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"The role of Peroxisome Proliferator Activated Receptors in Amyotrophic Lateral Sclerosis: potential mechanisms for neuroprotection"

Docente guida: Chiar.ma Prof.ssa Adriana Maggi

Coordinatore: Chiar.mo Prof. Alberto Panerai

Chiar.ma Prof.ssa Francesca Guidobono Cavalchini

Tesi di Dottorato di

Valeria Benedusi

Matricola R07856

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Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a chronic relentless neurodegenerative disorder also known as Lou Gehrig's disease or Charcot's sclerosis. It is the most common motor neuron disorder of the adult life, with an average onset at the age of 50-60 years. The incidence is uniformly scattered throughout the world and it is about 1-2 per 100.000 people, whilst the prevalence is 4-5 per 100.000 individuals. Males are slightly more commonly affected than females, with a ratio men to women of about 1,6/1. Affected people show the typical signs of both upper and lower motor neuron damage, i.e. clonus, hyperreflexia, spasticity, fasciculation, progressive muscle weakness, dysphagia. Symptoms progressively worsen till death, usually due to respiratory failure, within 1-5 years from the clinical onset (Bruijn et al., 2004; Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006; Strong, 2003; Valentine et al., 2005). The causes of this phenotype are the selective loss of cortical, brainstem and spinal cord motor neurons as well as the Wallerian degeneration of the descending axons with neurofilament accumulation. Dying neurons are characterized by the presence of protein aggregates including ubiquitinated inclusions, neurofilament-rich "hyaline conglomerated inclusions" and Bunina bodies, dense and refractile eosinophilic inclusions of lysosomal origin and cytoskeletal disorganization (Dal Canto and Gurney, 1995). These processes are often accompained by intense astrocytosis and microgliosis (McGeer and McGeer, 2002).

Some motor neurons are unaffected till very late stages of the disease, namely neurons of the Onuf's nucleus, deputed to control bladder evacuation, and those of the oculomotor nucleus (Strong et al., 2005).

Although ALS has been first described by the French physician Jean-Martin Charcot in the XIX century (Charcot, 1869), its aetiopathogenesis remains largely unknown. In the 90-95% of clinical cases, the disease occurs with no apparent genetic linkage (sporadic ALS, sALS), whilst in the remaining 5-10%, the disease is inherited (familial ALS, fALS). Importantly, sALS and fALS occurrences are indistinguishable in terms of both clinical signs and neuropathology .

Genetics of amyotrophic lateral sclerosis

Familial ALS is generally inherited as an autosomic dominant pathology, although a recessive form and an X-linked phenotype have been described. Extensive genetic analyses have been performed so far and have allowed the linkage between ALS and mutations in different genes and specific genetic loci.

Superoxide dismutase 1

The major breakthrough in the field was achieved by Rosen et al. (Rosen et al., 1993), who found that a number of families with fALS harbored mutations in the gene coding for the enzyme superoxide dismutase 1 (SOD1). As yet, more than 150 different ALS-associated point mutations in the sequence of the *SOD1* gene, located on chromosome 21q22.1, have been described (http://alsod.iop.kcl.ac.uk/Als/index.aspx Fig. 1a) and account for about 20% of fALS. SOD1 is an ubiquitously expressed enzyme of 153 amino acids. It is a metalloprotein, as it coordinates an atom of copper and one of zinc. The zinc allows the proper folding and stabilization of the protein, whilst the copper is necessary for its enzymatic activity. SOD1, indeed, acts as a homodimer to detoxify, through a 2-steps reaction, the superoxide anion produced during the cell metabolism. In particular, the catalytic copper is first reduced to Cu^{+} with oxygen formation and then it is oxidized to Cu^{2+} with the production of hydrogen peroxide (Fig. 1b).

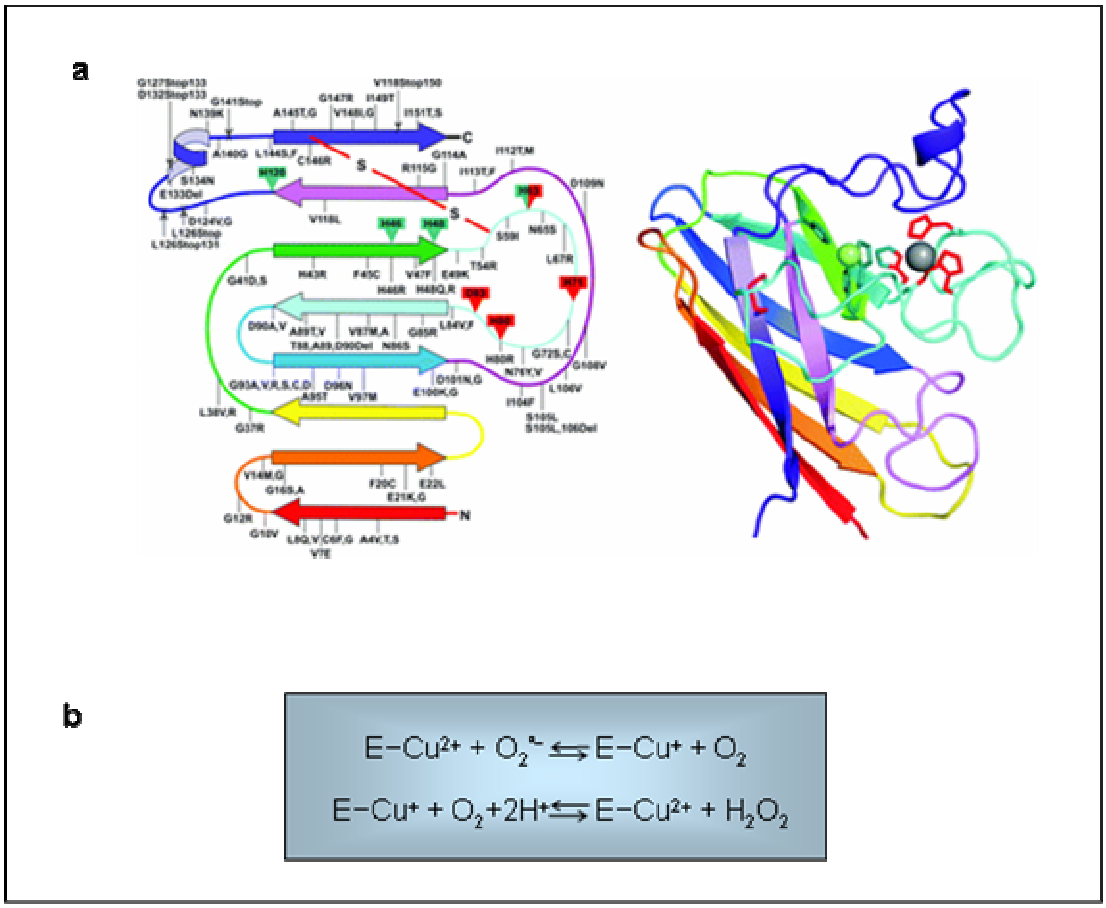

Fig.1 Human SOD1 structure and normal catalytic activity

(a) secondary structural representation of human SOD1 showing the locations of fALS-associated mutations (left) and a monomer of SOD1 (right) colored to match the drawing on the left (from (Selverstone Valentine et al., 2005)). (b) schematic representation of the two emi-reactions catalyzed by SOD1 (E = enzyme)

Most of the identified mutations generate amino acid substitution throughout the primary and the tertiary structure of the protein, whilst others give rise to truncated proteins. Generally, all these mutations confer an autosomal dominant phenotype with a 100% of penetrance, with some exceptions. Namely, SOD1-D90A[1] is usually dominant, although in Scandinavian populations it was reported to be recessive, implying that other genes may influence the clinical outcome. Therefore, different

[1] Mutations are conventionally expressed referring to the protein sequence, namely indicating the corrispondent aminoacid, its position and the aminoacid that substitutes it

mutations can give rise to phenotypic variations, such as different age of onset, disease progression or main affected district (Andersen, 2006).

The advent of transgenic mice allowed great advances in the field, including the confirmation of a causative relationship between the expression of mutant SOD1 (mtSOD1) and the disease. Of the more than 100 mutations in humans, 3 (SOD1-G85R, SOD1-G37R, and SOD1-G93A) have been extensively characterized in transgenic mouse models of ALS (Bruijn and Cleveland, 1996; Gurney et al., 1994; Ripps et al., 1995) (Wong et al., 1995). In these mice, the mutant human protein is ubiquitously expressed (under the control of the human or mouse SOD1 gene promoter) at levels equal to or several fold higher than the level of endogenous SOD1. Unlike the variable pattern in humans, weakness typically starts in the hind limbs between 3 and 12 months of age, depending on both the mutation and the level at which mtSOD1 is expressed. Hind limb weakness coincides with increased astrogliosis, activation of microglia, and loss of spinal cord motor neurons. Thus, pathology in these mice closely mimics many aspects of the human disease.

This amount of evidence and the fact that turning off mutant human SOD1 (hSOD1) expression in transgenic mice by *small interfering* RNAs (siRNAs) reversed the pathological phenotype, definitely confirmed a cause-effect relationship between mtSOD1 expression and ALS development (Ralph et al., 2005; Raoul et al., 2005; Saito et al., 2005; Wang et al., 2008; Xia et al., 2006).

Nevertheless, despite several years of intense research, it is still unclear as to how the mutations in the *SOD1* gene induce this disease. It was first hypothesized that mtSOD1s could lose their physiological activity, but this statement was proven untrue by studies performed on both human samples and transgenic animals. Indeed, dismutase activity level did not correlate with the age of disease onset or with the rapidity of progression in patients (Bowling et al., 1995; Cleveland et al., 1995). Moreover, transgenic mice expressing different mutant hSOD1s developed the pathological phenotype despite normal (Borchelt et al., 1994; Bruijn et al., 1997b 1995 #33) or even elevated (Gurney et al., 1994 1995 #27) enzymatic activity. Finally, genetic ablation of the *sod1* gene in transgenic mice neither caused motor neuron pathology nor affected the lifespan (Reaume et al., 1996). Therefore, mtSOD1 toxicity was later ascribed to a gain of an unknown function, rather than to

the loss of its enzymatic activity. The different pathogenetic hypotheses related to this issue will be discussed in details in the next sections.

Alsin

A juvenile form of ALS has also been described (Ben Amida et al., 1990). In these cases the disease is inherited as recessive trait and it is characterized by slow progression and a predominant upper motor neuron phenotype. In 2001, two groups independently identified the gene alsin in the locus 2q33 (*ALS2*) as the mutation site responsible for this type of pathology (Hadano et al., 2001; Yang et al., 2001). Since then, a total of 19 independent *ALS2* mutations from 17 families have been reported (Hadano et al., 2001; Hadano et al., 2007; Herzfeld et al., 2009; Mintchev et al., 2009; Shirakawa et al., 2009; Sztriha et al., 2008; Verschuuren-Bemelmans et al., 2008; Yang et al., 2001). These mutations are predicted to result in either premature termination of translation or substitution of an evolutionarily conserved amino acid in alsin, the *ALS2*-coded protein, leading to loss of its function. Alsin is a 184 KDa protein ubiquitously expressed, particularly in neurons, and localized to the cytoplasmic face of the endosomal membranes (Yamanaka et al., 2003). It contains three putative guanine nucleotide exchange factor (GEF) domains, involving Ras, Rab and Ran motifs. GTPases of the Ras subfamily regulate cellular signalling that couples extracellular signals to intracellular responses regulating vesicle transport and microtubule assembly. Although its function is not fully understood, it is known to act as an exchange factor for Rab5a in vitro (Otomo et al., 2003), which regulates endosomal trafficking and Rac1 activiy (Kanekura et al., 2004) and is involved in macropinocytosis-associated endosome trafficking and fusion (Kunita et al., 2007; Otomo et al., 2008) and neurite outgrowth (Jacquier et al., 2009; Jacquier et al., 2006; Otomo et al., 2008).

Because affected families displayed frameshift mutations that lead to aberrant proteins without detectable functional domains, it was initially postulated that this form of ALS might be simply due to the loss of alsin function. To verify this hypothesis, different lines of transgenic mice that do not express alsin were generated (Cai et al., 2005; Devon et al., 2006; Yamanaka et al., 2006). However, mice lacking *ALS2* do not recapitulate the complex disease phenotypes, despite

subclinical levels of motor dysfunction and axonal degeneration in aged animals (Cai et al., 2008; Hadano et al., 2006; Hadano et al., 2007). Thus, potentially important clinical implications raised from these observations, but the physiological functions of alsin and the molecular mechanisms underlying motor dysfunction resulting from *ALS2* deficiency remain to be clarified.

Dynactin

A variety of additional genes linked to other subtypes of ALS have been recently identified (reviewed in (Shaw, 2005)). In particular, a mutation in the gene encoding a subunit of dynactin was described in a family showing a phenotype characterized by early adulthood onset with respiratory difficulties (Puls et al., 2003). This finding corroborated previous studies in mice that develop motor neuron degeneration caused by disruption of normal dynactin/dynein function (LaMonte et al., 2002 2003 #247). As these proteins are deputed to the retrograde axonal transport of vesicle and organelles, this evidence highlighted the importance of this cellular function for proper motor neuron viability.

Senataxin

Missense mutations in *SETX* cause an autosomal dominant, juvenile onset motor neuron disease with distal muscle weakness and atrophy, normal sensation, pyramidal signs and a normal life-span. *SETX* encodes a 303 kDa DNA/RNA helicase domain with homology to human RENT1 and IGHMBP2 — two genes that encode proteins involved in RNA processing (Chen et al., 2004; Chen et al., 2006b). Altered RNA processing is implicated in two other inherited motor neuron diseases — i.e. spinal muscular atrophy (with mutations in the survival motor neuron gene) or a severe, infantile, distal spinal muscular atrophy with prominent respiratory dysfunction (SMARD; spinal muscular atrophy with respiratory distress, with mutations in IGHMBP2).

VAMP-associated protein B

The gene *VAPB* has six exons that encode a ubiquitously expressed 27.2 kDa homodimer, which belongs to a family of intracellular vesicle-

associated/membrane-bound proteins that are presumed to regulate vesicle transport.

Defects in this gene cause adult-onset, autosomal dominant ALS and atypical ALS (slowly progressive with tremors) but not frontotemporal dementia (for an example, see (Skibinski et al., 2005)).

TDP-43

TDP-43 (TAR DNA-binding Protein) is a 43 KDa protein encoded by the *TARDBP* gene located on chromosome 1 (Arai et al., 2006; Neumann et al., 2006). In normal conditions, TDP-43 is a nuclear protein which regulates transcription and RNA splicing, and participates in mRNA transport and stability (Moisse et al., 2009b; Strong et al., 2007) (Buratti and Baralle, 2008). Various missense mutations of TDP-43 gene have been identified (Gitcho et al., 2008; Sreedharan et al., 2008); (Kuhnlein et al., 2008; Rutherford et al., 2008); (Baumer et al., 2009; Corrado et al., 2008; Daoud et al., 2009; Del Bo et al., 2009; Kamada et al., 2009; Yokoseki et al., 2008); (Benajiba et al., 2009; Kirby et al., 2010; Xiong et al., 2010). Mutant TDP-43 could be involved in 3% familial ALS and 2.9% sporadic ALS (Sreedharan et al., 2008); (Lagier-Tourenne and Cleveland, 2009) (Daoud et al., 2009). The dysfunction of TDP-43 resulting from the mutation, which induced apoptosis and mislocalization of TDP-43, and caused shorter motor neuronal axons could also play an essential role in the pathogenesis of ALS (Sreedharan et al., 2008; Winton et al., 2008; Johnson et al., 2008; Kabashi et al., 2010; Chen-Plotkin et al., 2010). Many studies have shown that TDP-43 in tissues taken from cases of FTLD-TDP or ALS is often hyperphosphorylated, cleaved, ubiquitinated, mislocalized, and poorly soluble (Arai et al., 2006; Chen-Plotkin et al., 2010; Neumann et al., 2006) and TDP-43 aggregates are present in the cytosol and the nucleus of neuronal and glial cells of ALS patients, with the striking exception of patients with familial ALS caused by SOD1 mutations (Lagier-Tourenne and Cleveland, 2009). It is still not clear if these aggregates are a causative event in the pathogenesis of ALS or if they are a consequence of the pathological process but these features suggest various loss of function and gain of toxic functions for TDP-43 with a possible role in disease pathogenesis (Chen-Plotkin et al., 2010). In addition, upregulation of TDP-

43 expression with prominent cytosolic localization in motor neurons injured by degenerative processes such as ALS could represent an appropriate response to neuronal injury (Moisse et al., 2009a). The observation that the majority of inclusion-bearing cells in patients display nuclei devoid of TDP-43 led to the hypothesis that some of the deleterious effects of abnormal TDP-43 metabolism may reflect a loss of TDP-43 nuclear function (Igaz et al., 2008).

FUS/TLS

The identification of TDP-43 mutations in ALS patients was rapidly followed by the association of another RNA/DNA-binding protein, named FUS/TLS (fused in sarcoma/translocated in liposarcoma) with this disorder (Kwiatkowski et al., 2009; (Vance et al., 2009). Thirty mutations have now been reported in about 4% of familial ALS and in rare sporadic patients with no apparent familial history. The inheritance pattern is dominant except for one recessive mutation (H517Q) found in a family of Cape Verdean origin (Kwiatkowski et al., 2009). Most are missense mutations with a few exceptions.

Similar to TDP-43, FUS is ubiquitously expressed and it is mainly localized into the nucleus of both neuronal and glial cells (Aman et al., 1996; Andersson et al., 2008). However, post-mortem analysis of brain and spinal cord from patients carrying FUS/TLS mutations identified abnormal FUS/TLS cytoplasmic inclusions in neurons and glial cells (Belzil et al., 2009; Corrado et al., 2010; Kwiatkowski et al., 2009; Tateishi et al., 2009; Vance et al., 2009). These inclusions were reported to be immunoreactive for FUS/TLS as well as for other proteins, including GRP78/BiP, p62 and ubiquitin, but strikingly not for TDP-43, implying that neurodegenerative processes driven by FUS/TLS mutations are independent of TDP-43 mislocalization (Vance et al., 2009; (Suzuki et al., 2010; Tateishi et al., 2009).

The mechanisms that determine the FUS subcellular localization are not fully understood. Since the molecular weight of human FUS protein is 53,426 daltons and the cutoff for passive diffusion through the nuclear pore complex is estimated to vary between 40 and 60 kDa (Gerace, 1995; Peters, 2009; Rout et al., 2003), it is theoretically possible but likely difficult for FUS to migrate across the nuclear pore

complex by this route. In keeping, there is no classic nuclear localization sequence (NLS) reported or predicted in FUS and bioinformatic attempts revealed no classically predictable NLS (eg, PredictNLS server at cubic.bioc.columbia.edu/services/predictNLS; (Cokol et al., 2000)). However, it has been suggested that the C-terminus of FUS could work as NLS (Gal et al., 2010). In this context, it is interesting to note that most of the FUS mutations identified to date are clustered in the C-terminus of the protein (Kwiatkowski et al., 2009; Vance et al., 2009).

Similarities between the pathologies caused by TDP-43 and FUS mutants have been identified. Although the two proteins are not closely related, they share similar sequence elements, such as the RNA recognition motif (Ou et al.) domain and glycine-rich regions, which are common to RNA/DNA binding proteins (Lagier-Tourenne and Cleveland, 2009). Furthermore, similar to FUS, TDP-43 has been reported to play roles in RNA transcription, splicing, and transport (Buratti and Baralle, 2008; Volkening et al., 2009). Both proteins are normally mostly nuclear in neurons, and their molecular features in ALS encompass their mislocalization to the cytoplasm and the formation of protein inclusions (Arai et al., 2006; Kwiatkowski et al., 2009; Kwong et al., 2007; Neumann et al., 2006; Sreedharan et al., 2008; Vance et al., 2009).

In addition to the genes described above, several potential risk factors for ALS have also been identified. Among these, there are polymorphisms of the *neurofilament heavy chain* (NF-H), the *ciliary neurotrophic factor* (CNTF) and the *vascular endothelial growth factor* (VEGF) (Bruijn et al., 2004). Furthermore, Amyotrophic lateral sclerosis has also been associated to a mutation of the glial glutammate transporter EAAT2 (Trotti et al., 2001).

Pathogenesis of ALS

The discovery of mutations in the *SOD1* gene allowed the development of several mouse models of ALS. These transgenic animals represent the most useful tool to study the mechanisms underlying the pathology outcome. Since the clinical

presentation of fALS and sALS is indistinguishable, it is possible to hypothesize that, whatever is the triggering cause, the pathological process passes through common pathways. Thus, the disease mechanisms extrapolated from studies on mutant SOD1 transgenic mice may be of interest also for the sporadic form of the disease. Among the recognized pathogenetic hypotheses, there are oxidative stress, excitotoxicity, protein aggregates, mitochondrial damage, cytoskeletal disorganization, trophic factor deficiency, neuroinflammation and the detrimental actions of non neuronal cells.

Oxidative stress

Despite intense experimental efforts, it is still unclear as to how mtSOD1 can cause ALS. As described in the genetics section, SOD1 is an antioxidant enzyme whose toxicity seems to be due to the gain of a deleterious function.

On these bases, it was first hypothesized that the mutant protein could display anomalous activity, namely handling aberrant substrate, such as hydrogen peroxide (Wiedau-Pazos et al., 1996), the normal end-product of its catalytic activity. In this study, it was proposed that, *in vitro*, H_2O_2 could be transformed by mtSOD1 into the highly reactive hydroxyl radical, triggering a peroxidation cascade. However, the evidence of this phenomenon *in vivo* was not exhaustive because signs of peroxidation were found in hSOD1-G93A mice (Andrus et al., 1998; Hall et al., 1998a), but not in other models (Bruijn et al., 1997a).

Another proposed substrate for mtSOD1 was peroxynitrite, which is the end-product of the reaction between superoxide and nitric oxide (NO). Initially, it was suggested that the spontaneously formed peroxynitrite could be used by the mutant enzymes to cause protein nitration (Beckman et al., 1993). However, a more articulated hypothesis was later postulated, starting from the observation that some mtSOD1s bind zinc ions less efficiently (Crow et al., 1997) and that zinc depleted SOD1s induce NO-dependent cell death in cultured primary neurons (Estevez et al., 1999). It was therefore proposed that zinc deficiency, in the presence of bound copper, facilitates the reversal of the usual reaction, namely the conversion of oxygen into superoxide (Estevez et al., 1999). It is to mention that also the peroxynitrite-hypothesis was weakened by *in vivo* studies, mainly because the

pharmacological reduction of NO levels pharmacologically (Upton-Rice et al., 1999) or the disruption of the *n-nos* (Facchinetti et al., 1999) or *i-nos* genes (Son et al., 2001) did not delay disease onset or improve survival.

All the issues addressing the oxidative stress hypothesis have in common the requirement for the catalytic activity of SOD1, which relies on the presence of the copper ion in the active site. Therefore, it was speculated that disrupting copper loading *in vivo* could improve the pathological phenotype. To address this issue, two transgenic mouse models were generated. The first one was based on a previous finding, namely the discovery that in yeast SOD1 requires a specific chaperone, the copper chaperone for SOD1 (CCS) (Culotta et al., 1997), to load copper. Transgenic mice devoid of CCS were therefore generated and cross-bred with different transgenic mouse models of ALS (Subramaniam et al., 2002). Unfortunately, disease onset and progression was not modified, even if copper loading into SOD1 motor neurons was greatly reduced. The other model consisted in transgenic mice harboring a mtSOD1 in which all the histidines necessary to coordinate copper were replaced (Wang et al., 2003), but also in this case, the animals displayed an ALS-like phenotype.

A microarray study of motor neuronal cells expressing mutant SOD1 reported down-regulation of genes involved in the antioxidant response, including the transcription factor Nrf2 (nuclear erythroid 2-related factor 2), several members of the glutathione S-transferase family, and two peroxiredoxins (Kirby et al., 2005). Reduced Nrf2 messenger RNA (mRNA) and protein expression has more recently been reported in spinal cord neurons from ALS patients (Sarlette et al., 2008). Activation of Nrf2 leads to its translocation into the nucleus, where it interacts with the antioxidant-response element (ARE) to drive the expression of antioxidant enzymes (Nguyen et al., 2009); furthermore Nrf2 regulates also its own expression through an auto-regulatory loop (Kwak et al., 2002). Although it is currently unclear how Nrf2 expression comes to be down-regulated in ALS patients, it should be noted that cellular Nrf2 levels declined by over 50% in old rats (24 months) relative to young rats (3 months) (Suh et al., 2004). Given that increased age is one of the major known risk factors for developing ALS, it seems conceivable that the mechanism(s) involved in the age-related down-regulation of Nrf2 may also

contribute to the down-regulation observed in ALS patients. Down-regulation of Nrf2 expression in ALS may reduce the ability of the cells to remove ROS generated through normal cellular metabolism, resulting in a gradual increase in oxidative stress over time.

Excitotoxicity

Glutamate is the major excitatory amino-acid of the central nervous system (CNS). While normal glutamate levels mediate physiological neurotransmission, elevated concentrations trigger excitotoxicity, a form of neuronal cell death with its own features (reviewed in (Arundine and Tymianski, 2003)). In order to prevent this deleterious event, glutamate is normally cleared from the synaptic cleft by specific transporters, named EAAT1-5 (reviewed in (Danbolt, 2001)), expressed both on neurons and on the surrounding glia.

Several findings suggest a role for excitotoxicity in the pathogenesis of ALS, since studies on patients showed increased glutamate levels in cerebrospinal fluid (CSF) (Fiszman et al., 2010). This extracellular accumulation of neurotransmitter was proposed to be due to a reduced uptake of the amino acid and, therefore, expression and activity levels of glutamate transporters were then investigated.

Particular attention was focused on the astrocyte specific transporter EAAT2, which is responsible for about 90% of the extracellular glutamate removal (Miller et al., 1996, Tanaka, 1997 #49). Its presence is necessary for neuronal survival, as reducing EAAT2 expression levels by antisense nucleotide was described to cause neuronal demise (Rothstein et al., 1996). Interestingly, the selective loss of EAAT2 was reported in ALS patients (Rothstein et al., 1995) as well as in both transgenic mouse (Bendotti et al., 2001b; Bruijn et al., 1997b) or rat (Howland et al., 2002) models of the disease. Various mechanisms for EAAT2 loss have been proposed, from mRNA aberrant splicing (Lin et al., 1998), that was observed also in Alzheimer patients, to the recently proposed selective cleavage of this transporter by caspase-3 (Boston-Howes et al., 2006) and subsequent sumoylation of the EAAT2 fragment which accumulates into the nucleus of spinal cord astrocytes (Gibb et al., 2007). If this event is relevant to the pathogenetic cascade, increasing the expression levels of EAAT2 should improve the clinical course of the disease.

In keeping with this hypothesis, the overexpression of EAAT2 in hSOD1-G93A mice delayed the grip strength decline, even though paralysis onset and life span were not affected (Guo et al., 2003). Moreover, a wide screen of the US *Food and Drug Administration* (FDA)-approved drugs has been recently performed to identify clinically available molecules able to increase EAAT2 expression levels (Rothstein et al., 2005). Surprisingly, beta-lactam antibiotics have proven useful to reach this aim and their efficacy has been therefore tested *in vivo*. Ceftriaxone has been delivered to hSOD1-G93A transgenic mice and the treated mice displayed delayed paralysis onset and prolonged life-span, thus demonstrating that a pharmacological therapy based on increasing EAAT2 function may be neuroprotective (Rothstein et al., 2005). As antibiotics are clinically tested drugs, a clinical trial to evaluate the efficacy of ceftriaxone is currently ongoing.

A decrease in the transporter expression level is not the only possible cause of its functional impairment: mutations or post-transcriptional modifications may also alter its properties. According to this hypothesis, a mutation in the EAAT2 gene, leading to reduced activity, was recently identified in a patient with sporadic ALS (Trotti et al., 2001). Moreover, EAAT2 was demonstrated to be very sensitive to the oxidative stress generated by two endogenous oxidants implicated in ALS pathogenesis: H_2O_2 (Volterra et al., 1994) and peroxynitrite (Trotti et al., 1996). Taken together, these findings provide evidence that the oxidative stress and the excitotoxic hypothesis are interrelated and may be considered as two aspects of a unique pathogenic pathway.

It is well known that excitotoxicity can be triggered not only by increased extracellular glutamate concentrations, but also by alterations of the glutamate receptors that lead to an improved sensibility to the toxic action of the neurotransmitter. This could be the case of motor neurons in ALS. Indeed, these cells normally display a particular vulnerability to glutamate especially following the activation of AMPA receptors (Terro et al., 1998), a feature that may be further increased in ALS. In cell culture studies, mtSOD1 can increase glutamate toxicity acting by altering AMPA receptor subunit composition (Roy et al., 1998). Normally, AMPA receptors are Ca^{2+}-impermeable, as they display the properly edited GluR2 subunit, but may become Ca^{2+}-permeant upon defective editing of the

G/R site. Therefore the proper editing of GluR2 and the subunit composition are critic to prevent excessive Ca^{2+} intake and toxicity. In agreement with this proposal, samples from ALS patients displayed alteration in GluR2 editing especially in motor neurons (Kawahara et al., 2004; Kwak and Kawahara, 2005; Takuma et al., 1999). Furthermore an increase in GluR3 AMPA subunit expression in animal models of ALS has been demonstrated (Spalloni et al., 2004; Sun et al., 2006) leading to a reduction of the ratio between GluR2 and GluR3 which is likely to be harmful to neurons. Consistently, administrating GluR3 antisense protein nucleic acid (Rembach et al., 2004) or overexpressing GluR2 (Tateno et al., 2004) prolonged mice survival. Conversely, mice lacking the GluR2 subunit showed a reduced survival (Van Damme et al., 2005).

Finally, it is worth to cite that the only drug approved for ALS treatment is riluzole, which is an antiglutamatergic drug. This compound was able to prolong survival both in transgenic mice (Gurney et al., 1998) and in patients (Bensimon et al., 1994; Lacomblez et al., 1996; Miller et al., 1996).

Protein aggregates

The presence of abnormal protein aggregates is a common feature of different neurodegenerative disorders, namely prion diseases, Alzheimer's disease, Parkinson's disease, Huntington's chorea and even ALS. Their significance is still debated, as they could be either detrimental or beneficial (Soto, 2003). In ALS, the presence of different intracellular cytoplasmic inclusions is a typical hallmark of dying motor neurons (reviewed in (Strong et al., 2005)). Interestingly, in transgenic animals, hSOD1 aggregates were detected in both motor neurons and astrocytes (Dal Canto and Gurney, 1995) (Bruijn et al., 1998; Dal Canto and Gurney, 1995; Furukawa and O'Halloran, 2006; Gurney, 1994; Miller et al., 2004; Pasinelli et al., 2000). Furthermore, SOD1 aggregates were found also in ALS patients (Shibata et al., 1996; Shibata et al., 1994) that lack mutations in this enzyme (Forsberg et al., 2010). Mutant hSOD1 aggregates have been found in the brain on both the matrix and the cytoplasmic face of mitochondria (Cleveland and Liu, 2000; Manfredi and Xu, 2005; Pasinelli et al., 2004; Vande Velde et al., 2008b; Vijayvergiya et al., 2005). Since protein aggregates were detected before the onset of symptoms (Bruijn

et al., 1998) and selectively in ALS tissues (Durham et al., 1997), it is likely that protein misfolding could be part of mtSOD1 toxicity. Many hypotheses were formulated to explain this issue including the one suggesting that aggregates may sequester other proteins (Watanabe et al., 2001) or alter the proper function of cellular organelles, such as mitochondria (see below). Among the proteins detected in the aggregates, there were also ubiquitin and chaperone proteins. The former is normally responsible for proper intracellular proteolysis (Ciechanover, 2005) and its sequestration may lead to a reduced proteasome activity (Allen et al., 2003), which in turn alters the physiological protein turnover. The latter are a group of proteins that promote the correct folding of protein (Bukau et al., 2006). Some of them, the *heat-shock protein* 40 and 70 (HSP40 and HSP70), form insoluble aggregates with mtSOD1. Interestingly, reduction in the availability of HSP70 seems to mediate part of the toxicity induced by mtSOD1, as overexpression of the chaperone protein can ameliorate neuronal sufferance (Bruening et al., 1999). Furthermore, hSOD1-G93A transgenic mice treated with arimoclamol, a HSP70 inducer, displayed a prolonged life span (Kieran et al., 2004).

A recent work by Poletti and colleagues demonstrated that HspB8 is overexpressed in hSOD1-G93A NSC34 cells and in motor neurons of hSOD1-G93A mice. Their findings indicate that HspB8 increases mutant SOD1 solubility and clearance by activating the autophagic removal of the misfolded mutant SOD1 in NSC-34 cells.

Mitochondrial damage

Since mitochondrial morphological abnormalities were initially described in proximal axons as well as in the ventral horns of sALS patients (Hirano et al., 1984), several studies have been focused on this issue (reviewed in (Hervias et al., 2006)). Data obtained from autoptic samples did not provide conclusive evidence whether such alterations are causative or mere by-products of the degenerative mechanism. Therefore, further investigations to solve this question were performed both *in vitro* and in transgenic mouse models of fALS.

Mitochondrial depolarization, an indicator of respiratory chain dysfunction, and alterations in calcium homeostasis were observed in both neuroblastoma cells

transfected with the mtSOD1 (Carri et al., 1997) and primary neurons from hSOD1-G93A mice (Menzies et al., 2002).

Degenerating mitochondria were described in two different transgenic lines, namely those expressing either hSOD1-G93A (Dal Canto and Gurney, 1995) or hSOD1-G37R (Wong et al., 1995). Moreover, in hSOD1-G93A mice, the number of vacuolated mitochondria increased dramatically at the onset of the disease, when neuronal loss was still limited, suggesting that mitochondrial alterations may be a triggering event in the pathogenesis of fALS (Kong and Xu, 1998). Ultrastructural studies showed that vacuoles may derive from expansion of the intermembrane space (Bendotti et al., 2001a), with subsequent collapse and degradation of the inner membrane (Higgins et al., 2003; Xu et al., 2004).

Based on all these observations, the connection between mtSOD1 and degenerating mitochondria was investigated. Initially, the localization of both mutant and wild type SOD1 was determined in the mitochondria of mouse brain and spinal cord (Mattiazzi et al., 2002), particularly in motor neurons (Higgins et al., 2002; Jaarsma et al., 2001). Abnormal clustering of mitochondria was recently reported in motor axons in mutant SOD1 transgenic mice (Sotelo-Silveira et al., 2009). However, the fine ultrastructural SOD1 localization within the mitochondria is still debated and probably it may be affected by the techniques utilized in the experimental plan. Mattiazzi and colleagues first proposed that SOD1 concentrates in the intermembrane space of mitochondria (Mattiazzi et al., 2002), but other groups demonstrated the association of the mutant enzyme with the cytoplasmic face of the outer membrane. This may be mediated by the interaction with integral membrane components (Liu et al., 2004), such as the anti-apoptotic protein Bcl-2 (Pasinelli et al., 2004) or the cytoplasmic exposed face of voltage-dependent anion channel 1 (VDAC1) (Israelson et al., 2010). Also Vande Velde and colleagues recently demonstrated a strong binding of misfolded mtSOD1 to the cytoplasmic face of mitochondria (Vande Velde et al., 2008a).

Copper chaperone for SOD1 (CCS) is also partially localized in mitochondria (Sturtz et al., 2001) and assists in the entry and retention of SOD1 in mitochondria (Field et al., 2003). Increased localization of mutant SOD1 in mitochondria by CCS overexpression in the CCS/hSOD1-G93A double transgenic mice caused early

mitochondrial pathology and accelerated disease course (Son et al., 2007). The presence of SOD1 aggregates was detected also in the brain mitochondrial matrix (Vijayvergiya et al., 2005). Although mtSOD1 presence was demonstrated in other subcellular compartments in mice (Kikuchi et al., 2006), only the mitochondrial localization appeared to be sufficient to trigger cell death in neuroblastoma cells expressing mtSOD1 targeted to different organelles (Takeuchi et al., 2002).

The mechanisms underlying these abnormalities are still object of intense studies, because an exhaustive demonstration of the cause-effect relationship between mtSOD1, mitochondrial dysfunction and neurotoxicity has not yet been provided. However, a recent finding seems to corroborate the hypothesis that the mitochondrial localization might mediate neurotoxicity. Indeed, it was demonstrated in cell culture that dorfin overexpression is useful to reverse mtSOD1 toxicity (Niwa et al., 2002), by reducing its mitochondrial localization (Takeuchi et al., 2004). Dorfin is a RING finger-type E3 ubiquitin ligase and it is localized in inclusion bodies in both sALS and fALS. It seems that dorfin binds ubiquitin to mtSOD1, thereby enhancing its clearence (Niwa et al., 2002).

Morphological abnormalities are not the only reported mitochondrial alterations, but also different functional impairments were described. The role of mitochondria within the cell is twofold: they provide energy with the mitochondrial electron transport chain and they simultaneously compartmentalize several molecules that may trigger apoptosis if released in the cytosol. As both of these functions seem to be affected in ALS, the former will be discussed below, whilst the latter in the next section.

A reduction in enzymatic activity of the respiratory chain complexes (Jung et al., 2002; Mattiazzi et al., 2002 2005 #76) and a decreased capacity to consume oxygen and to synthesize ATP (Mattiazzi et al., 2002) were first demonstrated. Interestingly, the bioenergetic failure was then correlated to a reduction in mitochondrial calcium loading capacity (Damiano et al., 2006), thereby providing a link between alterations of these organelles and excitotoxicity (see above). Some of these effects (Damiano et al., 2006; Kirkinezos et al., 2005) were detected before the clinical onset of the disease. Elevated calcium can induce reactive oxygen species and oxidative stress in primary motor neurons isolated from hSOD1-G93A

mice (Kruman et al., 1999). Alternatively, calcium-mediated glutamate excitotoxicity might contribute to the mutant SOD1 toxicity in motor neurons.

Also the axonal transport of mitochondria is impaired in ALS; recent studies showed that both the anterograde and retrograde transport of mitochondria were altered by mtSOD1 in NSC-34 cells expressing mitochondria-targeted SOD1. In addition, mtSOD1 also caused mitochondrial fragmentation and impaired mitochondrial dynamics (Magrane et al., 2009; Magrane and Manfredi, 2009).

The mechanisms by which mtSOD1 disrupts axonal transport of mitochondria are not completely understood but several possible scenarios can be hypothesized. MtSOD1 can cause a partial loss of mitochondrial membrane potential and thus can change axonal transport of mitochondria (Miller and Sheetz, 2004); the elevated local calcium concentration induced by decreased mitochondrial buffering capability can promote detachment of kinesin heavy chain from microtubule (Chang et al., 2006; Wang and Schwarz, 2009; Yi et al., 2004). Furthermore, the activated kinases can phosphorylate kinesin subunits and affect anterograde transport (De Vos et al., 2000; Morfini et al., 2007; Morfini et al., 2009; Pigino et al., 2009; Shibata et al., 2008). Alternatively, activated MAP kinase can also phosphorylate neurofilaments and lead to their accumulation in axons (Ackerley et al., 2004; Tortarolo et al., 2003). Finally the aberrant interaction between mtSOD1 and the dineyn-dynactin complex could also disrupt retrograde transport of mitochondria (Strom et al., 2008; Zhang et al., 2007). The axonal tranport of mitochondria was shown to be impaired in either the anterograde direction (De Vos et al., 2007) or in both anterograde and retrograde directions (Magrane et al., 2009).

Therapeutics targeting mitochondrial functions were delivered to SOD1 transgenic mice. In particular, creatine, which improves the metabolic functions of the mitochondria, ameliorated the pathological phenotype, increasing the motor function (Klivenyi et al., 1999). Unfortunately, different clinical trials (Drory and Gross, 2002; Groeneveld et al., 2003; Shefner et al., 2004) failed to demonstrate any therapeutic effect of this drug on clinical course of human cases.

Cytoskeletal disorganization

The most abundant structural proteins in several types of motor neurons are neurofilaments, which are neuron-specific intermediate filaments appointed to the maintenance of the cell shape and axon diameter. The individual proteins are classified on the basis of their molecular mass into high- (NF-H), medium- (NF-M) and low- (NF-L) molecular weight neurofilaments. These subunits are assembled in the cell body and then transported down the axon by slow axonal transport with extensive phosphorylation during movement (Strong et al., 2005).

Several findings led to hypothesis that damage to neurofilaments might be involved in the pathogenesis of ALS. First, it was observed that neuronal loss affects especially the largest caliber and neurofilament-rich motor neurons, in both patients (Kawamura et al., 1981) and in transgenic mice (Bruijn et al., 1997b). Moreover, neurofilament accumulation in motor neuronal cell body is a hallmark of ALS pathology (Hirano et al., 1984). Finally, mutations in *NF-H* gene was recognized in both sALS and fALS patients (Al-Chalabi et al., 1999).

To further investigate the role of neurofilaments in the degenerative process, several transgenic mice harboring alterations in the filament subunits (reviewed in (Strong et al., 2005)) were generated. In some of these models, neurofilament overexpression was sufficient to cause axonopathy and selective motor neurons degeneration. Therefore, it was proposed that neurofilament subunit deletion in ALS models transgenic mice should be neuroprotective. According to this, eliminating NF-L in SOD1 transgenic mice significantly extended their lifespan (Williamson et al., 1998). Surprisingly, a positive effect on survival was achieved also by overexpressing NF-L and NF-H (Kong and Xu, 2000) or NF-H alone (Couillard-Despres et al., 1998). This apparent contradiction was explained by proponing a different role for the diverse subunits. NF-H was considered important to buffer toxic events in the perykarya and therefore its increase could afford further protection. On the contrary, NF-L slowed the slow axonal transport that is affected early in ALS (Williamson and Cleveland, 1999) and, consequently, its reduction may counteract this impairment.

Another intermediate filament that seems to be involved in ALS pathogenesis is peripherin, since it was detected in neurofilament aggregates in motor neurons of

ALS patients (Corbo and Hays, 1992). This protein is encoded by a single gene and possesses three splice variants, named on the basis of their molecular weight as peripherin 56-58-61. Both peripherin 58 overexpression and peripherin 61 expression seem to be toxic to motor neurons (Robertson et al., 2003).

In a recent study, a novel human peripherin transcript, resulting in a truncated peripherin (Per28), was found upregulated in a case of human ALS, and an antibody specific for Per28 stained the filamentous inclusions (Xiao et al., 2008). These studies suggest that missplicing of peripherin could lead to disease. Additional reports have also implicated mutations in peripherin with ALS (Gros-Louis et al., 2004; Leung et al., 2004). Several transgenic mice carrying alteration in peripherin gene were developed (reviewed in (Strong et al., 2005)) and displayed motor impairments. However, crossbreeding these lines with ALS models did not affect the course of the disease (Lariviere et al., 2003), suggesting that their actual role in ALS pathogenesis deserves further investigations.

Trophic factors deficiency

Trophic factors, sometimes referred to as neurotrophins, are a family of molecules that encourage the survival of the nervous tissue. As certain neuronal populations are strictly dependent on trophic factors supply for their survival, it was easily hypothesized that motor neurons demise in ALS might be due to their deficiency and several of them were investigated.

Initial attention was focused on *ciliary neurotrophic factor* (CNTF), as autoptic samples from ALS patients displayed a reduced level of this protein (Anand et al., 1995). Moreover, a mutation in the *CNTF* gene mutation has been related to a faster disease progression in a family carrying also a mutation in the *SOD1* gene (Giess et al., 2002). Despite these findings indicating an involvement of CNTF in ALS pathogenesis, other observations did not corroborate this hypothesis. In particular, lack of *CNTF* gene neither cause motor neuron disease in patients (Takahashi et al., 1994), nor modifies the clinical course of sALS or fALS carrying the SOD1-D90A mutation (Al-Chalabi et al., 2003).

More attention was given to other trophic factors, such as the *glial cell line-derived neurotrophic factor* (GDNF) and the *insulin-like growth factor-1* (IGF-1), as both

of them support motor neurons *in vivo* and *in vitro* (Elliott and Snider, 1996 1996 #253), perhaps activating different cell survival pathways (Bilak et al., 2001). On the basis of these considerations, these factors were delivered to transgenic mouse models of ALS in order to test their capability to slow down disease progression. Interestingly, hSOD1-G93A mice, which were injected with an adeno-associated virus (AAV) expressing IGF-1 (Kaspar et al., 2003), displayed a marked increase in their lifespan, even if the treatment was started after the onset of the disease. On the contrary, GDNF had only moderate effect. This discrepancy raised questions about the mechanism through which IGF-1 might exert its benefic effect and it was postulated that the real targets might not be motor neurons, but muscle fibers, as demonstrated by the neuroprotective effect of a non-releasable form of IGF-1 injected directly into skeletal muscle (Dobrowolny et al., 2005).

Surprisingly, another growth factor seems to be involved in motor neuron maintenance, namely the *vascular endothelial cell growth factor* (VEGF). Particular attention was focused on this protein as transgenic mice harboring a mutation in the hypoxia-responding element of the *VEGF* gene unexpectedly developed a progressive motor impairment (Oosthuyse et al., 2001). Moreover, these animals displayed all the hallmarks of ALS pathology, namely neurofilament accumulation in motor neurons, axonal degeneration and progressive muscle atrophy (Oosthuyse et al., 2001). Furthermore reduced levels of VEGF have been reported in the spinal cord of ALS patients (Brockington et al., 2006). This finding led to perform a clinical study to determine whether mutation in *VEGF* gene may be correlated to human cases of ALS. In the European population, sALS was associated with three polymorphisms in the promoter region of the gene, leading to reduced circulating levels of the protein. However, it is worth mentioning that none of these alterations was localized in the hypoxia responding element (Lambrechts et al., 2003). Therefore, VEGF may be only a disease modifier, whose effects on ALS pathogenesis are strictly dependent on other unknown genetical, enviromental or lifestyle factors, as the observed association does not exist in other groups, such as an English subpopulation (Lambrechts et al., 2003) or the North Americans (Chen et al., 2006a). Nevertheless, the role of VEGF as a neuroprotective tool was extensively investigated. Initial *in vitro* studies demonstrated that this trophic factor

is necessary for survival of motor neurons (Van Den Bosch et al., 2004). hSOD1-G93A mice crossbred with transgenic mice expressing reduced levels of VEGF died erlier due to more severe motor neuron degeneration (Lambrechts et al., 2003). Conversely hSOD1-G93A crossbred with mice overexpressing VEGF in neurons show delayed motor neuron loss, delayed motor impairment, and prolonged survival compared with SOD1-G93A single transgenics (Wang et al., 2007). hSOD1-G93A transgenic mice injected with a lentivirus encoding VEGF after the onset of symptoms showed delayed onset of paralysis, slower disease progression and increased lifespan (Azzouz et al., 2004).

Neuroinflammation

Neuroinflammation is a complex phenomenon characterized by the production of inflammatory mediators and by the activation of glial cells (reviewed in (McGeer and McGeer, 1995)), namely astrocytes and microglia, which undergo a morphological switch in response to CNS injuries. Though it is a shared feature of different neurological disorders, such as Alzheimer disease or Parkinson disease, it is still an incompletely understood manifestation and it is still debated whether it is beneficial or detrimental to the pathological process (Monk and Shaw, 2006).

Different groups detected increased levels of various inflammatory biochemical mediators, namely the inducible enzyme cycloxygenase 2 (COX2) or some cytokines, such as interleukin-1β or TNFα in ALS patients as well as in hSOD1-G93A transgenic mice (Alexianu et al., 2001; Elliott, 2001; Hensley et al., 2002; Yoshihara et al., 2002). Interestingly, in this mouse model, cytokine raise was reported at a presymptomatic age (Hensley et al., 2002).

As antiinflammatory compounds are among the most utilized drugs worldwide, some of these molecules were delivered to transgenic mice to test their capability to slow disease progression. In particular, a COX2 inhibitor, celecoxib, was orally administrated to hSOD1-G93A mice, thus obtaining a significant survival prolongation (Drachman et al., 2002). Unfortunately, despite this promising result in preclinical models, a recent clinical trial failed to demonstrate analogous usefulness to treat the human disease (Cudkowicz et al., 2006).

As previously mentioned, reactive gliosis is a typical ALS hallmark in both human cases (Kawamata et al., 1992) and transgenic animals (Bruijn et al., 1997b; Gurney et al., 1994; Wong et al., 1995). Furthermore, in hSOD1-G93A mice, microgliosis preceeded the onset of symptoms and progressively worsened in parallel to the development of motor function impairment (Alexianu et al., 2001; Hall et al., 1998b). This last observation gave rise to the possibility that other cell types besides motor neurons might be involved in ALS pathogenesis, a hypothesis that will be further discussed in the following paragraph.

Detrimental actions of non-neuronal cells

In the last few years, the contribution of non-neuronal cells to ALS pathogenesis has been first recognized, so that ALS is currently considered a non-cell autonomous disease. The exact contribution of each cellular population is however unidentified, although a potential pathogenetic role for both astrocytes (Barbeito et al., 2004) and microglia (Sargsyan et al., 2005) was postulated.

Physiologically, astrocytes exert several functions ranging from providing trophic and metabolic support to neurons, to modulating synaptic activity (reviewed in (Volterra and Meldolesi, 2005)). The hypothesis of astrocyte involvement in ALS pathogenesis is supported by several observations. As largely discussed in the excitotoxicity section, astrocytes regulate extracellular levels of glutamate through the action of their specific transporter EAAT2/GLT1, which is down regulated in ALS (see above for details). Moreover, mtSOD1 seems to induce directly cell damage in astrocytes, as observed in hSOD1-G85R transgenic mice, who display ubiquitin-positive mtSOD1 inclusions in these cells earlier than in neurons (Bruijn et al., 1997b).

To establish which is the actual cellular target of mtSOD1 toxicity, different transgenic mice lines were generated that express the pathological protein selectively in specific cellular populations of the CNS. Astrocyte-specific (Gong et al., 2000) mtSOD1 expression was unable to induce the pathological phenotype while neuronal-specific mtSOD1 expression was either not pathological (Pramatarova et al., 2001) or sufficient to induce the disease (Jaarsma et al., 2008). Furthermore, increasing the expression of mtSOD1 selectively in adult neurons did

not fasten the course of the disease in hSOD1-G93A mice (Lino et al., 2002). To investigate more accurately the contribution of different cell populations to ALS pathogenesis, an innovative experimental model was generated and characterized, namely chimeric mice harboring a mixture of normal cells and human mtSOD1 expressing cells (Clement et al., 2003). These mice showed an increased lifespan depending on the percentage of wild type cells they possessed. Moreover, mtSOD1-expressing motor neurons were rescued in the presence of a high percentage of non-transgenic glial cells. Conversely, signs of cell sufferance in adjacent wild type motor neurons accompanied the presence of transgenic non-neuronal cells (Clement et al., 2003).

Taken together, all these observations support the view that mtSOD1 toxicity implies the contribution of different cell types in ALS, although they do not provide detailed mechanistic insights, which were explored with *in vitro* studies. The first question was to determine if mtSOD1 might induce in non-neuronal cells sufficient alterations to trigger motor neuron sufferance. Initially, it was demonstrated that mtSOD1-expressing neurons might activate glial cells (Cassina et al., 2005; Ferri et al., 2004; Urushitani et al., 2006), which in turn may trigger motor neuron demise (Cassina et al., 2002; Pehar et al., 2004). In particular, activated microglia cells seems to be particularly detrimental to motor neurons, as they may induce neuronal demise through neurite beading by inhibition of mitochondrial respiration and axonal fast transport (Takeuchi et al., 2005), well described features in ALS histopathology.

To investigate more sharply the contribution of microglia *in vivo*, transgenic mice carrying a deletable mtSOD1 gene were generated (Boillee et al., 2006). In particular, animals harboring the hSOD1-G37R sequence flanked by a pair *LoxP* sequences (LoxSOD1-G37R), which allowed recognition and regulated deletion by the Cre recombinase, were produced. These mice, who developed fatal progressive ALS-like phenotype, were then cross-breeded with mice expressing Cre recombinase under the control of either a neuron- or microglial-specific promoter, in order to shut down the expression of the toxic protein selectively in these cell populations. The effects on the pathological course resulted different. In particular, reduced mtSOD1 levels in motor neurons delayed disease onset without affecting

disease progression at later stages, whilst diminishing the mutant SOD1 expression in microglia prolonged survival by slowing the rate of motor neurons impairment (Boillee et al., 2006). The therapeutic utility of reducing microglial activation was also tested with a pharmacological approach. In particular, transgenic mice were treated with the antibiotic minocycline (Kriz et al., 2002; Van Den Bosch et al., 2002; Zhu et al., 2002), a tetracycline derivative able to switch off microglial activation (Yrjanheikki et al., 1999). Clinical trials to validate the therapeutic efficacy of this molecule are currently underway. Taken together with the studies presented in the previous section, these results suggest that microglia and the inflammatory process may influence the course of the disease, rather than being a cause (McGeer and McGeer, 2002; Monk and Shaw, 2006). In keeping with this point of view, transgenic mice harboring the hSOD1-G37R gene under the control of the prion protein promoter (PrP:hSOD1-G37R) were generated (Wang et al., 2005). The use of this specific promoter drives high expression levels of mtSOD1 in neurons, astrocytes and muscles, but not in microglia or in the macrophage lineage. As these mice developed a pathological phenotype, it is possible to hypothesize that the expression of mtSOD1 in all the elements of the neuromuscular unit is sufficient to cause motor neuron disease (Wang et al., 2005).

Interestingly Yamanaka and colleagues demonstrated that diminishing the expression of hSOD1-G37R by a CRE-lox system did not affect the onset of the pathology but delayed microglial activation and slowed late disease progression indicating that mutant astrocytes are possible pharmacological targets to counteract the process of non–cell autonomous killing of motor neurons in ALS (Yamanaka et al., 2008). Conversely, another recent study demonstated astrocyte mtSOD1 loss in G85R mice delayed disease onset and prolonged the early phase of disease progression, without affecting the late phase, confirming SOD1 mutation-specific effects on ALS pathogenesis (Wang et al., 2010).

Mechanisms of cell death in ALS

The main pathological hallmark in ALS is motor neurons degeneration, presumably occurring through the aberrant activation of programmed cell death pathways. The explanation of the molecular steps leading to neuronal death may have important

therapeutic implications as this may uncover specific targets to interrupt the harmful cascade. In the following paragraphs, the main hypothesis about this topic will be discussed.

Apoptosis

Apoptosis is more often utilized as a synonymous of programmed cell death, given that one of its main characteristics is to be a highly regulated mechanism, though the meanings of these terms are not identical, Apoptosis is a common phenomenon induced either by specific insults mediated through so-called "death receptors" (external pathway) or by non-specific insults leading mitochondrial alterations and release of detrimental molecules, such as cyotchrome c (internal pathway). Both these pathways converge on the executioner phase of the process, which is driven by proteolitic enzymes called “caspases”.

An amount of biochemical and functional evidence of activation of the apoptotic pathways were provided in ALS (Przedborski, 2003), since the initial observation that the presence of mutations switches SOD1 function from an anti- to a pro-apoptotic role (Rabizadeh et al., 1995). However, as dying motor neurons do not fulfil the morphology of classic apoptosis, it is still controversial whether or not the identified mechanisms determine neurodegeneration (Guegan and Przedborski, 2003).

The Bcl-2 family of proteins plays a fundamental role in regulating apoptosis, as the balance between the pro- and the anti-apoptotic members determines the fate of the cell. Therefore, their possible involvement in ALS was explored. Initially, the protective action of the anti-apoptotic human protein Bcl-2 on motor neurons was tested in hSOD1-G93A mice overexpressing Bcl2 selectively in neurons (Kostic et al., 1997). Bcl2 overexpression lead to a delay in the onset of motor impairment and an increased lifespan (Kostic et al., 1997; Vukosavic et al., 2000) so this encouraging result stimulated a more accurate analysis of the different proteins of the Bcl-2 family and led to the observations that the expression of the antiapoptotic protein Bcl-2 was reduced during disease, whilst the pro-apoptotic Bax was upregulated (Vukosavic et al., 1999) and translocates from the cytosol to the mitochondria (Guegan et al., 2001). The consequence of these events is likely the

activation of the intrinsic apoptotic pathway, as cytochrome c release from mitochondria and caspase-9 activation was detected in parallel with the neurodegenerative process (Guegan et al., 2001).

Recently, efforts have been performed to understand the molecular link between mtSOD1 and apoptosis. A great advance in this field was produced by the demonstration that mtSOD1 interacts with Bcl-2 at the mitochondrial surface (Pasinelli et al., 2004): this interaction,, as it was more recently demonstrated by the same authors, converts Bcl2 into a toxic protein by uncovering its pro-apoptotic BH3 domain (Pedrini et al., 2010).

Taken together, these observations strongly argue in favor of the involvement of the intrinsic apoptotic pathway in ALS, but different studies provided evidence also for the activation of the extrinsic pathway. In particular, *in vitro* experiments demonstrated that motor neurons are sensitive to the toxicity of Fas, a death domain containing receptor (Raoul et al., 2002) and transgenic mice expressing different mtSOD1 displayed a chronic activation of this pathway (Ackerley et al., 2004; Tortarolo et al., 2003; Wengenack et al., 2004) and an increased vulnerability to it (Raoul et al., 2002). As previously mentioned, both the intrinsic and the extrinsic apoptotic pathway converge on the activation of the caspases. In ALS mouse models, the mRNA of some of these enzymes, namely caspase-1 (Guegan et al., 2002; Li et al., 2000; Pasinelli et al., 2000; Vukosavic et al., 2000), caspase-8 (Guegan et al., 2002), caspase –9 (Guegan et al., 2001) and caspase-12 (Wootz et al., 2004) were found upregulated (Hensley et al., 2002) and activated. Subsequent activation of effector caspases like caspase -3 (Vukosavic et al., 2000) and caspase-7 (Guegan et al., 2001) was detected in the spinal cords motor neurons of hSOD1-G93A mice. Furthermore, caspase-1 and –3 activation was revealed also in glial cell (Pasinelli et al., 2000), thereby suggesting that motor neurons degeneration might not be the only cell demise involved in ALS (Rossi et al., 2008).

As caspase activity implies so harmful consequences, the cell has to tightly control this event, regulating not only their activation, but also their functionality. In particular, different proteins are deputed to counteract their action, namely the Inhibitor of Apoptosis Proteins (IAPs). In ALS, evidence of disruption at this level of control was reported, especially regarding the X-linked inhibitor of the apoptosis

protein (XIAP) (Guegan et al., 2001 2002 #256, Wootz, 2006 #164). Consistently, upregulating XIAP *in vitro* (Ishigaki et al., 2002) and *in vivo* (Wootz et al., 2006) protects neurons from mtSOD1 toxicity.

These studies provided extensive evidence of the presence of apoptotic markers in ALS and therefore therapies targeted to different molecular steps of these pathways were subsequently delivered to transgenic mice to demonstrate whether they actually contribute to the pathogenesis of the disease.

Initially, hSOD1-G93A mice were intracerebroventriculary delivered with zVAD-fmk, a broad-spectrum inhibitor of caspases and a delay in the onset and mortality was observed (Li et al., 2000). Differently, intraspinal delivery of Bcl-2 through a recombinant adeno associated virus, failed to increase lifespan (Azzouz et al., 2000), despite the fact that genetic neuronal Bcl-2 overexpression positively influenced the course of the disease in transgenic mice (Kostic et al., 1997) and rasagiline, which induces Bcl-2 expression, also prolonged hSOD1-G93A mouse lifespan (Waibel et al., 2004). Consistently, the role of the mitochondrial pathway seems to be important in ALS pathogenesis as minocycline, which inhibits cytochrome c release, delayed the onset of symptoms and mortality in transgenic mice (Zhu et al., 2002). Unfortunately a phase III clinical trial revealed harmful effects of minocycline on ALS patients (Gordon et al., 2007)

ALS as a distal axonopathy: the "dying back" hypothesis

Despite the general belief that ALS pathology is a consequence of motor neuron loss, some observations from both transgenic mouse models and human autoptic samples do not perfectly agree with this conviction. Neuronal loss in hSOD1-G93A transgenic mice was normally reported at 80-90 days (Chiu et al., 1995). However, accurate analysis revealed that these animals displayed electromyographical alterations at 40 days of age (Kennel et al., 1996) and that denervation of motor neurons from muscles occurs in early stages of the disease pathogenesis prior to clinical symptoms in mtSOD1 animals (Gordon et al., 2009; Lemmens et al., 2007; Park and Vincent, 2008) with selective loss of fast-firing neuromuscular synapses at 50 (Frey et al., 2000; Pun et al., 2006). Evidence of neuromuscular junction impairment therefore appears long before motor neurons loss. Recently, a

systematic count of both hindlimb muscle end-plates and motor neurons were performed in the same animal model (Fischer et al., 2004) and a temporal sequence of detrimental events was identified that begins with denervation, followed by axonal loss and finally neuronal cell body degeneration. In the same study, autoptic specimens from a patient died for other causes pretty soon after the ALS diagnosis were analyzed. In this case, muscle fibers showed signs of denervation and reinnervation, with little axonal degeneration at the ventral roots level and no evidences of motor neurons sufferance (Fischer et al., 2004). This pattern suggests that ALS might be a "dying back" neuropathy where distal axonal degeneration occurs early during the disease, before neuronal loss and onset of symptoms.

In agreement with these findings, early selective degeneration of fast fatigable motor neurons axons seems to be a pathological event shared by different animal models, namely hSOD1-G93A and hSOD1-G85R transgenic mice. Furthermore, the process was partially reverted by the local application of CNTF, which is an axon-protective agent (Pun et al., 2006).

The "dying back" hypothesis has been recently strengthen by the demonstration that motor neuron death is not required for disease (Gould et al., 2006). In this study, hSOD1-G93A mice were cross-breeded with a transgenic line genetically devoid of Bax, one of the most important pro-apoptotic proteins. The progeny displayed a delayed onset of the symptoms and a prolonged lifespan. Interestingly, this positive effect was a consequence of the delay in neuromuscular denervation, rather than of motor neuron rescue from mtSOD1 toxicity. Indeed, these animals never showed a reduction of motor neurons number, even at the end stage of the disease (Gould et al., 2006).

Other mechanisms of cell death: paraptosis and autophagy

The morphological incongruences between the classic apoptosis output and the appearance of dying motor neurons raised further proposals on the mechanisms of cell death in ALS.

Paraptosis is a programmed cell death characterized by cytoplasmic vacuolization, late mitochondrial swelling, gene expression- and protein synthesis-dependency (Sperandio et al., 2000). Since these features were described in hSOD1-G93A

transgenic mice (Dal Canto and Gurney, 1994), an involvement of this pathway was postulated. Nevertheless, this supposition has never been confirmed by further studies.

Autophagy is a Greek term that means "eat itself" and in biology indicates a lysosomal pathway for degrading organelles and long-lived proteins, characterized by the formation of intracellular vacuoles called "autophagosomes". The significance of this mechanism is presently uncertain, as autophagy may be indeed a compensatory mechanism to counteract a nutritional starvation. On the contrary, excessive or aberrant activation of this pathway may lead to self-digestion of vital components with subsequent cell death. Evidence of a role for this pathway in ALS is only clues and relies on the presence of cytoplasmic vacuolization and disorganization of intracellular organelles in ALS transgenic mice (Dal Canto and Gurney, 1995). Recently, an *in vitro* study demonstrated that motor neurons exposed to chronic excitotoxicity switch their demise pathway from programmed cell death into an autophagic mechanism (Tarabal et al., 2005).

Therapy

Conventional

Despite intense research, ALS is still an incurable disease. Up to now patient care is mainly limited to improve the quality of life through symptomatic therapy and orthotic devices. The sole approved drug to counteract neurodegeneration is riluzole, which has only a modest effect on survival (Bensimon et al., 1994; Lacomblez et al., 1996; Miller et al., 1996).

As mentioned in the previous sections, basic research studies identified several drugs targeting different molecular pathways and some of them (listed in (Carri et al., 2006)) were successfully tested in animal models. Unfortunately, none of the investigated molecules was able to modify the clinical outcome of the human disease (reviewed in (Gordon, 2005) or http://www.als.net/OurResearch/ClinicalTrials.aspx). This strident contrast raises questions about the usefulness of transgenic models to test therapeutics, although the discrepancy in the outcome might be explained by different pharmacokinetics

properties of the drugs in humans and in mice, or to different routes of delivery. Moreover, in transgenic mice, the timing of drug intervention is often before the onset of the disease whereas ALS patients receive drug only after the diagnosis, which is drawn up sometimes late after symptom onset. Finally, transgenic mice are models of fALS and, therefore, drugs targeting SOD1 toxicity might not be useful to treat sALS, which is the majority of the occurrences (Rothstein, 2003).
A list of the ongoing trials is available online at the web sites http://clinicaltrials.gov or http://www.als.net/OurResearch/ClinicalTrials.aspx.

Gene therapy

One of the most promising therapeutic tools is represented by the use of some trophic factors, such as IGF-1 or VEGF (see above), although early trials have been controversial (Gordon, 2005), likely because of the poor delivery of these drugs to motor neurons. In the next years, it will be hopefully possible to use recombinant viruses to deliver the active molecule to the active site, as validated in animal models (Azzouz et al., 2004; Kaspar et al., 2003; Storkebaum et al., 2005).

Cell therapy

The aim of the abovementioned therapies is to stop the progression of the disease. The full functional recovery, indeed, is impossible after neuronal loss, since these cells are post-mitotic. A new hope to regenerate damaged neural tissues comes from the discovery of stem cells that are "self-renewing progenitor cells that can generate one or more specialized cell type". The utility of these cells is theorically threefold: (a) they might indeed regenerate motor neurons; (b) they might regenerate non-neuronal cells, considering that ALS is a non-cell autonomous disease (see above); (c) they might be used as bioreactors to vehiculate an *in situ* synthesis of therapeutic molecules (Klein et al., 2005). Presently, only a few trials have been presented using autologous transplantation of blood (Janson et al., 2001) or marrow mesenchimal stem cells (Mazzini et al., 2003). These preliminary studies aimed at testing the safety of the method and evidence of a clinical improvement is still lacking.

Current evidence sustains an anti-inflammatory and neuroprotective role of Peroxisome Proliferator Activated Receptors (PPARs) agonists in many neurodegenerative diseases like Multiple Sclerosis, Parkinson's disease, Alzheimer's disease and Amyotrophic Lateral Sclerosis (Heneka et al., 2005; Kiaei et al., 2005; Natarajan and Bright, 2002; Niino et al., 2001; Schutz et al., 2005; Shimazu et al., 2005; Yan et al., 2003; Zhao et al., 2005).

Particularly two independent groups recently demonstrated a neuroprotective acitvity of Pioglitazone in a mouse model of Amyotrophic Lateral Sclerosis (Kiaei et al., 2005; Schutz et al., 2005). These studies provided a strong evidence for a neuroprotective activity of Pioglitazone, a PPARγ agonist, in the hSOD1-G93A mice demonstrating that Pioglitazone administered before the onset of symptoms, improves the pathology outcome by extending the survival and delaying the onset, ameliorates the motor dysfunction, reduces the weight loss and attenuates the motor neuron death. Pioglitazone also reduces the microglial activation and the gliosis in the spinal cord, decreasing the production of proinflammatory mediators like iNOS, NF-kB and COX2. These studies suggest that Pioglitazone, which is currently used in the therapy of type II diabetes, could be a promising new drug for the treatment of ALS.

The Peroxisome Proliferator Activated Receptors

The Peroxisome Proliferator Activated Receptors (Berger et al., 2005) are members of the steroid/thyroid hormone nuclear receptor superfamily, belonging to the family of ligand dipendent transcription factors; they regulate different aspects of energy balance, lipid and lipoprotein metabolism and glucose homeostasis (Berger et al., 2005; Evans et al., 2004). The three different isoforms of PPARs which are known so far, α (NR1C1), β/δ (NR1C2) and γ (NR1C3), exhibit homology in their amino acid sequence and structure but differ in their ligand-binding domains, ligand specificity, tissue distribution and biological actions. The name "Peroxisome Proliferator Activated Receptors" is derived form the PPARα mediated peroxisome proliferation in rodent hepatocytes; PPARγ and PPARβ/δ activation does not elicit this response. PPARs are in an inactivated state bound to co-repressor proteins in the nucleus; upon binding to the ligand they heterodimerize with the retinoid X

receptors (RXRs) and form heterodimers that can regulate gene expression through different mechanisms, namely the ligand-dependent transactivation, the ligand-indipendent repression and the ligand-dependent transrespression (Ricote and Glass, 2007) **(Fig. 1)**.

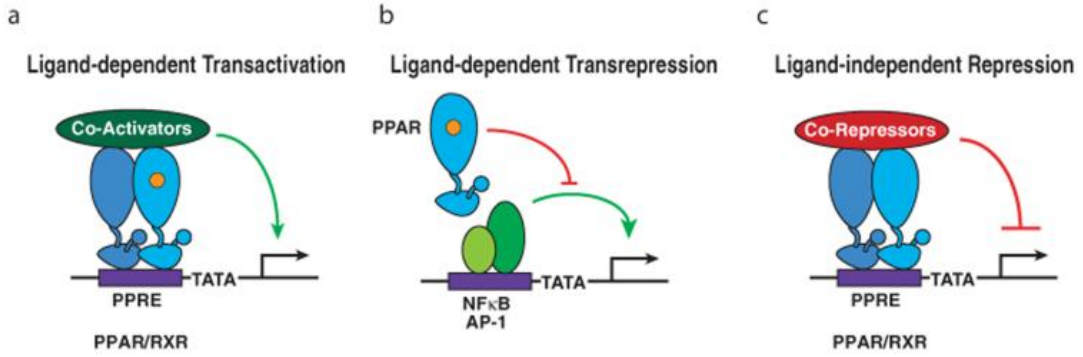

Fig. 1 Trancriptional activities of the Peroxisome Proliferator Activated receptors. PPARs can both activate and inhibit gene expression. (a) Ligand-dependent transactivation. PPARs activate transcription in a ligand-dependent manner by binding directly to specific PPAR-response elements (PPRE) in target genes as heterodimers with RXR. Binding of ligand leads to the recruitment of co-activator complexes that modify chromatin structure and facilitate assembly of the general transcriptional machinery to the promoter. (b) Ligand-dependent transrepression. PPARs repress transcription in a ligand-dependent manner by antagonizing the actions of other transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). (c) Ligand-independent repression. PPARs bind to response elements in the absence of ligand and recruit co-repressor complexes that mediate active repression. This complex antagonizes the actions of co-activators and maintains genes in a repressed state in the absence of ligand.
(Figure from Ricote M. and Glass C.K., Biochim. Biophys Acta. (2007) 1771(8):926-35.)

PPARs activate transcription in a ligand-dependent manner by binding directly to specific PPAR-response elements (PPRE) in the promoter region of target genes as heterodimers with RXR. The binding of agonists leads to the recruitment of co-activator complexes that modify chromatin structure and facilitate assembly of the general transcriptional machinery to the promoter. This activity enables PPARs to positively regulate gene networks involved in the control of lipid metabolism and glucose homeostasis in several tissues including adipose tissue, muscle and liver, ultimately influencing circulating lipid and glucose levels. In addition, PPARs also act directly to negatively regulate gene expression of proinflammatory genes in a ligand-dependent manner by antagonizing the activities of other transcription

factors, such as members of the nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) families (Devchand et al., 1996; Jiang et al., 1998; Lee et al., 2003; Marx et al., 1998; Ricote et al., 1998; Staels et al., 1998) (Fig. 1a). A major mechanism that underlies the ability of PPARs to interfere with the activities of these transcription factors has been termed transrepression and, unlike the transcriptional activity, it does not imply any interaction with the DNA (Fig. 1b). However despite the large amount of studies performed, the mechanisms whereby PPARs inhibit inflammatory gene expression are not completely understood. Recent studies using microarray analysis have shown that rosiglitazone inhibits only a subset of NF-κB target genes (Ogawa et al., 2005; Welch et al., 2003). This observation implies promoter-specificity in the mechanisms underlying transrepression. Many hypotheses have been suggested for the mechanisms of PPARs mediated transrepression, like the direct interaction of these receptors with other transcription factors, the regulation of *Mitogen-activated protein kinase* (MAPK) e *c-Jun N-terminal kinase* (JNK) signalling pathways, the interaction with co-regulators and the co-repressor-dependent transrepression; the exact mechanism of PPARs mediated transrepression, however, has not still been clarified (Ricote and Glass, 2007).

PPARs can also suppress the transcription of a target gene through a ligand indipendent repression mechanism (Fig. 1c); in this case they recruit co-repressors that do not allow the binding of co-activators (Ricote and Glass, 2007).

PPARs are composed by four domains with different functions: the DNA binding domain is highly conserved and is bound to the C-terminal region, which is responsible of the interaction with the ligand. The E/F domain is rather involved in the dimerization of PPARs with the RXRs and in the ligand-dependent transactivation. Finally the N-terminal domain is involved in the ligand-indipendent regulation of receptor activity (Kersten and Wahli, 2000); (Heneka et al., 2007). The interaction of agonists with their binding site determines a structural change which stabilizes the binding site of co-activators; the binding of antagonists, on the contrary, stabilizes a conformation promoting the binding of co-repressor complexes (Straus and Glass, 2007) (Yu and Reddy, 2007; Zoete et al., 2007).

It has been demonstrated that PPARs are sensors of different endogenous molecules (unsaturated fatty acids, eicosanoids and linoleic acid) produced by the metabolism of extracellular and intracellular fatty acids. It is difficult to establish which specific PPAR isoform is activated by these endogenous molecules *in vivo*, due to the low affinity of interaction and the low specificity of the ligands for the different isoforms. Furthermore, PPARs bind also to several synthetic compounds with an higher affinity and selectivity: among these, fibrates are PPARα agonists and are widely used in the treatment of hypertriglideridemia, while thiazolidinediones (TZD), like Rosiglitazone and Pioglitazone, are PPARγ ligands and are used in type II diabetes therapy (Fruchart et al., 1999; Ricote and Glass, 2007; Willson et al., 1996).

PPARα, β/δ and γ show distinct tissue distribution which mirrors their biological functions (Desvergne and Wahli, 1999; Heneka and Landreth, 2007; Willson et al., 2001). Though their expression has been more deeply analyzed in humans compared to rodents, data suggest that PPARα is expressed mainly in tissues dedicated to fatty acids catabolism like liver, heart, kidney, large intestine and skeletal. PPARα indeed has the primary functions of regulating energy homeostasis, stimulating fatty acids and cholesterol catabolism, regulating the gluconeogenesis and reducing the plasma levels of triglycerides (Bright et al., 2008; Heneka and Landreth, 2007). PPARβ/δ is ubiquitously expressed (Escher and Wahli, 2000), it is activated by Very Low Density Lipoproteins (VLDL)-derived fatty acids and by eicosanoids (i.e. prostaglandin A_1) and is involved in fatty acid oxidation in the muscle (Bright et al., 2008; Heneka and Landreth, 2007). PPARγ is abundantly expressed in the adipose tissue (white and brown) and, to a lesser extent, in the skeletal muscle, heart and liver, where it is activated by endogenous ligands like the 15-deoxy prostaglandin J2 (15-d PGJ2) (Tontonoz and Spiegelman, 2008) (Heneka and Landreth, 2007). The primary activity of PPARγ is to stimulate the differentiation of adipocytes and to control the distribution of lipid metabolites in the tissues. Furthermore PPARγ agonists are able to reduce the plasma levels of glucose, probably due to the PPARγ mediated modulation of endocrine factors (Gervois et al., 2004; Tontonoz and Spiegelman, 2008). This property allowed the

development of PPARγ agonists for the therapy of type II diabetes (Gauglitz et al., 2008; Kintscher and Goebel, 2009).

Anti-inflammatory role of PPARs

Besides the well studied activities of PPARs in metabolism and cell differentiation, many evidences suggest a possible role of these receptors in regulating the immune system. PPARs are indeed expressed in several immune cell types, including dendritic cells, macrophages and B and T lymphocytes. They are also present in epithelial cells where they play an essential role in the control of the immune response of mucous membranes. Worth to notice are the therapeutic effects demonstrated for some ligands of PPARα and PPARγ in many different models of inflammatory and autoimmune pathologies, namely Experimental Autoimmune Encephalitis (EAE) (Dunn et al., 2007), colitis (Cuzzocrea et al., 2004), allergic asthma (Woerly et al., 2003), edema and the carrageenan-induced pleuritis (Cuzzocrea et al., 2006; Straus and Glass, 2007). PPARβ/δ ligands have been less studied but they seem to be effective in EAE, too (Kielian and Drew, 2003).

The molecular basis of these activities, need to be further investigated in the light of their possible use in therapy.

It is well known that some ligand-dependent nuclear receptors, among these PPARs, can repress the expression of genes involved in the control of the inflammatory response through a ligand-dependent transrepression.

PPARγ is able to negatively control the expression of the inducible nitroxide synthase (iNOS) induced by lipopolysaccharide (LPS) in macrophages interfering with the elimination of the corepressor-complex from the promoter (Pascual et al., 2005). Furthermore, GW7845, a PPARγ ligand, represses the expression of genes coding for chemokines in LPS-stimulated macrophages (Ogawa et al., 2005).

PPARα and PPARβ/δ have also been demonstrated to reduce inflammatory gene expression with a ligand-dependent molecular mechanism, but the molecular details have not yet been fully characterized (Straus and Glass, 2007).

PPARγ is also expressed in dendritic cells where its activation inhibits the expression of membrane glycoproteins involved in promoting and maintaining inflammation (i.e. CD1a, CD40, CD83, CCR7), and of the costimulatory molecule

C80, of interleukin-12 (IL-12) and of other chemokines (Nencioni et al., 2002; Szatmari et al., 2006a; Szatmari et al., 2006b). Furthermore PPARγ seems to negatively regulate the immunogenicity of DC also in the absence of an exogenous ligand (Klotz et al., 2007).

PPARα and PPARγ are espressed both in the T and B cells; the activation of T cells determines a reduction in the expression of PPARα and an increase in the expression of PPARγ (Cunard et al., 2002; Jones et al., 2002).

Many studies demonstrated that PPARα agonists like fenofibrate and WY14643 are able to suppress the expression of Interferon γ (IFNγ) in T lymphocytes and the expression of interleukin-17 in activated splenocyte cultures (Cunard et al., 2002); the repression of IFNγ and IL-17 expression has never been observed in splenocyte cultures form PPARα -/- mice, thus it probably depends on PPARα (Lee et al., 2007).

A similar repressive activity of PPARγ agonists in human T cells and murine splenocytes has been observed (Cunard et al., 2002).

Furthermore, PPARα is able to suppress the Tumor Necrosis Factor α (TNFα) induced expression of adhesion molecules in the vessels, suggesting a possible role in inhibiting the recruitment of leukocytes to the inflammation site (Marx et al., 1999).

In summary, PPAR agonists play a pivotal role in the inhibition of the recruitment of inflammatory cells.

PPARs in the nervous system

Studies performed on the nervous system of rats demonstrated that all of the three isoforms of PPARs are espressed during late embryogenesis, with the prevalence of PPARβ/δ. Nevertheless, while PPARβ/δ mantains high levels of expression also during the postnatal period, the amount of the isoforms α and γ decreases after birth (Braissant et al., 1996).

The expression profile, which is isoform specific and highly regulated during development, suggests a possible role for PPARs in the CNS formation, even if this potential activity of PPARs has not been yet fully clarified.

The three isoforms of PPARs are all present in the brain and adult spinal cord. While PPARβ/δ is expressed in the neurons of different cerebral areas, PPARα and γ are localized in a lower number of regions. PPARs localization has been investigated also in non-neuronal cell cultures where PPARα is prevalently expressed in astrocytes, which present all the three isoforms in different amounts depending on the area and the age of the animal (Heneka and Landreth, 2007). PPARβ/δ is espressed also in immature oligodendrocytes where its activation promotes the differentiation, development and turn-over of myelin (Saluja et al., 2001). PPARγ is the dominant isoform in microglia (Cullingford et al., 1998).
The role of PPARs in the central nervous system is certainly inherent to lipid metabolism, even if this receptors are also implicated in cell migration and differentiation (Heneka et al., 2000; Inestrosa et al., 2005; Park et al., 2004b), in neuroinflammation and neurodegeneration (Heneka et al., 2000).

Regulation of glial cell activation by PPARs and role in neurodegenerative diseases

The CNS has always been considered an immune privileged site due to the capacity of the blood-brain barrier to prevent the immune cells from entrance and to protect the microenvironment from the changes in the blood levels of ions, amino acids, peptides and other substances. Nevertheless, resident glial cells like microglia and astrocytes are the effector of the innate immune system in the CNS and protect it from pathogens and injury. These cells readily activate to react to injury, modifying their morphology and their functions and proliferating. In particular microglia are normally quiescent but, when activated, can secrete pro-inflammatory cytokines, chemokines and oxygen reactive species, in addition to phagocyte pathogens and toxic debris. Astrocytes are involved in mantaining the extracellular environment optimal for neurons. Thus in physiogical conditions, they provide mechanic, trophic and metabolic support to neuronal cells. When challenged by toxic agents or pathogens, astrocyte, similarly to microglia, can become activated and produce nitric oxide and pro-inflammatory cytokines/chemokines. In such circumstances it has been hypothesized that glia remain cronically active and contribute to the neuronal damage typical of several CNS pathologies, including Multiple Sclerosis

(Smith et al., 2004; Sreedharan et al.), Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis and ictus (Drew et al., 2006).

Since the etiopathogenesis of most of these diseases is unknown, these pathologies represent an important challenge for the sanitary system due to the high social and economic expenses. The therapies available so far are most symptomatic and, therefore, the identification of new therapies is highly desirable.

Recent evidence suggests that PPARs agonists could have a therapeutic potential in the treatment of these pathologies, thanks to their anti-inflammatory properties (McGeer and McGeer, 2004; Szekely et al., 2004; Wahner et al., 2007).

All the three isoforms have been shown, indeed, to have an anti-inflammatory activity associated to the inhibition of glial activation.

PPARα

PPARα agonists have been demonstrated to reduce microglial and astrocyte activation *in vitro*. Gemfibrozil, a PPARα agonist, inhibits the induction by proinflammatory cytokines of NO and iNOS, NFkB and AP-1 in human astrocytes and murine primary astrocyte cultures (Pahan et al., 2002). Furthermore, gemfibrozil, when administrated with 9-cis-retinoic acid (RXR agonist), seems to inhibit the clinical symptoms of EAE in rodents (Lovett-Racke et al., 2004).

Fenofibrate, in combination with 9-cis-retinoic acid, inhibits microglial production of NO, TNFα, IL-1β e IL-6 in an additive manner (Xu et al., 2005) and showed positive effects in EAE and ictus therapy (Deplanque et al., 2003; Lovett-Racke et al., 2004).

PPARβ/δ

Alhtough the activity of PPARβ/δ ligands has been less investigated compared to the other two isoforms, GW0742 (PPARβ/δ agonist) was reported to inhibit NO production in microglia and astrocyte primary cultures (Polak et al., 2005). This compound has been succesfully studied also on EAE animal models where it has been found to delay the progression of the pathology and to slightly improve the symptoms (Polak et al., 2005).

Several PPARβ/δ agonists have shown efficacy in the treatment of ictus, reducing the ischemic area and the oxidative stress produced in the CNS by the stroke (Arsenijevic et al., 2006).

PPARγ

Many PPARγ agonists show an inhibitory activity on glial production of pro-inflammatory mediators. 15d-PGJ_2, for instance, inhibits the production of NO, IL-1β, and TNFα in murine microglial cultures (Koppal et al., 2000; Petrova et al., 1999), but has also been shown to reduce the T cell proliferation and IFNγ, IL-10 and IL-4 synthesis in EAE models (Diab et al., 2002; Niino et al., 2001). Many other studies on PPARγ ligands, like thiazolidinediones (Troglitazone, Ciglitazone, Pioglitazone) and PGA_2, led to the conclusion that these molecules, similarly to 15d-PGJ_2, act both with a PPARγ-dependent mechanism and inhibiting the I-kB kinase activity, with subsequent reduction of NF-kB activity (Diab et al., 2002; Giri et al., 2004; Storer et al., 2005). These drugs, particularly Pioglitazone, have been succesfully studied on animal models of EAE, ictus, AD, PD and ALS and showed a notably ability to inhibit and control the inflammatory response, delaying the onset of the pathology and prolonging the survival of the animal models (Heneka et al., 2005; Kiaei et al., 2005; Natarajan and Bright, 2002; Niino et al., 2001; Schutz et al., 2005; Shimazu et al., 2005; Yan et al., 2003; Zhao et al., 2005).

Many studies suggested that PPARγ agonists could positively influence EAE progression acting on the interaction between microglia and T cells and on T cells activation; the activity of these molecule seems to depend on the block of glial production of proinflammatory factors like NO, cytokines and chemokines (Diab et al., 2002; Kielian et al., 2004).

Recently, the group of Bernardo reported a PPARγ-mediated inhibitory effect of HCT1026, a non-steroidal anti-inflammatory drug (NSAID) derived from flurbiprofen, on the activation of rat primary cultures of microglia (Bernardo et al., 2005).

Ibuprofen, another NSAID, seems to improve pathology symptoms in animal models of AD, reducing glial activation, amyloid deposition and neuritic dystrophy with a PPARγ-dependent mechanism (Lim and Dey, 2000).

Generally, the activity of PPARγ agonists consists not only in the inhibition of microglial activation with subsequent reduction of the production of pro-inflammatory mediators, but also in the alteration of the expression of the same factors in astrocytes. Thus, it has been postulated that PPARγ agonists exert their neuroprotective activity by inhibiting the production of pro-inflammatory molecules, potentially neurotoxic, by monocytes and glial cells.

Still, it is mentioned that PPARs, and particularly PPARγ, are espressed also in neurons and their activation could directly influence the transport and the transduction of these cells as well as their differentiation (Cimini et al., 2005; Inestrosa et al., 2005; Park et al., 2004b; Smith et al., 2004). Furthermore it has been demonstrated also a PPARγ-mediated neuroprotective effect, particularly in animal models of ischemia, through mechanisms that directly target neurons (Uryu et al., 2002; Zhao et al., 2006). Activators of PPARγ, administered *in vivo* before or after the onset of ischemia, reduce ischemic damage (Ou et al., 2006; Shimazu et al., 2005; Sundararajan and Landreth, 2004; Tureyen et al., 2007; Victor et al., 2006). Recently Zhao and colleagues demonstrated that the expression of antioxidant genes catalase, SOD and GST and of genes that could improve neuronal resistance to ischemic and oxidative injury like LXRα and LPL, is reduced in neurons cultured from mice with selective neuronal PPARγ deficiency. Furthermore these mice showed significant increase in the infarct volume after cerebral ischemia (Zhao et al., 2009).

Izawa and colleagues recently demonstrated that pioglitazone regulates the energy metabolism also in the brain enhancing the oxidative metabolism of glucose in neurons (Izawa et al., 2009).

On these bases, it has been hypothesized that PPAR agonists could be able to exert a beneficial effect on the progression of chronic neurodegenerative diseases characterized by neuroinflammation, like ALS, inhibiting the glia-mediated neuroinflammatory process, directly protecting neurons, or both.

Further studies to clarify the mechanisms of action of these receptors are certainly necessary, as they could identify specific signal transduction pathways controlled by PPARs and possible new therapeutic targets.

AIM

During the last few years, a growing number of studies has demonstrated an anti-inflammatory activity for the PPARs agonists, which in several pathological instances have been able to decrease the production of proinflammatory genes, including cytokines and chemokines (Klotz et al., 2007; Pascual et al., 2005; Straus and Glass, 2007).

Based on these observations, the therapeutic impact of PPARs agonists has been more recently studied also in chronic neurodegenerative disorders characterized by neuroinflammatory processes, like Multiple Sclerosis, Alzheimer's disease, Parkinson's disease and Amyotrophic Lateral Sclerosis. In animal models of different neurodegenerative diseases, PPARs agonists proved to be efficacious in attenuating the manifestations of the pathology, and this effect was ascribed to their ability in reducing the production of proinflammatory mediators (Bright et al., 2008; Deplanque, 2004; Drew et al., 2006; Heneka et al., 2007; Hirsch et al., 2003; Sastre et al., 2006). Particular attention was focused on the PPARγ agonist Pioglitazone because of its capacity to penetrate the blood brain barrier. This compound was shown to be beneficial in many animal models of neurodegenerative diseases (Combs et al., 2000; Heneka et al., 2005; Kiaei et al., 2005; Lim and Dey, 2000; Schutz et al., 2005; Yan et al., 2003), including mice that reproduce several features of Amyotrophic Lateral Scerosis. Two independent groups demonstrated a neuroprotective acitvity of Pioglitazone on the hSOD1-G93A transgenic mouse model of ALS. In these studies, administration of Pioglitazone, before the onset of the symptoms, improved the motor performance and reduced the weight loss, attenuated motor neuron death and increased the survival delaying the onset. These effects were associated to reduced microglial activation and gliosis in the spinal cord as well as decreased production of proinflammatory mediators like iNOS, NF-kB and COX2.

As yet, different mechanisms of PPARs activation have been described, some of which directly related to gene transcription and other interfering with the activity of other transcription factors (Ricote and Glass, 2007), but the signalling pathways involved and the specific events responsible for their neuroprotective activity have not been clearly elucidated.

On these bases, the aim of my work was to study the transcriptional activity of the PPAR systems *in vivo*, in the central nervous system, throughout the course of ALS with the aim of clarifying the stage of the disease at which the activity of this class of receptors becomes relevant for the pathology. The comprehension of the molecular mechanisms that are responsible for their neuroprotective activity could then possibly lead to identify new targets for unprecedented therapeutic approaches.

METHODS

Animal models

hSOD1-G93A mice

The transgenic mouse model of ALS used in this study, the hSOD1-G93A animals, has been generated by Mark Gurney and his collaborators in 1994 (Gurney, 1994; Gurney et al., 1994). hSOD1-G93A mice ubiquitously express a high copy number of the glycine to alanine base pair mutation at the 93rd codon of the cytosolic human Cu/Zn superoxide dismutase gene (Gurney, 1994). These mice develop progressive motor neuron disease that recapitulates in many aspects the human pathology: the symptoms manifest around 90 days of age when the animals show hindlimb weakness, the development of tremors and loss of weight (Chiu et al., 1995; Gurney et al., 1994). Motor functions progressively decrease leading to complete paralysis and death at around 130-150 days of age. At the histopathological level, the phenotype is associated to selective degeneration of spinal motor neurons, protein inclusions in surviving motor neurons and significant loss of myelinated axons originating from the ventral horns of the spinal cord. Female hSOD1-G93A are infertile, so the transgenic mice hSOD1-G93A are maintained in hemizygosity in the mixed background C57Bl6/SJL by mating of hSOD1-G93A mice with wild type C57Bl6/SJL females.

PPRE-Luc mice

The transgenic mouse line PPRE-Luc, generated in our laboratory, is a reporter mouse ubiquitously expressing the gene coding for the firefly luciferase under the control of a promoter responsive to PPARs. This model allows to evaluate the activation of PPARs and represents a novel opportunity for the characterization of PPAR transcriptional activity in physiopathological conditions.

Transgenic PPRE-Luc mice were generated by pronuclear injection of the pMAR-PPRE5X-tk-Luc-MAR transgene, which contains copies of the PPAR responsive element (PPRE) and the minimal promoter of the thymidine kinase, into murine oocytes (Ciana et al., 2007). Matrix attachment regions (MAR) from chicken

lysozyme (Stief et al., 1989) flanking the constructs were previously described as elements indispensable to obtain the generalized expression of the reporter (Ciana et al., 2001).

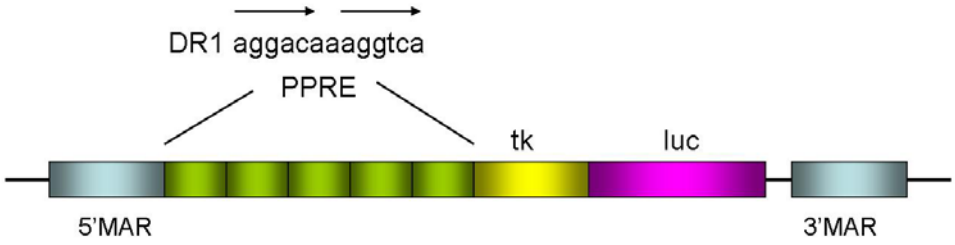

Fig. 1: pMAR-PPRE5X-tk-Luc-MAR transgenic construct: the selected PPRE is the most responsive to the PPARs and is repeated five times in tandem and cloned upstream the minimal promoter of thymidine kinase (tk), controlling luciferase expression. The MAR sequences flanking the construct are needed to obtain an ubiquitous expression of the reporter.

Generation of hSOD1-G93A+/-;PPRE-Luc+/- mice

Eterozygous hSOD1-G93A female mice were crossed with omozygous PPRE-Luc male mice to obtain hSOD1-G93A$^{+/-}$;PPRE-Luc$^{+/-}$ and hSOD1-G93A$^{-/-}$;PPRE-Luc$^{+/-}$ mice which were used for subsequent experiments.

Mice genotyping

Tail biopsies (1-2 mm) were lysed in 100 µl of lysis buffer (10 mM TRIS/HCl pH 9.0; 50 mM KCl; 0.45% Nonidet P40; 0.45% Tween 20; 0.1 mg/ml PK (proteinase K)) for 12 hours. The samples were then heated at 95°C for 10 min to inactivate the PK and then centrifuged at 13200 rpm for 20 min to precipitate the remaining tissue. The supernatant containing the genomic DNA was then used for subsequent PCR analysis.

The primers used for hSOD1-G93A amplification were:

5' SOD: 5' CAT CAG CCC TAA TCC ATC TGA 3'

3' SOD: 5' CGC GAC TAA CAA TCA AAG TGA 3'

IL-2 primers used in the same reactions as internal control were the following:

5' IL: 5' CTA GGC CAC AGA ATT GAA AGA TCT 3'

3' IL: 5' GTA GGT GGA AAT TCT AGC ATC ATC C 3'

The reaction mixture was the following: 0.4 μM primers, 200 μM dNTPs, 1X DNA Pol buffer and 32 U/ml DyNAzyme II DNA polimerasi. 0.5-1 μl of the supernatant of the lysed tail were added to 25 μl of reaction mixture.

After 35 cycles (30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C), the products were analyzed on 2% agarose gels stained with ethidium bromide in TAE buffer (0.04 M Trizma Base; 0.02 M acetic acid; 1 mM EDTA; 0.5 μg/ml ethidium bromide).

The amplicons of hSOD1-G93A and IL-2 were fragments of 250 bp and 320 bp long, respectively. The amplification of genomic DNA from transgenic animals produced both the bands, while the PCR of genomic DNA from non transgenic animals resulted only in the 320 bp IL-2 band.

Cellular models

The NSC-34 cells produced by Cashman et al. (Cashman et al., 1992) have emerged as the most promising alternative to primary motor neurons. NSC-34 is a hybrid cell line produced by fusion of motor neuron enriched, embryonic mouse spinal cord cells with mouse neuroblastoma. The NSC-34 cell line has many of the morphological, physiological and neurochemical properties of motor neurons [reviewed in (Matusica et al., 2008)], including motor neuron-like functional responses to numerous growth factors (He et al., 2002; Turner et al., 2004; Usuki et al., 2001). The majority of differentiated NSC-34 cells resemble cultures of motor neurons. Typically, these are large cells with multi-polar neurite projections, with a small percentage of the cells exhibiting a flat fibroblast-like morphology with short neuritic projections. Thus, the NSC-34 cells are the best available model for the *in vitro* investigation of many aspects of motor neuron biology, including studies on ALS. NSC-34 cells were grown in DMEM 10% Fetal Bovine Serum, L-Glutamine 2 mM, Sodium Pyruvate 1 mM, supplemented with Penicillin, streptomycin and anti-mycotic solution. Cells were splitted when the 70% confluence was reached.

Transfection of NSC-34 cells with the hSOD1-G93A expression vector

Cells were plated in 24-well plates until 70% of confluence. The medium was changed 2 hours before the transfection. For each well of a 24-well plate, 0.4 μg of pIRESGreen1 hSOD1-G93A or the empty vector were mixed with 3.6 μl of FuGene previously incubated with the growth medium (DMEM) for 5 min in a total volume of 20 μl, and left to incubate for 15 min at room temperature. The DNA-FuGENE complexes were then added to the cells. After 48 hours the cells were lysed and RNA wase xtracted for subsequent RT-PCR analysis.

Luciferase enzymatic assay

To quantify the luciferase activity in tissue extracts from luciferase reporter mice, animals were anesthetized with 78% ketamine and 15% xilazine in bdH_20 and sacrificed. The tissues of interest were collected and immediately frozen in dry ice and stored at -80°C. Tissues were omogenized in 200 μl of lysis buffer (100 mM KPO_4 pH 7.8, containing 1 mM dithiothreitol; 4 mM EGTA; 4 mM EDTA and 0.7 mM phenylmethylsulfonyl fluoride) using a TissueLyser (QIAGEN GmbH, Hilden, Germany) with 3 cycles of 10 sec with a 22 Hertz frequency. After one cycle of freezing-thawing to promote the breaking of cellular membranes the samples were centrifuged at 5900nrpm for 30 min at 4°C. The supernatants containing the protein extracts were then collected and used for subsequent luciferase enzymatic assay. 20 μl of tissue extract were added to the wells of a white 96 multiwell at 37°C, then the plate was positioned inside the luminometer (LUMAT LB 9501 Berthold) that injected sequentially 100 μl of a solution containing luciferine (470 μM Luciferine (Promega); 20 mM Tricine (Gibco); 0.1 mM EDTA (Merk); 1.07 mM $(MgCO_3)_4Mg(OH)$ x $25H_2O$ (Sigma); 2.67 mM $MgSO_4$ x $7H_2O$ (Merk) in bdH_2O pH 7,8 with 33.3 mM DTT (Boheringer Mannheim) and 530 μM ATP (Boheringer Mannheim) in each well. The luminometer detected the photon emission (RLU, Relative Luminescence Unit) coming from the enzymatic reaction between the luciferase inside the samples and the luciferine in the buffer 10 sec after having injected the substrate in each well of the plate.

The values of RLU were then normalized on the total protein content quantified with the Bradford Protein Assay.

Bradford Protein Assay

The Bradford Protein Assay is a colorimetric assay in which the interaction between the basic groups of the protein present in the sample and the Comassie reagent in an acidic environment leads to the development of a blue color. The intensity of the blue color is directly proportional to the amount of protein present in the sample.

The standard curve is based on serial dilutions of bovine albumine (BSA, Bovine Serum Albumine-Pierce) with the following concentration: 0,500-0,375-0,281-0,211-0,158-0,119-0,089-0,067 μg/ml.

200 μl of Comassie reagent were added to each well of a 96 well plate, then 4 μl of each point of the standard curve or of the unknown sample were added to the Comassie contained in each well. Every sample was analyzed in triplicate. The O.D. of each sample was read with a spectrophotmeter (Microplate reader, Bio-Rad) at the wave lenght of 595 nm.

The concentration of the samples were then obtained referring to the BSA standard curve.

Analysis of the expression of PPARα, PPARβ/δ and PPARγ target genes in the spinal cord of hSOD1-G93A mice

RNA extraction from spinal cord

hSOD1-G93A mice and their non-transgenic littermates were sacrificed and the spinal cord was immediately collected, frozen in dry ice and stored at -80°C.

The RNA was extracted using the RNA extraction kit RNeasy Mini kit (Qiagen) accordingly to manifacturer's instructions. Briefly:

1. the entire spinal cord was homogenized (5% w/v) in RLT Buffer with 1% β-mercaptoethanol using a Tissue Lyser (Qiagen) with 2 cycles of 15 sec with a frequency of 10 Hz.

2. 600 μl of the homogenized sample were transferred to a new tube and the remaining sample was stored at -80°C for potential further utilization.

3. The samples were then centrifuged for 3 min at 16100 x g and the supernatants were carefully removed by pipetting and transferred to a new microcentrifuge tube. Only these supernatants (lysates) will be used in subsequent steps.

4. 1 volume of 70% ethanol was added to the cleared lysate, and immediately mixed by pipetting.

5. up to 700 µl of the sample were transferred to an RNeasy spin column placed in a 2 ml collection tube. The samples were then centrifuged for 15 sec at 8000 x g . The flow-through was discarded and the procedure was repeated with the remaining lysate-ethanol mixture.

6. 350 µl of Buffer RW1 were added to the RNeasy spin column and the samples were centrifuged for 15 sec at 8000 x g to wash the spin column membrane. The flow-through was discarded.

7. 80 µl of DNase (Qiagen; resuspended in 550 µl of bdH2O and diluted 1:8 with RDD buffer (provided)) were added to each column and the samples were left to incubate for 15 min.

8. 350 µl of Buffer RW1 were added to the RNeasy spin column and the samples were centrifuged for 15 sec at 8000 x g to wash the spin column membrane. The flow-through was discarded.

9. the collection tube placed under the column was substituted with a new one and 500 µl of RPE buffer (provided, ethanol addiction is required at the first use, as indicated by the manufacturer) were added to the column and the samples were centrifuged for 15 sec at 8000 x g to wash the spin column membrane. The flow-through was discarded.

10. another washing step with RPE Buffer identical to the previous one was perfomed.

11. the columns were then placed on a new tube and centrifuged for 1 min at 16100 x g to dry the excess of RPE Buffer.

12. The columns were then placed on the collection tube and 40 µl of RNase free water (provided) were added to each column. The samples were then centrifuged for 15 sec at 8000 x g. The RNA is in the flow-through. To increase the yeld fo RNA, as suggested by the manifacturer, the flow-through was pipetted again on the membrane inside the column and the centrifugation step was repeated. The RNA

obtained was an aqueous solution that was stored at -80°C. The RNA concentration was quantified using a Nanodrop-1000 Spectrophotometer (Thermo Scientific).

RNA extraction from mouse motor neuron NSC-34 cell line

The NSC-34 cells transiently transfected with the hSOD1-G93A or the empty vector pIRESGreen1 were grown on 24 well plates, transfected and lysated 48h after the transfection. Briefly 5 wells of the 24 well plate were disrupted with 350 µl of RLT lysis buffer supplemented with 1% β-Mercaptoethanol. The collected lysate was then homogenized by 5 passages through a 21 gauge needle fitted to a RNAse-free syringe.

Then, the same procedure used for the spinal cord RNA extraction was followed (from step 4 to 12).

Reverse transcription and cDNA synthesis

1.5 µg of random primers (Promega) were added to 1 µg of RNA in a total volume of 15 µL. The samples were heated at 75°C for 5 min to allow RNA denaturation for random primers binding. 10 µl of the reaction mixture (dNTPs 1.25 mM, MMLV RT 20 U/ µl, 1X MMLV RT Buffer in bdH_2O) were added to each sample and they were left to incubate for 1 h at 37°C to allow first strand cDNA synthesis.

The samples were then heated to 75°C for denaturation and then placed on ice and stored at -80°C.

RT-PCR for PPARs target genes

The reaction mixture was the following: 0.8 µM primers, 200 µM dNTPs, 1X DNA Pol buffer and 10 U/ml GoTaq DNA polimerasi. 1 µl of cDNA was added to 24 µl of reaction mixture.

The primers used for each gene are outlined in Table 1.

Table 1

Gene	Primers
Medium chain Acyl CoA Dehydrogenase (MCAD)	FW: 5' GAAGAGTTGGCGTATGGG 3' REV 5' GCGGAGGGCTCTGTCACACA 3'
Acyl CoA synthetase long-chain family member 6 (Acsl6)	FW: 5' GAGGACAGGACAAAGGAGG 3' REV: 5' CACGACAATGCCAACCAAAAAG 3'
Lipoprotein lipase (LPL)	FW: 5' CACCGGGAGATGGAGAGCAAA 3' REV: 5' CCCAACTCTCATACATTCCC 3'
Catalase (Bernardo et al.)	FW: 5' CCTCGTTCAGGATGTGGTTT 3' REV: 5' GGCATCCCTGATGAAGAAAA 3'
Glutathione S-transferase 2 (Gsta2)	FW: 5' AAGACTGCCTTGGCAAAAGA 3' REV: 5' GCCAGTATCTGTGGCTCCAT 3'
Peroxisome Proliferator Activated Receptor gamma coactivator 1 alpha (PGC1alpha)	FW: 5' CTTCTTGCTCTTCCTTTAACTCTC 3' REV: 5' CTTTCTGCTTCTGCCTCTCTCTC 3'
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	FW: 5' ACGACCCCTTCATTGACC 3' REV: 5' TGCTTCACCACCTTCTTG 3'

The PCR reaction was optimized for each different gene; the linear range of amplification was calculated by performing the amplification at different cycles and then choosing the number of cycles comprised in the range in which the amplification was exponential. Particularly a large scale volume master mix of the PCR reaction was prepared and the proper amount of cDNA was added, then equal 25 µl aliquots were measured into 10 tubes. The reaction was initiated, with one tube removed every two cycles starting at 16 cycles.

The thermal profle used for each gene are outlined in Table 2.

Table 2

Gene	Thermal profile	Amplification fragment size
MCAD	(95°C 45 sec, 51 °C 45 sec, 72°C 1 min) x 27 cycles for spinal cords	167 bp
Acsl6	(95°C 45 sec, 55°C 45 sec, 72°C 1 min) x 26 cycles for spinal cords	240 bp
LPL	(95°C 45 sec, 55°C 45 sec, 72°C 1 min) x 30 cycles for spinal cords x 28 cycles for NSC34 cells	283 bp
Cat	(95°C 45 sec, 53°C 45 sec, 72°C 1 min) x 24 cycles for spinal cords x 30 cycles for NSC34 cells	352 bp
Gsta2	(95°C 45 sec, 53°C 30 sec, 72°C 1 min) x 39 cycles for spinal cords	345 bp
PGC1alpha	(95°C 1 min, 63°C 45 sec, 72°C 1min) x 27 cycles for spinal cords x 41 cycles for NSC-34 cells	402 bp
GAPDH	(95°C 1 min, 53°C 1 min, 72°C 1 min) x 22 cycles for spinal cords x 21 for NSC34 cells	691 bp

The products were analyzed on 3% agarose gels (1% for GAPDH, 2% for PGC1α) stained with ethidium bromide in TEB buffer (0.09M Tris; 0.096 M Boric acid; 0.01 M EDTA pH 8.4).

To perform a semi-quantitative assessment of the relative gene expression, 12,5 µl of each RT-PCR product was electrophoresed on agarose gel and stained with ethidium bromide. The optical density of each amplified band was calculated using the Gel Doc (BioRad) processing program and numerically expressed as the relative density in comparison to the optical density of the background. Furthermore, all results were normalized to the expression of the housekeeping gene Glyceraldehyde

3 phosphate dehydrogenase (GAPDH), which is constitutively expressed in all cells and serves therefore as an internal standard. Under these conditions, gross quantitative estimations were possible and broad differences in mRNA expression could be detected.

Nuclear and cytoplasmic protein fractionation from spinal cord

Frozen spinal cords were weighed and cut in small pieces with a scalpel on ice. The tissue was then omogenized in 3 ml per gram of tissue of hypotonic lysis buffer (20 mM Hepes pH 7.5; 5 mM NaF; 10 μM Na_2MoO_4; 0.1 mM EDTA; 1mM DTT; 0.01 % NP-40) and incubated on ice for 15 min. The samples were then centrifuged at 850 x g for 10 min at 4 °C.The supernatant obtained from this centrifugation represents the cytosolic fraction which was transferred to another tube.

The pellet was resuspended in 500 μl of hypotonic lysis buffer without DTT and NP-40. The samples were then incubated on ice for 15 min, then 50 μl of 10% NP-40 were added to the samples and the tubes were centrifuged at 14000 x g for 30 sec at 4°C. The supernatant obtained was the cytosolic fraction and was transferred to the tube together with the fraction previously isolated. The cytosolic fraction then was stored at -80°C. The pellet was then resuspended in 50 μL of extraction buffer (10 mM Hepes pH 7.9; 0.1 mM EDTA; 1.5 mM $MgCl_2$; 420 mM NaCl; 0.5 mM DTT; 0.5 mM PMSF; 1 μg/ml Pepstatin A; 1 μg/ml Leupeptin; 10 μg/ml Aprotinin; 20 mM NaF; 1 mM Beta-Glycerophosphate; 10mM Na_3OV_4; 25% Glycerol).

The samples were vortexed at high speed and incubated at 4°C in slow agitation for 30 min.

The samples were then centrifuged at 14000 x g for 10 min at 4°C, the supernatant was the nuclear fraction and was stored at -80°C.

The cytoplasmic and nuclear extracts were then quantified with the Bradford Protein assay and used for subsequent assay.

Quantitative assay for PPAR DNA binding

Nuclear presence of PPARα, PPARβ/δ and PPARγ was assayed using an enzyme-linked immunosorbent assay-based PPARα,-δ,-γ Transcription Factor Assay Kit

(Cayman Chemical). Nuclear proteins were extracted from spinal cords of hSOD1-G93A mice and non transgenic littermates according to the manufacturer's instructions and protein concentration was determined by the Bradford method using BSA as standard, as previously described. A double-stranded DNA sequence containing the PPAR response element was linked to the bottom of wells (96-well plate). PPARs within the nuclear fraction bound specifically to this sequence and isoforms were detected using primary antibodies directed against the individual PPARs. Clarified cell lysates were supplied for each PPAR isoform and acted as effective positive controls for PPAR DNA binding. Specificity of binding was also demonstrated using wells with no nuclear protein added and wells with positive control and an excess of consensus oligonucleotide (WT oligonucleotide) added which competes with the oligonucleotide bound to the wells. In these wells, no binding was detected. Binding activity was measured at 450 nm (minus the blank).

Experimental procedure:

The plate and the buffers were equilibrated at room temperature prior to opening.
The binding buffer (25% 4X Transcription Factor Binding Assay Buffer, 73% bdH_2O, 1& Reagent A, 1% 300mM DTT) was first added to the wells as follows:
Blank and non-specific binding wells: 100 µl
WT oligonucleotide plus positive control wells: 80 µl
Positive control or sample wells: 90 µl
Then 10 µl of WT oligonucleotide and 10 µl of positive control were added to the WT oligonucleotide plus positive control wells;
10 µl of positive control or of the samples to be analysed were added to the designated wells;
The plate was sealed with the cover provided and incubated over night at 4°C. The wells were emptied and washed 5 times with 200 µl of 1X Washing Buffer (provided, to be diluted 1:400 in bdH_2O 0,05% Tween-20).
100 µl of primary antibody specific for the individual PPARs diluted 1:100 in 1X Antibody Binding Buffer (ABB; provided, to be diluted 1:10 with bdH_2O) were added to each well except for the blank and the plate was incubated 1 h at room

temperature with agitation. The wells were emptied and washed 5 times with 200 µl of 1X Washing Buffer.

100 µl oh HRP-secondary antibody diluted 1:100 in 1X ABB were added to each well except for the blank and the plate was incubated for 1 h at room temperature. The wells were emptied and washed 5 times with 200 µl of 1X Washing Buffer.

100 µL of Developing Solution (provided) were added to each well and the plate was incubated with gentle agitation and protected from light, at room temperature, until the wells turn medium to dark.

After 45 min the reaction was stopped with 100 µl of Stop Solution (provided) added to each well and the plate was quickly positioned in a microplate reader (Microplate reader, Bio-Rad) and the colorimetric reaction was quantified at the wave lenght of 450 nm.

The data obtained were then expressed like medium optical density (O.D.)/µg of proteins.

Immunohistochemistry

hSOD1-G93A mice and non-transgenic littermates were sacrificed and the spine was fixed in parafomaldehyde 4% p/v in Phosphate buffered saline (PBS: 142 mM NaCl, 2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4 pH 7.4) for 24 h. The spinal cord was extracted and dehydrated through the following ethanol-xilene series:

Phosphate buffer (PB) 24h 2 times
70% ethanol 24 h
80% ethanol 24 h
95% ethanol 1 h 3 times
100% ethanol 1 h 3 times
Xilene 45 min 4 times

The tissue was then paraffin embedded and the lumbar tract was cut in 10 µm slices using a microtome (Boeckeler).

The sections were then deparaffinated trough the following ethanol-xilene series:
Xilene 5 min 3 times
100% ethanol 5 min 3 times
95% ethanol 5 min
75% ethanol 5 min 3
50% ethanol 5 min
The sections were the rehydrated in bdH_2O (5 min) and the antigen retrival was performed by boiling them in 10 mM citrate buffer pH 6 for 15 min in a microwave oven. The sections were then water-cooled for 30 min. The sections are then washed with bdH2O and permeabilized in TNT buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl (TN) 0.05 % Tween 20) for 5 min. The samples are then incubated in a blocking solution (TNB: 0,5% p/v of Blocking provided with the amplification kit Tyramide Amplification System-TSA (Perkin Elmer) in TN) for 1 h to saturate aspecific binding sites.
The sections were then incubated with the primary antibody diluted in TNB overnight.
Primary antibodies against PPARα, PPARβ/δ,PPARγ, SMI32 (neuronal marker which stains non-phosphorylated neurofilaments), GFAP (Glial Fibrillar Acidic Protein: astrocyte cyotskeletal marker) and *Licopersicum esculentum* Lectin (which stains microglia) have been used at the working dilutions indicated in the section "Materials".
The sections were then washed 3 times with TNT and incubated with the fluorescent secondary antibody diluted in TNB at room temperature and protected from the light. Secondary antibodies utilized and their working dilutions are indicated in the section "Materials".
The sections were washed 3 times with TNT and incubated with Hoechst 33342 1µg/mL in PBS for 15 min to stain the cellular nuclei.
The samples were finally washed 3 times with TNT and cover glass were mounted on the sections using the Dako mounting medium (Dako Mounting Medium).

Microscopy

The staining was captured with a 40X magnification using a Zeiss Axioskop 40, equipped with a digital camera (Nikon Coolpix 9900).
The immunofluorescence images in the ventral horns of the spinal cord sections were recorded.

Quantification of the nuclei positively stained for PPARs

The quantification of the number of nuclei positively stained for PPARs in the different cell types was performed manually by counting the number of cells which had a predominant nuclear staining compared to the cytoplasm in the ventral horns of spinal cord sections of hSOD1-G93A mice and, in the case of motor neurons, their non transgenic littermates. The results were reported as percentage of the total number of cells considered.

Quantification of the nuclear fluorescence of PPARγ

The quantification of the nuclear fluorescence of PPARγ was performed using ImageJ, a software from NIH. The application measures the signal intensity per pixel in a previously identified region of interest (ROI). The result is the mean of all the signals reported in that area. ROIs of 1188 pixels were created on the images of lumbar spinal cord sections previously stained for PPARγ and the neuronal marker SMI32. The fluorescence intensity of both PPARγ and the marker Hoechst 33342 were determined in the nucleus of motor neurons and non neuronal cells. Since Hoechst 33342 is a fluorescent stain which binds the DNA, we considered its intensity as a measure of the quantity of DNA. Since the nuclei of motor neurons are bigger that those of non neuronal cells, their DNA content is more diluted. Thus, the intensity of fluorescence coming from PPARγ labeling has been normalized on the intensity of the fluorescence signal of Hoechst 33342.

Statistical analyses

Statistical analysis data were obtained by means of analysis of variance (ANOVA) using GraphPad Software (PRISM) (San Diego, CA). Difference between groups was determined by Bonferroni comparision; a $P<0.05$ was considered statistically significant.

MATERIALS

Acetic acid	Riedel-de Haen
Agarose	Sigma Aldrich
Aprotinin	Sigma Aldrich
ATP	Boheringer Mannheim
BSA (Bovine serum albumin)	Pierce
Beta-glycerophosphate	Sigma Aldrich
Boric acid	Sigma Aldrich
Citric acid	Sigma Aldrich
Comassie	Sigma Aldrich
dNTPs (deoxynucleotide triphosphates)	Amersham Pharmacia Biotech AB
DTT (Ditiotreithol)	Sigma Aldrich
EDTA (ethylendiamminotetraacetic acid)	Sigma Aldrich
EGTA (ethylene glycol tetraacetic acid)	Sigma Aldrich
Ethanol, absolute	Merck
Ethidium bromide	Sigma Aldrich
FuGene	Roche
Glycerol	Fluka
Hepes	Sigma Aldrich
Hoechst 33342	Sigma Aldrich
Hygromycin B	Sigma Aldrich
KCl	Merck
Ketamine	Intervet Productions
K_2HPO_4	Merck
KH_2PO_4	Merck
$MgCl_2$	Merck

$(MgCO_3)_4$ $Mg(OH)_2$ x 2 H2O	Sigma Aldrich
$MgSO_4$ x 7H2O	Merck
Mounting medium	Dako
Leupeptin	Sigma Aldrich
Licopersicum esculentum Lectin	Sigma Aldrich
Luciferine	Promega
NaCl	Sigma Aldrich
NaF	Farmitalia Carlo Erba
Na_2HPO_4	Merck
NaH_2PO_4	Merck
Na_2MoO_4	Merck
Na_3OV_4	Sigma Aldrich
NP-40 (Nonidet P-40)	Sigma Aldrich
Paraffin	Merck
Paraformaldehyde	Fluka
Pepstatin A	Sigma Aldrich
(PMSF)Phenylmethylsulfonil fluoride	Sigma Aldrich
Primers	Sigma Aldrich
Proteinase K	Sigma Aldrich
Random primers	Promega
MMLV Retrotranscriptase (H-)point mutant	Promega
Sodium citrate	Sigma Aldrich
Taq GoTaq polimerase	Promega
Taq polimerase	GeneSpin
Transcription Factor PPARs assay	Cayman
Tricine	Gibco
TRIS HCl	Sigma Aldrich
Trizma base	Sigma Aldrich
TSA Amplification kit	Perkin Elmer
Tween 20	Sigma Aldrich
Xilazine	Rompun (Bayer)
Xilene	Merck

Primary antibodies:

Neurofilament H Non-Phosphorylated (SMI 32) Monoclonal Antibody (1:500)	Sternbergen Monoclonals Incorporated)
Anti-PPARα polyclonal antibody (1:100)	Affinity Bioreagents
Rabbit serum anti-PPARβ (1:300)	Kind gift from Dr. Herve Schon – University of Metz - France
Anti-PPARγ polyclonal antibody (1:100)	Affinity Bioreagents
Mouse anti-GFAP 488 conjugated (1:1000)	Chemicon

Secondary antibodies

Goat anti-mouse 488 (1:200)	Molecular Probes
Donkey anti-rabbit Cy3 (1:400)	Jackson Immuno Research

RESULTS

As mentioned in the "Introduction" section, increasing evidence demonstrates that PPARγ plays neuroprotective and anti-inflammatory roles in various neurodegenerative diseases, including ALS (Heneka and Landreth, 2007). Furthermore several indications suggest that PPARs are implicated in a number of signaling transduction pathways potentially involved in neuronal activity and survival, suggesting that dysfunction of these receptors may influence the neuronal pathophysiology. However the specific mechanisms by which PPARs exert their neuroprotective roles remain to be further elucidated.

Analysis of PPAR transcriptional activity in the spinal cord of hSOD1-G93A mice

To investigate the activity of Peroxisome Proliferator Activated Receptors in the central nervous system during the progression of Amyotrophic Lateral Sclerosis, we decided to analyse the transcriptional activity of these receptors in the spinal cord of hSOD1-G93A mice during the course of the disease taking advantage of the reporter PPRE-Luc mouse line (Ciana et al., 2007), available in our laboratory, in which the luciferase reporter gene is expressed under the control of a promoter responsive to PPARs.

Omozygous female PPRE-Luc mice (PPRE-Luc +/+) were crossed with eterozygous male hSOD1-G93A+/- animals, obtaining emizygous PPRE-Luc mice (PPRE-Luc +/-) transgenic or non transgenic for the human mutated SOD1 (hSOD1-G93A).

The animals were sacrificed at the critical stages of the pathology: 30 days (pre-symptomatic stage), 75 days (intermediate stage in which the motor neuron starts to detach form the motor plaque), 100 days (onset of the symptoms) and end stage, i.e. when the mice are unable to right themselves within 30 sec when being placed on their side. Since the endstage of hSOD1-G93A mice is around 120-140 days of age, non-transgenic mice were sacrificed at 30 days, 75 days, 100 and 130 days as controls. The cervico-thoracic and thoraco-lumbar spinal cord, the motor cortex, the hippocampus, the thalamus and the cerebellum were collected. We collected also the liver, the kidney and the lung to verify possible influences by the disease in the peripheral organs, which are not involved in the pathological process. The harvested tissues were immediately frozen at -80°C until subsequent analysis. To reduce the variability linked to the gender we focused our studies only on female mice. Five mice per each stage of the pathology were analysed.

The data showed that, in non-transgenic mice, PPAR activation is fairly constant throughout the progression of the pathology in all CNS areas taken into consideration, with the exception for non- statistically significant differences. The hSOD1-G93A mice showed a similar trend in the spinal cord, motor cortex and cerebellum until the onset of the pathology at 100 days, then luciferase activity increases abruptly and significantly at the end stage ($p<0.001$). The hippocampus and thalamus showed non-significant decreases in luciferase activity from 30 to 100 days, then the signal increased at the end stage ($p<0.001$) **[Fig. 1]**.

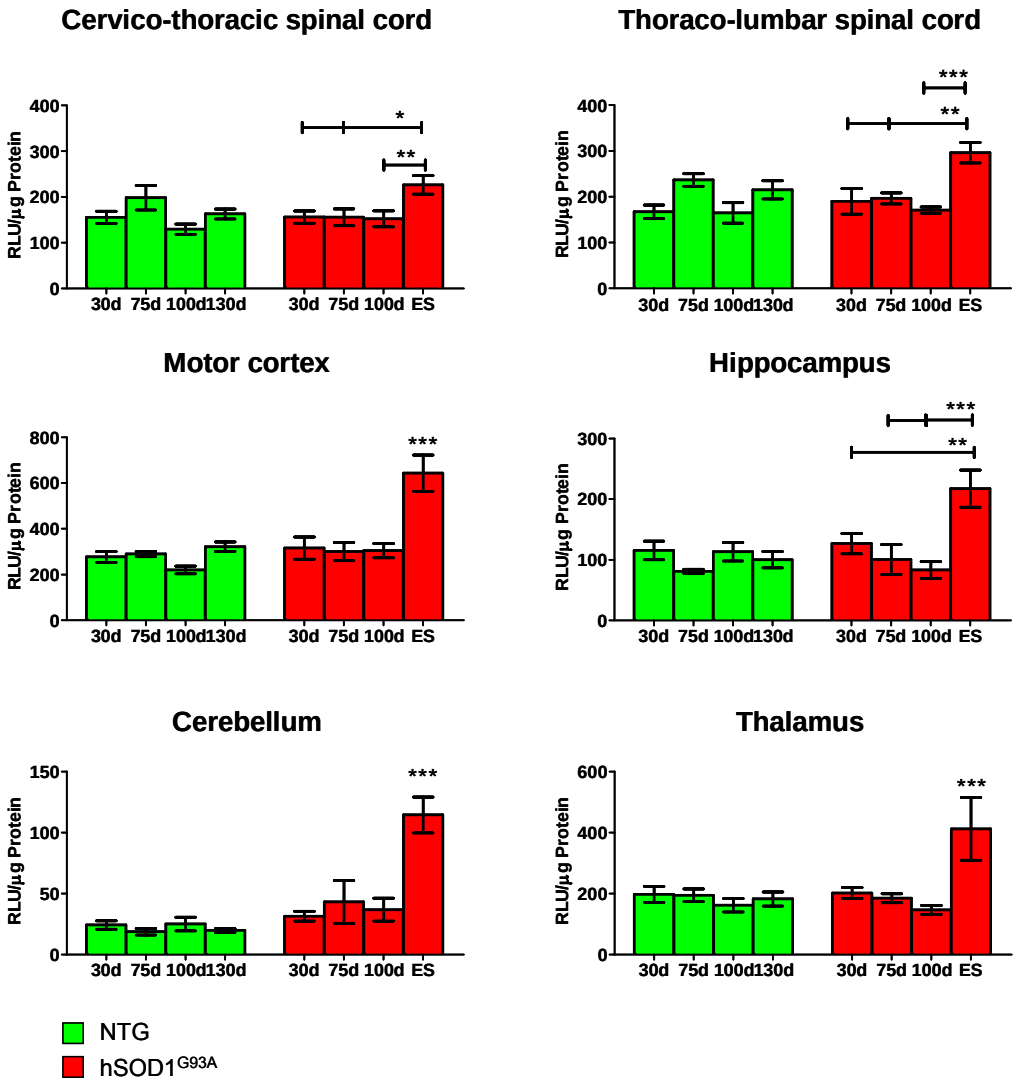

Fig. 1: Evaluation of PPARs activity in the different CNS areas of hSOD1-G93A mice at the different stages of the pathology. The cervico-thoracic and thoraco-lumbar spinal cord, the motor cortex, the cerebellum, the hippocampus and the thalamus of female PPRE-Luc +/- mice transgenic or not for the mutated (G93A) human SOD1 were harvested at the critical stages of the pathology: 30, 75, 100 days (d=days), end stage (ES). The luciferase activity was normalized to the total protein content. The data are expressed as RLU (Relative Luminescence Unit)/µg of total proteins ± SEM. Two-way analysis of variance (ANOVA) followed by Bonferroni Post Test was used to compare the means of the different stages in the transgenic and non transgenic groups. *=p<0,05 , **=p<0,01 , ***=p<0,001.

The luciferase activity in the peripheral tissues was variable but we never observed the increase in luciferase activity observed in the CNS of hSOD1-G93A mice at the endstage. **[Fig. 2].**

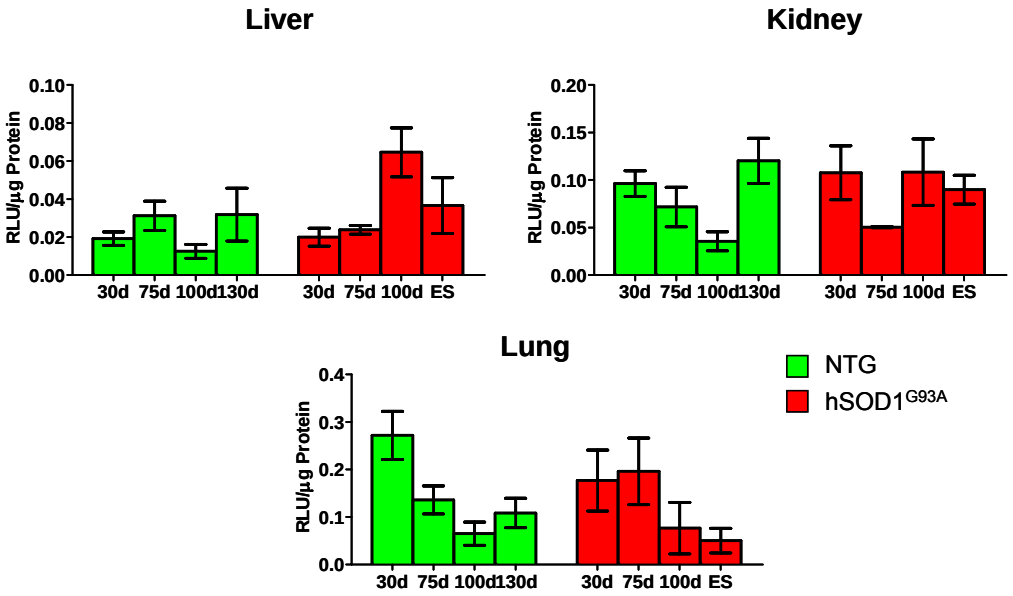

Fig. 2: Evaluation of PPARs activity in the peripheral organs of hSOD1-G93A mice at the different stages of the pathology. The liver, the kidney and the lung of female PPRE-Luc +/- mice transgenic or non-trangenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology,i.e. 30, 75, 100 days (d=days), end stage (ES). Luciferase activity was normalized to the total protein content. The data are expressed as RLU (Relative Luminescence Unit)/µg of total proteins ± SEM. The data were analyzed using GraphPad Software (PRISM) (San Diego, CA). Two-way analysis of variance (ANOVA) followed by Bonferroni Post Test was used to compare the means of the different stages in the transgenic and non transgenic groups. *=p<0,05 , **=p<0,01 , ***=p<0,001.

The increase in PPAR activity which is detectable only in the CNS of hSOD1-G93A mice was a very interesting data that we decided to further investigate.

Influence of starvation on PPAR transcriptional activity

PPARs are known to be widely involved in the regulation of multiple metabolic processes. Therefore, we decided to verify whether the starvation that the hSOD1-G93A mice experience in the last period of their life, due to dysphagia and complete paralysis which prevents them from reaching for food and water, could influence PPARs activity The effect of starvation was analysed on selected CNS areas (spinal cord and cerebellum) and peripheral organs (liver and kidney) of PPRE-Luc +/- mice.

PPRE-Luc female mice were divided into two groups: one group was fed *ad libitum* (*ad libitum*, al) while the other group was deprived of food and water for 48 h (starvation, s). The animals were sacrificed and the spinal cord, the cerebellum, the liver and the kidney were collected and immediately stored at -80°C for subsequent luciferase enzymatic assay.

The results show that the luciferase activity in the spinal cord and cerebellum is constant between the two experimental groups **[Fig. 3]**.

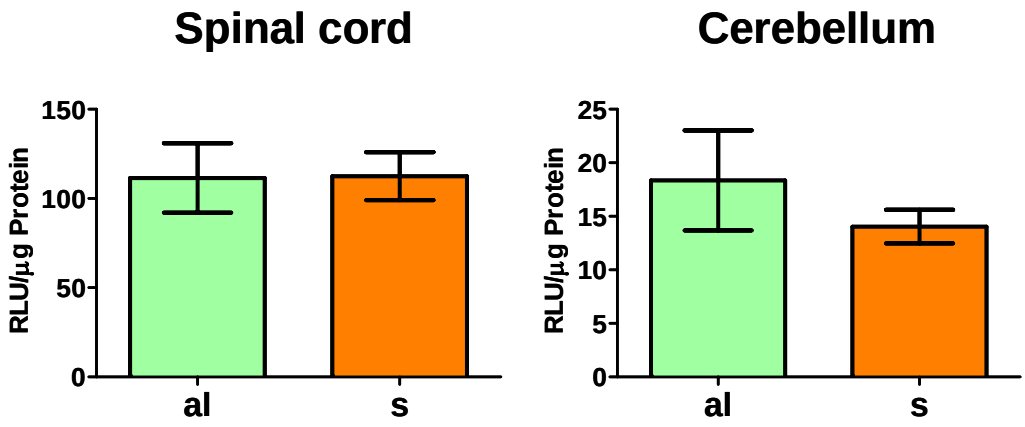

Fig. 3: Evaluation of PPARs activity in the spinal cord and cerebellum of PPRE-Luc +/- female mice after starvation. Female PPRE-Luc +/- mice were fed ad libitum (al) or starved (s) for 48 h. Luciferase activity was normalized on the total protein content. The data are expressed as RLU

(Relative Luminescence Unit)/µg of total proteins ± SEM. The data were analyzed using GraphPad Software (PRISM) (San Diego, CA). T test was used to compare the means of the different stages in the transgenic and non transgenic groups. No statistical significance resulted.

In the liver of starved animals, luciferase activity decreases slightly compared to controls, while no significant variability was detected in the kidneys **[Fig. 4]**.

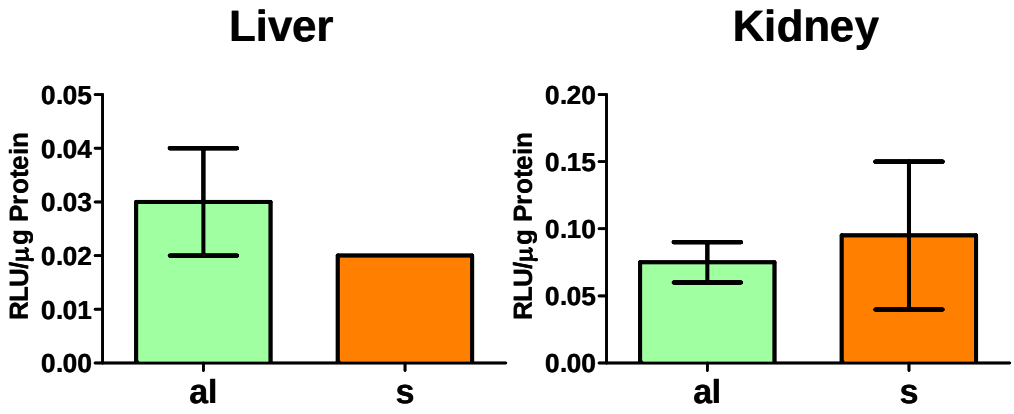

Fig. 4: Evaluation of PPARs activity in the liver and kidneys of PPRE-Luc +/- female mice after starvation. Female PPRE-Luc +/- mice were fed ad libitum (al) or starved (s) for 48 h. The luciferase activity was normalized on the total protein content. The data are expressed as RLU (Relative Luminescence Unit)/µg of total proteins ± SEM. The data were analyzed using GraphPad Software (PRISM) (San Diego, CA). T test was used to compare the means of the different stages in the transgenic and non transgenic groups. *=p<0,05.

The results obtained led us to conclude that the starvation does not influence PPAR activity neither in the peripheral organs of PPRE-Luc +/- mice nor in the CNS areas analysed, particularly the spinal cord which is the tissue primary compromised in ALS. On these bases, we concluded that the increase in PPAR activity detected in the PPRE-Luc+/-;hSOD1-G93A female mice was not dependent on nutritional defects.

Quantitative assay for PPARs DNA binding

PPARs activation implies their translocation into the nucleus where they bind to the responsive elements in the promoter of target genes and regulate gene transcription. To evaluate if the nuclear translocation of PPARα, PPARβ/δ and PPARγ is modulated during the progression of the disease and if an increased nuclear translocation could be responsible for the increase in PPARs transcriptional activity at the end stage of the disease, we decided to quantify the amount of PPARα, PPARβ/δ and PPARγ in the nuclear fraction of spinal cord homogenates from hSOD1-G93A mice and non transgenic littermates at the different stages of the pathology, using an ELISA-based Transcription Factor assay specific for each isoform of PPARs. Female hSOD1-G93A and non transgenic littermates were sacrificed at 30, 75, 100 days or at the end stage and the spinal cords were collected.

We found that the presence of PPARα in the nucleus undergoes a progressive non-significant decrease in the spinal cord of hSOD1-G93A mice during the course of the disease and does not mimic the increase at the end stage seen with the luciferase assay **[Fig. 5]**.

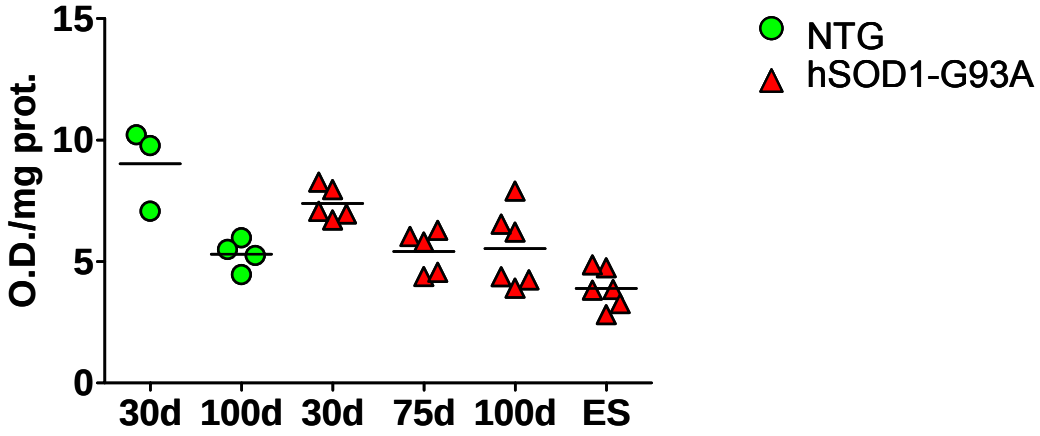

Fig. 5: Evaluation of the nuclear presence of PPARα in the spinal cord of hSOD1-G93A and non transgenic mice. Spinal cords of female PPRE-Luc +/- mice transgenic or non-transgenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology, i.e. 30, 75, 100 days (d=days), end stage (ES). The nuclear fraction was utilized for an ELISA-based assay. The data were expressed as optical density (O.D.)/mg of total proteins ± SEM. The data were analyzed using GraphPad Software (PRISM) (San Diego, CA). Two-way analysis of variance (ANOVA) followed by Bonferroni Post Test was used to compare the means of the different stages in the transgenic and non transgenic groups. *=p<0,05 , **=p<0,01 , ***=p<0,001.

The nuclear presence of PPARβ/δ decreases at the onset of symptoms, i.e. 100 days of age, and then increases but not significantly at the end stage **[Fig. 6]**.

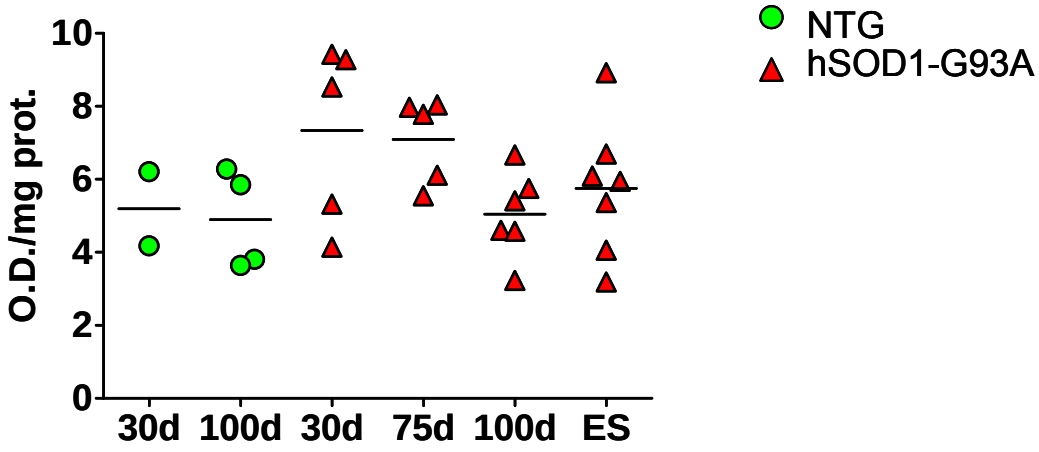

Fig. 6: Evaluation of the nuclear presence of PPARβ/δ in the spinal cord of hSOD1-G93A and non transgenic mice. The spinal cords of female PPRE-Luc +/- mice transgenic or non transgenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology, i.e. 30, 75, 100 days (d=days), end stage (ES). The nuclear fraction was utilized for an ELISA-based assay. The data were expressed as optical density (O.D.)/mg of total proteins ± SEM. The data were analyzed using GraphPad Software (PRISM) (San Diego, CA). Two-way analysis of variance (ANOVA) followed by Bonferroni Post Test was used to compare the means of the different stages in the transgenic and non transgenic groups. The statistical analysis does not show any significance.

The assay on the nuclear presence of PPARγ shows a decrease until 100 days of age and then a non significant increase at the end stage **[Fig. 7]**.

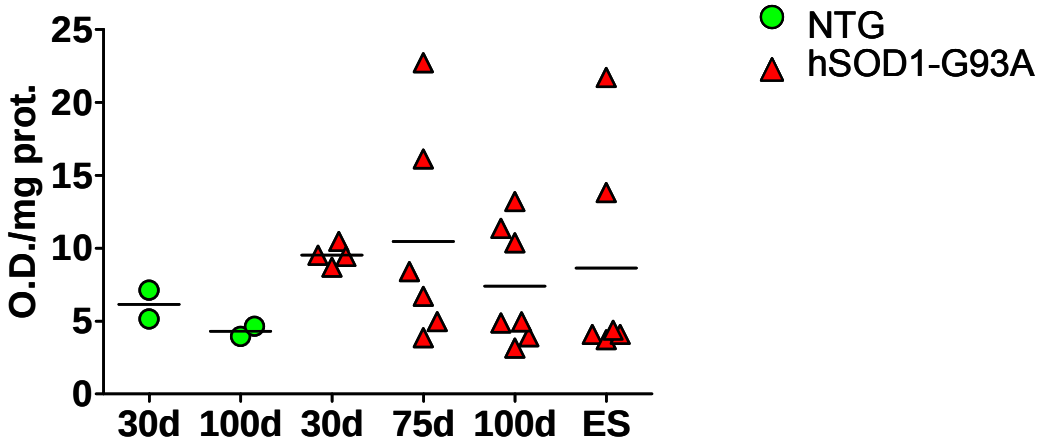

Fig. 7: Evaluation of the nuclear presence of PPARγ in the spinal cord of hSOD1-G93A and non transgenic mice. The spinal cords of female PPRE-Luc +/- mice transgenic or non transgenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology, i.e. 30, 75, 100 days (d=days), end stage (ES). The nuclear fraction was utilized for an ELISA-based assay. The data were expressed as optical density (O.D.)/mg of total proteins ± SEM. The data were analyzed using GraphPad Software (PRISM) (San Diego, CA). Two-way analysis of variance (ANOVA) followed by Bonferroni Post Test was used to compare the means of the different stages in the transgenic and non transgenic groups. The statistical analysis does not show any significance.

In summary, these data reveal that the worsening of the pathology does not cause an increased PPAR translocation into the nucleus. These assays have been performed on lysates from the entire spinal cord and, therefore, they do not provide any information on the different neural cell types. We postulated that the increase of PPARs activity at the end stage of the pathology could be due to an enhanced presence of the PPARs only in selected cell types. Furthermore ligand-dependent

effects derived from the interaction of PPARs with cofactors and regulatory molecules cannot be excluded.

Analysis of the subcellular localization of PPARα, PPARβ/δ and PPARγ in motor neurons, astrocytes and microglia in the spinal cord of hSOD1-G93A mice

To study extensively the expression and subcellular localization of the isoforms of PPARs in the different cell types of the spinal cord, we decided to perform immunohistochemical analyses on lumbar sections of spinal cords of hSOD1-G93A mice at different stages of the pathology.

Motor neurons

The cell type primarily involved in ALS is represented by motor neurons and, thus, we initially focused on these cells. Sections were double-stained with SMI32, an antibody specific for the non-phosphorylated neurofilaments, and antibodies directed against the isoform of PPAR to be analysed. The cell nuclei were stained with Hoechst 33342, a fluorescent dye that binds to the DNA.

The results obtained show that PPARα is localized predominantly in the nucleus or equally distributed between the nucleus and the cytoplasm in motor neurons **[Fig. 8]**.

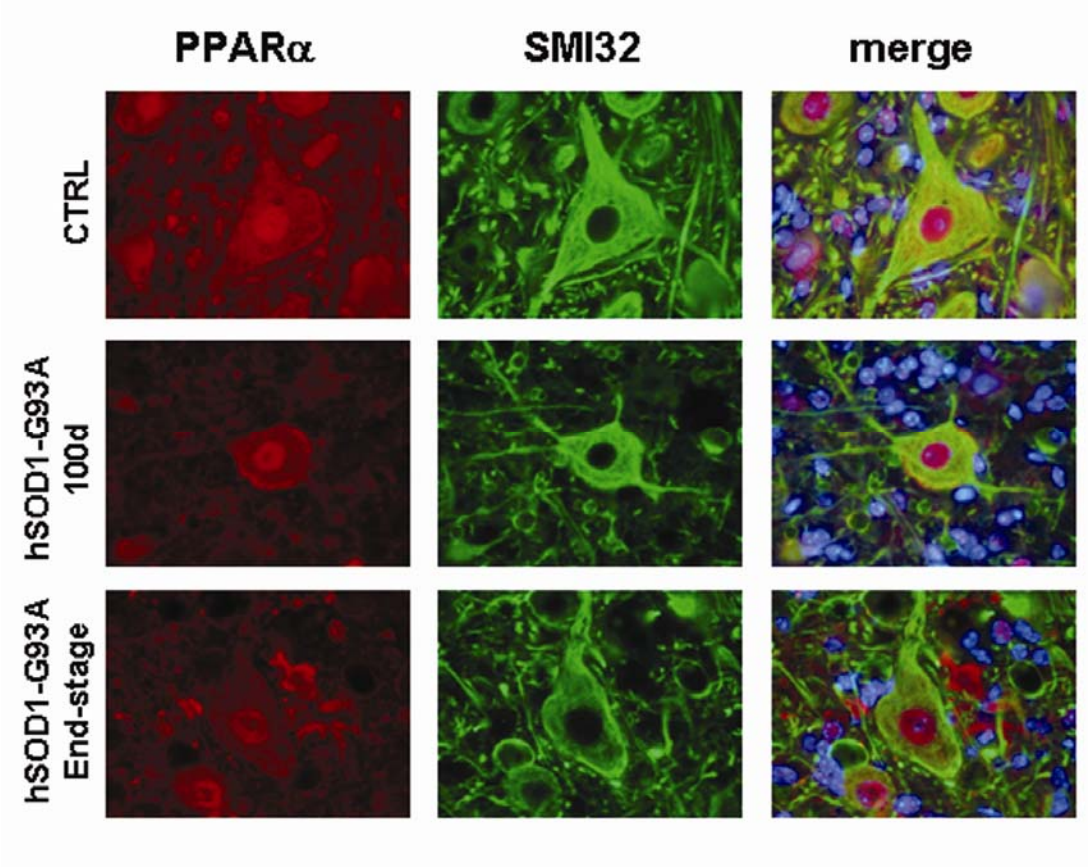

Fig. 8 PPARα shows a cytoplasmic and nuclear distribution in motor neurons. Spinal cord sections of female hSOD1-G93A mice at 30 days, 100 days and end stage were stained with an anti-PPARα antibody(red) and SMI32 antibody (green). Nuclei are stained with Hoechst 33342 (blue).

PPARβ/δ is localized both in the nucleus and cytoplasm of motor neurons but it is more abundant into the nucleus **[Fig. 9]**.

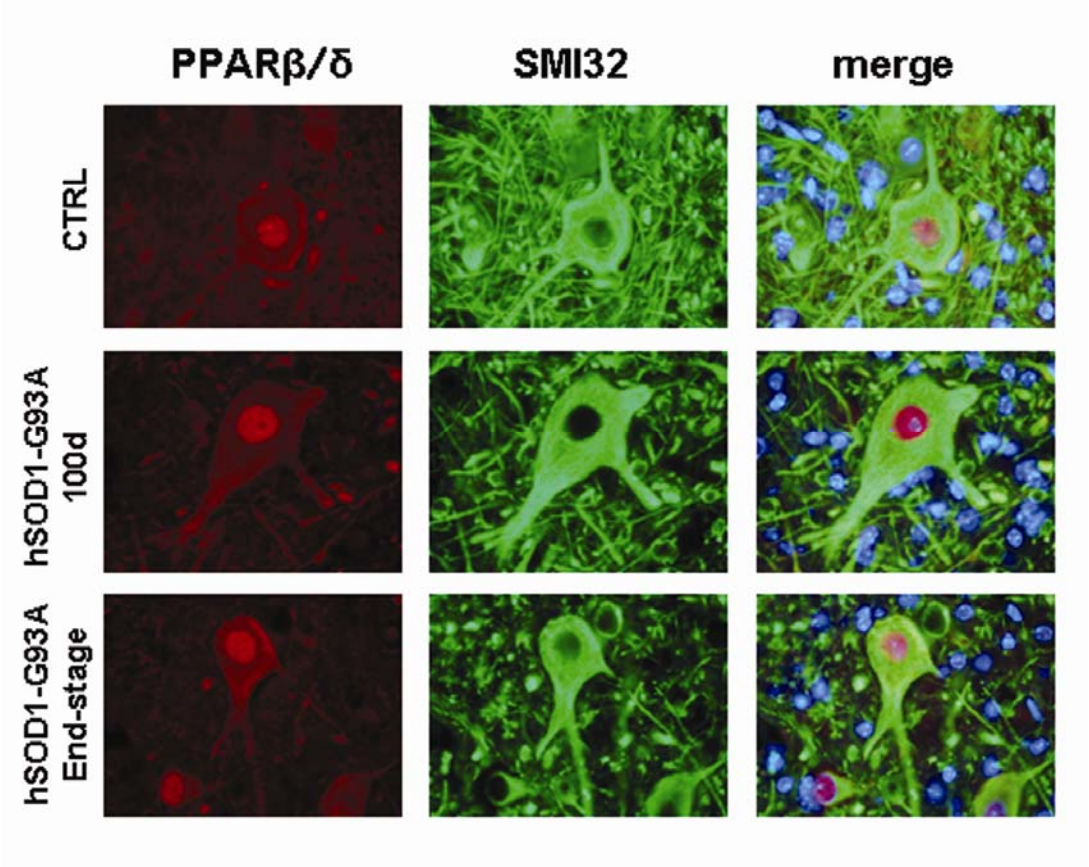

Fig. 9 PPARβ/δ shows a cytoplasmic and nuclear distribution. Spinal cord sections of female hSOD1-G93A mice at 30 days, 100 days and end stage were stained with an anti-PPARβ/δ rabbit serum (red) and SMI32 antibody (green). Nuclei are stained with Hoechst 33342 (blue).

PPARγ is exclusively localized in the nuclei of motor neurons **[Fig. 10]**.

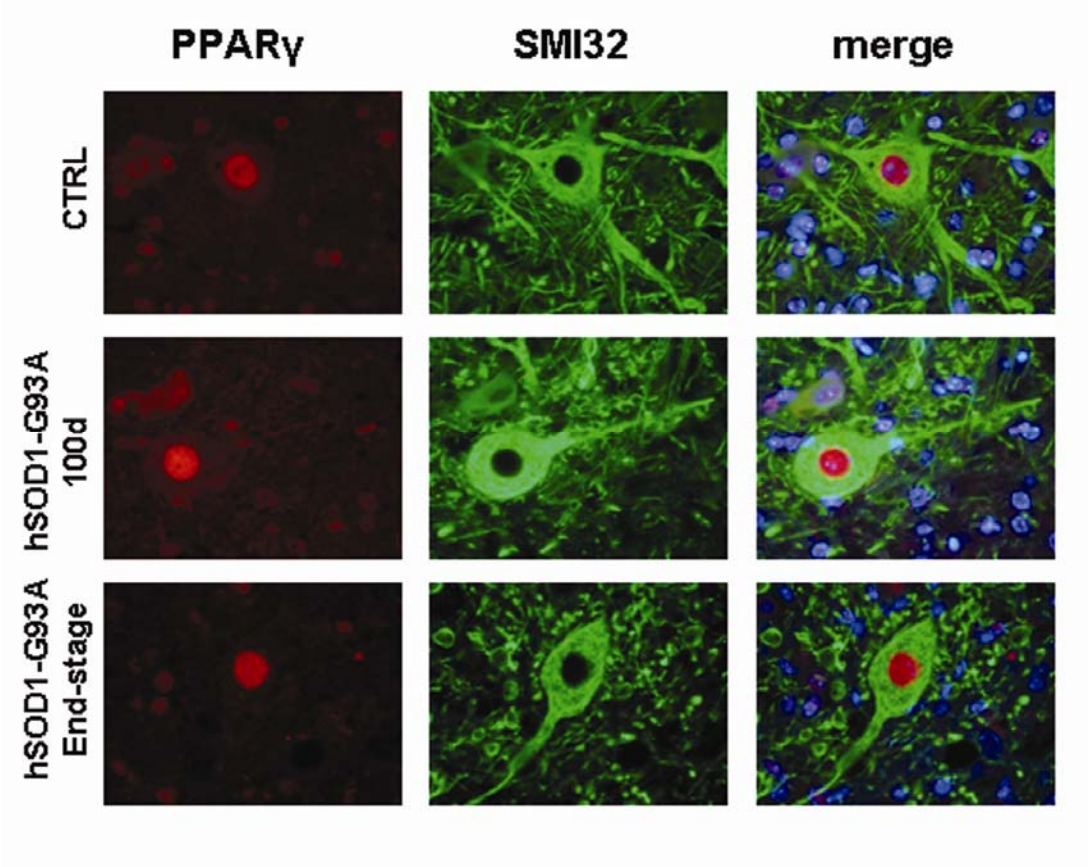

Fig. 10 PPARγ shows an exclusively nuclear distribution. Spinal cord sections of female hSOD1-G93A mice at 30 days, 100 days and end stage were stained with an anti-PPARγ antibody (red) and SMI32 antibody (green). Nuclei are stained with Hoechst 33342 (blue).

Astrocytes

Sections were double-stained with GFAP, an antibody directed to Glial fibrillar acidic protein which is widely used as an astrocytic marker, and antibodies directed against the isoform of PPAR to be analysed. The cell nuclei were stained with Hoechst 33342. PPARα **[Fig. 11]**, PPARβ/δ **[Fig. 12]** and PPARγ **[Fig. 13]** are localized in the nucleus of astrocytes, but PPARα is more abundantly expressed, while PPARβ/δ has the lowest expression.

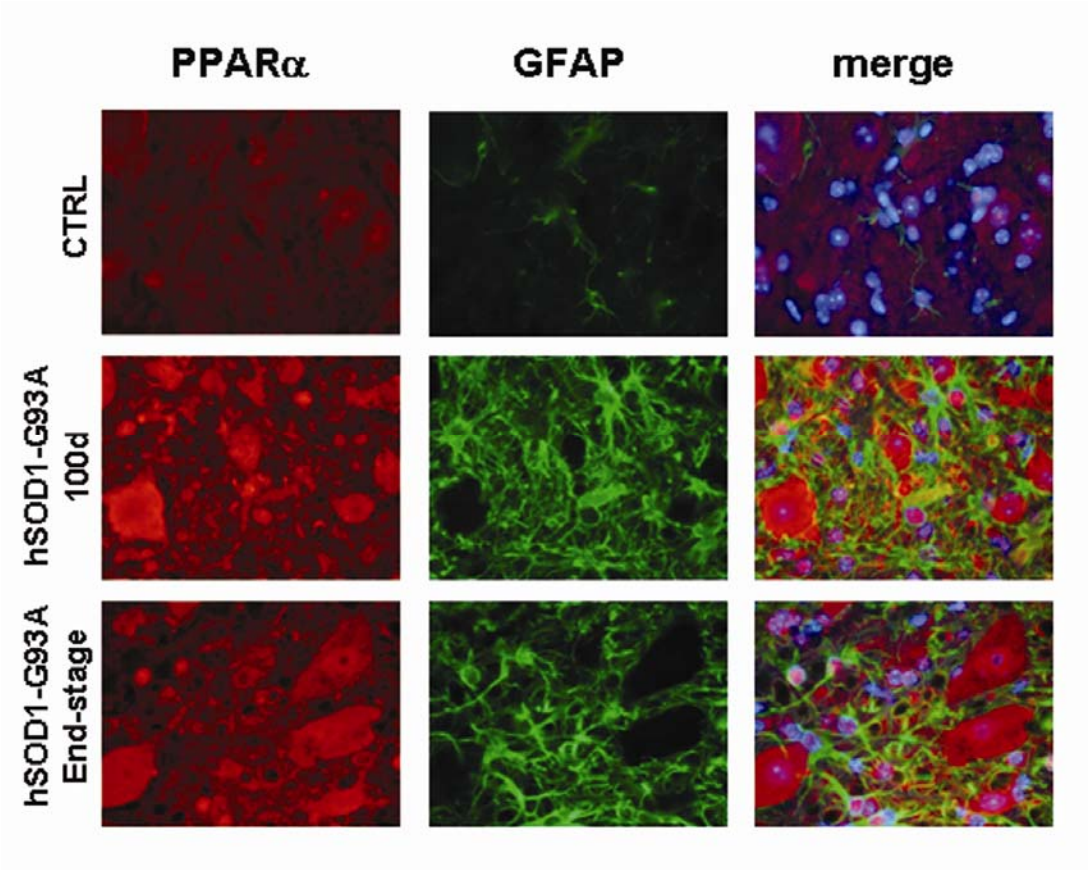

Fig. 11 PPARα shows a nuclear distribution in astrocytes. Spinal cord sections of female hSOD1-G93A mice at 30 days, 100 days and end stage were stained with an anti-PPARα antibody(red) and anti-GFAP antibody (green). Nuclei are stained with Hoechst 33342 (blue).

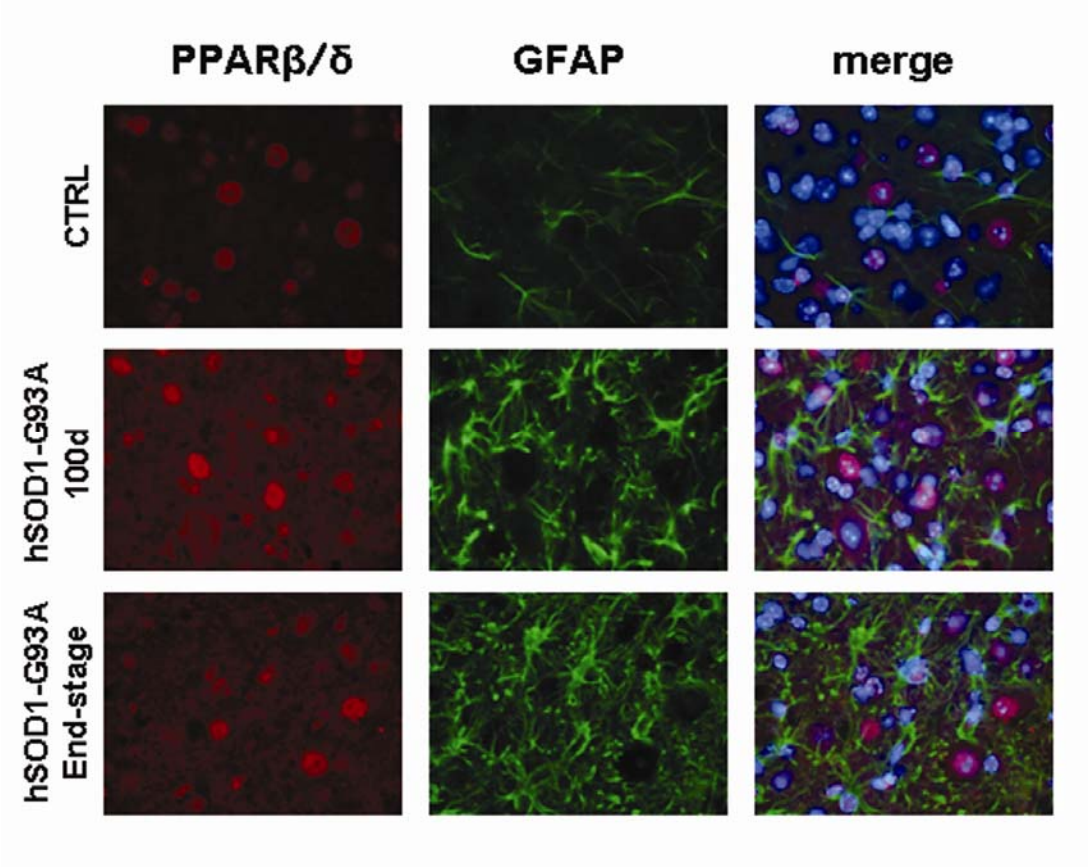

Fig. 12 PPARβ/δ shows a low expression and a nuclear distribution in astrocytes. Spinal cord sections of female hSOD1-G93A mice at 30 days, 100 days and end stage were stained with an anti-PPARβ/δ rabbit serum (red) and anti-GFAP antibody (green). Nuclei are stained with Hoechst 33342 (blue).

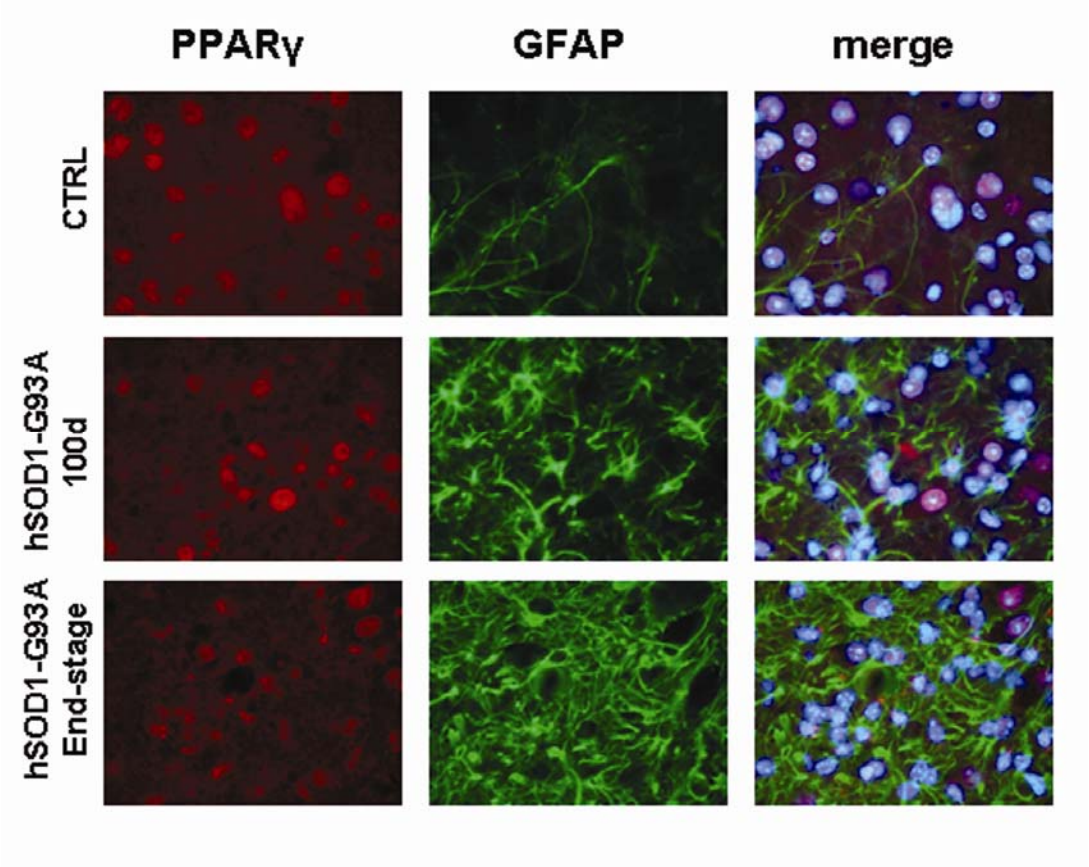

Fig. 13 PPARγ shows a nuclear distribution in astrocytes. Spinal cord sections of female hSOD1-G93A mice at 30 days, 100 days and end stage were stained with an anti-PPARγ antibody (red) and anti-GFAP antibody (green). Nuclei are stained with Hoechst 33342 (blue).

Microglia

Sections were double-stained with Tomato lectin, which recognizes microglia, and antibodies directed against the isoform of PPAR to be analysed. The cell nuclei were stained with Hoechst 33342. PPARα **[Fig. 14]** and PPARβ/δ **[Fig. 15]** were undetectable in microglia cells. PPARγ **[Fig. 16]** shows a nuclear localization.

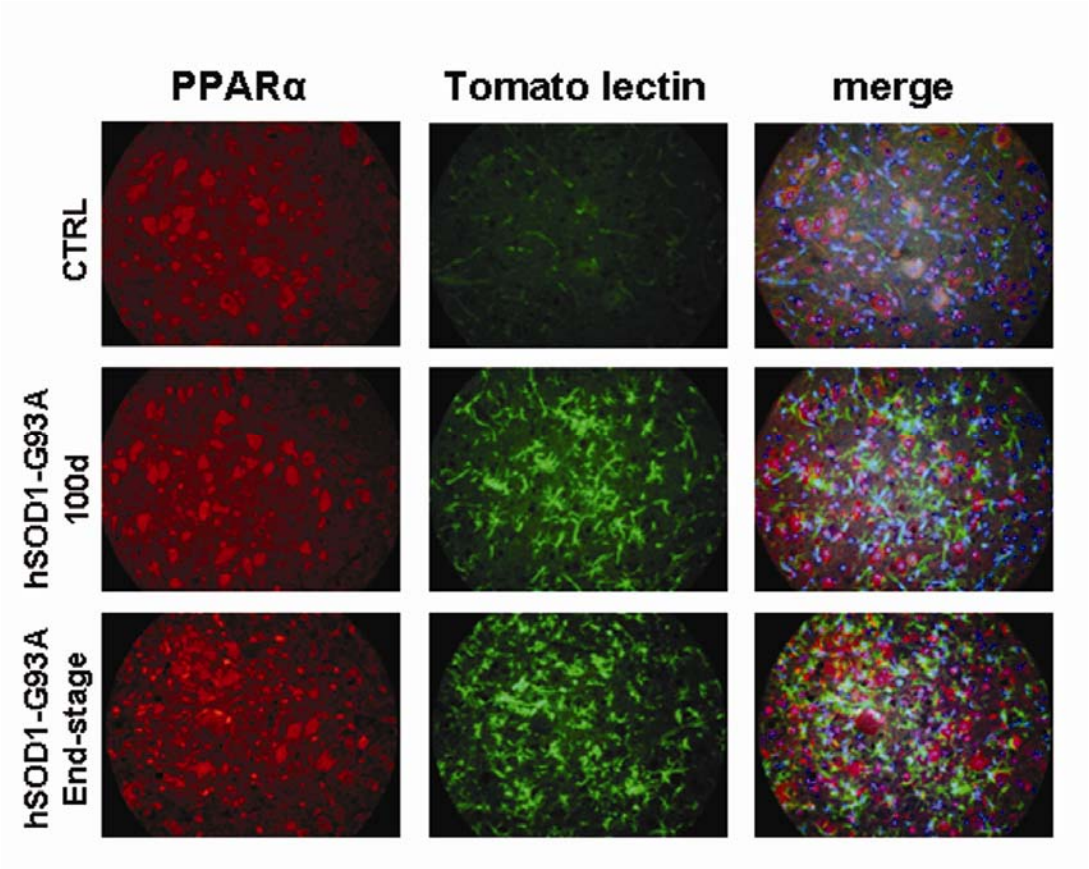

Fig. 14: PPARα is undetectable in microglia. Spinal cord sections of female hSOD1-G93A mice at 30 days, 100 days and end stage were stained with an anti-PPARα antibody (red) and Tomato lectin (green). Nuclei are stained with Hoechst 33342 (blue).

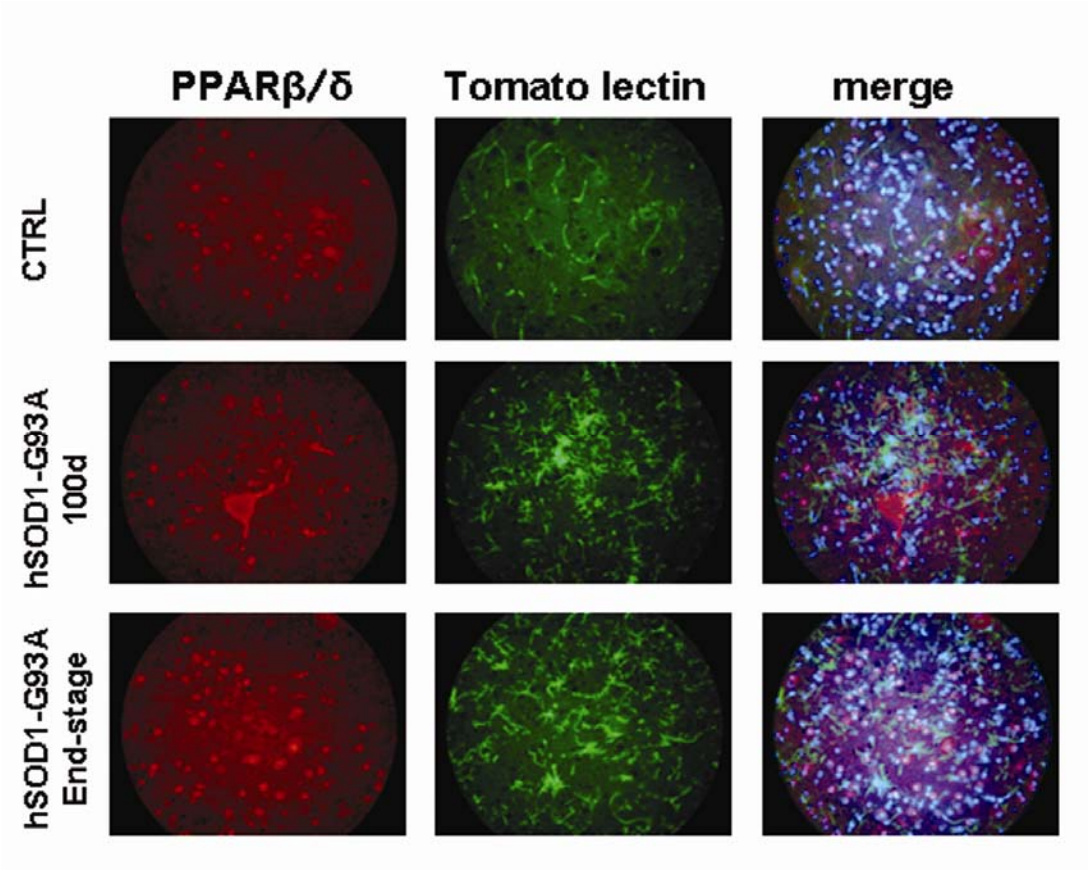

Fig. 15: PPARβ/δ is undetectable in microglia. Spinal cord sections of female hSOD1-G93A mice at 30 days, 100 days and end stage were stained with an anti-PPARβ/δ rabbit serum (red) and Tomato lectin (green). Nuclei are stained with Hoechst 33342 (blue).

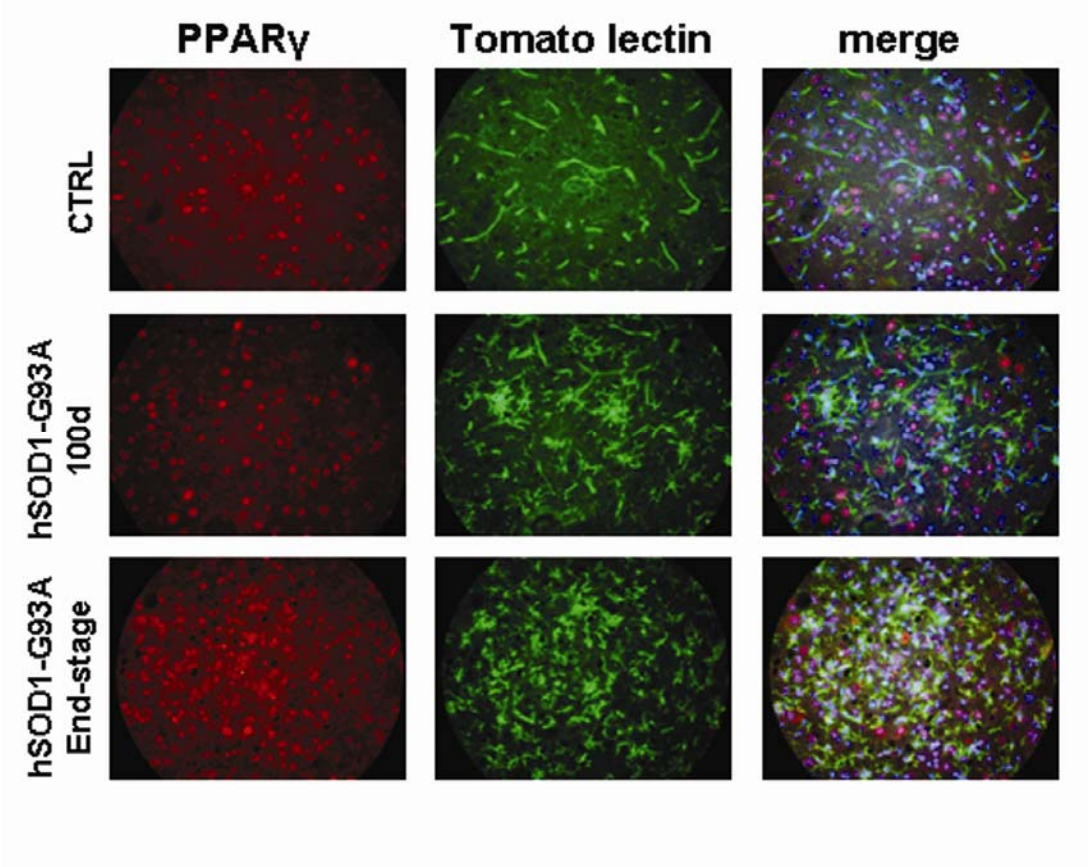

Fig. 16 PPARγ shows a nuclear distribution in microglia. Spinal cord sections of female hSOD1-G93A mice at 30 days, 100 days and end stage were stained with an anti-PPARγ antibody (red) and Tomato lectin antibody (green). Nuclei are stained with Hoechst 33342 (blue).

Evaluation of cells with immunopositive nuclei for PPARs during the pathology

We next decided to evaluate the number of neuronal and glial cells showing PPARα, PPARβ/δ or PPARγ in the nucleus during the progression of the disease in order to identify the specific cell type(s) in which PPAR activity is modulated.

Images of spinal cord ventral horns from hSOD1-G93A mice stained for both PPARs and cell specific markers were taken and motor neurons, astrocytes and microglia with PPARα, PPARβ/δ or PPARγ nuclear expression were counted.

The results show that none of the three isoforms of PPARs increases their nuclear translocation in the different cell types at the end stage of the disease compared to the earlier ages **[Fig. 17, 18, 19]**, confirming the results obtained with the ELISA-based Transcription Factor Assay.

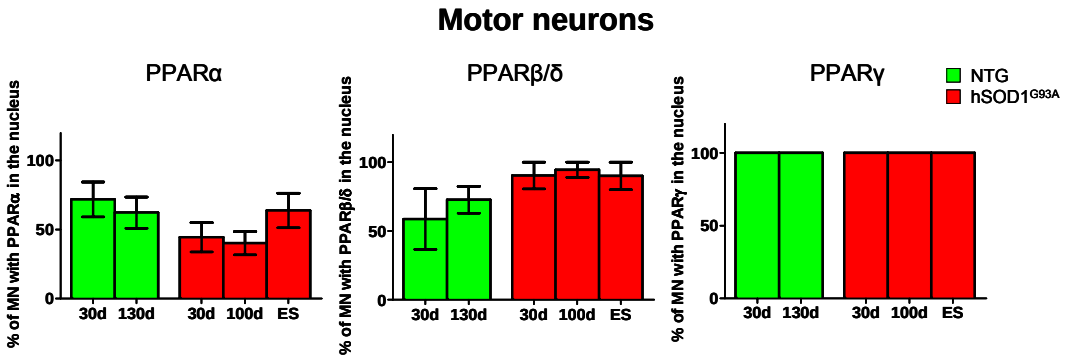

Fig. 17 Motor neurons with PPARα, PPARβ/δ and PPARγ predominantly localized in the nucleus are expressed as percentage of the total number of motor neurons considered.

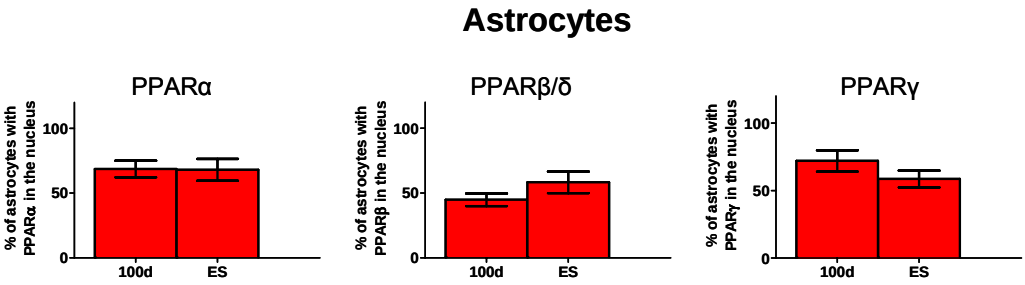

Fig. 18: Astrocytes with PPARα, PPARβ/δ and PPARγ predominantly localized in the nucleus are expressed as percentage of the total number of astrocytes considered.

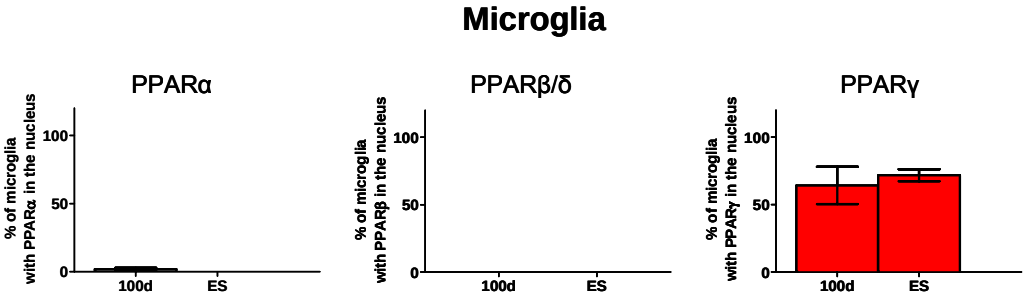

Fig. 19 Microglia with PPARα, PPARβ/δ or PPARγ predominantly localized in the nucleus are expressed as percentage of the total number of microglia considered.

In summary, the results obtained so far show a strong activation of PPAR transcriptional activity at the end stage of the disease. The data obtained with the Transcription Factor Assay on the entire spinal cord and then with the immunohistochemical analysis on the specific cell types show that the increase in PPAR transcriptional activity at the end stage of the disease is not due to an increased translocation into the nucleus, suggesting that ligand-dependent mechanisms and/or different molecules recruitment are involved.

Analysis of the expression of the isoform-specific PPAR target genes in the spinal cord of hSOD1-G93A mice

We next sought to identify the isoform of PPARs responsible for the increase of luciferase activity at the end stage of hSOD1-G93A mice by analysing the expression of the target genes of PPARα, PPARβ/δ and PPARγ in spinal cords of hSOD1-G93A mice by semi-quantitative RT-PCR. We identified a target gene for each isoform of PPARs, i.e. medium chain acyl-CoA dehydrogenase (MCAD) for PPARα (Cullingford et al., 2002) Acyl CoA synthetase long chain 6 (Acsl6) for PPARβ/δ (Basu-Modak et al., 1999) and lipoprotein lipase (LPL) for PPARγ (Victor et al., 2006).

The RT-PCR analysis of MCAD, Acsl6 and LPL showed that MCAD **[Fig. 20]** and Acsl6 **[Fig. 22]** levels remain fairly constant throughout the disease while LPL expression slightly increases at the onset of the disease (100 days), and further increases at the end stage **[Fig. 21]**.

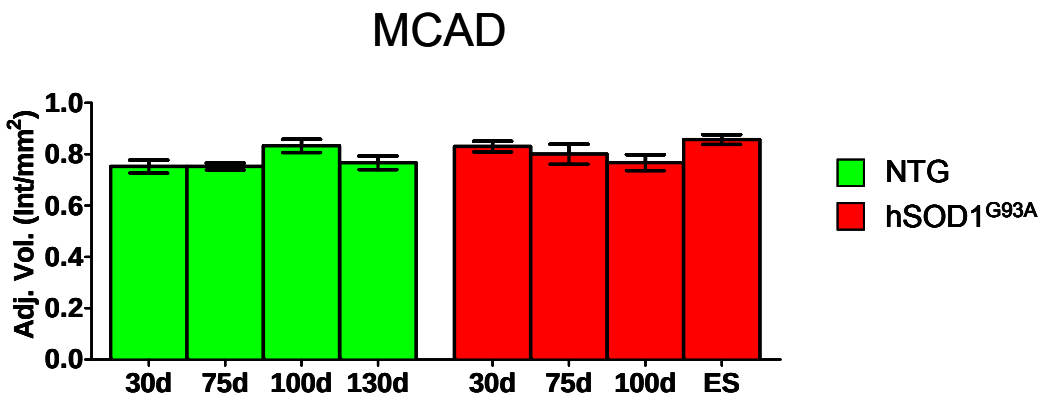

Fig. 20: RT-PCR analysis of MCAD expression in the spinal cord of hSOD1-G93A mice. The spinal cords of female PPRE-Luc +/- mice transgenic or non-transgenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology, i.e. 30, 75, 100 days (d=days), end stage (ES). The RNA was extracted, reverse transcribed to cDNA, and used as template for RT-PCR.

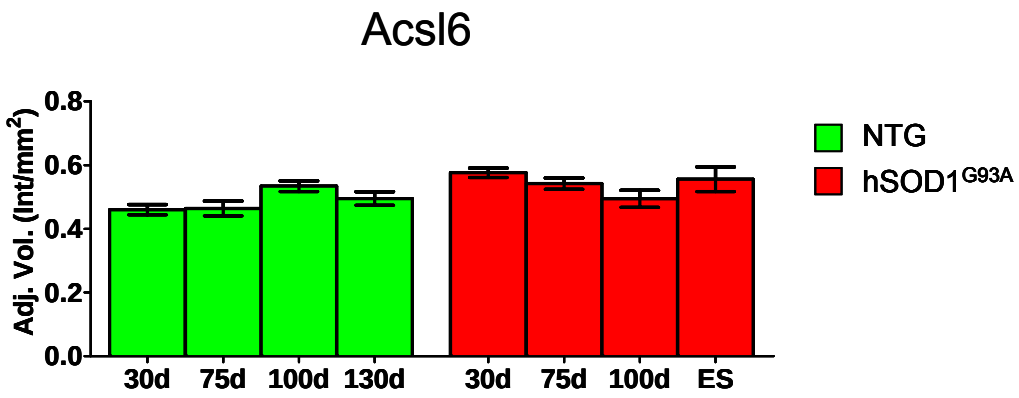

Fig. 21: RT-PCR analysis of Acsl6 expression in the spinal cord of hSOD1-G93A mice. The spinal cords of female PPRE-Luc +/- mice transgenic or non-transgenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology, i.e. 30, 75, 100 days (d=days), end stage (ES). The RNA was extracted, reverse transcribed to cDNA, and used as template for RT-PCR.

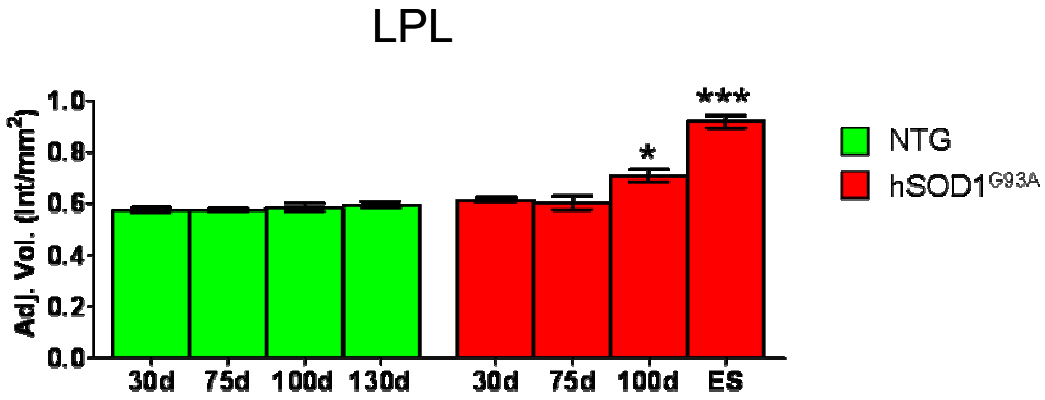

Fig. 22: RT-PCR analysis of LPL expression in the spinal cord of hSOD1-G93A mice. The spinal cords of female PPRE-Luc +/- mice transgenic or non-transgenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology, i.e. 30, 75, 100 days (d=days), end stage (ES). The RNA was extracted, reverse transcribed to cDNA, and used as template for RT-PCR.

These results suggested that PPARγ is the isoform involved in the increase of PPARs activity at the end stage of the disease.

To confirm this observation we decided to analyse the expression of other PPARγ target genes and we selected genes involved in the antioxidant system, like Catalase (Bernardo et al.; Okuno et al., 2008) and Glutathione S-transferase alpha 2 (Gsta2) (Park et al., 2004a), as well as the Peroxisome Proliferator Activated Receptor γ Coactivator α (PGC1α), a coactivator of PPARγ which is known to be regulated by PPARγ itself (Hondares et al., 2006).

The RT-PCR analysis of the expression of Cat, Gsta2 and PGC1α showed that Cat **[Fig. 23]** and PGC1α **[Fig. 25]** show a similar trend of reduction till the onset of the disease, 100 days, then the levels of PGC1α slightly increase while the Cat expression increases in a significant manner at the end stage of the disease. Gsta2

expression remains fairly constant till the end stage when it increases significantly **[Fig. 24]**.

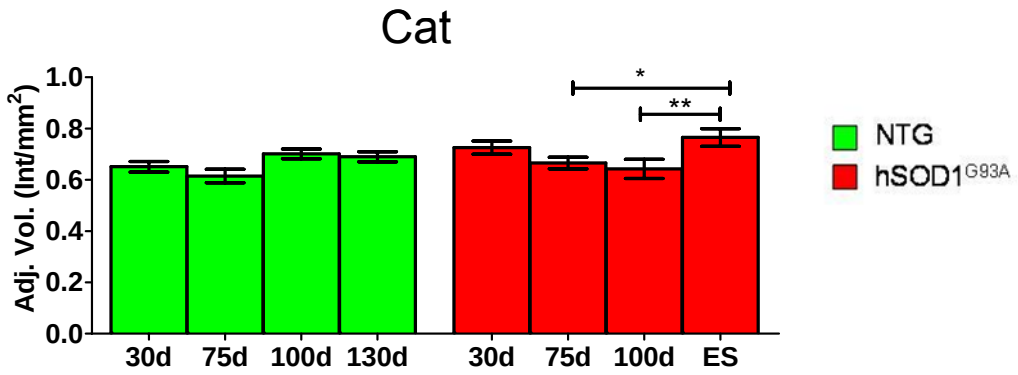

Fig. 23: RT-PCR analysis of Cat expression in the spinal cord of hSOD1-G93A mice. The spinal cords of female PPRE-Luc +/- mice transgenic or non-trangenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology, i.e. 30, 75, 100 days (d=days), end stage (ES). The RNA was extracted, reverse transcribed to cDNA, and used as template for RT-PCR.

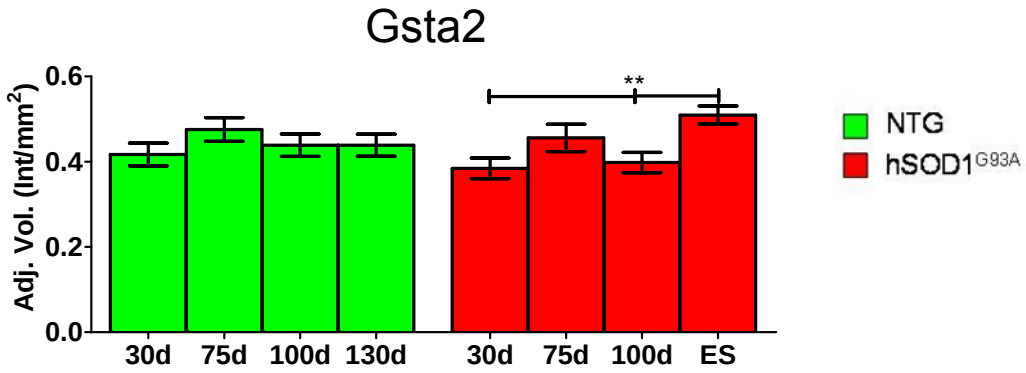

Fig. 24: RT-PCR analysis of Gsta2 expression in the spinal cord of hSOD1-G93A mice. The spinal cords of female PPRE-Luc +/- mice transgenic or non-trangenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology, i.e. 30, 75, 100 days (d=days), end stage (ES). The RNA was extracted, reverse transcribed to cDNA, and used as template for RT-PCR.

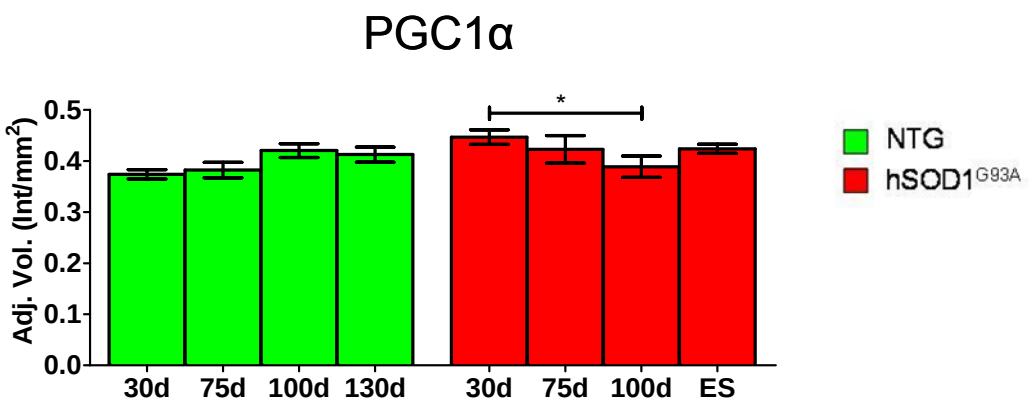

Fig. 25: RT-PCR analysis of PGC1α expression in the spinal cord of hSOD1-G93A mice. The spinal cords of female PPRE-Luc +/- mice transgenic or non-trangenic for the mutant (G93A) human SOD1 were harvested at the critical stages of the pathology, i.e. 30, 75, 100 days (d=days), end stage (ES). The RNA was extracted, reverse transcribed to cDNA, and used as template for RT-PCR.

These results further confirmed our hypothesis that PPARγ activation increases at the end stage of the disease.

Quantification of the nuclear fluorescence of PPARγ

Based on the abovementioned observations, we decided to identify the cell type involved in the increase in PPARγ activity observed in the hSOD1-G93A mice at the end stage of the disease. The nuclear staining of PPARγ in the lumbar sections of spinal cord of nontransgenic and hSOD1-G93A mice seemed to be much more intense in the nuclei of motor neurons compared to the nuclei of non-neuronal cells. Therefore, we decided to confirm this observation by quantifying the intensity of the fluorescence of the signal obtained with the immunostaining for PPARγ and normalizing it versus the intensity of the nuclear staining obtained with Hoechst 33342. The reason why we used this approach is because the DNA content in the

nuclei of motor neurons is more diluted when compared to that of the non neuronal cells, due to the bigger size of motor neuronal nuclei. A normalization on Hoechst 33342 signal would take into account the component of the larger magnitude of motor neuronal nuclei. The quantification of fluorescence intensity was obtained using the NIH software ImageJ.

The results obtained show that the intensity of the fluorescence signal of PPARγ staining normalized to the intensity of Hoechst 33342 staining is much more elevated in the nuclei of motor neurons compared to the nuclei of non-neuronal cells **[Fig. 26]**. These results demonstrate that PPARγ is abundant in motor neurons and led us to hypothesize that the motor neurons are the most likely cell type to be involved in the increase in PPARγ activity at the end stage.

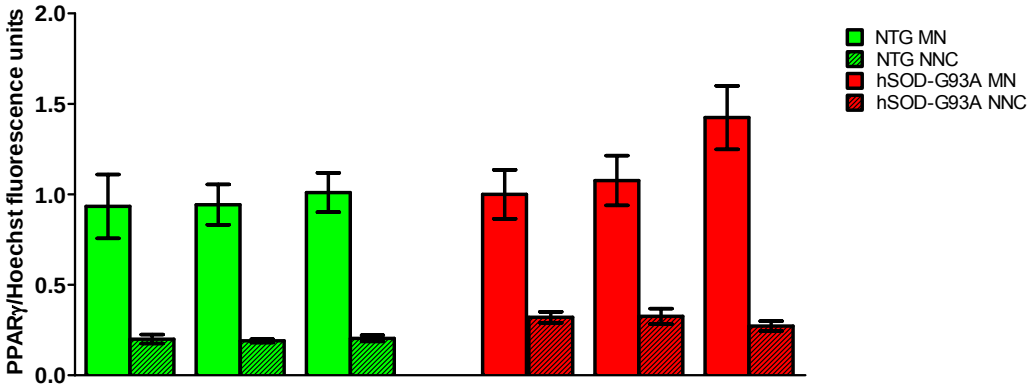

Fig. 26: Quantification of the intensity of PPARγ staining in the nuclei of motor neurons (MN) and non neuronal cells (NNC) in lumbar spinal cord sections stained for PPARγ and the nuclear dye Hoechst 33342 from non transgenic (NTG) and hSOD1-G93A mice.

Analysis of the expression of PPARγ target genes in the NSC-34 motor neuron cell line

On the basis of the high amount of PPARγ identified in the nuclei of motor neurons, we decided to focus on this cell type and to switch to a *in vitro* system. Thus, we investigated the expression of the PPARγ target genes in the NSC-34 cells, an immortalized mouse motor neuron cell line.

RT-PCR analysis of LPL, Cat and PGC1α transcripts in NSC-34 cells transiently transfected with an expression vector coding for hSOD1-G93A showed that the presence of the mutant protein significantly increases the levels of the PPARγ target genes taken into consideration **[Fig. 27].** Gsta2 expression could not be analysed in this cellular model due to its low expression which renders it undetectable by RT-PCR.

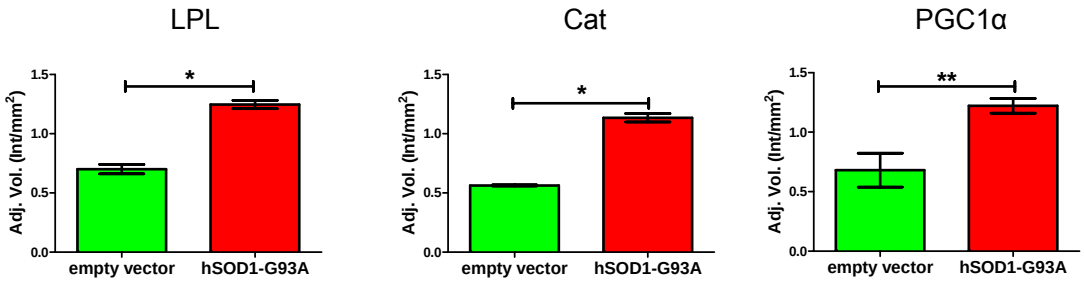

Fig. 27: RT-PCR analysis of LPL, Cat and PGC1α expression in the NSC-34 cells transiently transfected with an expression vector encoding hSOD1-G93A or an empty vector. The RNA was extracted, reverse transcribed to cDNA, and used as template for RT-PCR.

In summary, our results demonstrate an increase of PPARγ activity at the end stage of the disease that, in our hypothesis, could represent a late attempt to counteract the neurodegenerative process. Pharmacologically anticipating the activation of PPARγ could result in a delay of the onset of the pathology and, thus, ameliorate

the disease outcome. Our results on NSC-34 cells transiently transfected with a vector encoding for hSOD1-G93A clearly indicate that motor neurons are the cell type involved in the activation of PPARγ during the degenerative process.

DISCUSSION

The vast majority of neurodegenerative disorders are adult-onset, incurable diseases. Understanding the pathogenetic mechanisms underlying these disorders and finding molecules apt to correct such processes are, therefore, among the hottest topics of biomedical research. The need of effective therapy is also an urgent social need, especially in western countries, where the incidence of these conditions is growing up as a consequence of the aging of the populations and the increased lifespan.

Amyotrophic Lateral Sclerosis is one of the most common adult-onset neurodegenerative diseases characterized by progressive degeneration of upper and lower motor neurons leading to paralysis and death due to respiratory failure within 3-5 years from the onset. The incidence and prevalence of ALS are 1-2 and 4-6 per 100,000 each year, respectively, with a lifetime ALS risk of 1/600 to 1/1,000 (Pasinelli and Brown, 2006). Only one drug, riluzole, has proved effective in extending the lifespan of patients with ALS, but only by 3-6 months (Bensimon et al., 1994; Lacomblez et al., 1996). For this reason the development of effective therapies for this pathology is highly invocated, but to date all attempts to develop novel treatments have failed. In this context, two recent reports on the neuroprotective activity of the PPARγ agonist Pioglitazione in ALS mice result of considerable interest: in these studies, two independent groups demonstrated that Pioglitazone, an agent which is currently used in therapy for the treatment of type II diabetes, is neuroprotective in a mouse model of Amyotrophic Lateral Sclerosis, the hSOD1-G93A transgenic mice. Pioglitazone treatment started before the appearance of the symptoms, improved the motor performance and reduced the weight loss, attenuated motor neuron death and increased the survival. In addition, Pioglitazone reduced microglial activation and gliosis in the spinal cord, decreasing the production of pro-inflammatory mediators, such as iNOS, NF-kB and COX2 (Kiaei et al., 2005; Schutz et al., 2005).While the activity of the PPARs has been extensively characterized in peripheral organs, due to their well-known involvement in different metabolic pathways, the functions of the different isoforms in the central nervous system have been investigated only in the last few years (Bright et al., 2008; Heneka and Landreth, 2007). Nevertheless, the beneficial properties of

the PPARs towards the diseases of the CNS has recently gained more and more consideration based on the anti-inflammatory and neuroprotective activities recently demonstrated in a variety of animal models of neuroprotective disease, like Multiple Sclerosis, Parkinson's disease, Alzheimer's disease and Amyotrophic Lateral Sclerosis (Heneka et al., 2005; Kiaei et al., 2005; Natarajan and Bright, 2002; Niino et al., 2001; Schutz et al., 2005; Shimazu et al., 2005; Yan et al., 2003; Zhao et al., 2005).

On this ground, we decided to investigate the transcriptional activity of PPARs in the the central nervous system of the hSOD1-G93A mouse line, a well-characterized animal model of Amyotrophic Lateral Sclerosis, with the aim of identifying the stage of the disease at which the activity of PPARs becomes relevant to the pathology. To this end, we took advantage of the transgenic mouse PPRE-Luc, available in the laboratory, in which the reporter gene luciferase is expressed under the control of a promoter responsive to PPARs (Ciana et al., 2007). Thus, we crossed the PPRE-Luc mice with the hSOD1-G93A animals to obtain mice that are heterozygous for the PPRE-Luc transgene and heterozygous or null for the hSOD1-G93A transgene. These mice represent a invaluable tool to investigate the transcriptional activity of PPARs during the progression of the disease, because luciferase activity is taken as a surrogate of PPAR activity and can be more easily measured. The analysis of the enzymatic activity of luciferase in the spinal cord and the brain areas of PPRE-Luc;hSOD1-G93A mice shows an abrupt increase of PPAR activity at the end stage of the disease in the spinal cord, which is the organ principally involved in the pathology, and in all the brain areas analysed. We demonstrated that this phenomenon clearly depends on the pathology because it is not shared by the peripheral organs (e.g. kidney and liver). Furthermore, it is not dependent on the metabolic modifications induced from the starvation that the animals experience during the last days of their life when they are almost completely paralysed and, thus, unable to reach food and water. We suggest that the increase in PPAR activity at the end stage of the disease could represent a compensatory mechanism aimed at counteracting the intense neurodegenerative process which takes place at this time. We subsequently decided to further investigate this mechanism by identifying the isoform(s) responsible for the

increase of PPARs activity at the last stage of the disease and the cell type(s) involved. We first analysed the nuclear translocation of PPARα, PPARβ/δ and PPARγ in the spinal cord of hSOD1-G93A mice with an ELISA-based Transcription Factor Assay. The results obtained from these experiments showed that the overall nuclear presence of the different isoforms of PPARs does not change during the course of the disease. In order to obtain a cell specific information about the distribution of PPARs in the spinal cord, we next analysed the localization of PPARα, PPARβ/δ and PPARγ by immunohistochemistry on sections from the lumbar spinal cord of hSOD1-G93A mice at the different stages of the pathology using primary antibodies for the specific isoforms of PPARs and cell specific markers.

Our stainings revealed that all the three isoforms of PPARs are expressed in spinal cord motor neurons; PPARα and PPARβ/δ are localized prevalently into the nucleus but show also a cytoplasmic staining, while PPARγ is exclusively nuclear.

All the three isoforms are present also in astrocytes where they are exclusively nuclear and, in keeping with data published by other groups, PPARα is the most abundant isoform (Heneka and Landreth, 2007). Only PPARγ was detectable in microglia, and was localized into the nucleus. Accordingly to the literature microglia should express also PPARα and PPARβ/δ but PPARγ is the dominant isoform (Cullingford et al., 1998).

Immunohistochemical analysis confirms that the increase in PPAR activity at the end stage of the disease is not dependent on the increase in the nuclear presence of the receptors in the different cell types of the spinal cord, suggesting that it possibly derives from ligand-dependent effects and/or the differential recruitment of co-regulators. To identify the specific isoform whose activity is important during the pathology we analysed the expression of isoform-specific target genes, i.e. MCAD for PPARα (Cullingford et al., 2002), Acsl6 for PPARβ/δ (Basu-Modak et al., 1999) and LPL for PPARγ (Victor et al., 2006). Only the expression of LPL abruptly increases at the end stage of the disease strongly suggesting that the increase in luciferase activity detected at the later stage of ALS is due to the activation of PPARγ. To confirm this result we analysed other PPARγ target genes, i.e. Catalase (Okuno et al., 2008), Glutathione S-tranferase alpha-2 (Park et al.,

2004a) and Peroxisome Proliferator Activated Receptor gamma coactivator 1-alpha (Hondares et al., 2006). The RT-PCR analysis of the expression of Cat, Gsta2 and PGC1α showed that Cat and PGC1α show a similar trend of reduction till the onset of the disease, 100 days, then the levels of PGC1α slightly increase while the Cat expression increases in a significant manner. Gsta2 expression remains fairly constant till the end stage when it increases significantly. LPL, the first PPARγ target gene analysed in our work, is a lipase which transfers lipids to tissues by the hydrolysis of triglycerides in chylomicrons and VLDL particles (Davies, 1994). Its function in the brain has not been yet fully characterized but it was demonstrated that it transports membrane components such as lipids, cholesterol and vitamin E to neurons (Ben-Zeev et al., 1990; Nunez et al., 1995). Furthermore, LPL stimulates the endocytosis of lipids in neuronal cells (Paradis et al., 2003). Thus, it is reasonable to hypothesize that the increase in LPL expression at the end stage of the disease could lead to enchanced endocytosis of lipoproteins limiting the propagation of lipid peroxidation in the highly oxidative environment that is typical of ALS and possibly toxic for motor neurons (Keller et al., 1999). Furthermore, in keeping with our results, another group demonstrated an increase in LPL levels in the context of a wide analysis of differentially expressed genes in the spinal cord of hSOD1-G93A mice at the onset of the pathology (Chen et al., 2010). Catalase is an anti-oxidant enzyme which catalyzes the decomposition of hydrogen peroxide to water and oxygen while PGC1α is a transcriptional co-activator of PPARγ which has also been demonstrated to be transcriptionally regulated by PPARγ itself (Hondares et al., 2006). PGC1α has been reported to play a protective role on mitochondrial biogenesis. Interestingly, recent data showed that the levels of genes controlling cellular bioenergetics, expressed in response to PGC1α activation, are under-expressed in PD patients. Furthermore, activation of PGC1α was described to block dopaminergic neuronal loss in animal models of PD (Zheng et al., 2010). It is not surprising that catalase and PGC1α tend to decrease during the progression of the disease when the anti-oxidant system and the mitochondrial function become progressively impaired. Conversely, their increase at the end stage of the disease is probably dependent on PPARγ activation in an attempt to provide a late protective reaction. Glutathione S-transferase alpha-2 belongs to the family of Glutathione S-

transferases which detoxify a wide variety of compounds, such as xenobiotics or endogenous compounds like peroxidised lipids, by conjugating reduced glutathione to the electrophilic center of the substrate (Berhane et al., 1994). Its PPARγ-dependent activation at the last stage of the disease could represent a protective action directed to scavenge the peroxidised lipid that are present in the degenerated nervous tissue.

Several lines of evidence indicate that the activity of PPARγ is involved in a wide variety of biological processes. It follows that the pharmacological activation of PPARγ in the context of Amyotrophic Lateral Sclerosis could represent a sort of "multi-targeted" approach that could address different mechanisms that are de-regulated in the neurodegenerative process. In their studies, Kiaei and Schutz (Kiaei et al., 2005; Schutz et al., 2005) slowed the progression of the pathology by reducing neuronal cell death and ameliorating the symptoms with a chronic treatment with Pioglitazone started before the onset of the pathology. In view of the data obtained in the course of this thesis, we postulate that the protective effect of Pioglitazone could be due to an anticipation of the protective reaction that we observe at the end stage of the pathology. Pharmacologically bursting this multi-faceted protective reaction could help counteracting the degenerative process before overt neuronal death takes place. Furthermore, it could also contribute to maintain in an active status the compensatory mechanisms of the CNS that have possibly been triggered in response to the early neurodegenerative events, but that fail soon when neuronal dysfunction reaches a critical threshold.

On these bases we decided to further investigate the mechanisms of PPARγ activation at the end stage of the disease by identifying the cell type involved.

The analysis of the fluorescence intensity into the cellular nuclei of lumbar spinal cord sections stained for PPARγ demonstrated that the intensity of the receptor signal is greater in motor neurons than in non- neuronal cells. This result is in line with the demonstration provided by Sarruf and colleagues that the levels of PPARγ are markedly reduced in both whole brain and hypothalamus of neuron-specific PPARγ knock out mice, thus indicating that neurons are the predominant source of PPARγ in the central nervous system (Sarruf et al., 2009). This data led us to hypothesize that motor neurons could be the most likely cell type involved in the

activation of PPARγ at the end stage of the disease *in vivo*. Thus, we decided to analyse the expression of the PPARγ target genes previously analysed in the spinal cords of hSOD1-G93A mice in an immortalized motor neuronal cell line, the NSC-34 cells. NSC-34 is a hybrid cell line produced by fusion of motor neuron enriched, embryonic mouse spinal cord cells with mouse neuroblastoma (Cashman et al., 1992) and represents the best available model for the studies on motor neuron biology.

The expression of LPL, Cat and PGC1α in NSC-34 cells transiently transfected with the hSOD1-G93A-encoding expression vector is significantly increased as compared to the NSC-34 cells transfected with the empty vector. These data clearly confirm the involvement of motor neurons in PPARγ activation at the last stage of the disease; future studies will be aimed to further elucidate the molecular mechanisms of PPARγ protective activity on motor neurons in ALS.

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