernatant medium from NSC-34 cells stably expressing $hSOD1^{wt/G93A}$, and that this protein was associated with exosomes.

Observations from several authors have also identified SOD1 present in the extracellular medium from different cell types (Mondola et al., 1996; Mondola et al., 2003; Turner et al., 2005; Urushitani et al., 2006). It has also been reported that both wild-type and mutant SOD1 species are detected in CSF of both transgenic rats carrying human SOD1 (Turner et al., 2005) and ALS patients with the SOD1 mutation (Jacobsson et al., 2001). Nevertheless, the present work is the first report that describes SOD1 secretion in NSC-34 cells associated with exosomes.

The presence of $hSOD1^{wt}$ in the exosomes and hence in the extracellular medium of NSC-34 cells could be of biological relevance by protecting the cells against the physiological production of reactive oxygen intermediates present outside the plasma membrane surface. This is supported by studies using chimeric mice with mixed populations of cells expressing either endogenous or transgenic mutant $SOD1^{G93A}$ or $SOD1^{G37R}$, where MN expressing transgenic SOD1 failed to degenerate if they were adjacent to large numbers of supporting cells (such as astrocytes and glia) without the mutant protein (Clement et al., 2003). These findings sustain the protective role of a wild-type environment in the ALS pathology.

Mutant hSOD1, although present in the exosomes, seemed not to be incorporated to the same extent as $hSOD1^{wt}$, and, consequently, might be entrapped inside the cell possibly contributing to deleterious effects. Turner et al. (2005) have also observed that SOD1 was less abundant in the supernatant media from NSC-34/$hSOD1^{G93A}$ cells when compared with

NSC-34/hSOD1^{wt} cells. There has been evidence that the toxicity of SOD1 mutants is non-cell autonomous, that is, it requires mutant damage not just within MN but also to non-neuronal cells (Pramatarova et al., 2001; Lino et al., 2002; Clement et al., 2003; Monk and Shaw, 2006). Concomitantly, in ALS there has been growing evidence of a role for inflammation, and the activation of microglia (Monk and Shaw, 2006). The presence of mutant SOD1, even though to a smaller extent, in the extracellular media via exosomes could be acting as an inflammation trigger. Urushitani et al. (2006) have shown that extracellular SOD1 mutants cause microgliosis and neuron death, while wild-type SOD1 caused suppression of microglial activation in BV2 cells. The same authors (Urushitani et al., 2007) have observed therapeutic effects of immunization with mutant SOD1 in mice models of ALS with late onset and moderate levels of mutant SOD1. Mondola et al. (2004) reported that extracellular SOD1 specifically interacts in a dose-dependent manner with the cell surface membrane of SK-N-BE cells and modulates intracellular calcium-dependent signalling pathways.

Based on the results presented here the exosomes produced by NSC-34 cells can interact with the same cells, and possibly mutant SOD1 can pass onto neighbouring cells, transferring its toxicity. This result supports the notion of a role for extracellular mutant SOD1 in the pathogenesis of ALS. Exosomes could, in this sense, represent an additional means of communication between cells. Besides, the capacity of exosomes to fuse with acceptor cells like neurons, astrocytes or microglia, has to be further investigated, so that one can be sure that they can act as a way to transfer cytosolic proteins like SOD1 between different cells (Thery et al., 2001).

The role of the exosomes in neurodegenerative diseases has still to be further explored, but in Alzheimer's disease β-amyloid peptides, that are intracellularly generated, were shown to be released to the extracellular space, where they accumulate, in association with exosomes (Rajendran et

al., 2006). These vesicles can act as a way to mediate intercellular transfer of Aβ. Similarly, infectious prion protein mediates its intercellular transfer via exosomes, bypassing cell-cell contact in the dissemination of prions. Indeed, non-infected cells when incubated in the presence of cell culture media from infected cells became infected, showing that non-infected cells uptake prion protein associated with exosomes (Fevrier et al., 2004). Furthermore, exosomes originating from either non-neuronal or neuronal cells can transmit infection between cell lines and also produce clinical prion disease when inoculated into recipient mice (Vella et al., 2007).

This is, as far as we know, the first time that SOD1 was observed associated with exosomes in the NSC-34 ALS cell model. This evidence suggests a new cellular mechanism contributing for dissemination of mutant SOD1 toxicity in ALS pathogenesis.

5.6. Acknowledgements

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Chapter 6

General discussion and conclusions

6. General discussion and conclusions

6.1. General discussion and perspectives

ALS is a complex disease with multiple causes, and current hypotheses for the underlying biology represent noncompeting mechanisms that are likely to converge in various unfortunate patterns to mediate selective MN degeneration (Figure 2, Chapter 1).

Since the identification of autosomal-dominant mutations in the SOD1 gene in ALS patients, intensive genetic and molecular research has been conducted, and several cellular and animal models of FALS have been generated. Nevertheless, open questions regarding pathological mechanisms remain unanswered, and no effective treatment has emerged (Pasinelli and Brown, 2006).

Given the clinical similarity between both familial and sporadic cases of ALS, putative pathogenic mechanisms mediated by mutant SOD1 may be also relevant in understanding SALS (Vucic and Kiernan, 2009).

In this study, the importance of mutant SOD1 overexpression in mammalian cells as a model to study pathological characteristics of FALS, such as fragmentation of the Golgi apparatus, mutant protein aggregation, alterations in the levels of specific glycoproteins and SOD1 secretion via exosomes was shown. Additionally, these models were used to test possible therapeutic agents.

6.1.1. Cellular models overexpressing mutant SOD1 to study ALS

To better understand the biology of ALS, over the last decade, researchers have come to rely on various laboratory-based models. Models of disease initially consisted of pure MN cultured *in vitro*, and slices of the SC. Purified neuronal cultures allow analysis of many aspects of neuronal function without the influence of neighboring neurons and/or glia. However,

MN have proved exceptionally difficult to isolate, they have short term survival potential, and dissociation methods are restricted to embryonic tissues. Although the search effort for a suitable alternative to primary MN, the NSC-34 cells produced by Cashman et al. (1992) have emerged as the most promising alternative to primary MN (Muyderman et al., 2009).

As referred throughout this Thesis several cellular models have been used to study the pathological mechanisms associated with ALS. In fact, non-neuronal cells, such as HEK (human embryonic kidney) cells overexpressing wild-type or mutant SOD1, were used to demonstrate that wild-type SOD1 has direct effects on the aggregation of mutant SOD1 with a tendency to slow the process (Prudencio et al., 2009b), to study the interaction between wild-type and mutant SOD1 (Witan et al., 2008) and also to elucidate the role of disulfide crosslink in the aggregation of mutant SOD1 (Karch and Borchelt, 2008). Furthermore, COS-7 cells (simian fibroblasts) overexpressing mutant SOD1 showed protein aggregates formation in association with the ER, induced ER stress (Tobisawa et al., 2003) and the unfolded protein response (Atkin et al., 2006). They have also been used to study the secretion of misfolded mutant SOD1 by interaction with chromogranins (Urushitani et al., 2006). NIH3T3 (mouse fibroblasts) cells were used to show that proteasome inhibition enhanced the secretion of mutant SOD1 with or without chromogranins (Urushitani et al., 2006) and that mutant SOD1 co-immunoprecipitated with Hsp70, Hsp40 and αβ-crystallin (Shinder et al., 2001).

In addition, neuronal cells such as, Neuro2a (mouse neuroblastoma, N2a) overexpressing human mutant SOD1 showed that this protein specifically interacted with chromogranins (Urushitani et al., 2006), and induced ER stress (Oh et al., 2008). Furthermore, in these cells post-translational modifications of SOD1 have been studied (Furukawa et al., 2008), intracytoplasmic aggregates were found to inhibit neurite outgrowth (Takeuchi et al., 2002), and mutant SOD1 aggregates were present within

ER and Golgi compartments (Urushitani et al., 2008). They have been used to study apoptosis pathways (Pasinelli et al., 1998) and mutant SOD1 toxicity (Witan et al., 2008) associated with ALS, or to reveal that mutant SOD1 co-immunoprecipitated with the anti-apoptotic protein Bcl-2 (Pasinelli et al., 2004). Also, SH-SY5Y cells (human neuroblastoma) have been used to study apoptosis and mitochondrial damage (Cozzolino et al., 2006), or impaired glutamate transport and increased vulnerability to oxidative stress (Sala et al., 2005) induced by mutant SOD1.

Recently, it has been proposed that a human ALS model based on human embryonic stem cell derived MN, in coculture with human astrocytes is a robust and invaluable model to study ALS disease and to initiate species-specific drug development assays (Marchetto et al., 2008; Karumbayaram et al., 2009).

Nonetheless, the NSC-34 cell line has become the cell of choice for the study of ALS disease mechanisms (Muyderman et al., 2009). It is a mouse neural hybrid cell between neuroblastoma and SC MN from enriched primary cultures, which has many of the morphological, physiological and neurochemical properties of MN, including generation of action potentials, expression of neurofilament proteins, acetylcholine synthesis, storage and release, and MN-like functional responses to numerous growth factors. The majority of differentiated NSC-34 cells resemble cultures of MN. Typically, these are large cells with multi-polar neurite projections, with a small percentage of the cells exhibiting a flat fibroblast-like morphology with short neuritic projections (Cashman et al., 1992). Several studies have highlighted the suitability of the NSC-34 cells for transient and stable genetic transfection, and stably transfected lines have been created as models of FALS to study mitochondrial dysfunction (Menzies et al., 2002), cytosolic proteome (Allen et al., 2003; Kirby et al., 2005), effect of vascular endothelial growth factor on MN death (Li et al.,

2003), apoptosis (Sathasivam et al., 2005b) and Golgi apparatus fragmentation (Chapter 2, Gomes et al., 2008).

Other different systems have also been used to elucidate the mechanisms involved in ALS. Indeed, the baker's yeast, *Saccharomyces cerevisiae*, has been widely used to screen for interacting proteins and to study basic cellular mechanisms involved in protein-misfolding diseases, including Parkinson's (Outeiro and Lindquist, 2003; Cooper et al., 2006), Huntington's (Duennwald et al., 2006), Alzheimer's (Bagriantsev and Liebman, 2006), and Creutzfeldt–Jakob diseases (Ma and Lindquist, 1999). In ALS, it has been used to screen for proteins interacting with mutant SOD1 (Kunst et al., 1997; Urushitani et al., 2006), and its expression was shown to decrease mitochondrial electron transport in yeast (Gunther et al., 2004). It has also been used to study other mutations such as VAPB (Suzuki et al., 2009) and TDP-43 (Johnson et al., 2008; Kim et al., 2009).

The worm *Caenorhabditis elegans* has been used as a valuable *in vivo* model system to study proteins involved in neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Link, 1995; Waxman and Giasson, 2009). In ALS, it has been used to study mutant SOD1 protein aggregation (Witan et al., 2008; Gidalevitz et al., 2009; Wang et al., 2009), oxidative stress (Oeda et al., 2001) and also other mutations such as VAPB (Tsuda et al., 2008).

Zebrafish (*Dania rerio*) is an organism that is becoming a promising tool for drug discovery (Zon and Peterson, 2005) and, recently, it has been used to model neurodegenerative diseases such as tauopathies (Paquet et al., 2009). In ALS, overexpression of several mutants of human SOD1 in zebrafish embryos induced a specific motor axonopathy, and it reveals the potential of vascular endothelial growth factor for the treatment of ALS (Lemmens et al., 2007). It has also been used to study other mutations, such as those found in Alsin (Gros-Louis et al., 2008).

Drosophila melanogaster has proven to be instrumental in modeling various neurodegenerative diseases, including polyglutamine expansion diseases, α-synuclein-linked Parkinson disease, and tauopathies (Marsh and Thompson, 2006). In ALS, it has been used to uncover cell-autonomous injury by SOD1 to MN *in vivo*, as well as non-autonomous effects on glia (Watson et al., 2008). It has also been used for the study of the VAPB mutation (Tsuda et al., 2008).

Additionally, the development of rodent animal models has interest to unravel the pathogenesis and treatment of neurodegenerative diseases. Identification of pathogenic alleles of SOD1 has led to the generation of transgenic mice (Julien and Kriz, 2006) and rat (Nagai et al., 2001; Howland et al., 2002) models for the study of ALS. In these models, the mutant human protein is ubiquitously expressed at levels equal to or several fold higher than the level of endogenous SOD1, and leads to late-onset, progressive neurodegenerative disease that is quite similar to the human illness (Gros-Louis et al., 2006; Turner and Talbot, 2008a). Although, animal models have been useful and largely contributed to the insights into the pathomechanisms of MN degeneration they have their limitations. Some of their limitations are inherent to the animal's anatomy, physiology, longevity and genetics. There is clearly scope for developing further animal models that more closely resemble the human disease (Goodall and Morrison, 2006; Julien and Kriz, 2006).

Recently, it was described the first spontaneous animal model for ALS. Awano and colleagues (Awano et al., 2009) described that the progression and distribution of lesions in canine degenerative myelopathy are similar to those reported for the upper MN dominant onset form of ALS. They consistently contained cytoplasmic inclusions that stained with anti-SOD1 antibodies similar to those found in ALS patients and rodent models with *SOD1* mutations. More strikingly, genome-wide association revealed an E40K missense mutation in SOD1 protein in these dogs. Making them

potential animal models for ALS without very high levels of mutant *SOD1* expression. The canine model may prove to be particularly valuable for evaluating therapeutic interventions.

Nowadays, there is an universal consciousness towards the reduction and replacement of the animals used to perform toxicological tests and for drug testing (Abbott, 2005). Cellular models are then becoming very relevant, not only for the understanding of the pathological mechanisms associated with diseases, as referred before, but also to high-throughput screening assays for drug discovery. These cellular models are animal-free systems that have the advantage of high reproducibility, cheaper and easy handling. In this context, the work described in this Thesis supports the feasibility and usefulness of cellular models that mimic the pathological mechanisms associated with ALS such as Golgi apparatus fragmentation (Chapter 2) and aggregate formation (Chapter 4). Further, it enabled to evaluate the therapeutic potential of the chemical chaperone, trehalose to help prevent protein aggregate formation (Chapter 4). It allowed also to describe SOD1 secretion associated with exosomes (Chapter 5) important in the context of toxicity spread and the primary cultures described (Chapter 3) can be used to study the crosstalk between the different cellular models involved in this non-cell autonomous disease.

6.1.2. Morphology of Golgi apparatus and intracellular protein trafficking in cellular models of ALS

As referred in Chapter 1, the atrophy and fragmentation of neuronal Golgi apparatus (Figure 1J, Chapter 1) is consistently reported in spinal MN of SALS, mutant SOD1-linked FALS (Mourelatos et al., 1990; Fujita et al., 2000) and Guamanian ALS/parkinsonism dementia complex individuals (Mourelatos et al., 1994). The fragmentation consists of dispersion of the normal network of large and irregular elements of the Golgi apparatus into numerous shortened disconnected cisternae (Gonatas et al., 2006). Similar

Golgi apparatus pathology has been observed in other neurodegenerative conditions such as Parkinson's disease (Gosavi et al., 2002), Alzheimer's disease (Stieber et al., 1996), spinocerebellar ataxia type 2 (Huynh et al., 2003), Creutzfeldt-Jacob disease (Sakurai et al., 2000), among others (Fan et al., 2008). Importantly, lesions of the Golgi apparatus were detected in spinal MN of presymptomatic transgenic $SOD1^{G93A}$ mice, suggesting that the Golgi apparatus is targeted early in disease progression (Stieber et al., 2000). We have also reported that the expression of mutant $SOD1^{G93A}$ in NSC-34 cells caused increased Golgi fragmentation prior to signs of apoptosis, where neither apoptotic nuclei, activation of caspase-3 nor loss of mitochondrial transmembrane potential were detected (Chapter 2, Gomes et al., 2008).

The structural and functional integrity of the Golgi apparatus is maintained by different proteins and structures that include microtubules and microtubule-associated proteins, the actin-associated cytoskeleton, the Golgi matrix proteins and proteins that ensure the targeting and fusion of transport vesicles to the correct compartment (Gonatas et al., 2006). It is believed that the Golgi structure has evolved to sense and transduce specific stress signals (Hicks and Machamer, 2005) such as apoptosis (Nakagomi et al., 2008).

Pro-apoptotic caspase-2, element of the cell death machinery, has been shown to be localized to the Golgi apparatus in mammalian cells (Mancini et al., 2000). Hence, caspase-2 might initiate apoptosis after irreparable stress to the secretory pathway (Mancini et al., 2000) suggesting an early role for caspase-2 activation at the Golgi (Hicks and Machamer, 2005). It would be interesting to determine if upon mutant SOD1 induced Golgi fragmentation, described in Chapter 2, caspase-2 would be activated. Several reports also involve caspase-mediated cleavage of proteins implicated in Golgi structure, such as GRASP65 (Lane et al., 2002), golgin-160 (Maag et al., 2005), GM130 (Walker et al., 2004),

giantin (Lowe et al., 2004), p115 (Chiu et al., 2002), dynein and dynactin (Lane et al., 2001) in causing such damage, which result in irreversible fragmentation. Nevertheless, as referred the morphology of the fragmented Golgi in ALS is different from the morphology of the fragmented Golgi as the result of apoptosis (Gonatas et al., 2006; Chapter 2, Gomes et al., 2008). Besides, the levels of three Golgi matrix proteins, GRASP55, p115 and GM130, were found at the same levels for the different cell lines (Chapter 2, Gomes et al., 2008). This supports the notion that Golgi is an early target in the disease process.

The fragmentation of Golgi apparatus in neurodegenerative diseases is probably caused by a variety of mechanisms involving the interactions between mutant proteins and one or more proteins that are involved in the maintenance of the structure of the Golgi apparatus. It is quite possible that membranes of the Golgi apparatus, or key proteins mediating the anchorage of Golgi membranes with microtubules, may be early targets of the toxic function of the mutated SOD1 protein (Fan et al., 2008). It would be interesting to evaluate if mutant SOD1 interacts with any of the proteins involved in Golgi maintenance and structure, such as microtubules using co-immunoprecipitation techniques, or the yeast two-hybrid approach.

Golgi apparatus fragmentation may lead to functional impairment of intracellular protein trafficking essential for secretion and axonal transport in neurons as well as in non-neuronal cells (Nakagomi et al., 2008). In fact, α-synuclein expression blocked ER-to-Golgi vesicular trafficking (Gosavi et al., 2002; Cooper et al., 2006), and similarly, misfolded growth hormone caused Golgi apparatus fragmentation and disrupted ER-to-Golgi traffic (Graves et al., 2001). In ALS, transfection of CHO cells with mutant SOD1 induced Golgi apparatus dispersion and dysfunction of the secretory pathway (Stieber et al., 2004). Here, we have observed that NSC-34 cells overexpressing mutant $SOD1^{G93A}$ showed decreased differentiation and

proliferation capacities (Chapter 2, Gomes et al., 2008), which could be due to the increased fragmentation of the Golgi apparatus reported in these cells. However, mutant SOD1 overexpression in NSC-34 cells did not lead to intracellular accumulation of β–TP or EPO (Chapter 4). Furthermore, when the glycosylation profile of secreted β–TP was analyzed no differences between cells overexpressing wild-type or mutant SOD1 were observed. Therefore, the fragmentation of the Golgi apparatus observed did not have impact on the secretion or *N*-glycosylation of at least two secretory glycoproteins.

On the other hand, overexpression of mutant SOD1 led to decreased levels of the secretory glycoproteins β–TP and EPO, as well as the plasma membrane glycoprotein L1. It is possible that mutant SOD1 triggered ER stress that caused reduced synthesis of those glycoproteins. Since, ER stress at its initial stage causes a reduction of global protein synthesis (Harding et al., 2002).

There have been reports of increased ER stress in ALS, and, it was reported that mutant SOD1 linked to FALS, but not wild-type, aggregated in association with the ER, induced ER stress (Tobisawa et al., 2003), and specifically impaired ER-associated protein degradation (Nishitoh et al., 2008). Recently, evidence was provided that the unfolded protein response was present in $SOD1^{G93A}$ rodent model where the protein disulphide isomerase was up-regulated in SC and physically associated with mutant SOD1 inclusions in these animals, and also in NSC-34 cells (Atkin et al., 2006). Similarly, up-regulation of the full spectrum of unfolded protein response markers in lumbar cord tissue from human SALS patients was also demonstrated (Atkin et al., 2008).

In the cellular model studied it would be interesting to analyze the status of ER stress upon mutant SOD1 expression by evaluating up-regulation of specific markers such as ER transmembrane kinase receptors, inositol requiring kinase 1 and PKR-like ER kinase, or ER

chaperones such as protein disulphide isomerase or immunoglobulin binding protein, by Western blot (Atkin et al., 2008). Similarly, as it was described that ER stress can activate autophagic degradation of protein aggregates (Hoyer-Hansen and Jaattela, 2007), it would be interesting to evaluate markers of autophagy in these cells, such as increase in microtubule-associated protein 1 light chain 3 (LC3) by immunofluorescence microscopy or through the conversion from LC3-I into LC3-II by Western blot (Pasquali et al., 2009).

6.1.3. Reducing mutant SOD1 aggregation in cellular models of ALS

The aggregation of misfolded proteins leads to cellular degenerative processes that ultimately cause neuronal death. This kind of disturbances is well characterized for neurodegenerative diseases like Alzheimer's, Parkinson's and Huntington's diseases. In ALS, intracellular inclusions have been described in both SALS and FALS as well as aggregation of mutant SOD1 in FALS (Chattopadhyay and Valentine, 2009).

The presence of protein aggregates in the NSC-34 cells transfected with hSOD1^{G93A} and the fact that trehalose was successfully used to reduce protein aggregation and insolubility (Chapter 4) could be the starting point for a future strategy to search for agents that can prevent the abnormal aggregation of mutant SOD1.

Trehalose belongs to a group of small-molecular weight compounds, known as compatible solutes or osmolytes. They may have, at least *in vitro*, an auxiliary function in improving protein stability. Most of the compatible solutes of microorganisms are neutral or zwitterionic and, include amino acids and amino acid derivatives, sugars, sugar derivatives and polyols, betaines and the ectoines. Some are widespread in microorganisms, such as trehalose (Santos and da Costa, 2002).

The structure of the apo-SOD1 is strikingly similar to those implicated in amyloid disease (Nordlund and Oliveberg, 2006), so

compounds that are known to act as chaperones preventing protein aggregation could be used to prevent the formation of mutant SOD1 aggregates in ALS by stabilizing mutant protein. Indeed, trehalose, a chemical chaperone (Davies et al., 2006) prevented *de novo* formation of aggregates in the FALS cell model (Chapter 4). Accordingly, it has been used successfully to reduce aggregate formation in a transgenic mouse model of Huntington disease (Tanaka et al., 2004), to inhibit the formation of fibrillar aggregates of insulin (Arora et al., 2004), to reduce Aβ aggregation and cytotoxicity related to Alzheimer's disease (Liu et al., 2005), to impair prion protein aggregation and to protect prion-infected cells against oxidative damage (Beranger et al., 2008), and in cell models of oculopharyngeal muscular dystrophy (Davies et al., 2006). Another approach could be the design of new compounds in order to reduce mutant SOD1 protein aggregates and toxicity, it was reported that new trehalose-conjugated pentapeptides inhibited β–amyloid fibrillogenesis and toxicity toward pure cultures of rat cortical neurons (De Bona et al., 2009).

Similarly, the compatible solutes ectoine and hydroxyectoine were also shown to strongly inhibit the Alzheimer's Aβ42 amyloid formation *in vitro* and reduce the toxicity to human neuroblastoma cells (Kanapathipillai et al., 2005), suggesting that they could be potential candidates of anti-amyloid therapeutics for treating neurodegenerative diseases involving protein aggregates, such as ALS. Additionally, mannosylglycerate was shown to act as an efficient suppressor of aggregation of denaturated protein (Faria et al., 2003). The authors hypothesise that *in vivo* mannosylglycerate could play an important role in the prevention of aggregation of nascent or partially unfolded proteins. Indeed, this compound inhibited β–amyloid peptide aggregation and neurotoxicity in human neuroblastoma cells (Ryu et al., 2008). Ectoine and mannosylglyceramide also inhibited aggregation and neurotoxicity of a prion peptide in human neuroblastoma cells SH-SY5Y (Kanapathipillai et

al., 2008). Due to their properties in preventing protein aggregation, which is common in the pathology of neurodegenerative diseases, compatible solutes are good candidates as potential therapeutic agents.

In cells with an excess of misfolded proteins and where the capacities of chaperones and of the ubiquitin-proteasome pathway have been exceeded, it has been hypothesised that these protein aggregates are targeted for destruction via autophagy (Pasquali et al., 2009). In this context, it seems plausible that drugs targeted to improve autophagy could have beneficial effects to prevent protein aggregates in neurodegenerative diseases such as ALS (Sarkar and Rubinsztein, 2008). There are reports in which induction of autophagy reduced toxicity of α-synuclein (Webb et al., 2003), tau (Ravikumar et al., 2004) and polyglutamine proteins (Berger et al., 2006) in cellular and *in vivo* models. In fact, in ALS autophagy clearance of mutant SOD1 seems to play a role (Chapter 1), and it is believed that there is a defective autophagy pathway in the disease (Fornai et al., 2008). It has been reported that trehalose is an autophagy enhancer that accelerates the clearance of mutant huntingtin and α-synuclein (Sarkar et al., 2007). The decrease of mutant SOD1 aggregation in the presence of trehalose found in this Thesis (Chapter 4) could also suggest that trehalose acted through the induction of autophagy. In this context, it would be interesting to analyse autophagy markers in this FALS cellular model before and after treatment with trehalose. Another compound that is known to stimulate autophagy clearance of protein aggregates in low doses is lithium, which in ALS was shown to be neuroprotective, delayed disease onset and duration, and augmented the life span of the G93A mouse model of ALS (Fornai et al., 2008). In human patients affected by ALS, lithium was found to delay disease progression and is currently being tested in phase II/III trials as a potential therapeutic agent (Table 2, Chapter 1) (Eisen, 2009).

As referred, the cellular models described in this Thesis can be used to identify small molecules that inhibit the formation of cytotoxic mutant SOD1 aggregates via high-throughput screening, either by quantifying aggregate formation by fluorescence microscopy, or by monitoring detergent insolubility by Western blot (Chapter 4). Indeed, high-throughput screening initiatives have led to the discovery of compounds that inhibit the aggregation of expanded polyglutamine proteins (Zhang et al., 2005; Bodner et al., 2006) or prion protein (Bertsch et al., 2005).

6.1.4. Crosstalk between neuronal and non-neuronal cells via exosomes in ALS

The toxicity induced by SOD1 mutants in MN seems to be non-cell autonomous, as mutant damage occurs not just within MN but also in non-neuronal cells, suggesting that neuronal death depends, at least in part, on a contribution from surrounding astrocytes and possibly other cell types (Van Den Bosch and Robberecht, 2008). The rapidly progressing nature of this disease indicates that some circulating or secreted factors may transit between MN and their neighboring cells in a timely fashion. Accordingly, astrocytes that express mutant SOD1 secrete factors that are toxic to MN, but specific factors have not been identified yet (Clement et al., 2003; Di Giorgio et al., 2007; Nagai et al., 2007; Di Giorgio et al., 2008; Marchetto et al., 2008; Yamanaka et al., 2008).

Recently, it was shown that cytosolic mutant SOD1 can reach the outside of neurons via interaction with chromogranins (Urushitani et al., 2006) or via exosomes (Chapter 5, Gomes et al., 2007).

Exosomes are derived from the fusion of multivesicular bodies with the plasma membrane and extracellular release of the intraluminal vesicles. Different cell types that include neurons, astrocytes (Faure et al., 2006) and microglial cells (Potolicchio et al., 2005) were shown to secrete exosomes.

Indeed, evidence suggesting that exosomes are an intercellular mode of communication are increasing (Keller et al., 2006), as well as their role in the spread of toxicity associated with neurodegenerative diseases, such as Alzheimer's disease (Rajendran et al., 2006) and prion diseases (Fevrier et al., 2004). In this latter, non-infected cells were shown to uptake prion protein associated with exosomes and they became infected when incubated in the presence of cell culture media from infected cells (Fevrier et al., 2004). It is possible that other neurodegenerative-associated proteins, which were detected in extracellular fluids, such as culture media and CSF, such as α-synuclein or Tau (El-Agnaf et al., 2003; Vandermeeren et al., 1993), may also be contained in exosomes. Supporting this hypothesis was the observation that aggregated α-synuclein is secreted through an exocytic pathway distinct from the ER-Golgi (Lee et al., 2005). Cell-to-cell transmission of α-synuclein aggregates was found concomitant to caspase-3 activation in recipient neurons (Desplats et al., 2009), as well as neurotoxicity, microglial activation and release of pro-inflammatory cytokines from astrocytes (Lee, 2008).

It is proposed that once released from a cell exosomes could fuse with membranes of neighboring cells, transferring exosomal molecules from one cell to another, then constituting a way to spread and transfer exosomal infectivity to cells of different origin (Vella et al., 2007). It seems that the exosome pathway provides proteins an escape pathway out of the cell, favoring their spread in the extracellular environment and contributing to subsequent neurodegeneration, and is not simply removing unwanted, excess protein through lysosomal degradation (Vella et al., 2008).

As far as ALS is concerned, we reported for the first time that mutant SOD1 is secreted in association with exosomes in a cellular model of FALS (Chapter 5, Gomes et al., 2007). Further work must be done to establish if exosomes constitute a mean of communication between cells involved in ALS. Nevertheless, NSC-34 cells, modeling MN, released

exosomes and preliminary results shown in Chapter 5 indicated that these exosomes interacted with other NSC-34 cells, although it will be necessary to evaluate if mutant SOD1 would be toxic once inside the recipient cell, triggering the apoptotic pathway for instance. Additionally, it will be interesting to evaluate if exosomes containing mutant SOD1 have the capacity to interact and fuse with astrocytes and microglia triggering astrogliosis or microgliosis, which could result in the release of oxygen radicals, inflammatory cytokines such as TNF-α or interleukins. Furthermore, it would be valuable to determine if astrocytes and microglial cells produce exosomes containing mutant SOD1 capable of transferring toxicity to neuronal cells such as NSC-34 cells. In light of these, primary cultures described in Chapter 3 could be used to study toxicity transfer between different cell types via exosomes containing mutant SOD1.

6.2. General conclusions

The work described in this Thesis allowed the identification of different pathological features in cellular models of FALS associated with mutant $SOD1^{G93A}$, such as alterations in the Golgi morphology, mutant SOD1 aggregation, and the identification of one mechanism for SOD1 secretion.

Mutant $SOD1^{G93A}$ caused increased Golgi apparatus fragmentation independent of apoptosis; this can explain the decreased cell differentiation and proliferation observed for the cells expressing mutant SOD1.

Primary cultures of SC cells from rat embryos $SOD1^{G93A}$ resemble the SC environment and constitute a model of FALS, in which the interactions between the different cell types involved in ALS can be studied.

Overexpression of mutant $SOD1^{G93A}$ caused decreased levels of the concomitantly expressed glycoproteins β-TP, EPO and L1, possibly due to ER stress.

Trehalose, a known chemical chaperone, prevented *de novo* formation of mutant SOD1 aggregates in the FALS cell model.

In NSC-34 cells, mutant SOD1 is secreted via exosomes; these may be a way of cell-to-cell transfer of toxicity.

In summary, the work described in this Thesis provides evidence that cellular models are important tools for the study of pathogenic mechanisms in FALS, and can be further applied as tools for the search of possible therapeutic agents.

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