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Mestrado em Biologia Molecular e Genética

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Dissertação orientada por:
Doutora Alexandra Marçal, Instituto de Medicina Molecular, Lisboa
Professora Doutora Margarida Ramos, Faculdade de Ciências da Universidade de Lisboa

Gonçalo Luis Monteiro Ramos

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O presente trabalho foi realizado na Unidade de Farmacologia e Neurociências, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa.

Aos meus Avós.

Ao meu Avô, ***José Augusto Gonçalves Ramos,***

cuja rectidão de carácter, princípios, disciplina,

dedicação e amor sempre me influenciaram.

Que orgulho tenho em ti!

"Consistency is the last refuge of the unimaginative."

- Oscar Wilde

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Resumo

A Esclerose Lateral Amiotrópica (ELA) é uma doença progressiva e fatal caracterizada pela degeneração selectiva dos neurónios motores do córtex motor, tronco cerebral e medula espinal, que provoca atrofia muscular, paralesia e morte por falha respiratória. A etiologia da doença continua desconhecida, mas com um consenso de que o dano dos neurónios motores é causado por uma rede de processos patológicos complexos. Os mecanismos envolvidos na degeneração dos neurónios motores são melhor conhecidos num subtipo da doença causada por mutações na enzima superóxido dismutase 1 (SOD1). Esta enzima actua na eliminação de radicais livres de oxigénio e na ELA o processo de degeneração neuronal deve-se a um ganho de função da SOD1. A adenosina tem uma função importante na modulação da transmissão sináptica no SNC e SNP, actuando a dois níveis: inibitório, modulado pelos receptores do subtipo A_1 e excitatório, mediado pelos receptores do subtipo A_{2A}. É conhecido que a expressão dos receptores A_1 e A_{2A} da adenosina está alterada nalgumas doenças neurodegenerativas, mas o seu papel na ELA é ainda muito pouco conhecido.

O objectivo deste trabalho foi determinar o efeito da ELA na expressão proteica e de mRNA dos receptors A_1 e A_{2A} da adenosina no decurso da doença. O modelo de murganhos transgénicos para o gene SOD1 humano com a mutação G93A foi usado neste trabalho. Os níveis proteicos e de mRNA de ambos os receptores foram quantificados através das técnicas de immunoblotting e PCR quantitativo em tempo real, respectivamente. Foram estudados diferentes tecidos do SNC e SNP, nomeadamente, córtex e medula espinal (apenas immunoblotting) e nervo frénico-diafragama, de animais selvagens e portadores da doença nas fases pre-sintomática (4-6 semanas) e sintomática (13-14 semanas).

Resultados deste estudo indicaram níveis proteicos não alterados nos SNC e SNP do receptor A_1 ao longo da progressão da doença. No entanto, observou-se uma sobreexpressão dos receptores A_{2A} no córtex na fase pre-sintomática e um decréscimo na fase sintomática. Os outros tecidos mantiveram-se inalterados no que se refere aos receptores A_{2A} em ambas as fases da doença. A avaliação da expressão de mRNA no diafragma não revelou quaisquer alterações em ambos os receptores da adenosina durante a progressão da doença. Assim, no que se refere aos receptores da adenosina em ELA, as primeiras alterações parecem ocorrer logo no início da doença nos receptores A_{2A} do SNC.

Palavras-chave: Esclerose Lateral Amiotrópica (ELA); mutação $SOD1^{G93A}$; receptor A_1 da adenosina; receptor A_{2A} da adenosina.

Abstract

Amyothrophic Lateral Sclerosis (ALS) is a progressive and fatal disease categorized by a selective degeneration of motor neurons from the cerebral cortex, brainstem and spinal cord that provokes muscle atrophy, progressive paralysis and death due to respiratory failure. The etiology of most ALS cases remains unknown but there is a current consensus that motor neuron degeneration is caused by a complex interaction between multiple pathogenic processes. The mechanisms of motor neuron degeneration are best understood in the subtype of disease caused by mutations in the enzyme superoxide dismutase 1. This enzyme is enrolled in the degradation of free oxygen radicals and in ALS neuronal damage is due to its gain-of-function. Adenosine has a central role as a neuromodulator of the CNS and PNS synaptic transmission. Adenosine acts at two levels: inhibitory through the subtype A_1 receptor and excitatory through the subtype A_{2A} receptor. Variation on the expression of A_1 and A_{2A} receptors has been identified in some neurodegenerative diseases, but their role in ALS is not yet understood.

The objective of this work was to determine the effect of ALS on the protein and mRNA expression of A_1 and A_{2A} adenosine receptors through disease progression. The transgenic model of mice carrying the human SOD1 gene with the G93A mutation was used in this work. Protein and mRNA levels of both receptors were quantified through immunblotting and quantitative real time PCR, respectively. Different tissues of the CNS and PNS, namely cortex and spinal cord (immunoblotting only) and phrenic nerve-diaphragm were studied in wild-type and transgenic mice in the pre-symptomatic (4-6 weeks) and symptomatic (13-14 weeks) phases of the disease.

Results from this study indicate unaltered A_1 receptor protein levels at the CNS and PNS through disease progression. However, there is an overexpression of A_{2A} receptors in the cortex of pre-symptomatic mice and a decrease in the symptomatic phase. The A_{2A} receptors are unaltered in the other tissues in both phases of the disease. The mRNA evaluation does not reveal significant alterations in both adenosine receptors during disease progression. Thus, regarding adenosine receptors in ALS, the first changes seem to occur early in the disease at the CNS in A_{2A} receptors.

Key words: Amyotrophic Lateral Sclerosis (ALS); $SOD1^{G93A}$ mutation; A_1 adenosine receptor, A_{2A} adenosine receptor.

Abbreviations list

A_1R – A_1 adenosine receptor

A_{2A}R – A_{2A} adenosine receptor

A_{2B}R – A_{2B} adenosine receptor

A_3R – A_3 adenosine receptors

ADP – Adenosine Diphosphate

ALS – Amyothrophic Lateral Sclerosis

ATP – Adenosine Triphosphate

BSA – Bovine Serum Albumin

CNS – Central Nervous System

CTRL – Control (referring to Wild-type endogenous SOD1 mouse model)

DEPC – Diethylpyrocarbonate

dNTP - Deoxynucleotide Triphosphates

DTT – Dithiothreitol

ECL - Enhanced Chemiluminescence

EDTA - Ethylenediaminetetracetic Acid

ER – Endoplasmic Reticulum

FTD – Frontotemporal Dementia

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

LMN – Lower Motor Neuron

MND – Motor Neuron Diseases

NADH - Nicotinamide Adenine Dinucleotide Hidrogenase

NMJ – Neuromuscular Junction

NP-40 – Nonyl Phenoxypolyethoxylethanol

PBS - Phosphate Buffered Saline

PD – Parkinson's Disease

PMSF – Phenylmethanesulfonyl Fluoride

PNS – Peripheral Nervous System

PST – Pre-Symptomatic phase mice

PVDF – Polyvinylidene Difluoride

RIPA - Radio-Immunoprecipitation Assay

ROS – Reactive Oxygen Species

RPM - Revolutions Per Minute

SDS - Sodium Dodecyl Sulfate

SOD1 – Superoxide Dismutase 1 (referring to transgenic mouse model for human SOD1 with G93A mutation)

ST – Symptomatic phase mice

TBS – Tris Buffered Saline

TBS-T - Tris-Buffered Saline with Tween

TDB – Tail Digestion Buffer

UMN – Upper Motor Neuron

INTRODUCTION

1. Amyotrophic Lateral Sclerosis

1.1. Historical background

It was in the latter half of the 19th century that the initial steps towards the unraveling of one of the most common motor neuron diseases (MND) were accomplished. Using clinical cases and autopsy material, a technique known as "anatomo-clinical method", the famous French neurobiologist and physician Jean-Martin Charcot (Figure 1), showed that it could be possible to correlate anatomical lesions in the nervous system by the presence of clinical signs (Goetz *et al.*, 1995; Goetz, 2000 and Rowland, 2001). In this context, his first major contribution was in 1865 (Charcot, 1865) when he presented a case of a young woman who developed profound weakness and showed increased muscle tone, with contractures of all extremities, despite she had no intellect or sensory abnormalities, and her urinary control was normal. At the autopsy study, Charcot found specific and isolated lateral column degeneration in the spinal cord:

"On careful examination of the surface of the spinal cord, on both sides in the lateral areas, there are two brownish-gray streak marks produced by sclerotic changes. These grayish bands begin outside the line of insertion of the posterior roots, and their anterior border approaches, but do not include, the entrance area of the anterior roots. They are visible throughout the thoracic region and continue, though greatly thinning out, up to the widening point of the cervical cord. Below, they are barely visible in the thoracolumbar region. Transverse sections taken at different levels allow one to see that the lateral columns have in their most superficial and posterior regions, a gray, semitransparent appearance, rather gelatinous.... At no point does the diseased tissue penetrate the gray matter which remains unaffected." (Charcot, 1865).

Figure 1 | Jean-Martin Charcot (1825-1893). Jean-Martin Charcot was a French neurobiologist and physician that first characterized Amyotrophic Lateral Sclerosis.

In a second apparently unrelated observation (Charcot & Joffroy, 1869) with his colleague, Joffroy, they found pediatric cases of infantile paralysis in which the spinal cord lesions were systematically limited to the anterior horns of the grey matter. Thus raised the hypothesis that the spinal cord motor system was organized into two parts, and that lesions

affecting each part cause different clinical signs. These conclusions became the pillars of modern neurology: when gray matter motor nuclei are damaged, weakness is associated with muscular atrophy in the body areas supplied by those cells, whereas when white lateral column damage occurs, weakness is associated with progressive contractures and spasticity. Charcot's achievement to make sense of these evidences, led him for the first time in 1874 (Charcot, 1874) to use the term Amyotrophic Lateral Sclerosis to refer to this disorder and stated that:

"I do not think that elsewhere in medicine, in pulmonary or cardiac pathology, greater precision can be achieved. The diagnosis as well as the anatomy and physiology of the condition "amyotrophic lateral sclerosis" is one of the most completely understood conditions in the realm of clinical neurology." (Charcot, 1887).

ALS first became known as Charcot's sclerosis but in North America the term "ALS" is used interchangeably with "Lou Gehrig's disease" in memory of the famous baseball player who died of the disease in 1941. The word Amyotrophic comes from the Greek language. "A" means no, "Myo" refers to muscle, and "Trophic" means nourishment – "No muscle nourishment". When a muscle has no nourishment, it atrophies or wastes away. "Lateral" identifies the areas in a person's spinal cord where portions of the nerve cells that signal and control the muscles are located. As this area degenerates it leads to scarring or hardening ("sclerosis") in the region (The ALS Association, 2010).

1.2. Epidemiological and Clinical features of ALS

After 140 years, ALS is the most common adult-onset motor neuron disease. With a uniform worldwide incidence (frequency of new cases per year) of approximately 1-2 per 100 000 individuals and a prevalence (the proportion of affected individuals in the population) of 4-6 per 100 000. It affects people of all races and ethnic backgrounds and more commonly men than women (the male:female ratio is 3:2) (Kiernan *et al*., 2011). There are a few exceptions with higher frequency of cases, such as Guam (Reed *et al.*, 1975), the Kii Peninsula of Japan (Kimura, 1965) and the southern lowlands of western New Guinea (Gajdusek & Salazar, 1982). Although 90-95% of cases have been classed as sporadic ALS (SALS) with no apparent genetic link, in the remaining 5-10% of instances the disease is inherited in an autosomal dominant manner, referred as familial ALS (FALS). The mean age of onset is 45-60 years in both forms of ALS. The primary hallmark is the degeneration of the upper motor neurons (UMN) of the motor cortex and of the lower motor neurons (LMN), which extend through the brainstem and spinal cord to innervate skeletal muscles. Clinical

presentation (figure 2) varies but most commonly consists of progressive muscle weakness, fasciculations (twitching muscles), atrophy and spasticity (the persistent contraction of certain muscles, which causes stiffness and interferes with gait, movement or speech). However ALS clearly spares cognitive ability, sensation, and autonomic nervous functions, like eye movement and control of urinary sphincters. It is less well recognized that at least 30% of small interneurons in the motor cortex and spinal cord also degenerate (Cleveland & Rothstein, 2001). Generally fatal within 1-5 years of onset, ALS culminates in death from respiratory failure because of denervation of respiratory muscles and diaphragm. The causes of almost all occurrences of the disease remain unknown (Pasinelli & Brown, 2006; Andersen & Al-Chalabi, 2011).

Regrettably there is no primary theraphy for this disorder and the single drug approved for use in ALS, Rilutek® (riluzole), acting through inhibition of pre-synaptic glutamate release, only slightly prolongs survival for a few months (Bensimon *et al.*, 1994). Symptomatic measures (for example, feeding tube and respiratory support) are the mainstay of management of this disorder in later stages of disease.

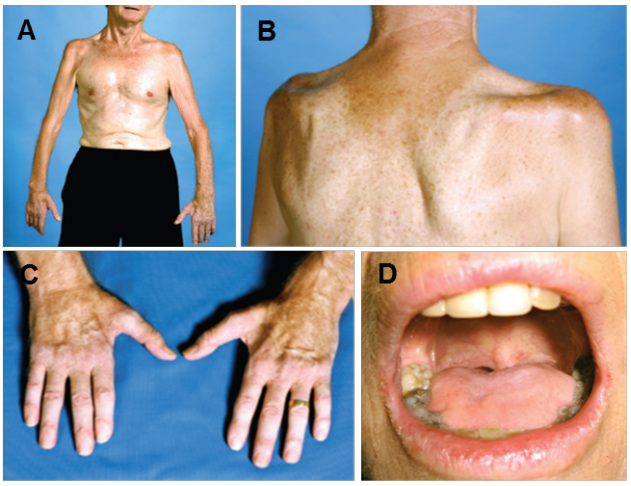


Figure 2 | Clinical features of muscle wasting in patients with ALS. (A) Proximal and symmetrical upper limb wasting results in an inability to lift arms against gravity. (B) Recessions above and below the scapular spine, indicating wasting of supraspinatus and infraspinatus muscles, as well as substantial loss of deltoid muscle. (C) Disproportionate wasting of the thenar muscles combined with the first dorsal interossei. (D) Substantial wasting of the tongue muscles. Note the absence of palatal elevation present on vocalisation. Difficulty in mouth opening and swallowing (extracted from Kiernan *et al.,* 2011).

1.3. Superoxide dismutase 1 mutation

The identification of some of the genetic subtypes of ALS (table 1) has established key molecular and pathogenic mechanisms, which are applicable not only to the minority of cases that carry FALS mutations, but also to SALS more broadly. However, the discovery of Cu/Zn superoxide dismutase's (SOD1) role in FALS (Rosen *et al.*, 1993) offered the first insight to unravel ALS. The authors reported that mutations in this enzyme occur in an autosomal dominant manner in adult-onset ALS (ALS1) and account for 2-3% of ALS cases and about 15%-20% of instances of FALS.

Table 1 | Reviewed genes associated with familial ALS. (adapted from Ferraiuolo *et al.*, 2011).

Genetic ALS subtype	Chromosomal locus	Gene (gene symbol)
Oxidative stress		
ALS1	21q22	Superoxide dismutase 1 (SOD1)
RNA processing		
ALS4	9q34	Senataxin (SETX)
ALS6	16p11.2	Fused in sarcoma (FUS)
ALS9	14q11.2	Angiogenin (ANG)
ALS10	1p36.2	TAR DNA-binding protein (TARDBP)
Endossomal trafficking and cell signalling		
ALS2	2q33	Alsin (ALS2)
ALS11	6q21	Polyphosphoinositide phosphatase (FIG4)
ALS8	20q13.3	Vesicle-associated protein-associated protein B (VAPB)
ALS12	10p13	Optineurin (OPTN)
Glutamate excitotoxicity		
ND	12q24	D-amino acid oxidase (DAO)
Ubiquitin/protein degradation		
ND	9p13-p12	Valosin-containing protein (VCP)
ALSX	Xp11	Ubiquilin 2 (UBQLN2)
Cytoskeleton		
ALS-dementia-PD	17q21	Microtubule-associated protein tau (MAPT)
Other genes		
ALS5	15q15-q21	Spatacsin (SPG11)
ALS-FTD	9p13.3	σ Non-opioid receptor 1 (SIGMAR1)
ALS-FTD	9q21-q22	Chromosome 9 open reading frame (C9ORF72)
Unknown genes		
ALS3	18q21	Unknown
ALS7	20ptel-p13	Unknown

SOD1 dismutates free oxygen radicals into O_2 and hydrogen peroxide (H_2O_2). H_2O_2 is then converted to H_2O by either catalase or glutathione peroxidase (Nicholls & Ferguson, 2002). The SOD1 gene comprises five exons that encode 153 evolutionarily conserved amino acids which, together with a catalytic copper ion and a stabilizing zinc ion, form a subunit. Through covalent binding, pairs of these subunits form the SOD1 homodimers (Cleveland & Rothstein, 2001).

Following linkage analysis, in 1993, using modern genetic mapping methods and with DNAs from patients suffering from familial ALS, Rosen and colleagues (1993) identified 11 missense mutations in the SOD1 gene in 13 of 18 pedigrees with high-penetrance dominantly inherited FALS. Since then, 166 SOD1 mutations have been reported (figure 3) to be associated with ALS, plus 8 silent mutations and 9 intronic variants, presumed to be nonpathogenic. Of the 166 disease associated mutations, 147 are of the missense type. The remaining 19 mutations are nonsense and deletion mutations that result in a change in

length of the SOD1 polypeptide (Cleveland & Rothstein, 2001; Turner & Talbot, 2008). The pathological effects of SOD1 mutations are not thought to result from loss of dismutase activity but rather from gain-of-function effects through which the protein acquires one or more toxic properties. This theory is supported by several lines of evidence, including the absence of motor neuron degeneration in hSOD1-null mice and its occurrence in transgenic mice overexpressing mutant forms of SOD1, irrespective of residual dismutase activity (Gurney *et al.*, 1994).

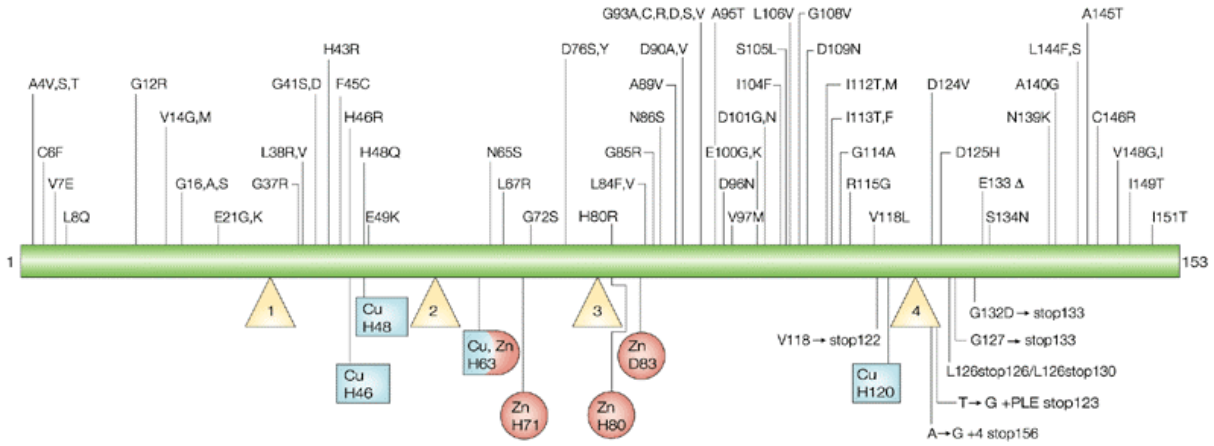


Figure 3 | Mutations causing ALS. So far, 166 mutations have been linked to ALS throughout the 153 SOD1 aminoacid polypeptide length (Adapted from Cleveland & Rothstein, 2001).

1.4. SOD1 mouse models

1.4.1. SOD1 overexpressing and knockout models

Mice deficient for SOD1 were generated by targeted gene deletion. Homozygote SOD1 knockout mice were viable and appeared to develop without any obvious motor abnormalities (Ho *et al.*, 1998; Reaume *et al.*, 1996). Hence, disruption of SOD1 alone appeared to be insufficient to cause spontaneous motor neuron degeneration in mice without injury or challenge. SOD1 knockouts are repeatedly reported to be normal or healthy which is interpreted as a major defeat for a loss-of-activity hypothesis for SOD1 mutations. However, SOD1 null mice develop chronic age-related peripheral axonopathy, denervation muscle atrophy and accelerated sarcopenia which confers significant locomotor deficits (Flood *et al.*, 1999; Shefner *et al.,* 1999; Muller *et al.,* 2006). It seems that a loss-of-function cannot be completely excluded from a pathogenic mechanism of all SOD1 mutants.

Similarly with other diseases, increased dosage of SOD1 was also tested. Transgenic mice overexpressing human $SOD1^{WT}$ were generated (Epstein *et al.,* 1987). This model was characterized by hypotonia, hindlimb neuromuscular pathology (Avraham *et al.,* 1988, Avraham *et al.,* 1991; Rando *et al.,* 1998), muscular dystrophy, vacuolar pathology, axonal

loss and motor neuron degeneration were described in spinal cords of aged animals (Dal Canto & Gurney, 1995; Jaarsma *et al.*, 2000). No lines of transgenic human $SOD1^{WT}$ mice have succumbed to ALS symptoms to date, although animals appear to undergo prolonged subclinical motor neuron degeneration.

1.4.2. SOD1 mutant transgenic model

In ALS research, the mainstay has been a mouse that bears the human gene for the known mutation of SOD1 associated with familial ALS. The mouse bearing this kind of mutated gene was the first laboratory model clearly linked to ALS based on a known cause of the disease. The interpretation of this particular model requires some consideration of wild-type SOD1 overexpressing and knockout mice, described above (Turner & Talbot, 2008). The discovery of SOD1 mutations in FALS was promptly followed by the generation of transgenic mice constitutively expressing mutant human SOD1 genes (Gurney *et al.*, 1994). These transgenic constructs typically involve 12-15 kb human genomic fragments encoding SOD1 (harboring a 93 Gly – Ala substitution, from which $SOD1^{G93A}$ designation derives) driven by the human endogenous promoter and regulatory sequences. Despite vast differences in transgene copy number, steady-state transcript and protein levels, dismutase activity and neuropathology, the mutations induce fatal symptoms strongly indicative of ALS with different disease latencies and progression rates. Crucially, the disease phenotype of transgenic mice expressing hSOD1 mutants on a background of endogenous enzyme argued for a dominant gain-of-function mechanism in toxicity (Gurney *et al.*, 1994). Transgenic $SOD1^{G93A}$ mice are principally used in ALS research because of abundant expression, stability and activity in the CNS. Mice develop hindlimb tremor and weakness at around 3 months detected by locomotor deficits progressing to hyper-reflexia, paralysis and premature death after 4 months (Gurney *et al*., 1994). Pathologically, neuromuscular junctions degenerate around 47 days which appears selective for fast-fatiguable axons (Pun *et al.,* 2006). Proximal axonal loss is prominent by 80 days coinciding with motor impairment and a severe (50%) dropout of lower motor neurons, is evident at 100 days (Fisher *et al.,* 2004). This retrograde sequence of neurodegeneration has led to an attractive proposal that ALS may be a distal axonopathy, described later.

At present, 12 different human SOD1 mutants have been expressed in mice. These include nine missense and three C-terminally truncated variants (Turner & Talbot, 2008).

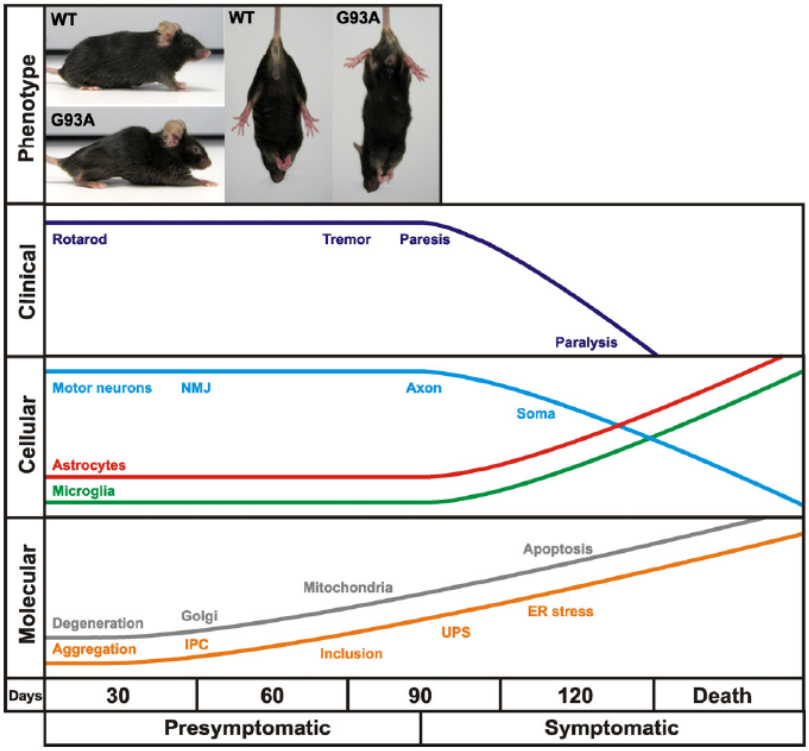


Figure 4 | Time course of clinical and neuropathological events in the high copy number transgenic SOD1^{G93A} mice. Mice develop hindlimb tremor, weakness and locomotor deficits at about 3 months which is preceded by distal synaptic and axonal degeneration. This progresses into fatal paralysis about 1 month later concomitant with spinal motor neuron loss and reactive gliosis. A sequence of mutant SOD1 aggregation into insoluble protein complexes (IPC), inclusion bodies modified by the ubiquitin-proteasome system (UPS) and subcellular degeneration in motor neurons may underlie the phenotype. (extracted from Turner & Talbot, 2008).

1.5. Pathogenic mechanisms of ALS

Despite the fact that a number of genes have now been linked to ALS, the exact pathogenic mechanisms are still largely unclear. Our current understanding of the pathology of ALS is largely based on studies of ALS-associated gene mutations. Because the clinical and pathological profiles of sporadic and familial ALS are similar, it can be predicted that insights from studies of ALS-causing gene mutations apply to sporadic ALS. The mechanisms underlying neurodegeneration in ALS are multifactorial and operate through inter-related molecular and genetic pathways (figure 5). Specifically, neurodegeneration in ALS might result from a complex interaction of several factors including: cytoplasmic misfolded protein aggregates, glutamate excitotoxicity, mitochondrial dysfunction, oxidative stress, disruption of axonal transport process, endoplasmic reticulum stress and neuroinflammation. Other factors equally important, which are not going to be described in detail, are endossomal trafficking dysregulation, transcription and RNA processing

impairements and the role of non-neuronal cells (Boillée *et al.*, 2006; Dion *et al.,* 2009; Redler & Dokholyan, 2012).

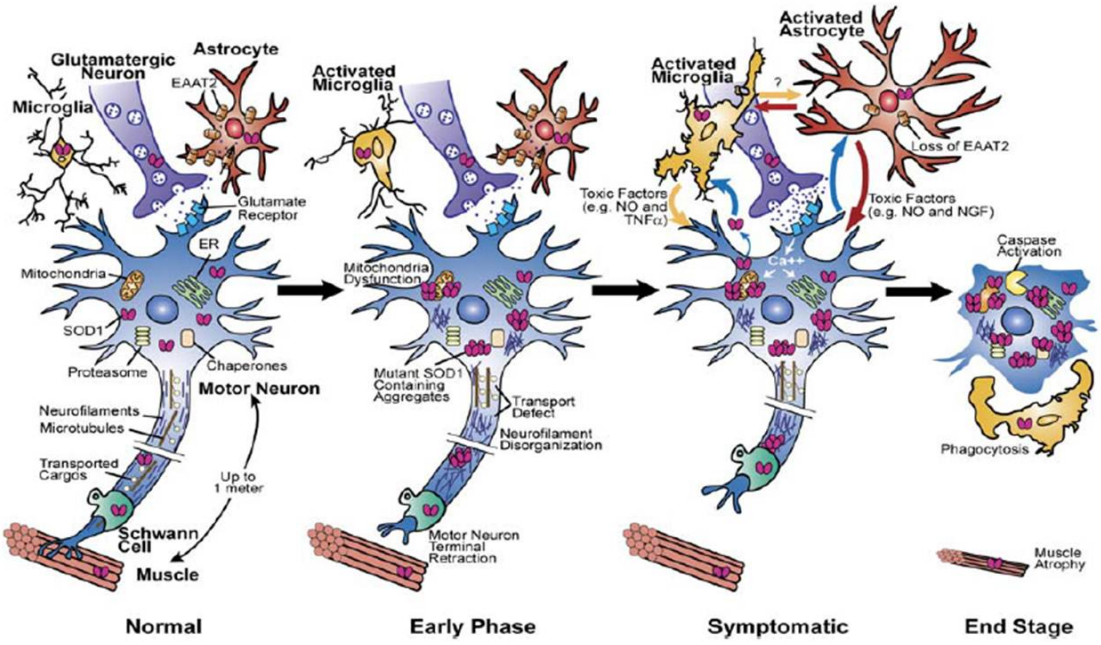


Figure 5 | Schematic evolution of motor neuron degeneration during the course of SOD1 mutant ALS disease. Four stages are defined (normal, early phase, symptomatic, and end stage). Toxicity is non-cell-autonomous, produced by a combination of damage incurred directly within motor neurons that is central to disease initiation and damage within non-neuronal neighbors, including astrocytes and microglia, whose actions amplify the initial damage and drive disease progression and spread. Selective vulnerability of motor neurons to ubiquitously expressed mutant SOD1 is determined by the unique functional properties of motor neurons (e.g., they are very large cells with large biosynthetic loads, high rates of firing, and respond to glutamate inputs) and damage to their supporting cells in the neighborhood. (extracted from Boillée *et al.*, 2006).

1.5.1. Protein misfolding and aggregation

Protein misfolding and aggregation are prominent features of ALS. Aspects of toxicity can arise either through aberrant chemistry, mediated by the misfolded aggregated mutants, or through loss or sequestration of essential cellular components; for example, by saturating the protein-folding chaperones and/or the protein-degradation machinery. Consistent with the latter, the aggregates are intensely immunoreactive with antibodies to ubiquitin, a feature common not only to all instances of disease in mice, but also to many human examples (Clement *et al.,* 2003; Henkel *et al.,* 2006; Cassina *et al.,* 2008). Partial inhibition of the proteasome is sufficient to provoke large aggregates in non-neuronal cells that express SOD1 mutants, leading to the proposal that proteasome activity could be limiting by combating such aggregates and moreover, that undue proteasomal attention to aberrantly folded forms of SOD1 could compromise the removal of even more important components (Guo *et al.*, 2003).

1.5.2. Mitochondrial dysfunction and oxidative stress

Mitochondria, have developed defenses to detoxify superoxide ($O_2^{\bullet-}$) generated by the respiratory chain, a highly reactive molecule that contributes to oxidative stress and has been implicated in a number of diseases and aging (Turrens,1997; Barja, 1999). Multiple studies have shown that oxidative stress interacts with, and potentially exacerbates, other pathophysiological processes that contribute to motor neuron injury, including excitotoxicity (Rao & Weiss, 2004), mitochondrial impairment (Duffy *et al*., 2011), protein aggregation (Wood *et al.*, 2003), endoplasmic reticulum stress (Kanekura *et al.*, 2009), and alterations in signaling from astrocytes and microglia (Sargsyan *et al.*, 2005; Blackburn *et al.*, 2009). The most important are the Cu/Zn-superoxide dismutase (SOD1) and the manganese superoxide dismutase (Mn-SOD or SOD2). Age-related diseases, like neurodegenerative diseases, are associated with increased mitochondrial production of $O_2^{\bullet-}$ and H_2O_2. In ALS, there are evidences that these two reactive oxygen species (ROS) can generate highly reactive radicals, like $OH^{\bullet}$, which will modify all kinds of macromolecules including lipids (Shibata *et al*., 2001), proteins (Shaw *et al.*, 1995), nuclear and mitochondrial DNA (Fitzmaurice *et al.*, 1996), and RNA species (Beal, 2005; Chang *et al.*, 2008). Defective respiratory chain function associated with oxidative stress has also been found in tissue from patients with ALS at earlier stages of the disease. Dysfunction of components of the mitochondrial respiratory chain is also evident in the spinal cord of $SOD1^{G93A}$ transgenic mice at disease end stage (Mattiazzi *et al.*, 2002). Furthermore, mutant SOD1 insoluble aggregates could directly damage the mitochondrion through: swelling, with expansion and increased permeability of the outer membrane and intermembrane space, leading to release of cytochrome c and caspase activation; inhibition of the translocator outer membrane (TOM) complex, preventing mitochondrial protein import; and aberrant interactions with mitochondrial proteins such as the anti-apoptotic BCL2 (Pasinelli & Brown, 2006; Ferraiuolo *et al.,* 2011).

1.5.3. Excitotoxicity

Excitotoxicity, results from excessive influx of calcium cations through the over-stimulation of post-synaptic glutamate receptors which may be caused by increased synaptic levels of glutamate, or by increased sensitivity of the post-synaptic neuron energy homeostasis or glutamate receptor expression. This increase of calcium can activate enzymes such as phosphatases, proteases, lipases and endonucleases, causing protein and lipid alterations in cell membranes, generation of ROS, and mitochondrial damage and dysfunction (Corona *et al.,* 2007). Decreased levels of the glutamate transporter EAAT2 (excitatory aminoacid transporter 2) are found in both human patients and in mutant SOD1

transgenic rodents (Rothstein *et al.*, 1995). Thus, excitotoxicity may be involved in modulation of disease progression.

1.5.4. Impaired axonal transport

Motor neurons are highly polarized cells with long axons, and axonal transport is required for delivery of essential components, such as RNA, proteins and organelles, to the distal axonal compartment, which includes synaptic structures at the neuromuscular junction (NMJ). The main machinery for axonal transport uses microtubule-dependent kinesin and cytoplasmic dynein molecular motors, which mediate transport towards the NMJ (anterograde transport) and towards cell body (retrograde transport), respectively. Defects in either supply or clearance of material within an axon can lead to neuronal death. Axonal transport becomes impaired due to neurofilament disorganization via activation of protein kinases that phosphorylate neurofilament proteins (Pasinelli & Brown, 2006).

1.5.5. Endoplasmic reticulum stress

ER stress is an important pathway to cell death in ALS (Atkin *et al.*, 2006; Atkin *et al.*, 2008), and is triggered very early in $SOD1^{G93A}$ transgenic mice (Saxena *et al.*, 2009). ER stress is triggered when misfolded proteins accumulate within the ER lumen, inducing the unfolded protein response (UPR). Although the initial phases of the UPR aim to promote cell survival, prolonged or severe ER stress triggers the apoptotic phase of the UPR. Up--regulation of the three UPR sensor proteins, PERK, ATF6 and IRE1, have been observed both at the symptom onset and at disease end stage of $SOD1^{G93A}$ transgenic mice, implying the involvement of ER stress in disease mechanisms (Atkin *et al.*, 2006; kikuchi *et al.*, 2006). The ER chaperone, protein disulphide isomerase (PDI), was found to co-localize with mutant SOD1 inclusions in both cellular and animal models of ALS and overexpression of PDI decreased mutant SOD1 aggregation, ER stress, and apoptosis (Walker *et al.,* 2010).

1.5.6. Neuroinflamation

Neuroinflammation is characterized in ALS by the appearance of reactive microglial and astroglial cells (Neusch *et al.,* 2007; Van de Bosch *et al.,* 2008), suggesting a non-cell autonomous process (Clement *et al.*, 2003). In ALS, reactive astrocytes produce nitric oxide and peroxynitrite, and trigger mitochondrial damage and apoptosis in motor neurons (Cassina *et al.,* 2008). Astrocytes may also contribute to damage motor neurons through excitotocicity. Furthermore, microglial cells are reported to be activated in the brain and spinal cord of patients with ALS, as well as mutant SOD1 transgenic mice. In fact activated

microglia were detected before motor neuron loss (Henkel *et al.,* 2006). Damage within motor neurons is enhanced by injury from microglial cells via an inflammatory response that accelerates disease progression (Barbeito *et al.,* 2004).

1.6. Where does ALS begin?

Despite Charcot's initial observation of concomitant UMN and LMN pathological changes in ALS, the question of where ALS begins has not been established. Resolution of this question might enhance the understanding of the pathophysiology of ALS and has diagnostic and therapeutic importance (Meininger, 2011). Two theories have been proposed to explain which are the initial steps of ALS disease but more importantly where do they take place. The "dying-forward" hypothesis proposes that ALS is mainly a disorder of corticomotoneurons mediating anterograde degeneration of anterior horn cells. Support to this hypothesis includes:

- Transneuronal degeneration in ALS is an active excitotoxic process in which live but dysfunctional corticomotoneurons, originating in the primary motor cortex, drive the anterior horn cell into metabolic deficit. When this is marked, it will result in more rapid and widespread loss of lower motor neurons (reviewed here Eisen & Weber, 2001).
- Expression of $SOD1^{G93A}$ mutation induces energy dysfunction in discrete CNS motor regions long before motor neuron degeneration occurs (Browne *et al.,* 2006).
- Asymptomatic 60 day-old mice have lost approximately 9%, 12% and 14% of their corticospinal, bulbospinal and rubrospinal projections, respectively. 90 day-old mice that display the first clinical signs have lost approximately 30%, 33% and 33% of their corticospinal, bulbospinal and rubrospinal projections, respectively. Mice aged 110 days that have severe clinical signs, have lost approximately 53%, 41% and 43% of their corticospinal, bulbospinal and rubrospinal projections, respectively. (Zang & Cheema, 2002).

The prevailing and best documented proposal however, is the "dying-back" hypothesis in which motor neuron loss in ALS involves retrograde degeneration within the muscle cells or at the NMJ. Support for the dying-back hypothesis includes:

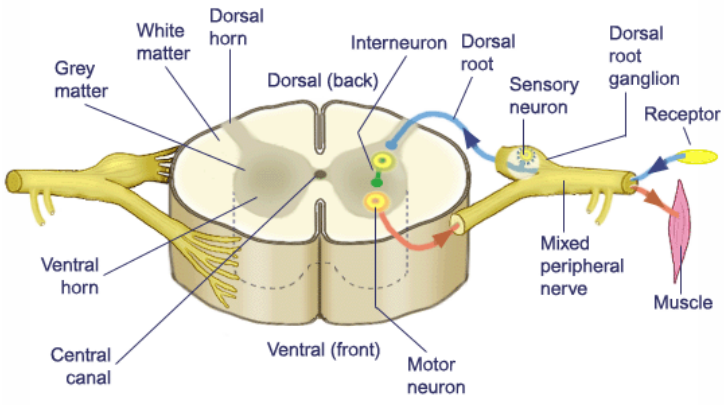
- A quantitative analysis demonstrating denervation at the NMJ by day 47, followed by severe loss of motor severe loss of motor axons (approximately 60%) from ventral roots between days 47 and 80, and loss of α-cell bodies from the lumbar spinal cord after day 80 (Fisher *et al.*, 2004).
- Transgenic mice with skeletal muscle-restricted expression of hSOD1 gene develop neurologic and histopathologic phenotypes consistent with ALS. Muscle restricted expression is sufficient to cause dismantlement of NMJ and distal axonopathy (Dobrowolny *et al.*, 2008, Wong & Martin, 2010).

- Magnetic Resonance Imaging (MRI) studies showed a significant reduction in muscle mass that parallels reduction in fiber diameter and muscle atrophy from week 8. Evidences of neurodegeneration in the brainstem detected only from week 10 (Marcuzzo *et al*., 2011).

2. The motor nervous system

The somatic portion of the nervous system is composed of two major types of nerve cells which connect the spinal cord to the periphery (figure 6). These are primary sensory neurons (or afferent neurons), which relay input from the periphery to the spinal cord, and spinal cord motor neurons (or efferent neurons) which convey motor outflow from the spinal cord to the periphery. Motor neurons can be divided in two groups: UMN, which originate in the motor region of the cerebral cortex or the brain stem and carry motor information down to the final common pathway, and LMN, connecting the brainstem and spinal cord to muscle fibers. Axons of spinal cord motor neurons pass to the periphery to innervate striated muscle. Even for most other reflexes, such as the withdrawal reflex, there is at least one other neuron (an interneuron - Renshaw cells) interposed in the circuit (Kandel *et a*l., 2000; Marieb & Hoehn, 2007).

Skeletal muscle is a form of striated muscle tissue existing under the control of the somatic nervous system. Some examples of this type of muscle are respiratory muscles and the diaphragm, severely targeted in patients with ALS disease (Marieb & Hoehn, 2007).



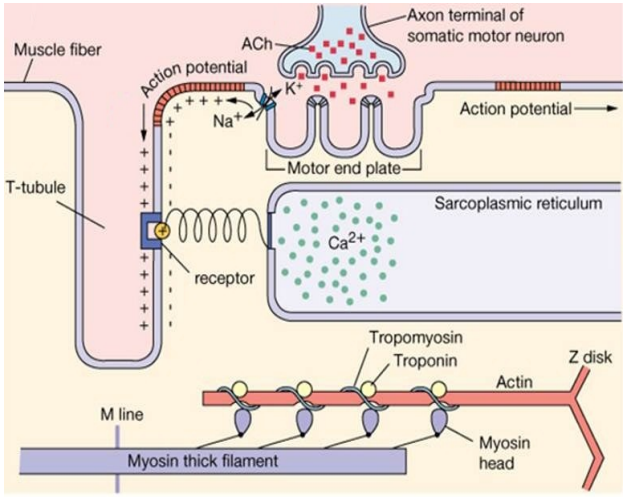
(http://davidsciencestuff.tripod.com/id1.html)

Figure 6 | Somatic Component of the Peripheral Nervous System. The peripheral nervous system can be subdivided into somatic and autonomic components. The somatic nervous system contains motor nerves and sensory nerves innervating skin and muscle. The soma (cell bodies) of motor nerves and sensory nerves are located in the gray matter of the anterior horn of the spinal cord and in the dorsal root ganglia, respectively.

2.1. Motor neurons and neuromuscular synaptic transmission

The phenomenon of neuronal cross-talk is often termed neurotransmission and it is mediated by neurotransmitters. Neurotransmitters are endogenous chemicals that transmit signals from a neuron to a target cell across a synapse allowing the brain to communicate with the rest of the body (Marieb & Hoehn, 2007).

The nerve terminal is responsible for neurotransmitter release and stores it in small, uniformly sized vesicles. Synapses between motor neurons typically use glutamate or GABA as their neurotransmitters, while the NMJ uses acetylcholine exclusively. Glycine is also present in the interneurons of the spinal cord. Arrival of an action potential at the motor neuron ending leads to an instant opening of voltage-gated Ca^{2+} channels with a subsequent abrupt increase in intracellular calcium concentration (Fagerlund & Eriksson, 2009; Martyn *et al.,* 2009). This increased calcium concentration triggers a cascade of intracellular signaling events leading neurotransmitter-containing vesicles to migrate, dock, fuse to the surface of the nerve, rupture and discharge the specific neurotransmitter through the synaptic cleft to the receptive post-synaptic component, either a neuron or the NMJ. The energy required for these processes is generated by a large population of mitochondria present in the cytoplasm. At the NMJ (figure 7) the nicotinic acetylcholine receptors (nAChRs) in the sarcolemma, activated by the released acetylcholine, respond by opening their channels for influx of sodium ions into the muscle to depolarize it (Hughes *et al.,* 2006).



(adapted from http://faculty.pasadena.edu)

Figure 7 | Structure of the Neuromuscular Junction. Schematic representation of the adult NMJ with the three main components: pre-synaptic nerve terminal, synaptic cleft and post-synaptic membrane. Stimulation of a motor nerve results in the release of acetylcholine from vesicles at the pre-synaptic membrane; acetylcholine diffuses and binds to post-synaptic receptors, producing depolarization of the sarcolemma and leading to an action potential.

3. **Adenosine**

Purinergic research has been demonstrated to be potentially fruitful on neurodegenerative disorders such as ischemia, neuropatic pain, multiple sclerosis, Parkinson's, Alzheimer's and Huntington's diseases (Burnstock, 2008a,b) and might accordingly provide novel clues also for ALS. The validation that purinergic research could indeed meet ALS comes from the general notions that 1) microglia, astrocytes and degenerating neurons, commonly release and promptly respond to both ATP and adenosine; 2) extracellular ATP secreted at high concentrations is toxic to neurons and activates microglia and astrocytes, being thereafter degraded to adenosine creating neuroinflammation; 3) neuron toxicity, microglia and astrocyte activation are all common

features to ALS (Volonté *et al.*, 2011). Adenosine is a ubiquitous nucleoside, present and being released from apparently all cells, including neurons and glia. It comprises a molecule of adenine attached by a glycosidic bound to a ribose sugar molecule. Perhaps as a result of their ubiquitous nature, purines have also evolved as important molecules for both intracellular and extracellular signaling, roles that are distinct from their activity related to energetic metabolism, as adenosine diphosphate (ADP) and adenosine triphosphate (ATP), and synthesis of nucleic acids (Khakh and Burnstock, 2009).

Unlike ATP, which may function as a neurotransmitter in some brain areas, adenosine is neither stored nor released as a classical neurotransmitter. It does not accumulate in synaptic vesicles, being released from the cytoplasm into the extracellular space in a calcium independent process through a nucleoside transporter. The adenosine transporters also mediate adenosine reuptake, being the direction of the transport dependent on the concentration gradient at both sides of the membrane. As it is not exocytotically released, adenosine behaves as an extracellular signalling molecule that modulates synaptic transmission. Using G-protein coupled mechanisms, that not only lead to changes in second messenger levels but also to regulation of ion channels, such as calcium and potassium channels, adenosine modulates neuronal activity, pre-synaptically by inhibiting or facilitating transmitter release, post-synaptically by affecting the action of other neurotransmitters and non-synaptically by hyperpolarizing or depolarizing neurons and/or exerting non-synaptic effects (*e.g.* on glial cells). Adenosine, therefore, belongs to the group of neuromodulators, endogenous substances released at the synaptic cleft that influence the release (pre-synaptic modulation) or the action (post-synaptic modulation) of the neurotransmitters (Sebastião & Ribeiro, 2000; Ribeiro & Sebastião, 2010).

3.1. Adenosine receptors

Adenosine receptors are a class of specific purinergic receptors with adenosine as the endogenous ligand. There are four adenosine receptor subtypes among vertebrates, which have been cloned and characterized to date (table 2): adenosine A_1, A_{2A}, A_{2B} and A_3 receptors that belong to the G-protein coupled receptors (GPCRs) family. (Fredholm *et al*., 1994, Fredholm *et al.,* 2001). These receptors are also known as P1 receptors, from the P1 (adenosine selective)/P2 (ATP selective) nomenclature (Burnstock, 1978). A_1R and A_3R are coupled to $G_{i/o}$ inhibitory proteins while $A_{2A}R$ and $A_{2B}R$ are coupled to G_s excitatory proteins (Linden, 2001; Ribeiro *et al.*, 2002). Neuromodulation by adenosine is exerted through activation of high-affinity adenosine receptors (A_1 and A_{2A}) which are probably of physiological importance, and of low-affinity adenosine receptors (A_{2B}), which might be relevant in pathological conditions. The A_3R is a high-affinity receptor in humans, but it has a

low density in most tissues (Ribeiro & Sebastião, 2010). However, much of the data on coupling to other G-proteins are from transfection experiments and it is not known if such coupling is physiologically important. There are evidences that $A_{2A}R$ may be coupled to different G-proteins in different areas (Kull *et al.,* 2000). Other authors (Auchampach *et al.*, 1997) found that one adenosine receptor may also be coupled with more than one G-protein.

Table 2 | Adenosine receptors in the central nervous system and their properties (Dunwiddie & Masino, 2001).

Receptor Type	G Protein	Adenosine Affinity[a]	Effects	Major Therapeutic Potential	High Abundance	Medium/ Low Abundance
A_1	$G_{i/o}$	70 nM	Inhibits adenylyl cyclase Inhibits Ca^{2+} channels Activates GIRKS Activates PLC	Activation: seizure suppression Neuroprotection Spinal analgesia	Hippocampus Neocortex Cerebellum Spinal cord	Amygdala Olfactory bulb Striatum Thalamus Substantia nigra
A_{2A}	$G_{s/olf}$	150 nM	Activates adenylyl cyclase Inhibits Ca^{2+} channels	Inhibition: Parkinson's disease Activation: anti-inflammatory action	Striatum Olfactory bulb	Hippocampus Neocortex Thalamus
A_{2B}	G_s	5100 nM	Activates adenylyl cyclase Activates PLC	Inhibition: anti-asthmatic		Uniform low level expression
A_3	G_{i3}, G_q	6500 nM	Inhibits adenylyl cyclase Activates PLC Increases intracellular Ca^{2+}	Inhibition: anti-inflammatory action		Hippocampus Cerebellum

GIRKS, G-protein–dependent inwardly rectifying K+ channels; PLC, phospholipase C.

3.2. Adenosine receptors distribution and interactions

Within the CNS, the A_1R has the highest expression in the brain cortex, cerebellum, hippocampus and dorsal horn of spinal cord, coupled to activation of K^+ channels (Trussell & Jackson, 1985) and inhibition of Ca^{2+} channels (Macdonald *et. al.*, 1986), both of which would inhibit neuronal activity. The $A_{2A}R$ is expressed at high levels in only a few regions of the brain (striatum has the higher expression) and is primarily linked to the activation of adenylate cyclase (Sebastião & Ribeiro, 1996). The $A_{2B}R$, which also activates adenylate cyclase, is thought to be fairly ubiquitous in the brain (Dixon *et al.*, 1996), but it has been difficult to link this receptor to specific physiological or behavioral responses (Feoktistov & Biaggioni, 1997). The A_3R is also somewhat poorly characterized, but apparently has intermediate levels of expression in the human cerebellum and hippocampus and low levels in most of the brain (Fredholm *et al.*, 2001). It has been reported to uncouple A_1 and metabotropic glutamate receptors via a protein kinase C–dependent mechanism (Dunwiddie *et al.*, 1997; Macek *et al.*, 1998), and thus, one of its functions may be to modulate the activity of other receptors. Both A_1 and A_{2A} receptors are predominantly, but not exclusively,

located pre-synaptically (Rebola *et al.*, 2003; Baxter *et al.*, 2005; Rebola *et al.*, 2005a; Rebola *et al.,* 2005b). Previous evidence indicates that A_1R and $A_{2A}R$ may be co-localized in the same nerve terminals (Correia-de-Sá *et al.*, 1991; Lopes *et al.*, 1999a; Rebola *et al*., 2005b; Pousinha *et al*., 2010). Moreover, both receptors were shown to form a heteromeric complex in co-transfected cultured cells (Ciruela *et al.*, 2006). It is believed that A_1R or $A_{2A}R$ are preferentially activated as a function of the source and amount of adenosine (Sebastião & Ribeiro, 2009). A_1R are preferentially activated by adenosine generated intracellularly, released through adenosine transporters, and $A_{2A}R$ are activated preferentially by adenosine generated extracellularly (Cunha *et al.*, 1996), due to the action of ectonucleotidases upon ATP release. The relative density of A_1R or $A_{2A}R$ adenosine receptors in sub-regions of the same brain area may differ. Whenever the two receptors co-exist, we can ask about their relative importance, i. e. the hierarchy of one receptor with respect to the other. This may change with neuronal activity, the age and even on other molecules that are in the vicinity of the site of action and that may be relevant for the production or inactivation of the ligand (Sebastião & Ribeiro, 2009). High frequency of neuronal firing favours ATP release (Cunha *et al.*, 1996) and adenosine formed from released adenine nucleosides seems to prefer $A_{2A}R$ activation (Cunha *et al.*, 1996) which may be due to the geographical distribution of ecto-5-nucleotidases and $A_{2A}R$. $A_{2A}R$ activate adenosine transport, which in the case of high neuronal activity and ATP release is in the inward direction. This induces a decrease in extracellular adenosine levels and a reduced ability of A_1R to be activated by endogenous extracellular adenosine. By themselves, $A_{2A}R$ are able to attenuate A_1AR activation (Cunha *et al.*, 1994) which may further contribute to a decreased activity of A_1R receptors under high frequency neuronal firing. The ability of adenosine receptors to inhibit synaptic transmission is attenuated by the protein kinase C (PKC) activation (Sebastião & Ribeiro, 1990) and a similar mechanism appears to be involved in the $A_{2A}R$-mediated attenuation of A_1R responses (Lopes *et al.,* 1999). Adenosine receptors are also present in the peripheral nervous system, either autonomic or somatic, especially at the motor nerve endings. There are reports that human and rat skeletal muscle express both mRNA and protein of adenosine receptors (Dixon *et al.,* 1996; Lynge & Hellsten, 2000).

OBJECTIVES

The present work was designed to determine the effect of the $SOD1^{G93A}$ mutation on the expression of A_1 and A_{2A} adenosine receptors of the ALS transgenic mouse model for the human gene SOD1, through disease progression (pre-symptomatic and symptomatic phases). As described above ALS's primary hallmark is the degeneration of the upper motor neurons of the motor cortex and of the lower motor neurons, which extend through the brainstem and spinal cord to innervate skeletal muscles at the neuromuscular junction level (Boilléee *et al.*, 2006). Therefore, three different tissues were analyzed, *i.e.* the motor cortex, spinal cord and phrenic nerve-diaphragm (herein referred as diaphragm). This approach covered both CNS and PNS adenosine receptor expression. Adenosine is known to modulate various physiological functions of most tissues, including skeletal muscles (Hespel & Richter, 1998). This is the main reason why adenosine is the target of this work.

Therefore, to achieve this main goal I formulated three specific questions:

Aim 1: Are the protein levels of A_1 and A_{2A} receptor changed in the motor cortex, spinal cord and the neuromuscular junction throughout disease progression? To answer this question the immunoblotting technique was used.

Aim 2: Are the mRNA levels of A_1 and A_{2A} receptor altered in the neuromuscular junction throughout disease progression? To answer this question quantitative Real-Time PCR technique was used.

Aim 3: Is the primary pathological feature, regarding the expression of adenosine receptors, observed in the central or in the peripheral nervous system? Specifically, I tried to establish throughout disease progression, if the first event related to adenosine receptor alterations occurs in the motor cortex, spinal cord or at the neuromuscular junction level.

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MATERIALS & METHODS

1. Breedings and housbandry

Transgenic B6SJL-Tg(SOD1-G93A)1Gur/J males and wild-type B6SJLF1/J females were purchased from Jackson Laboratory (USA; Stock No. 002726 and 100012, respectively) and a colony was established at the rodent facility (Instituto Medicina Molecular). Since transgenic female line has a very high incidence of non-productive matings (Leitner *et al.,* 2009), mice were maintained on a background B6SJL by breeding transgenic males with non-transgenic females in a rotational scheme. Animals were handled according to European Community Guidelines and Portuguese Law on Animal Care (2010/63/UE). At time of weaning, littermates were identified through ear punching and separated in different cages according to their gender. This system is a permanent procedure that attributes to each hole a number and allows individual identification of mice. Moreover, this method does not require anesthesia, guarantee animal welfare, and the tissue removed by the ear punch can be used for DNA analysis, phasing out the requirement of an additional procedure for genotyping (Costa & Antunes, 2010). All animals were housed 4-5 mice per cage, under a 12h light/12h dark cycle, and received food and water *ad libitum*.

2. Mice genotyping

Using the tissue removed by ear punching, as described above, the mice DNA was isolated by adding TDB (50 mM KCl, 10 mM Tris-HCl pH=9.0, 0.1% Triton X-100, 0.15 mg/mL proteinase K) followed by an overnight incubation at 56°C. Additionally a heat proteinase K inactivation was performed during 15 min at 95°C. After a 2 min centrifugation to remove debris a PCR reaction was prepared. Primers against interleukin-2 precursor (internal positive control) and human SOD1 transgene were raised (see annexe II, table 7). For a total of 25 µL the following components were added per sample: 0,2 mM dNTP mix, 10X DreamTaq Buffer containing $MgCl_2$, 1.5 U DreamTaq DNA polymerase (Fermentas®), 1.33 µM of the transgene SOD1 primer, 0.75 µM of the control primer, water and 200-500 ng DNA template (BioRad® C1000 Thermal Cycler, see annexe I, table 4 for PCR conditions). Both PCR products and DNA ladder (1 kb gene ruler, Fermentas®) were loaded in a 2% agarose gel and an electrophoretic migration took place. With a Red Safe dyed gel it was possible to inspect bands in a transiluminator (Molecular Imager® Gel Doc™ XR System) and distinguish between wild-type SOD1 individuals and human SOD1 transgenics.

3. Tissue extraction and dissection

Male or female, wild-type and transgenic, F2 mice (4-6 and 13-14 weeks old) were anesthetized under isoflurane atmosphere before being decapitated. The brain was rapidly removed from the brain cavity and dissected free in ice-cold PBS 1X (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) in order to separate the motor cortex (rich in A_1R) from the striatum (rich in $A_{2A}R$) to avoid contamination. Additionally, the spinal cord and the diaphragm were extracted. The different tissues were either homogenized or separately frozen at -80°C until further use, as described below.

4. Protein quantification

4.1. Total protein homogenates

Frozen tissue (cortex, striatum, spinal cord and diaphragm) was placed in 800 µL of RIPA buffer (1 M Tris pH 8.0, 0.5 M EDTA pH 8.0, 5 M NaCl, 0.1% SDS, 10% NP-40, 50% Glycerol), supplemented with protease inhibitors (Roche™ tablet and PMSF were added just prior to use), and homogenized in a Potter-Elvehjem homogenizer with a Teflon piston. The samples were then centrifuged at 3.5 rpm (1000 *x g*) during 10 minutes at 4°C, the supernatant was collected and corresponds to the whole tissue lysate. Protein was quantified using the BioRad™ Dc Protein assay Kit based on Lowry (1951), due to the high levels of detergents in the lysis buffer, by measuring the absorbance at 750 nm.

4.2. Gel electrophoresis and immunoblotting

In order to assess the protein levels of A_1 and A_{2A} adenosine receptors in the motor cortex, spinal cord and in the diaphragm throughout disease progression the immunoblotting technique was used. After homogenate preparation and protein quantification, the appropriate volume of each sample (corresponding to 30 µg and 50 µg of protein to A_1R and $A_{2A}R$ quantification, respectively) was diluted in water and loading buffer 5X (350 mM Tris HCl, 30% Glycerol, 10% SDS, 600 mM DTT and 0.012% Bromophenol blue, pH 6.8) performing a total volume of 25 µL to apply *per* lane. The striatum (5 µg) was used as positive internal control for $A_{2A}R$ due to its high content in this adenosine receptor. The cortex was used as positive control for A_1R since it has high levels of this protein. As endogenous control, α-tubulin was used because it proved to be a reliable reference control. Prior to loading, the samples were boiled at 95°C for 5 minutes. Under reducing and denaturing conditions an electrophoretic migration took place, with both molecular weight marker and

samples, in a 10% resolving (and 5% stacking) gel concentration. A standard running buffer 1X (25 mM Tris Base, 190 mM Glycine and 0.1% SDS) was used. Submerged in transfer buffer 1X (25 mM Tris Base, 190 mM Glycine and 20% Methanol), the separated gel proteins were then transferred to PVDF membranes, through a 250 mA electrical current for 90 minutes. A Ponceau Red staining followed in order to check for success of transfer confirming that no air bubbles have formed between the gel and membrane. At this point membranes were cut into pieces separating above α-tubulin proteins from the receptor ones. Membranes were blocked with 5% non-fat dry milk for 1 hour, washed with TBS-T (200 nM Tris Base, 1.5 M NaCl and 0.1% Tween-20, pH 7.6) and incubated with primary antibodies overnight at 4°C. Primary antibodies were diluted in 3% BSA, 0.02% NaN_3 and TBS-T. After washing again for 30 minutes, the membranes were incubated with secondary antibody, for 1 hour at room temperature, and washed again (see table 1 for antibodies information). Finally, a chemoluminescent detection method was performed with ECL western blot detection reagent (GE Healthcare™) using X-Ray films (Fujifilm™). Western blots densitometry was determined with Image J software and normalized to the respective α-tubulin band density. ImageJ Gel analyzer options: Uncalibrated OD; Inverted Peaks; Plot lanes (ImageJ v.1.47c, Wayne Rasband, National Institutes of Health, USA) (according to Miller, 2010; McLean, 2011).

Table 3 | Antibodies used in this study. Primary and secondary antibodies and related conditions used in the immunoblotting experiments for individual proteins. All primary antibodies were diluted in 3% BSA with 0.02% NaN_3 and secondary antibodies in 5% non-fat dry milk.

Protein	Predicted protein size	Primary antibody	Animal	Dilution	Secondary antibody	Dilution
A_1R	37 kDa	Thermo Scientific™ (PA1-041A)	Rabbit	1:1000	Sta. Cruz Biotechnology™: goat anti-mouse (SC-2005); goat anti-rabbit (SC-2004)	1:10000
$A_{2A}R$	42 kDa	Upstate™ (05-717)	Mouse	1:1000		1:10000
α-Tubulin	50 kDa	Abcam™ (ab4074)	Rabbit	1:20000		1:20000

5. mRNA quantification

5.1. Total RNA homogenates

Frozen tissue (diaphragm) was placed in 1000 µL QIAzol Lysis Reagent (according to Quiagen™ RNeasy Lipid Tissue Mini Kit protocol) and homogenized in a Potter-Elvehjem homogenizer with a Teflon piston. The subsequent steps were followed from the same protocol and total RNA was eluted in 40 µL RNase-free water supplied. The concentration of total RNA was determined by measuring the absorbance at 260 nm in NanoDrop ND-1000 (Thermo Scientific™).

5.2. cDNA synthesis and qRT-PCR

Quantitative Real-Time PCR technique was used to evaluate the mRNA expression levels of A_1 and A_{2A} adenosine receptors in the diaphragm throughout disease progression. cDNA was obtained using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™) according to manufacturer's protocol. For a total volume of 20 µL in each reaction tube, the following components were mixed: 0.5 mM dNTP, Random hexamears (50 ng), DEPC-treated water, 1X RT buffer, 5 mM $MgCl_2$, 10 mM DTT, 25 U of SuperScript™ II reverse transcriptase and 2-5 µg RNA template. Minus RT controls were performed for each sample, in which DEPC-treated water was added instead of SuperScript™ II reverse transcriptase. The reverse transcription of all samples took place in Bio Rad C1000 Thermal Cycler (see annexe I, table 5). These PCR products were quantified through qRT-PCR in Rotorgene 6000 (Quiagen™) using SYBR® Green master mix method (Applied Biosystems™). Specific primers against A_1R, $A_{2A}R$ and β-actin DNA sequence (see annexe I, table 6) were used in this reaction. β-actin was used as a reference gene to normalize target gene results. For a total volume of 25 µL in each reaction tube, these components were added: 2x SYBR® Green master mix, 5 µM primer solution, water and cDNA template. Non Template Controls (NTC) were performed for each primer, in which DEPC-treated water was added instead of cDNA templates.

In order to make valid comparisons between different samples it is important to determine the primer amplification efficiency. Ideally, amplification efficiencies for control and target primer should be roughly equal. However, the amplification efficiency for a specific pair of primers is affected by differences in primer binding sites, the sequence of the amplification product, and PCR product sizes, and thus should be determined experimentally. The efficiency equation is:

$$\mathrm{E} = 10^{(-1/\mathrm{M})} - 1$$

where E is the efficiency of the reaction and M refers to the slope of the plot of C_t value versus the log of the input template amount. A slope between −3.6 and −3.1 corresponds to an efficiency between 90% to 110% (which corresponds to a value of E between 0.9 and 1.1) (Fraga *et al*., 2008). The C_t (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (***i.e.*** exceeds background level). C_t levels are inversely proportional to the amount of target nucleic acid in the sample (*i.e.* the lower the C_t level the greater the amount of target nucleic acid in the sample)**.** The results for β-actin and $A_{2A}R$ were calculated based in the efficiency obtained from calibration curve analysis (see annexe III) and determined for each target gene using serial dilutions (1:5) of cDNA of pre-symptomatic diaphragm homogenate. The presented results are fold change values calculated with the Pfaffl equation (Pfaffl, 2001). Therefore, they reflect the difference in adenosine receptors mRNA expression between CTRL and SOD1 animals. Values equal to

1 (=1) are indicative of same CTRL and SOD1 expression; fold change above the unit (>1) mean that SOD1 has a higher expression in relation to the CTRL; and values below 1 (<1) mean that SOD1 has a lower expression when compared to CTRL.

The Pfaffl equation to calculate fold change values (Pfaffl, 2001) is:

$$\boldsymbol{r}\textbf{atio (fold change)} = \frac{\mathbf{E_{target}}^{\mathbf{\Delta Ct_{target}(control-sample)}}}{\mathbf{E_{reference}}^{\mathbf{\Delta Ct_{reference}(control-sample)}}}$$

in which C_t reference corresponds to the number of amplification cycles obtained in the qRT-PCR for β-actin triplicates, and C_t target for target gene triplicates, both belonging to the same animal sample. β-actin fold change results were calculated considering the efficiency (E=1,01), slope (M=-3,288) and the correlation coefficient (R^2=0,99) of the calibration curve. $A_{2A}R$ efficiency is 1,11, slope is -3,089 and correlation coefficient 0,99. For A_1R, due to insufficient levels of mRNA expression in the diaphragm required to obtain a calibration curve, data from spinal cord experiments (laboratory results, unpublished data) were used. Efficiency for A_1R calibration curve is 1,0, slope is -3,351 and correlation coefficient 0,99. Melting point analysis was always performed after each qRT-PCR run as a quality control step (Fraga *et al*., 2008). Melting point analysis is used to distinguish target amplicons from PCR artifacts such as primer-dimer or mis-primed products. Specificity is confirmed by the presence of a unique peak in the melting curve (see annexe III).

6. Statistics

For statistical evaluation of the data, Graphpad PRISM® 5 (San Diego, California, USA) software was used. To assess the significance for protein quantification between CTRL and SOD1 mice for each tissue, the Student's *t*-test was used (with the Welch's correction). The significance of diaphragm mRNA levels between the pre-symptomatic and suymptomatic phases were assessed with a Student's *t*-test (with the Welch's correction). The values presented, for protein and mRNA quantification, are mean ±SEM of *n=5* experiments. Values of $p<0.05$ were considered to be statistically significant.

RESULTS

Quantification of A_1 and A_{2A} receptor protein levels

Regarding A_1R protein levels in pre-symptomatic animals (figure 8A) there is no significant difference (*t*-test, p=0,8396) between wild-type mice (henceforward named as CTRL) and $hSOD1^{G93A}$ transgenic mice (now on referred as SOD1) for the motor cortex (CX). The same observation is true for the spinal cord (SC) and the diaphragm (DP) (*t*-test p=0,6383 and p=0,8984, respectively). Although not significant, slight differences are noted in the SC where SOD1 animals show a decrease in A_1R protein expression.

For the symptomatic phase (figure 8B) no significant difference in A_1R is observed between SOD1 and CTRL mice for the CX (*t*-test, p=0,9974). For the SC an increase in A_1R is registered for SOD1 mice, despite not statistically significant (*t*-test, p=0,5143). For the DP there is a decrease in A_1R protein expression of SOD1 when compared to CTRL animals (*t*-test, p=0,6676).

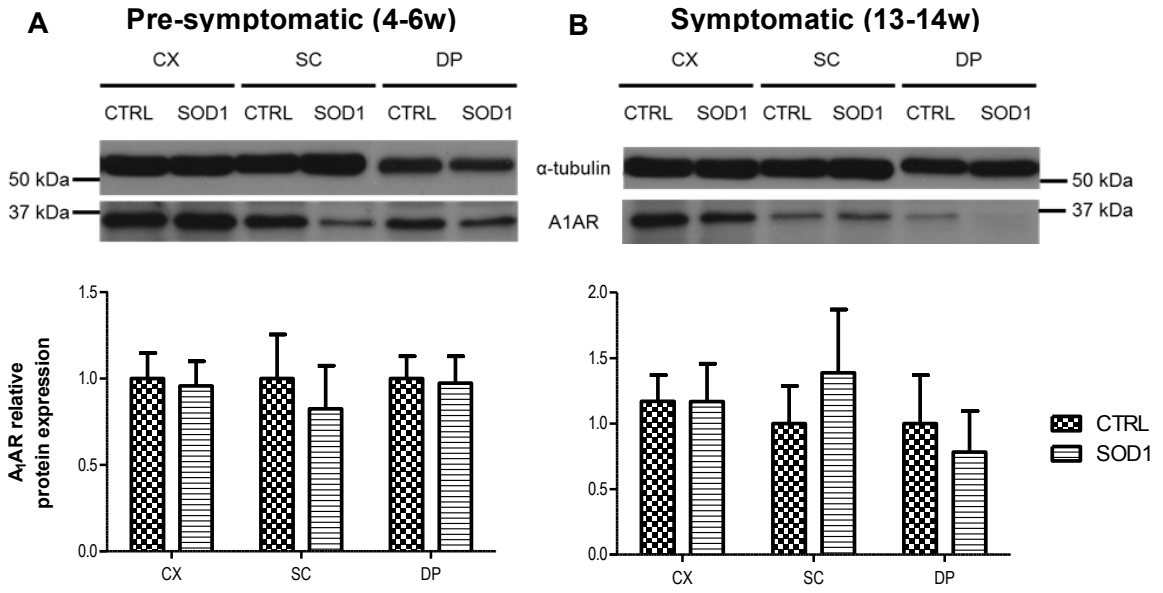

Figure 8 | Immunoblot analysis of the expression levels of A_1 adenosine receptor in control and hSOD1 mutants. (A) Immunoreactivity of A_1AR was performed in motor cortex, spinal cord and diaphragm homogenates of pre-symptomatic and (B) symptomatic animals. A_1AR runs at ~37 kDa in the western blot. As a loading control α-tubulin was used (~55 kDa). Graphs represents the mean quantification of A_1/tubulin intensity ratio of n=5. Error bars indicate the standard error and statistical comparison was performed by *t*-test. CTRL, wild-type control; SOD1, transgenic SOD1; CX, cortex; SC, spinal cord; DP, diaphragm.

With respect to $A_{2A}R$ protein expression in the pre-symptomatic animals (figure 9A) the CX shows an accentuated increase in SOD1 when compared to CTRL animals. However, this difference is not statistically significant (t-test, p=0,1560). For the other tissues, SC and DP, no significant are observed between SOD1 and CTRL animals (*t*-test, p=0,7083 and p=0,3724, respectively).

Concerning the symptomatic animals (figure 9B), once again, the CX has the highest difference, although not significant (*t*-test, p=0,3409) with SOD1 showing a decreased expression of $A_{2A}R$ in relation to CTRL mice. For the other tissues, SC and DP, no significant differences are observed between SOD1 and CTRL mice (p=0,6722 and p=0,3305, respectively), despite in both cases a minor decrease in $A_{2A}R$ protein expression is detected.

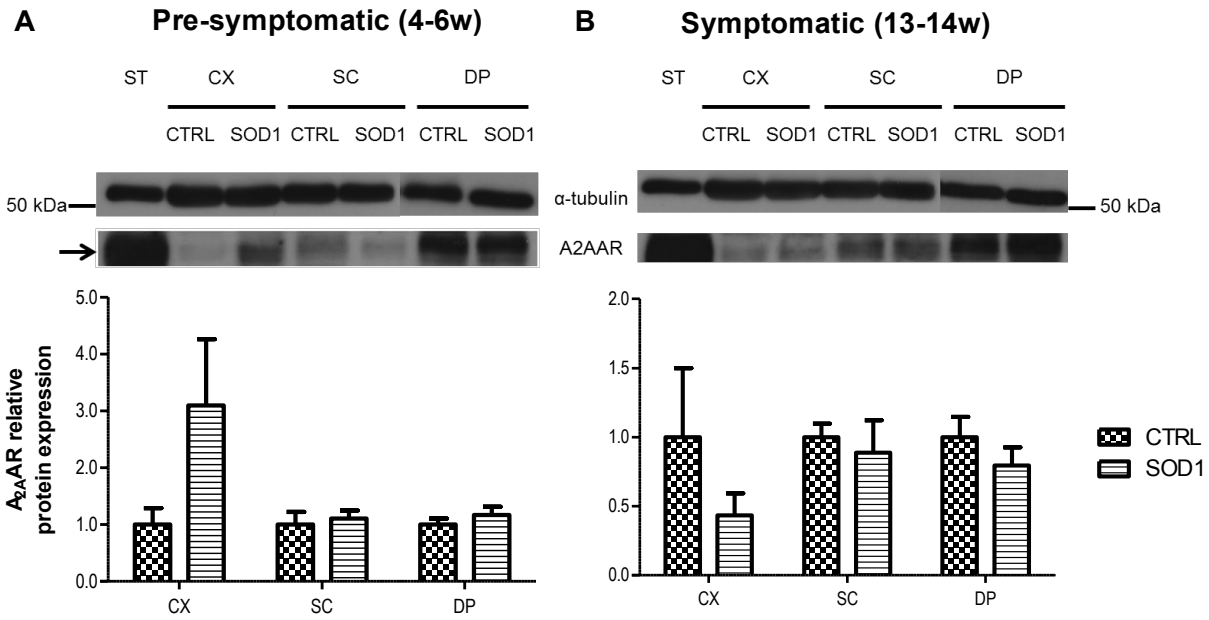

Figure 9 | Immunoblot analysis of the expression levels of A_{2A} adenosine receptor in control and hSOD1 mutants. (A) Immunoreactivity of $A_{2A}AR$ was performed in motor cortex, spinal cord and diaphragm homogenates of pre-symptomatic and (B) symptomatic animals. $A_{2A}AR$ runs at ~42 kDa in the western blot. As a loading control α-tubulin was used (~55 kDa). Graphs represents the mean quantification of A_{2A}/tubulin intensity ratio of n=5. Error bars indicate the standard error and statistical comparison was performed by *t*-test. CTRL, wild-type control; SOD1, transgenic SOD1; ST, striatum; CX, cortex; SC, spinal cord; DP, diaphragm.

Quantification of A_1 and A_{2A} receptor mRNA levels

The A_1R mRNA expression in the diaphragm (figure 10) of pre-symptomatic SOD1 animals is 1,2 in relation to CTRL which reflects no significant difference (*t*-test, p= 0,9138). For the symptomatic phase. SOD1 animals present a 1,6 fold change in A_1 mRNA expression, but this increase in SOD1 mice is not statistically significant (*t*-test, p=0,4012). Furthermore, although not significant (*t*-test, p=0,3900), it is possible to observe that, A_1R mRNA expression slightly increases in SOD1 mice through disease progression.

Regarding $A_{2A}R$ mRNA expression in pre-symptomatic animals, it is possible to see a fold change of 1,0. This indicates that SOD1 are expressing $A_{2A}R$ as CTRL mice, so the differences at this phase of the disease are not significant (*t*-test, p=0,7713). For the symptomatic phase SOD1 mice show no significant difference (*t*-test, p=0,3638) in adenosine $A_{2A}R$ mRNA expression, with a fold change of 0,9. Moreover, there is no significant alteration (*t*-test, p=0,6205) of $A_{2A}R$ mRNA expression through disease progression.

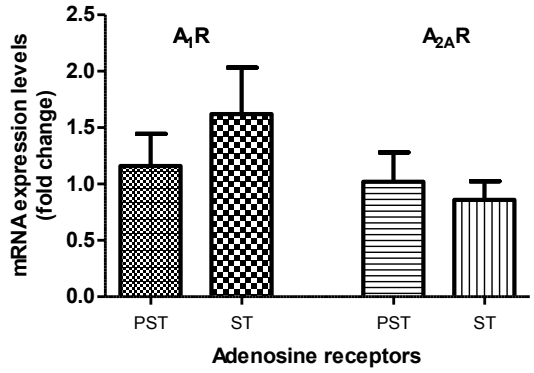

Figure 10 | Quantitative RT-PCR analysis of the expression levels of A_1 and A_{2A} adenosine receptor in diaphragm for hSOD1 mutants throughout disease. mRNA of adenosine receptors expression was performed in whole diaphragm homogenates of pre-symptomatic and symptomatic animals. As an endogenous control β-actin was used. Graph represents the fold change (FC) values of normalized A_1 and A_{2A}/β-actin intensity ratio of n=5. Fold change values were calculated according to Pfaffl equation. If fold change value is equal to 1 (FC=1), it means that expression of SOD1 is the same as in CTRL animals. FC values higher (FC>1) than 1 mean a mRNA increased expression in SOD1 comparing to CTRL mice, and lower than 1 (<1) a decreased mRNA expression. Error bars indicate the standard error and statistical significance was performed by *t*-test analysis. PST, pre-symptomatic phase; ST, symptomatic phase.

Primary pathological feature, regarding the expression of adenosine receptors

Results from this study (figure 11) indicate unaltered A_1 receptor protein levels at the CNS and PNS through disease progression. However, there is an overexpression (not statistically significant) of A_{2A} receptors in the cortex of pre-symptomatic mice and a decrease in the symptomatic phase. The A_{2A} receptors are unaltered in the other tissues in both phases of the disease. The mRNA evaluation does not reveal significant alterations in both adenosine receptors during disease progression. Thus, regarding adenosine receptors in ALS, the first changes seem to occur early in the disease at the CNS in A_{2A} receptors

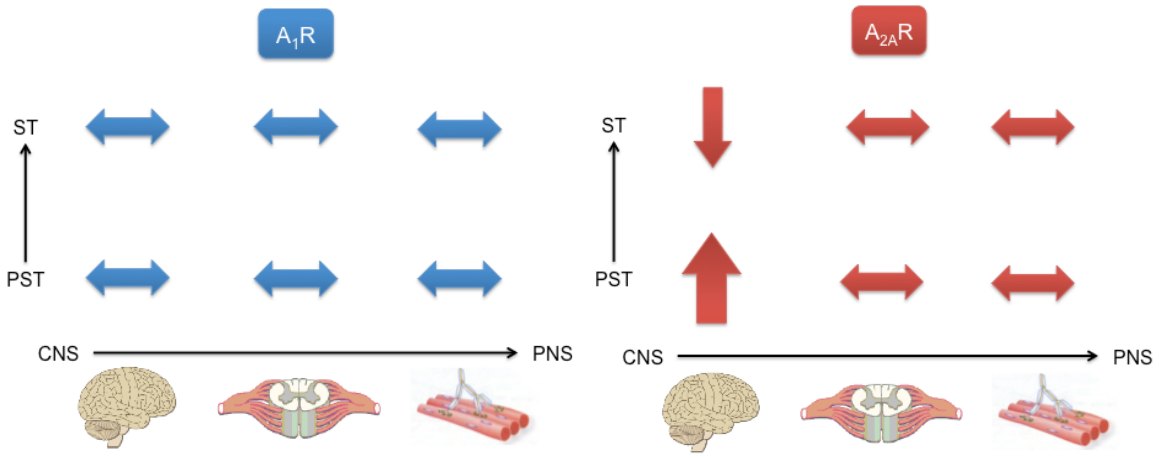

Figure 11 | Schematic representation A_1 and A_{2A} adenosine receptor variation in the CNS and PNS of ALS SOD1^{G93A} transgenic mice throughout disease progression. PST, pre-symptomatic phase; ST, symptomatic phase.

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DISCUSSION

Amyotrophic Lateral Sclerosis related research offered only one drug with very modest disease modifying results. Then, it is urgent to identify specific biomarkers for the disease diagnostic and treatment targets. In this scenario, extracellular purines are powerful physiopathological molecules, signaling to most cell types and directing cell-to-cell communication networks. Their role has increasingly been recognized in several neurodegenerative and neuroinflammatory conditions such as ischemia, neuropatic pain, multiple sclerosis, Parkinson's disease, Alzheimer's disease and Huntington's disease (Amadio *et al.*, 2011). However, the involvement of adenosine receptors in ALS is still poorly understood.

The results from this work show that A_1 adenosine receptor protein levels are not altered in this particular model of ALS, at specific periods of the disease time course (pre-symptomatic and early symptomatic). These results are consistent across all tested tissues from the CNS and PNS, *i.e*. motor cortex, spinal cord and phrenic nerve-diaphragm. A first suggestion immediately rises that the G93A mutation, in SOD1 gene, is not affecting any key pathways of A_1 receptors expression like transcription, translation, protein migration, membrane integration and others. Contrary to our findings in ALS mice, Albasanz *et al.,* (2007), observed that A_1 receptor protein levels were increased in the cortex of Alzheimer´s disease patients. Therefore, it would be interesting, in the future, to evaluate if the function of these receptors is conserved in ALS. The registered oscillations (that are not statistically significant) can be explained by animals intrinsic variability or due to the number of replicas used.

The $A_{2A}R$ protein expression is increased in the cortex of pre-symptomatic animals, but not in the other tissues (spinal cord and diaphragm). Up-regulation of A_{2A} receptors was also found in the frontal cortex of Pick's and Alzheimer's disease patients (Albasanz *et al.,* 2006, 2007). Recent findings indicate a potential crosstalk between TNF receptors and A_{2A} receptors in Parkinson´s Disease (Varani *et al.*, 2010). According to these authors, high levels of TNF increase the expression of A_{2A} receptors. TNF is related to neuroinflammation and neuronal damage and it is produced in higher levels by adult $hSOD1^{G93A}$ microglial cells (Boilléee *et al.*, 2006). In the symptomatic phase, the results for the cortex of transgenic mice point to a down regulation of A_{2A} receptors. However, these are unchanged in the spinal cord and diaphragm of these animals. Once again, the SOD1 mutation is not affecting receptor expression or transport, but it is not possible to know with this work if they are functionally active.

Analysis of A_1R and A_{2A} mRNA expression in the diaphragm also show no differences in both phases of the disease, which is in accordance with the protein expression profile

obtained for the diaphragm adenosine receptors. Alterations in the mRNA expression of adenosine receptors in the phrenic nerve are more critical to detect because transcription takes place at the C3-C5 spinal segments, which contain most of phrenic motor neurons somas. mRNA transcripts are then transported through the long axons by active transport through cellular cytoskeleton to the neuromuscular junction. Either mRNA and protein expression results are from total diaphragm homogenates, which means that we can be overanalyzing adenosine receptors in muscles and vasculature expression more than in phrenic nerve terminals. But, although some authors (Dixon *et al.*, 1996; Lynge & Hellsten, 2000) report that human and rat skeletal muscle express both mRNA and protein of adenosine receptors, their levels are low, especially in what concerns the A_1 receptor.

Results from our group (unpublished data) indicate that A_1 and A_{2A} receptor mRNA levels are also unaltered in the spinal cord of transgenic mice during disease progression. In the cortex, although the mRNA expression of A_1 receptors is unchanged the mRNA levels of A_{2A} receptors is significantly increased in the pre-symptomatic phase. These findings are in accordance with the protein level results obtained in this work for both adenosine receptors through disease progression. Interestingly, in their study on Alzheimer´s disease, Albasanz *et al*. (2007) found that the increased protein levels of both adenosine receptors were not associated with an increase in the mRNA levels.

Taken together, results from this study and from our laboratory (unpublished data) indicate no alterations of A_1 receptor protein or mRNA levels in $SOD1^{G93A}$ mice both in CNS and PNS during ALS progression. A_1 receptor has long been known to mediate neuroprotection, mostly by blockade of calcium influx, which results in inhibition of glutamate release and reduction of its excitatory effects at a postsynaptic level (Dunwiddie & Masino, 2001; Ribeiro *et al*., 2003; Fredholm *et al.*, 2005; Stone, 2005). One of the pathological features of ALS is an excessive influx of Ca^{2+} during repetitive firing of glutamate receptors on motor neurons due to the loss of the EAAT2 glutamate transporter especially at the symptomatic phase (Boillée *et al*., 2006; Turner & Talbot, 2008). The unchanged levels of A_1 receptors point to a deficient neuroprotection by A_1 receptors at this stage of the disease. This may be because the A_1 receptors neuroprotective role is outweighed by the excitotoxicity caused by glutamate release. Another possible explanation is that, although the A_1 receptors expression is unaltered their function to modulate synaptic transmission may be compromised. However, the role of A_{2A} receptor in neuroprotection is not so clear, probably because of its lack of abundance in brain regions other than the striatum (Albasanz *et al.,* 2008). Data from the present study and from our laboratory (unpublished data) show that A_{2A} receptor protein or mRNA levels in $SOD1^{G93A}$ mice are up-regulated in the cortex (CNS) but not in the spinal cord or phrenic nerve-diagphram (CNS/PNS) through ALS progression.

Adenosine A_{2A} receptors can modulate the release of several neurotransmitters, such as acetylcholine, glutamate, and noradrenaline. The release of GABA might be either enhanced or inhibited by A_{2A} receptor activation. Additional functions of these receptors include modulation of neuronal excitability, synaptic plasticity, as well as locomotor activity and behavior. The ability of A_{2A} receptors to interact with other receptors such as adenosine A_1 receptors, CGRP receptors, metabotropic glutamate receptors and nicotinic autofacilitatory receptors, increases the range of possibilities used by adenosine to interfere with neuronal function and communication (Sebastião & Ribeiro, 1996). Blocking A_{2A} receptor function using antagonists or deleting the A_{2A} receptor gene results in a decrease in the extent of neuronal damage in adult animals (Albasanz *et al*., 2006). Beneficial effects evoked by A_{2A} receptor antagonists may be caused by blockade of presynaptic A_{2A} receptors which are stimulatory on glutamate release (Abbracchio & Cattabeni, 1999). However, the mechanisms behind this neuroprotection remain unknown (Cunha, 2005).

Considering our results, that A_{2A} receptors have an up-regulated protein expression in the cortex of ALS SOD1 transgenic mice and that they are associated with neuronal damage, an increase in A_1 receptor levels was expected as a neuroprotective compensatory mechanism of the system against A_{2A} receptor effects, but this was not the case here. In fact, in their study on Alzheimer´s disease, Albasanz *et al*. (2007) found increased protein levels of both adenosine receptors connected with a functional increase in A_1 and A_{2A} receptor-mediated response, but not with an mRNA expression increase. Processes associated with adenosine receptors functionality, like post-transcriptional and translational modifications, are beyond the scope of this work and cannot be predicted, *i.e.* even when the expression mechanisms are not altered further research is needed to unravel the adenosine receptors capacity to modulate synaptic transmission. However, some authors (*e.g*. Volonté *et al*., 2011) have identified some SOD1 caused impaired molecular features such as aberrant axonal transport, cytoskeleton alterations, mitochondrial dysfunction, protein misfolding and aggregation and altered RNA metabolism that could compromise functional processes related to adenosine receptors neuromodulation.

In conclusion, the major findings of this study indicate unaltered A_1 receptor protein levels at the CNS and PNS through disease progression. However, there is an overexpression of A_{2A} receptors in the cortex of pre-symptomatic mice and a decrease in the symptomatic phase, being unchanged in the other tissues at both phases of the disease. Thus, regarding adenosine receptors in ALS, the first changes seem to occur early in the disease at the CNS in A_{2A} receptors. Since A_{2A} receptor high expression can be found only in the cortex of ALS mice, I hypothesize that this work supports the dying forward theory which proposes that ALS originates primarily in the motor neurons of the cortex, *i.e.* it starts in the CNS and progressively affects the PNS.

REFERENCES

Abbracchio M.P. & Cattabeni, F. Brain adenosine receptors as targets for therapeutic intervention in neurodegenerative diseases. Ann NY Acad Sci. 1999; 890: 79–92.

Albasanz, J.L., Rodriguez, A., Ferrer, I. & Martin, M. Adenosine A2A receptors are up-regulated in Pick's disease frontal cortex. Brain Pathol. 2006; 16: 249–255.

Amadio, S., Apolloni, S., D'Ambrosi, N., & Volonté, C. Purinergic signalling at the plasma membrane: A multipurpose and multidirectional mode to deal with amyotrophic lateral sclerosis and multiple sclerosis. J Neurochem 2011; 116: 796–805.

Andersen, P.M. & Al-Chalabi, A. Clinical genetics of amyotrophic lateral sclerosis: What do we really know? Nature Reviews, Neurology. Advance online publication. 2011; 1-13.

Arosio, B., Mastronardi, L., Gussago, C., Nicolini, P., Casè, A., Ziglioli, E. & Bergamaschini, L. Adenosine A2A Receptor and IL-10 in Peripheral Blood Mononuclear Cells of Patients with Mild Cognitive Impairment. International Journal of Alzheimer's Disease. 2011; Volume 2011: Article ID 484021, 6 pages.

Atkin, J.D.; Farg, M.A.; Turner, B.J.; Tomas, D.; Lysaght, J.A.; Nunan, J.; Rembach, A.; Nagley, P.; Beart, P.M.; Cheema, S.S.; et al. Induction of the unfolded protein response in familial amyotrophic lateral sclerosis and association of protein-disulphide isomerase with superoxide dismutase 1. J. Biol. Chem. 2006, 281, 30152–30165.

Atkin, J.D.; Farg, M.A.; Walker, A.K.; McLean, D.; Tomas, D.; Horne, M.K. Endoplasmic reticulum stress and induction of the unfolded protein response in human sporadic amyotrophic lateral sclerosis. Neurobiol. Dis. 2008, 30, 400–407.

Avraham, K.B., Schickler, M., Sapoznikov, D., Yarom, R. & Groner, Y. Down's syndrome: abnormal neuromuscular junction in tongue of transgenic mice with elevated levels of human Cu/Zn-superoxide dismutase. Cell 1988; 54: 823–829.

Avraham, K.B., Sugarman, H., Rotshenker, S. & Groner, Y. Down's syndrome: morphological remodelling and increased complexity in the neuromuscular junction of transgenic CuZn-superoxide dismutase mice. J. Neurocytol. 1991; 20: 208–215.

Barbeito, L.H.; Pehar, M.; Cassina, P.; Vargas, M.R.; Peluffo, H.; Viera, L.; Estevez, A.G.; Beckman, J.S. A role for astrocytes in motor neuron loss in amyotrophic lateral sclerosis. Brain Res. Brain Res. Rev. 2004, 47, 263–274.

Barja, G. Mitochondrial oxygen radical generation and leak: sites of production in state 4 and 3, organ specificity, and relation to aging and longevity. Journal of Bioenergetics and Membranes. 1999; 31(4): 347-366.

Baxter, R.L., Vega-Riveroll, L.J. Deuchars, J. & Parson, S.H. A_{2A} adenosine receptors are located on pre-synaptic motor nerve terminals in the mouse. Synapse. 2005; 57: 229-234.

Beal, M.F. Less stress, longer life. Nature Medicine.2005; 11(6): 598-599.

Bensimon, G., Lacomblez, L. & Meininger, V. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. N. Engl. J. Med. 1994; 330: 585–591.

Blackburn, D., Sargsyan, S., Monk, P. N. & Shaw, P. J. Astrocyte function and role in motor neuron disease: a future therapeutic target? Glia 2009; 57: 1251–1264.

Boilléee, S., Velde, C.V. & Cleveland, D.W. ALS: A disease of motor neurons and their nonneuronal neighbors. Neuron. 2006; 52:39-59.

Browne, S.E., Yang, L., DiMauro, J-P., Fuller, S.W., Licata, S.C. & Beal, M.F. Bioenergetic abnormalities in discrete cerebral motor npathways presage spinal cord pathology in the G93A SOD1 mouse model of ALS. Neurobiology of Disease, 2006; 22: 599-610.

Burnstock, G. A basis for distinguishing two types of purinergic receptors. In: R.W. Straub & L. Bolis (eds) Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. 1978; pp. 107–118. Raven Press, New York.

Burnstock, G. Purinergic signalling and disorders of the central nervous system. Nat Rev Drug Discov 2008a; 7: 575–590.

Burnstock, G. Unresolved issues and controversies in purinergic signalling. J. Physiol 2008b; 586: 3307–3312.

Cassina, P.; Cassina, A.; Pehar, M.; Castellanos, R.; Gandelman, M.; de Leon, A.; Robinson, K.M.; Mason, R. P.; Beckman, J. S.; Barbeito, L.; et al. Mitochondrial dysfunction in SOD1G93Abearing astrocytes promotes motor neuron degeneration: prevention by mitochondrial-targeted antioxidants. J. Neurosci. 2008, 28: 4115–4122.

Chang, Y. et al. Messenger RNA oxidation occurs early in disease pathogenesis and promotes motor neuron degeneration in ALS. PLoS ONE 2008; 3: e2849.

Charcot, J-M. & Joffroy, A. Deux cas d'atrophie musculaire progressive avec lésions de la substance grise et de faisceaux antérolatéraux de la moelle épinière. Arch Physiol Norm Pathol 1869; 1:354–367; 2:628–649; 3:744–757.

Charcot J-M. Les Leçons du Mardi: Policlinique. Paris: Bureaux du Progrès Médical; 1887.

Charcot, J-M. Sclérose des cordons latéraux de la moelle épiniere chez une femme hystérique atteinte de contracture permanente des quatre membres. Bull Soc Med (Paris) 1865; p24–35

Charcot, J-M. Sclérose latérale amyotrophique. In: Oeuvres Complètes. Paris: Bureaux du Progrès Mèdical; 1874; Vol 2, p249–266. (In English: Amyotrophic lateral sclerosis: symptomatology.In: Sigerson G, translator. Lectures on diseases of the nervous system. London: New Sydenham Society; 1881; p192–204).

Ciruela, F., Casadó, V., Rodrigues, R.J., Luján, R., Burgueno, J., Canals, M., Borycz, J., Rebola, N., Goldberg, S.R., Mallol, J., Cortés, A., Canela, E.I., López-Giménez, J.F., Milligan, G., Lluis, C., Cunha, R.A., Ferré S. & Franco, R. Pre-synaptic control of striatal glutamatergic neurotransmission by adenosine A1–A2A receptor heteromers. J Neurosci 2006; 26: 2080–2087.

Clement, A.M.; Nguyen, M.D.; Roberts, E.A.; Garcia, M.L.; Boillee, S.; Rule, M.; McMahon, A.P. Doucette, W.; Siwek, D.; Ferrante, R.J.; et al. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. Science 2003, 302: 113–117.

Cleveland, D.W. & Rothstein, J.D. From Charcot to Lou Gehrig: Deciphering selective motor neuron death in ALS. Nature Reviews 2001; 2:806-819.

Corona, J.C.; Tovar-y-Romo, L.B.; Tapia, R. Glutamate excitotoxicity and therapeutic targets for amyotrophic lateral sclerosis. Expert Opin. Ther. Targets 2007, 11, 1415–1428.

Correia-de-Sá , P., Sebastião, A.M. & Ribeiro, J.A. Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve ending of the rat. Br J Pharmacol 1991; 103: 1614–1620.

Costa, A. & Antunes, L. Handbook of laboratory animals: mice, rats and rabbits. Série didáctica Ciências Aplicadas 419. Vila Real, 2011

Cunha, R.A. Neuroprotection by adenosine in the brain: from A1 receptor activation to A2A receptor blockade. Purinergic Signal. 2005; 1: 111–134.

Cunha, R.A., Johansson, B., van der Ploeg, I., Sebastião, A.M., Ribeiro, J.A. & Fredholm, B.B. Evidence for functionally important adenosine A2a receptors in the rat hippocampus. Brain Res. 1994; 649: 208–216.

Cunha, R.A., Vizi, E.S., Ribeiro, J.A. & Sebastião, A.M. Preferential release of ATP and its extracellular catabolism as a source of adenosine upon high- but not low frequency stimulation of rat hippocampal slices. J Neurochem 1996; 67: 2180–2187.

Dal Canto, M.C., Gurney, M.E. Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing wild type human SOD: a model of familial amyotrophic lateral sclerosis (FALS). Brain Res. 1995; 676: 25–40.

Dion, P.A., Daoud, H. & Rouleau, G.A. Genetics of motor neuron disorders: new insights into pathogenic mechanisms. Nature Reviews, Genetics. 2009; 10: 769-782.

Dixon, A.K., Gubtiz, A.K., Sirinathsinghji, D.J.S., Richardson, P.J. & Freeman, T.C. Tissue distribution of adenosine receptor mRNAs in the rat. British Journal of Pharmacology. 1996; 118: 1461-1468.

Dobrowolny, G., Aucello, M., Rizzuto, E., Beccafico, S., Mammucari, C., Bonconpagni, S., Belia, S., Wannenes, F., Nicoletti, C., Prete, Z.D., Rosenthal, N., Molinaro, M., Protasi, F., Fanò, Sandri, M. & Musarò A. Skeletal Muscle is a primary target of SOD1 G93A-mediated toxicity. Cell Metabolism. 2008; 8: 425-436.

Duffy, L.M. *et a*l. The role of mitochondria in the pathogenesis of amyotrophic lateral sclerosis. Neuropathol. Appl. Neurobiol. 2011; 37: 336–352.

Dunwiddie T.V., Diao L., Kim H.O., Jiang J.L. & Jacobson K.A. Activation of hippocampal adenosine A3 receptors produces a desensitization of A1 receptor-mediated responses in rat hippocampus. J Neurosci 1997; 17: 607–614.

Dunwiddie, T.V. & Masino, S.A. The role and regulation of adenosine in the central nervous system. Ann. Rev. Neurosci. 2001; 24: 31–55.

Eisen, A. & Weber, M. The Motor Cortex and Amyotrophic Lateral Sclerosis. Muscle Nerve 2011; 24: 564–573.

Epstein, C.J., Avraham, K.B., Lovett, M., Smith, S., Elroy-Stein, O., Rotman, G., Bry, C. & Groner, Y. Transgenic mice with increased Cu/Znsuperoxide dismutase activity: animal model of dosage effects in Down syndrome. Proc. Natl. Acad. Sci. U.S.A. 1987; 84: 8044–8048.

Fagerlund M.J. & Eriksson, L.I. Currents concepts in neuromuscular transmission. British Journal of Anaesthesia 2009; 103:108-114.

Feoktistov, I. & Biaggioni, I. Adenosine A2B receptors. Pharmacol Rev. 1997 Dec; 49(4): 381-402.

Ferraiuolo, L., Kirby, J., Grierson, A.J., Sendtner, M. and Shaw, P.J. Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. Nature Reviews 2011; 7:616-630.

Fisher, L.R., Culver, D.G., Tennant, P., Davis, A.A., Wang, M., Castellano-Sanchez, A., Khan, J., Polak, M.A. & Glass, J.D. Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. Experimental Neurology 2004; 185: 232-240.

Fitzmaurice, P. S. et al. Evidence for DNA damage in amyotrophic lateral sclerosis. Muscle Nerve 1996; 19: 797–798.

Flood, D.G., Reaume, A.G., Gruner, J.A., Hoffman, E.K., Hirsch, J.D., Lin, Y.G., Dorfman, K.S. & Scott, R.W. Hindlimb motor neurons require Cu/Zn superoxide dismutase for maintenance of neuromuscular junctions. Am. J. Pathol. 1999; 155: 663–672.

Dean Fraga, D., Meulia, T. & Fenster, S. Current Protocols Essential Laboratory Techniques. Real-Time PCR. John Wiley and Sons, Inc. 2008.

Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P., & Williams, M. Nomenclature and classification of purinoceptors. Pharmacol Rev 1994; 46:143–156.

Fredholm, B.B., Chen, J.F., Cunha, R.A., Svenningsson, P. & Vaugeois, J.M. Adenosine and brain function. Int Rev Neurobiol. 2005; 63:191–270.

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K-N. & Linden, J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol. Rev.2001; 53: 527–552.

Gajdusek, D.C.; Salazar, A.M. Amyotrophic lateral sclerosis and parkinsonian syndromes in high incidence among the Auyu and Jakai people of West New Guinea. Neurology 1982; 32, 107-126.

Goetz, C.G. Amyotrophic lateral sclerosis: early contributions of Jean-Martin Charcot. Muscle Nerve. 2000; 23:336-343.

Goetz, C.G., Bonduelle, M. & Gelfand, T. Charcot. Constructing Neurology. Oxford, England: Oxford University Press; 1995.

Guo, H.; Lai, L.; Butchbach, M.E.; Stockinger, M.P.; Shan, X.; Bishop, G.A.; Lin, C.L. Increased expression of the glial glutamate transporter EAAT2 modulates excitotoxicity and delays the onset but not the outcome of ALS in mice. Hum. Mol. Genet 2003, 12: 2519–2532.

Gurney, M.E., Pu, H., Chiu, A.Y., Dal Canto, M.C., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y.W., Deng, H.X., Chen,W., Zhai, P., Sufit, R.L., Siddique, T., Motor neuron degeneration in mice that express a human Cu,Zn Superoxide Dismutase Mutation. Science 1994; 264:1772-1775.

Henkel, J.S.; Beers, D.R.; Siklos, L.; Appel, S.H. The chemokine MCP-1 and the dendritic and myeloid cells it attracts are increased in the mSOD1 mouse model of ALS. Mol. Cell. Neurosci. 2006, 31: 427–437.

Hespel, P. & Richter, E.A. Role of adenosine in regulation of carbohydrate metabolism in containing muscle. Adv. Exp. Med. Biol. 1998; 441: 97-106.

Ho, Y.S., Gargano, M., Cao, J., Bronson, R.T., Heimler, I. & Hutz, R.J. Reduced fertility in female mice lacking copper-zinc superoxide dismutase. J. Biol. Chem. 1998; 273: 7765–7769.

Hughes, B.W., Kusner, L.L. & Kaminski, J. Molecular architecture of the neuromuscular junction. Muscle & Nerve 2006; 33: 445-461.

Jaarsma, D., Haasdijk, E.D., Grashorn, J.A., Hawkins, R., van Duijn, W., Verspaget, H.W., London, J. & Holstege, J.C. Human Cu/Zn superoxide dismutase (SOD1) overexpression in mice causes mitochondrial vacuolization, axonal degeneration, and premature motoneuron death and accelerates motoneuron disease in mice expressing a familial amyotrophic lateral sclerosis mutant SOD1. Neurobiol. Dis. 2000; 7: 623–643.

Kandel, E., Schwartz, J. & Jessell, T. Principles of Neural Science. 4th edition McGraw-Hill. 2000.

Kanekura, K., Suzuki, H., Aiso, S. & Matsuoka, M. ER stress and unfolded protein response in amyotrophic lateral sclerosis. Mol. Neurobiol. 2009; 39: 81–89.

Khakh, B.S. & Burnstock, G. The double life of ATP. Sci. Am. 2009 301(6): 84-92.

Kiernan, M.C., Vucic, S., Cheah, B.C., Turner, M.R., Eisen, A., Hardiman, O., Burrell, J.R. & Zoing, M.C. Amyotrophic lateral sclerosis. Seminar. The Lancet 2011; 377:942-955.

Kikuchi, H.; Almer, G.; Yamashita, S.; Guegan, C.; Nagai, M.; Xu, Z.; Sosunov, A.A.; McKhann, G.M., 2nd; Przedborski, S. Spinal cord endoplasmic reticulum stress associated with a microsomal accumulation of mutant superoxide dismutase-1 in an ALS model. Proc. Natl. Acad. Sci. USA 2006, 103, 6025–6030.

Kimura K. Studies of amyotrophic lateral sclerosis in the Kozagawa district in the Kii Peninsula. Jpn. Wakayama Med J. 1965; 9: 177 – 92.

Kull, B., Svenningsson, P. & Fredholm, B.B. Adenosine A_{2A} receptors are colocalized with and activate Golf in rat striatum. Mol. Pharmacol. 2000; 58: 771–777.

Leitner, M., Menzies, S. & Lutz, C. Working with ALS mice: Guidelines for preclinical testing & colony management. The Jackson Laboratory 2009.

Linden, J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. Annu. Rev. Pharmacol. Toxicol. 2001; 41: 775–787.

Lopes LV, Cunha RA, Ribeiro JA (1999) Cross talk between A_1 and A_{2A} adenosine receptors in the hippocampus and cortex of young adult and old rats. J Neurophysiol 1999; 82: 3196–3203.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., "Protein Measurement with the Folin Phenol Reagent," *Journal of Biological Chemistry,*1951, 193: 265-275.

Lynge, J. & Hellsten, Y. Distribution of adenosine A_1, A_{2A} and A_{2B} receptors in human skeletal muscle. Acta physiol. Scand. 2000; 169:283-290.

MacDonald, R.L., Skerritt, J.H. & Werz, M.A. Adenosine agonists reduce voltage-dependent calcium conductance of mouse sensory neurones in cell culture. J Physiol. 1986 Jan; 370: 75-90.

Macek T.A., Schaffhauser H. & Conn P.J. Protein kinase C and A3 adenosine receptor activation inhibit pre-synaptic metabotropic glutamate receptor (mGluR) function and uncouple mGluRs from GTP-binding proteins. J Neurosci 1998; 18: 6138–6146.

Marieb, E.N. & Hoehn, K. Human anatomy and physiology. 7th Edition Pearson, Benjamin Cummings. 2007.

Marcuzzo, S., Zucca, I., Mastropietro, A., Rosbo, N.K., Cavalcante, P., Tartari, S., Bonanno, S., Preite, L., Mantegazza, R. & Bernasconi, P. Hind limb muscle atrophy precedes cerebral neuronal degeneration in G93A-SOD1 mouse model of amyotrophic lateral sclerosis: a longitudinal MRI study. Experimental Neurology 2011; 231: 30-37.

Martyn, J.A.J., Fagerlund, M.J. & Eriksson, L.I. Basic principles of neuromuscular transmission. Anaesthesia 2009; 64:1-9.

Mattiazzi, M. et al. Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. J. Biol. Chem. 2002; 277: 29626-29633.

McLean, A. Densitometry of western blots using Image J software. Bennett Lab (NRL): Revised: 2011 03 12.

Meininger, V. ALS, what new 144 years after Charcot? Archives Italiennes de Biologie. 2011; 149: 29-37.

Miller, L. Densitometry of western blots using Image J software. November 2010.

Muller, F.L., Song, W., Liu, Y., Chaudhuri, A., Pieke-Dahl, S., Strong, R., Huang, T.T., Epstein, C.J., Roberts II, L.J., Csete, M., Faulkner, J.A. & Van Remmen, H. Absence of Cu,Zn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. Free Radic. Biol. Med. 2006; 40: 1993–2004.

Neusch, C.; Bahr, M.; Schneider-Gold, C. Glia cells in amyotrophic lateral sclerosis: new clues to understanding an old disease? Muscle Nerve 2007, 35, 712–724.

Nicholls, D.G. & Ferguson, S.J. Bioenergetics 3. 2002. Academic Press, Elsevier Imprint.

Pasinelli, P. & Brown, R.H. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nature Reviews 2006; 7:710-723.

Pfaffl, M. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 2001; 29:2002-2007.

Pousinha, P.A., Diogenes, M.J., Ribeiro, J.A. & Sebastião, A.M. Triggering of BDNF facilitatory action on neuromuscular transmission by adenosine A2A receptors. Neurosci Lett 2006; 404: 143–147.

Pun, S., Santos, A.F., Saxena, S., Xu, L. & Caroni, P. Selective vulnerability and runing of phasic motorneuron axons in motorneuron disease alleviated by CNTF. Nat. Neuroscience 2006; 9: 408-419.

Rando, T.A., Crowley, R.S., Carlson, E.J., Epstein, C.J. & Mohapatra, P.K. Overexpression of copper/zinc superoxide dismutase: a novel cause of murine muscular dystrophy. Ann. Neurol. 1988; 44: 381–386.

Rao, S.D. & Weiss, J.H. Excitotoxic and oxidative cross-talk between motor neurons and glia in ALS pathogenesis. Trends Neurosci. 2004; 27: 17–23.

Reaume, A.G., Elliott, J.L., Hoffman, E.K., Kowall, N.W., Ferrante, R.J., Siwek, D.F.,Wilcox, H.M., Flood, D.G., Beal, M.F., Brown Jr., R.H., Scott, R.W. & Snider, W.D. Motor neurons in Cu/Zn superoxide dismutasedeficient mice develop normally but exhibit enhanced cell death after axonal injury. Nat. Genet 1996; 13: 43–47.

Rebola, N., Rodrigues, R.J., Lopes, L.V., Richardson, P.J., Oliveira, C.R. & Cunha, R.A. Adenosine A1 and A2A receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. Neuroscience. 2005b; 133(1): 79-83.

Rebola, N., Canas, P.M., Oliveira, C.R., Cunha, R.A. Different synaptic and subsynaptic localization of adenosine A2A receptors in the hippocampus and striatum of the rat. Neuroscience. 2005a; 132(4): 893-903.

Rebola N., Sebastião A.M., Mendonca A., Oliveira C.R., Ribeiro J.A. & Cunha R.A. Enhanced adenosine A2A receptor facilitation of synaptic transmission in the hippocampus of aged rats. J Neurophysiol 2003; 90: 1295–303.

Redler, R.L. & Dokholyan, N.V. The complex molecular biology of Amyotrophic Lateral Sclerosis (ALS). Progress in Molecular Biology and Translational Science. 2012; 107: 215-262.

Reed DM, Torres JM, Brody JA. Amyotrophic lateral sclerosis and parkinsonism-dementia on Guam, 1945–1972. II. Familial and genetic studies. Am J Epidemiol 1975;101: 302–10.

Ribeiro,J.A., Sebastião, A.M. & Mendonça, A. Adenosine receptors in the nervous system: pathophysiological implications. Progress in Neurobiology. 2003; 68: 377-392.

Ribeiro, J.A., Sebastiao, A.M., de Mendonca, A. Participation of adenosine receptors in neuroprotection. Drug News Perspect. 2003; 16: 80–86.

Ribeiro, J.A. & Sebastião, A.M. Modulation and metamodulation of synapses by adenosine. Acta Physiol 2010; 199:161-169.

Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., Rahmani, Z., Krizus, A., McKenna-Yasek, D., Cayabyab, A., Gaston, S.M., Berger, R., Tanzi, R.E., Halperin, J.J., Hertzfeldt, B., Van den Bergh, R., Hung,W.Y., Bird, T., Deng, G., Mulder, D.W., Smyth, C., Laing, N.G., Soriano, E., Pericak-Vance, M.A., Haines, J., Rouleau, G.A., Gusella, J.S., Horvitz, H.R. & Brown, R.H. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993; 362: 59–62.

Rothstein, J.D.; Van Kammen, M.; Levey, A.I.; Martin, L.J.; Kuncl, R.W. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. Ann. Neurol. 1995, 38, 73–84.

Rowland, L.P. How amyotrophic lateral sclerosis got its name. Arch neurol 2011; 58:512-515.

Sargsyan, S.A., Monk, P.N. & Shaw, P.J. Microglia as potential contributors to motor neuron injury in amyotrophic lateral sclerosis. Glia 2005; 51: 241–253.

Saxena, S.; Cabuy, E.; Caroni, P. A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice. Nat. Neurosci. 2009, 12, 627–636.

Sebastião, A.M. & Ribeiro, J.A. Adenosine A_2 receptor-mediated excitatory actions on the nervous system. Prog. Neurobiol. 1996; 48:167–189.

Sebastião, A.M. & Ribeiro, J.A. Adenosine receptors and the central nervous system. Handbook of Experimental Pharmacology. Springer-Verlag Berlin Heidelberg 2009; 193: 471-534.

Sebastião, A.M. & Ribeiro, J.A. Fine-tuning neuromodulation by adenosine. TiPS 2000; 21:341-346.

Sebastião, A.M. & Ribeiro, J.A. Interactions between adenosine and phorbol esters or lithium at the frog neuromuscular junction. Br J Pharmacol 1990; 100: 55–62.

Shaw, P. J., Ince, P. G., Falkous, G. & Mantle, D. Oxidative damage to protein in sporadic motor neuron disease spinal cord. Ann. Neurol. 1995; 38: 691–695.

Shefner, J.M., Reaume, A.G., Flood, D.G., Scott, R.W., Kowall, N.W., Ferrante, R.J., Siwek, D.F., Upton-Rice, M. & Brown Jr., R.H. Mice lacking cytosolic copper/zinc superoxide dismutase display a distinctive motor axonopathy. Neurology 1999; 53: 1239–1246.

Shibata, N. et al. Morphological evidence for lipid peroxidation and protein glycoxidation in spinal cords from sporadic amyotrophic lateral sclerosis patients. Brain Res. 2001; 917: 97–104.

Stone, T.W. Adenosine, neurodegeneration and neuroprotection. Neurol Res. 2005; 27: 161–168.

The ALS Association, 1275 K Street NW - Suite 1050 - Washington, DC 20005. (Website: http://www.alsa.org/) 2010.

Trussell, L.O. & Jackson, M.B. Adenosine-activated potassium conductance in cultured striatal neurons. Proc Natl Acad Sci U S A. 1985 Jul; 82(14): 4857-61.

Turner, B.J. & Talbot, K. Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. Progress in Neurobiology2008; 85:94-134.

Turrens, J. Superoxide Production by the mitochondrial respiratory chain. Bioscience Reports, 1997; 17(1): 3-8.

Van Den Bosch, L.; Robberecht, W. Crosstalk between astrocytes and motor neurons: what is the message? Exp. Neurol. 2008, 21:, 1–6.

Varani, K., Vincenzi, F., Tosi, A., Gessi, S., Casetta, I., Granieri, G., Fazio, P., Leung, E., MacLennan, S., Granieri, E. & Borea, P.A. A_{2A} adenosine receptor overexpression and functionality, as well as TNF-alpha levels, correlate with motor symptoms in Parkinson's disease. FASEB J. 2010 Feb; 24(2): 587-98.

Volonté, C., Apolloni, S., Carrì, M. & D'Ambrosi, N. ALS: Focus on purinergic signalling. Pharmacology & Therapeutics 2011; 132: 111-122.

Walker, A.K.; Farg, M.A.; Bye, C.R.; McLean, C.A.; Horne, M.K.; Atkin, J.D. Protein disulphide isomerase protects against protein aggregation and is S-nitrosylated in amyotrophic lateral sclerosis. Brain 2010, 133, 105–116.

Wong, M. & Martin, L.J. Skeletal muscle-restricted expression of human SOD1 causes motor neuron degeneration in transgenic mice. Human Molecular Genetics, 2010; 1-19.

Wood, J.D., Beaujeux, T.P. & Shaw, P.J. Protein aggregation in motor neurone disorders. Neuropathol. Appl. Neurobiol. 2003; 29: 529–545.

Zang, D.W. & Cheema, S.S. Degeneration of corticospinal and bulbospinal systems in the superoxide dismutase 1 G93A G1H transgenic mouse model of familial amyotrophic lateral sclerosis. Neuroscience Letters 2002; 332: 99–102.

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ANNEXES

Annexe I

Table 4 | Thermocycler PCR conditions for genotyping protocol.

Step	Temperature ºC	Time	Number of cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	
Annealing	62	30 sec	29
Extension	72	30 sec	
Final Extension	72	10 min	1
Storage	4	forever	-

Table 5 | Thermocycler cDNA sysnthesis protocol. (*) Add 2x reaction mix + Superscript II RT.
RT - reverse transcriptase.

Step	Temperature, ºC	Time	Number of cycles
RNA denaturation	65	5 min	1
Pause (*)	4	2 min	1
RNAaseOUT step	25	10	1
Reverse transcription	42	50 min	1
RT inactivation	70	15 min	1
RNaseH	37	20 min	1
Storage	4	forever	-

Table 6 | Rotorgene thermocycler conditions for qRT-PCR.

Step	Temperature, ºC	Time	Number of cycles
AmpliTaq Gold DNA Polymerase activation	95	10 min	1
Denaturation	95	15 sec	
Annealing/Extension/Reading Cycling A Green	60	25 sec	45
Melting curve (72-95ºC)	72	90 sec	1
	each degree to 95	5 sec	1

Annexe II

Table 7 | Primers used in this study for genotyping and qRT-PCR. **(*)** β-actin primers kindly provided by Tiago Outeiro laboratory.

Primer type	Sequence (5' to 3')	Assay
hSOD1 Forward	CAT CAG CCC TAA TCC ATC TGA	Genotyping
hSOD1 Reverse	CGC GAC TAA CAA TCA AAG TGA	
Internal positive ctrl Forward	CTA GGC CAC AGA ATT GAA AGA TCT	
Internal positive ctrl Reverse	GTA GGT GGA AAT TCT AGC ATC ATC C	
β-actin Forward (*)	CTC TCA GCT GTG GTG GTG AA	qRT-PCR
β-actin Reverse (*)	AGC CAT GTA CGT AGC CAT CC	
A_1 Forward	TCG GCT GGC TAC CAC CCC TTG	
A_1 Reverse	CCA GCA CCC AAG GTC ACA CCA AAG C	
A_{2A} Forward	ATT CCA CTC CGG TAC CGG TAC AAT GG	
$A2_A$ Reverse	AGT TGT TCC AGC CCA GCA T	

Annexe III

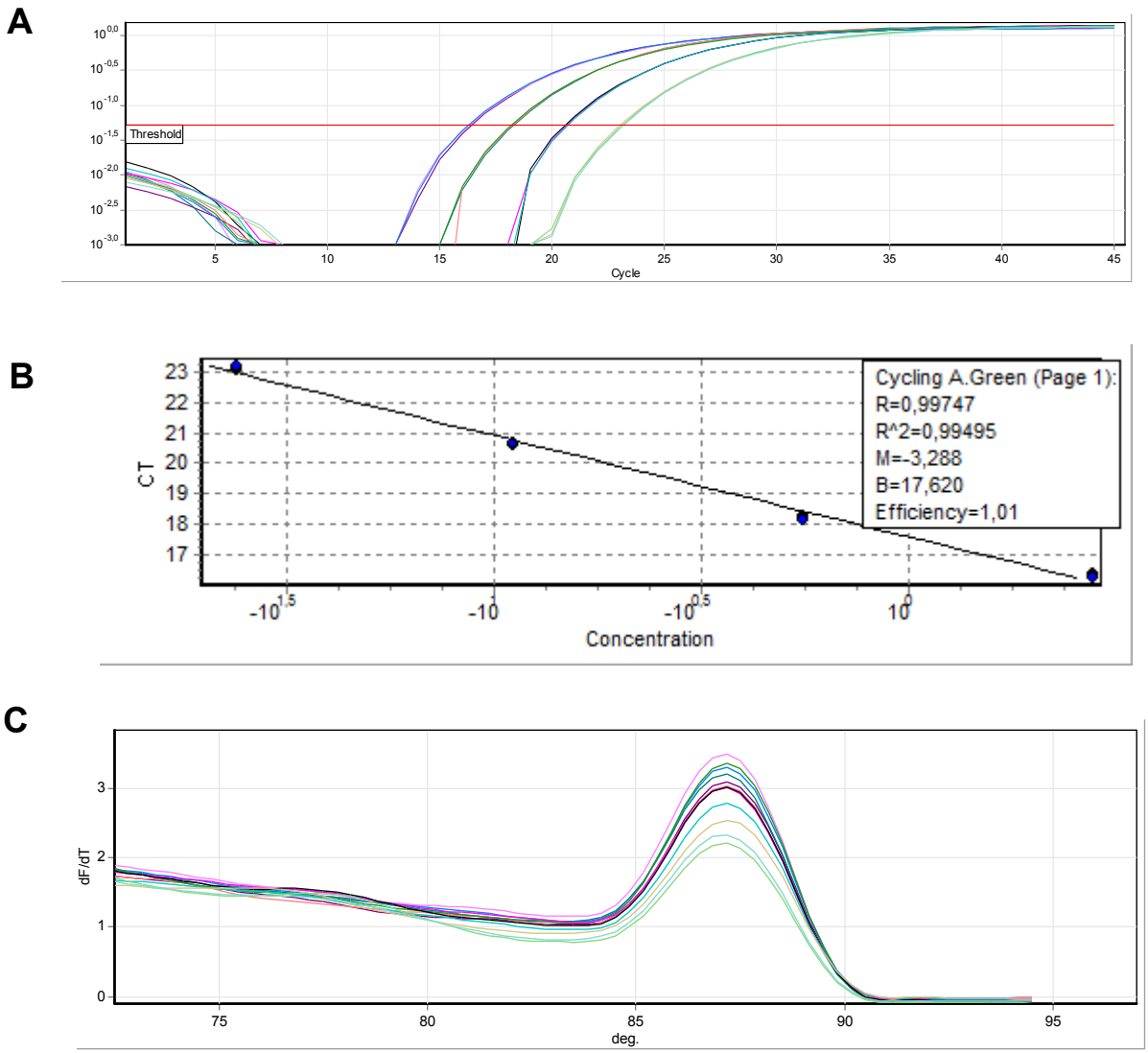

Figure 12 | qRT-PCR calibration curve and quality control using SYBR Green method for β-actin mRNA quantification. (A) Amplification performed in Rotorgene 6000 for triplicated serial dilutions of diaphragm (1:5; 1:25; 1:125, 1:625) regarding β-actin reference gene. Threshold was adjusted in both linear and logarithmic plots, to allow detection of product while it is still in the exponential phase, and finally set as showed for determination of C_t values. NTC and RT- controls show no amplification. **(B**) Calibration curve based on the C_t values extracted from previous analysis and plotted with an efficiency (E) of 1,01, slope (M) of -3,288 and a R^2 of 0,99. **(C)** Melting curve for β-actin primers shows a single peak, representative of a single species of DNA molecule in the reaction. Note that NTC and RT- controls did not show any amplification spikes (not shown). C_t, cycle threshold.

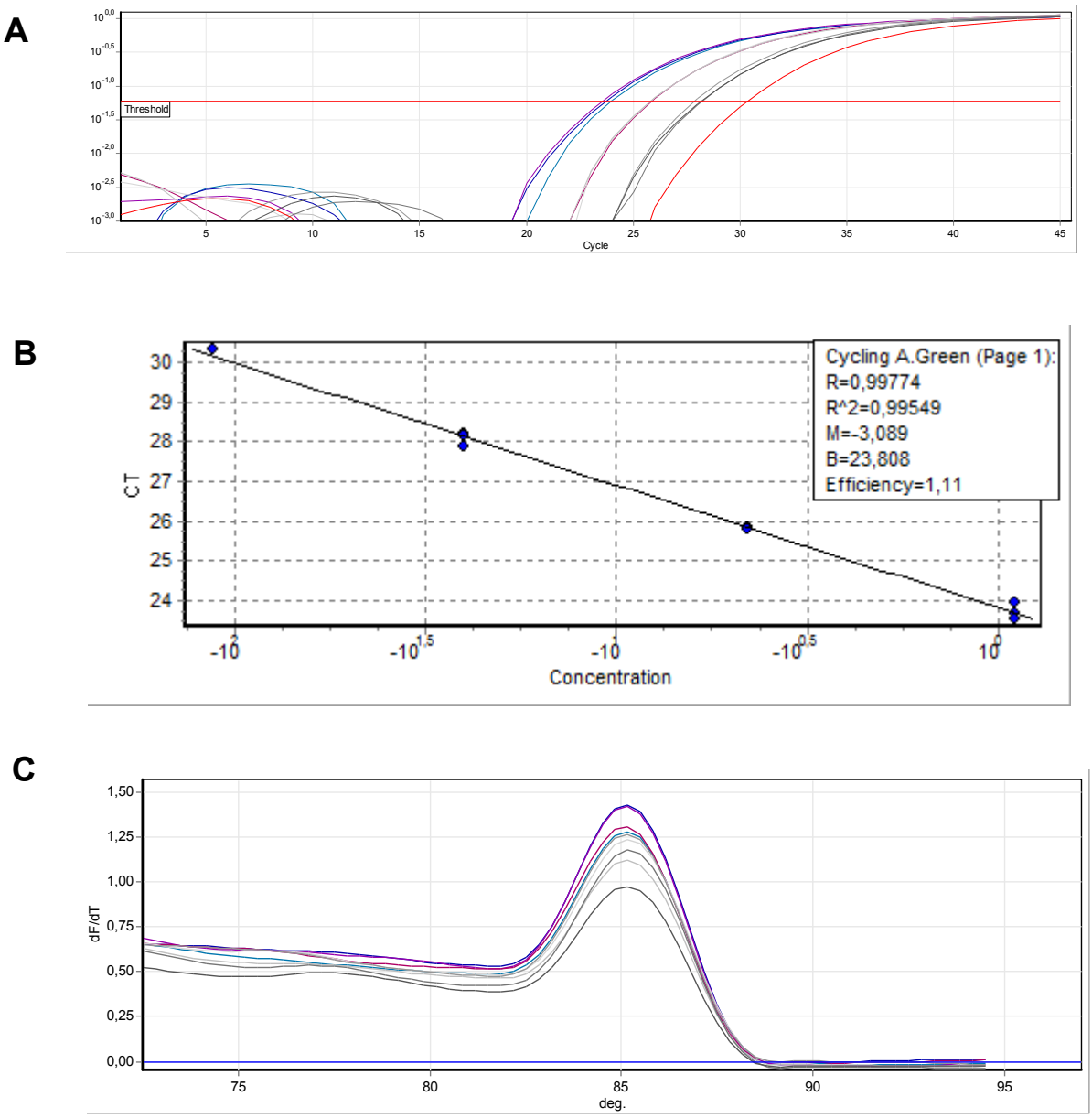

Figure 13 | qRT-PCR calibration curve and quality control using SYBR Green method for $A_{2A}R$ mRNA quantification. (A) Amplification performed in Rotorgene 6000 for triplicated serial dilutions of diaphragm (1:5; 1:25; 1:125, 1:625) regarding $A_{2A}R$ target gene. Threshold was adjusted in both linear and logarithmic plots, to allow detection of product while it is still in the exponential phase, and finally set as showed for determination of C_t values. NTC and RT- controls show no amplification. **(B**) Calibration curve based on the C_t values extracted from previous analysis and plotted with an efficiency (E) of 1,11, slope (M) of -3,089 and a R^2 of 0,99. **(C)** Melting curve for $A_{2A}R$ primers shows a single peak, representative of a single species of DNA molecule in the reaction. Note that NTC and RT- controls did not show any amplification spikes (not shown). C_t, cycle threshold.

Annexe IV

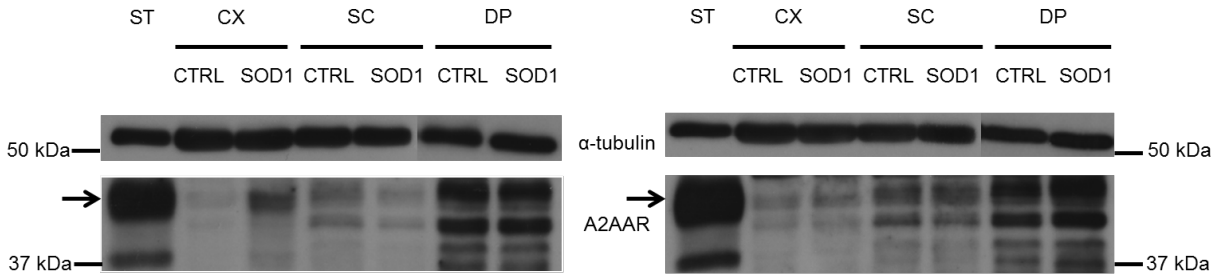

Figure 14 | Immunoblot analysis of the expression levels of A_{2A} adenosine receptor in control and hSOD1 mutants (complete gel image). This image shows the diffuse pattern of A_{2A}R protein bands in immunoblotting gels and consequently gives an idea of the optimization problems. (A) Immunoreactivity of A_{2A}AR was performed in motor cortex, spinal cord and diaphragm homogenates of pre-symptomatic and (B) symptomatic animals. A_{2A}AR runs at ~42 kDa in the western blot. As a loading control α-tubulin was used (~55 kDa). Graphs represents the mean quantification of A_{2A}/tubulin intensity ratio of n=5. Error bars indicate the standard error and statistical comparison was performed by *t*-test. CTRL, wild-type control; SOD1, transgenic SOD1; ST, striatum; CX, cortex; SC, spinal cord; DP, diaphragm.