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ENDOTHELIN-1 IS OVER-EXPRESSED IN AMYOTROPHIC LATERAL SCLEROSIS AND INDUCES MOTOR NEURON CELL DEATH

TESI DI DOTTORATO

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1. INTRODUCTION

1.1. Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that selectively targets motor neurons (MNs) of cerebral cortex, brainstem and spinal cord, leading to paralysis of voluntary muscles (Shaw and Ince, 1997). Currently, there are approximately 25.000 patients with ALS in the USA, with a median age of onset of 55 years, with a typical disease course of one to five years. The incidence (frequency of new cases per year) and prevalence (the proportion of affected individuals in the population) of ALS are 1-2 and 4-6 per 100.000 each year, respectively (Boillée et al., 2006). In Europe, there are approximately 1.5-2.5 new cases per 100.000 people. In Italy, the SLA is one of the pathologies reported to the National Register of rare diseases. It is believed that people who are living with ALS in Italy are more than 4.000, a number that corresponds to about 6-8 cases /100.000 inhabitants (data from the Italian Ministry of Health). Approximately 10% of cases of

ALS are inherited in a dominant manner (and referred to as familial ALS; FALS), although recessive pedigrees have been described. To date, 12 disease loci have been reported to be associated with typical ALS or atypical motor neuron disease and two loci with ALS with frontotemporal dementia (ALS-FTD) (**Tab. 1** by Ticozzi et al., 2011). The first pathogenic mutations causing FALS were identified in 1993 in the *superoxide dismutase 1 (SOD1)* gene, encoding for the Cu/Zn superoxide dismutase, a cytoplasmic enzyme responsible for the catabolism of superoxide radical to hydrogen peroxide and molecular oxygen (Rosen et al., 1993). *SOD1* mutations are the most frequently identified causes of FALS, accounting for ~20% of all patients. To date, there is no conclusive explanation on how mutations in the *SOD1* gene cause ALS. Initially, it was hypothesized that mutations would impair the enzymatic activity of the protein, thus resulting in increased cellular levels of reactive oxygen species, oxidative stress and neuronal death. Recently, several studies showed that mutant SOD1 is prone to misfolding and forms cytoplasmic aggregates which may lead to cell death by sequestering other cytoplasmic proteins essential

for neuronal survival. An exciting step forward in ALS genetics is represented by the recent discovery of mutation in the *TAR DNA binding protein (TARDBP)* and *fused in sarcoma/traslocated in liposarcoma (FUS)* genes, representing ~5% of all FALS cases each. *TARDBP* and *FUS* genes encodes for a DNA/RNA binding protein that has been demonstrated to play a role in several biological processes, including gene transcription, splicing regulation, transport and stabilization of mRNA molecules. TARDBP and FUS, which normally localizes to the cell nucleus, in ALS are absent from the nuclei of neurons and form cytoplasmic inclusions, suggesting a nucleo-cytoplasmic redistribution of the proteins. Thus, it has been hypothesized that TARDBP and FUS mutations may contribute to ALS pathogenesis through the formation of cytoplasmic inclusions and / or the loss of the physiological nuclear functions of the protein (Ticozzi et al., 2011). Pathogenetic mutations in seven other genes (*ALS2, SETX, SPG11, VAPB, ANG, FIG4,* and *OPTN*) account for < 5% of families. Usually those mutations are found in isolate pedigrees, often with atypical ALS phenotypes. Lastly, variants in several other genes have been suggested to be

associated with FALS, although the data are still inconclusive (Ticozzi et al., 2011).

Most incidence (90%) of ALS is sporadic (SALS), that is, without an obvious genetic component. Age and gender are documented SALS risk factors (the male:female ratio is 3:2) (Boillée et al., 2006). The causes of most cases of sporadic ALS are yet undefined. Investigations have identified multiple perturbations of cellular function in ALS motor neurons, incriminating: oxidative stress, neuroinflammation, dysregulated interaction with neighboring glial cells, excessive excitatory tone, mitochondrial dysfunction, dysregulated endosomal trafficking, endoplasmic reticulum stress, protein misfolding, impaired energy production, abnormal calcium metabolism, altered axonal transport, dysregulated transcription and RNA processing and activation of proteases and nucleases (as described in **Fig. 1** by Ferraiuolo et al., 2011). Several factors are proposed to trigger these fenomena, including latent infections by viral and non-viral agents, toxins (for example, insecticides and pesticides) and autoimmune reactions (Ferraiuolo et al., 2011; Pasinelli and Brown, 2006).

Sporadic and familial ALS produce similar pathological hallmarks, including progressive muscle weakness, atrophy and spasticity, each reflecting the degeneration and death of upper and lower motor neuron. However, muscles that control eye movements and the urinary sphincters are spared. Respiratory failure causes death, which typically occurs within five years of developing this debilitating condition (Boillée et al., 2006).

Regrettably, there is no primary therapy for this disorder and the single drug approved for use in ALS, riluzole, only slightly prolongs survival (Pasinelli and Brown, 2006).

1.2. Reactive gliosis

ALS is regarded as a MN-specific disorder, but increasing evidence indicates that non-neuronal cells play a significant role in disease pathogenesis (Van Den Bosch and Robberecht, 2008). Astrocytes represent the largest cell population in the central nervous system (CNS). They closely interact with neurons to provide structural, metabolic and trophic support and actively participate in modulating neuronal excitability and

neurotransmission by controlling the extracellular levels of ions and neurotrasmitters. *In vitro*, astrocytes exert potent trophic influences on motor neuron through a variety of proteins and low molecular weight molecules (Barbeito et al., 2004). Astrocytes respond to CNS damage by proliferating and adopting a reactive phenotype characterized morphologically by hypertrophic nuclei and cell bodies and elaboration of distinct long and thick processes with increased content of glial fibrillary acidic protein (GFAP). In addition, reactive astrocytes express a wide variety of markers such as cytoskeleton proteins, cell surface and matrix molecules, protease, protease inhibitors and several growth factors and cytokines.

Reactive astrogliosis is not merely a marker of neurophatology, but play essential roles in orchestrating the injury response. It is not clear if activated astrocytes mediate a protective action or amplify neuron death. Activated astrocytes may participate in the death of MNs by several mechanisms including release of inflammatory mediators such PGE2, cytokines (IL-1β, TNFα, IL-6, TGF-β1), reactive oxygen species, NO and excitatory amino acids (glutamate, ATP and serine),

reduction of lactate release, activation of pro-NGF-p75 receptor signalling, reduction of expression and activity of glutamate reuptake transporters EAAT2, increasing of glutamate efflux from astrocytes and modulation of the Ca^{2+} permeable AMPA receptor GluR2 subunit expression in MNs which could potentiate excitotoxic cell death of motor neurons (Ferraiuolo et al., 2011; Philips and Robberecht, 2011; Boillée et al., 2006). After activation by injury, astrocytes and microglia release factors that recruit other astrocytes and microglia to the injury site. This recruitment can lead to glial scar formation, which has the potential to block the growth and maturation of neural progenitors and also impedes neovascularization, thus inhibiting recovery after injury (Fawcett and Asher, 1999).

Since gliosis also occurs in a variety of conditions such as cerebral ischemia, Alzheimer's disease, Parkinson's disease, frontotemporal dementia and Huntington' disease, it has long been suggested to be a non-specific response of glial cell to injury and often it is not considered as a primary pathogenic element in ALS. On the other hand, recent evidence indicates the existence of other molecular mechanisms by which activated

astrocytes may contribute to either the death of neurons or to their survival in response to damage. Understanding the interactions between neurons and glia in ALS may help to explain the progressive nature of ALS. Accordingly, astrocytes and reactive astrocytes are increasingly recognized as potential targets for novel therapeutic strategies in a variety of CNS pathological conditions.

1.3. Endothelins

Enormous progress has been made in characterizing molecular mechanisms underlying reactive astrocyte functions, and a number of signalling molecules released by astrocytes is being defined. Several endogenous agents have been implicated as possible mediators of astrogliosis, including endothelins. In 1988, the endothelial cell-derived peptide endothelin (ET) was isolated from porcine aortic endothelial cells (Yanagisawa et al., 1988) as a 21 amino acid cyclic peptide, with two disulphide bridges joining the cysteine amino acids (positions 1–15 and 3–11). The C-terminal end contains the amino acids that bind to the

receptor, the N-terminal end determines the peptide's binding affinity to the receptor. There appears to be at least 2 other endothelin isoforms including endothelin-2 (ET-2) and endothelin-3 (ET-3), which differ from ET-1 in two and six amino acid residues, respectively (**Fig. 2** by Khimji et al., 2010).

The endothelin peptides are produced through a set of complex molecular processes (**Fig. 3** by Khimji et al., 2010): preproendothelins are synthesized via transcriptional activation of the preproendothelin gene. The translational product is a 203-amino acid peptide known as preproendothelin which is cleaved at dibasic sites by furin-like endopeptidases to form big endothelins; these biologically inactive 37- to 41-amino acid intermediates are cleaved at Trp21–Val22 by a family of endothelin-converting enzymes (ECE) to produce mature ET-1.

Since its discovery, ET-1 has been implicated in a wide number of physiological systems and ET peptides and ET receptors have been localized in a wide variety of tissues including: kidney, lung, liver, cardiovascular, renal, gastrointestinal, pituitary, adrenal, nasal, immune, hepatic, genitourinary, endocrine systems and brain (Watts, 2009; **Fig.4**

by Nelson et al., 2003). The precise role that endothelins play in the CNS is not clear; it is however clear that ET-1 is expressed in various regions and different cell types of the brain including Purkinjie, cortical, paraventricular and supraoptic nuclei hypothalamic neurons (Giaid et al., 1989), cerebellum, motor neurons in the spinal cord and in peripheral ganglia (Prasanna et al., 2011), in endothelial cells (Yoshimoto et al., 1990) and astrocytes (MacCumber et al., 1990). Expression in these key areas of the brain suggests that ET-1 has a neuromodulatory role in the CNS.

At this time, only two classes are recognized as ET receptors, the ET_A receptor and ET_B receptor which are G protein-coupled receptors belonging to the rhodopsin superfamily (Class A), possessing a 7α helical transmembrane domain, an extracellular amino terminus and an intracellular carboxy terminus. In many instances, endothelin receptors are found in tissues that are in close proximity to the site of ET synthesis and release, suggesting autocrine or paracrine effect of peptide (Prasanna et al., 2011). ET_A receptors are recognized by their relative insensitivity to stimulation by the agonist ET-3, whereas

ET-2 and ET-1 have similar affinities. Antagonists selective for subtype A of ET receptor include BQ123, ZD4054, atrasentan, darusentan, ambrisentan and sitaxsentan. Selective agonists of this receptor have been poorly defined. The pharmacology of the ET_B receptor is somewhat richer than that of the ET_A receptor in that multiple agonists of the ET_B receptor are recognized, including sarafotoxin 6c (S6c) and IRL1620. Selective antagonists of the ET_B receptor include BQ788, A192621, RES7011 and IRL2500. Unlike the ET_A receptor, all three endogenous ET peptide (ET-1, ET-2 and ET-3) have similar affinity for the ET_B receptor (Watts, 2009).

ET-1 signaling is extremely complicated and ET receptor activation leads to diverse cellular responses. In one of the canonical signaling pathways, the dissociated G-protein subunit activates phospholipase C (PLC). Activation of PLC leads to the formation of inositol triphosphate (IP3) and diacylglcerol (DAG). IP3 then diffuses to specific receptors on the endoplasmic reticulum and releases stored Ca^{2+} into the cytosol. This causes a rapid elevation in intracellular Ca^{2+}, which in turn causes cellular contraction. Also ET stimulation induces activation of PLD,

yielding phosphatidic acid (PA) which is dephosphorylated to DAG which activates protein kinase C (PKC). Further signalling pathway induced by the ET receptor include stimulation of arachidonic acid production and prostaglandin release and this occurs as a result of activation of phospholipase A2. In addition, endothelin-1 stimulates protein tyrosine kinases (PTK) such as FAK and RAS which induces the RAF/MEK/MAPK pathway, subsequently stimulating transcription of protooncogenes such as c-FOS, c-MYC, c-JUN, in turn activating cell growth and metastasis (**Fig. 5** by Khimji et al., 2010; Schinelli, 2006).

Previous studies have shown that both CSF and plasma levels of ET-1 are low in the normal CNS but significantly increase following neurological disorders such as virus infection, subarachnoidal hemorrhage (Blomstrand et al., 1999), traumatic brain injury (Salonia et al., 2010), infarcts, transient forebrain ischemia, stroke (Lampl et al., 1997), Alzheimer's disease, astrocytic tumors (Stiles et al., 1997) and inflammatory diseases (Schinelli, 2006).

Expression levels of Endothelins and their receptors are massively up-regulated in activated astrocytes and microglia, and

are possibly implicated in initiating and sustaining reactive gliosis, via an autocrine mechanism (Koyama and Michinaga, 2012; Gadea et al., 2008; Ehrenreich et al., 1991) promoting cell proliferation, mitogenesis, hypertrophy, stimulation of DNA synthesis, (Couraud et al., 1991; Teixeira et al., 2000; Gadea et al., 2008), cytoplasmic expansions of stellate astrocytes, re-organization of cytoskeletal actin filaments (Koyama and Baba, 1999), production of VEGF-A (which stimulate astrocytic proliferation) (Koyama et al., 2012) and formations of focal adhesions (FAs) (which regulate cell adhesion, migration and proliferation) (Koyama and Michinaga, 2012). The ET-1 may contribute to elevating extracellular glutamate levels through promotion of efflux of glutamate via reversal of the Na^+ and/or K^+ glutamate transporters (Sasaki et al., 1997), inhibition of glial glutamate transporter expression and decrease of glutamate uptake (Leonova et al., 2001). The ET-1 inhibits gap junctional communication between astrocytes through downregulation of the expression of connexin43 (CX43), the main protein forming gap junction channels in astrocytes which is involved in multiple functions of astrocytes, such as intracellular and extracellular

ionic homeostasis, metabolic trafficking, proliferation, neuroprotection, propagation of death signals and cell swelling (Herrero-Gonzalez et al., 2009; Giaume and McCarthy, 1996). ET-1 acts as local pro-inflammatory factor in various inflammatory conditions both in the periphery and in the brain and influences the release of neuroinflammatory mediators such PGE2, NO and COX2 in glial cells (Koyama et al., 1999; Filipovich and Fleisher-Berkovich, 2008). Thus, while it is still unclear if reactive astrogliosis is beneficial or detrimental to neuronal survival and regeneration, there is evidence to suggest that ET's actions on reactive glial cells may promote neurotoxicity within the injured CNS (Peters et al., 2003). Conversely, other studies demonstrate that ET mediates pro-survival effects by stimulating the astrocytic production of neurotrophic factors such GDNF, NGF, NT-3 and BDNF through ET_B receptor (Koyama and Michinaga, 2012) and the neuroprotective endocannabinoids (anandamide and 2-Arachidonoyl glycerol) that may promote neuronal viability and re-generation of injured nerve cells (Koyama et al., 2003; Walter and Stella, 2003).

ET-1 is implicated in many CNS pathologies that involve reactive gliosis (Hasselblatt et al., 2001, 2003; Egnaczyk et al., 2003; Sofroniew, 2005); however, its involvement in ALS has not been studied. It is known that both ET-1 and its receptors ET_A and ET_B are expressed in the spinal cord under normal conditions (Giaid et al., 1989; Peters et al., 2003) and that, compared to physiological conditions, there is a significant increase in ET-1 levels in plasma and in the CNS following spinal cord trauma and brain injury (Uesugi et al., 1996). In addition, a whole genome expression study has shown that the mRNA of ET-1 is up-regulated in the cortex of patients with ALS (Lederer et al., 2007).

2. AIM OF WORK

The aim of my thesis is to study the expression of ET-1 in the spinal cord of G93A mouse model of ALS and ALS patients. I also studied the effects of ET-1 exposure on MN survival by using a well characterized *in vitro* model of mixed spinal cord cultures consisting of MNs and astrocytes bearing a stellate phenotype, which mimic the reactive gliosis observed in ALS. I report that ET-1 is toxic for MNs through a mechanism that involves ET_A and ET_B receptors and is mediated by reactive astrocytes.

3. MATERIALS AND METHODS

3.1. Mixed spinal cord cultures

Mixed spinal cord cultures (MSCs) were prepared from 13- to 15-days-old Wistar rat embryos as previously described with minor modifications (D'Antoni et al., 2011). Spinal cords were dissected in HBSS (Gibco–Invitrogen); meninges and dorsal horns were removed and spinal cords were digested and incubated in trypsin 0.05% (Gibco–Invitrogen) for 5 min at 37°C. Trypsin was inactivated with FBS (Gibco–Invitrogen), and spinal cords were washed three times with HBSS and then dissociated by gentle pipetting. Cell suspension (40 µl) was plated on an area of approximately 50 mm^2 onto 35 mm cell culture dishes (Nunc) coated with poly-L-ornithine (10mg/ml, Sigma) at a density of about 80.000 cells/dish in DMEM with 10% of FBS and 1% P/S. Cell density was lower than previously established (D'Antoni et al., 2011) and resulted in a slightly higher percentage of MNs survival without substantial changes in the percentage of glial cells (see results). Three hours later 2 ml

of Neurobasal Medium (Gibco–Invitrogen) supplemented with 1% P/S, 10% FBS, 2% HS (Horse serum, Gibco–Invitrogen), 0.1 mg/ml conalbumin (Sigma), 20 nM progesterone (Sigma), 0.1 mM putrescine (Sigma), 0.1 mM IBMX (3-isobutyl-1-methylxanthine, Sigma), 1% G5 supplement (Gibco–Invitrogen) and 10 μM forskolin (Tocris) (NB-G5) were added to the 40 μl of plating medium. We also used Neurobasal Medium containing 2% B27 supplement (Gibco–Invitrogen), 1% P/S and 0.5% Glutamax (Gibco–Invitrogen) (NB-B27 serum free). In some experiments, cells were treated with AraC (cytosine β-D-arabinofuranoside) 5 μM starting at the 3 days in vitro (DIV). At 9 DIV 600 μl of culture medium was replaced with fresh medium.

3.2. Pharmacological Treatments

MSCs were treated with different concentrations (10 nM, 100 nM, 200 nM, 500 nM and 1 μM) of ET-1 (Bachem) for 48 hours and with a fixed 100 nM ET-1 concentration for different lengths of time (8, 24, 48 and 72 hours). The toxic effect of ET-1

on MNs was investigated in the presence of the selective antagonists of receptors A, BQ-123 (2 μM, Tocris), and B, BQ-788 (200 nM-2 μM, Tocris).These treatments were performed 2 hours before adding ET-1 (100 nM for 48 hours). We also administered two selective agonists of the receptor B, ET-3 (100 nM, Bachem) and Sarafotoxin S6C (10 nM, Bachem) for 48 hours. In some experiments cells were treated for 48 hours with GYKI 52466 20 μM (a selective non-competitive AMPA receptor antagonist) in the presence or absence of ET-1. The effect of ET-1 was evaluated by directly counting the number of surviving MNs visualized by immunocytochemistry with the SMI32 antibody (see immunocytochemistry section). MNs were identified as SMI32-positive large (>20 μm) cells with prominent neuritic arborization and a single long axon. Percentage of cell death was calculated as a difference between percentage of surviving neurons under control condition (100%) and percentage of surviving neurons after treatments.

3.3. AMPA toxicity

MSCs with or without a pre-treatment with ET-1 10-100 nM for 48 hours were exposed at DIV 14 to the glutamate receptor agonist AMPA (50 μM, Tocris) for 15 min in Locke solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 5.4 mM glucose, 5.8 mM HEPES and 3.6 mM $NaHCO_3$, pH 7.4), washed twice and then maintained for 20 hours at 37°C in a fresh defined medium (Neurobasal medium containing 1% P/S, 20 nM progesterone, 0.1 mM putrescine, 0.1 mM IBMX, 1% G5 supplement and 10 μM forskolin). The excitotoxic effect of AMPA was quantified by directly counting the number of surviving MNs visualized by immunocytochemistry with the SMI32 antibody.

3.4. Immunocytochemistry

MSCs were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature (RT). Cultures were incubated with PBS containing 0.2% Triton for 10 min at RT and then with the appropriate blocking solution [PBS containing 4% normal goat

serum (Vector) and 4% bovine serum albumin (Sigma)] for 30-45 min at RT with gentle shaking. Subsequently, cultures were incubated o.n. at 4°C or for 2 hours a RT with the following primary antibodies: SMI32, a non-phosphorylated neurofilament-H (mouse, 1:4000, Steinberger Monoclonals, Baltimore, MD, USA); anti-microtubule-associated protein 2 (MAP2) (mouse, 1:1000, Steinberger Monoclonals, Baltimore, MD, USA); anti-glial fibrillary acid protein (GFAP) (rabbit, 1:1000, Dako Cytomation). For double-labelling experiments, cultures were co-incubated with anti-GFAP clone GA5 (mouse, 1:1000, Millipore) or SMI32 antibodies (mouse, 1:4000, Steinberger Monoclonals, Baltimore, MD, USA) together with anti-ET_A receptor antibody (rabbit, 1:250, Santa Cruz Biotechnology) or anti-ET_B receptor antibody (rabbit, 1:250, Santa Cruz Biotechnology). After washing, cultures were incubated for 45 min at RT with corresponding anti-mouse and anti-rabbit fluorescent secondary antibodies. Hoechst 33258 (0.4 μg/ml, Sigma) was used for nuclear counterstaining. Images were analyzed on a Zeiss (Imager.D2) fluorescence microscope and captured with an Axiovision Imaging System. Images of double-labelling

experiments were obtained using a Zeiss confocal laser-scanning microscope (LSM 510 Meta).

3.5. Western blotting

MSCs at 15 DIV were scraped in ice-cold lysis buffer (40 mM Tris–HCl pH 6.8, 1 mM EDTA, 5 mM EGTA, 20 μg/ml leupeptin, 0.1 mM PMSF, 20 μg/ml aprotinin, 1.5% SDS) and kept on ice for 15 min. Cells were centrifuged at 1000 × g for 15 min, to remove nuclear material and cell debris. Spinal cords of control and G93A mice at different ages (30, 100 and 130 days) were weighed and homogenized in 10% (w/v) buffer containing 50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 0.1mM phenylmethylsulfonyl fluoride (PMSF), 0,5% Sodium Deoxycholate, 1% NP40, 1mM Sodium orthovanadate and protease inhibitor cocktail (Roche). The homogenate was centrifuged for 15 min at 20800 x g (4°C) to separate the nuclear pellet and cellular debris. Protein concentration was determined by using the bicinchoninic acid method (BCA kit; Pierce, Rockford, IL). Then, proteins were boiled for 5 min in 4x

denaturing sample buffer and loaded onto 8% or 12% (only for ET-1 assessment in extracts of spinal cord). SDS-polyacrylamide gels. The separated proteins were blotted onto nitrocellulose membranes (Hybond Cextra, Amersham, Buckingumshire, UK). The filters, blocked for 4 hours in 5% non-fat dried milk in TTBS (100 mM Tris, 0.68 M NaCl, and 0.1% Tween 20), were then incubated o.n. with the following primary antibodies: anti-actin (mouse, 1:1000, Cell Signaling), anti-GFAP (mouse,1:100, Chemicon), anti-MAP2 (mouse, 1:2000, Millipore) anti-ET_A receptor antibody (rabbit, 1:200, Santa Cruz Biotechnology) and anti-ET_B receptor antibody (rabbit, 1:200, Santa Cruz Biotechnology). For ET-1 detection membranes were blocked o.n. in 5% non-fat dried milk in TBS (100 mM Tris, 0.68 M NaCl, and 0.1%) and then o.n with ET-1 antibody (goat, 1:200, Santa Cruz Biotechnology). After washing, membranes were incubated for 1 hour with the appropriate secondary antibodies conjugated with horseradish peroxidase (1:10000, Amersham or Santa Cruz Biotechnology for ET-1 detection). In the experiments aimed at evaluating the expression of ET_A and ET_B receptors in MSCs, lysates from mouse brain and testis were used

as positive control because of the relative higher levels of ET_B and ET_A receptors in these two tissues respectively (Hagiwara et al., 1993; Elshourbagy et al., 1993; Fantoni et al., 1993; Maggi et al., 1995). The signal was revealed by a chemiluminescent detection method (ECL plus, Amersham) and quantified by computer-assisted densitometry using the VersaDoc 4000 Imaging System (Biorad).

3.6. Statistical analysis

Data were statistically analysed using t-test or one-way analysis of variance (One-Way ANOVA) followed by post hoc Holm-Sidak method.

3.7. ALS patients: Tissue preparation and Immunohistochemistry

Post-mortem material was obtained at autopsy from 12 sporadic ALS patients at the Department of Pathology of the Academic Medical Center (University of Amsterdam). All patients fulfilled the diagnostic criteria for sporadic ALS (sALS;

El Escorial criteria; Brooks et al., 2000), were reviewed independently by two neuropathologists. The diagnosis of ALS was confirmed according to standard histopathological criteria (Ince et al., 1998; Piao et al., 2003). The group included 6 patients with rapid disease progression and short-term survival < 18 month (ALS-st) and 6 patients with slow disease progression and long-term survival > 48 months (ALS-lt) (see supplementary **Table 1**). All ALS patients died for respiratory failure. The control spinal cord tissue was obtained from 6 patients (4 males and 2 females) who died from a non-neurological disease (cause of death: myocardial infarction, renal failure, pulmonary embolism). Both ALS and control patients included in the study displayed no signs of infection before death. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes and approval was obtained from the relevant local ethical committees for medical research. All autopsies were performed within 12 hours after death. After removal of the spinal cord, 0,5 cm thick slices, taken from the cervical (C7), thoracic (T4 and T8) and lumbar (L1) levels were fixed in 10 % buffered formalin.

Paraffin-embedded tissue was sectioned at 6 μm and mounted on pre-coated glass slides (StarFrost, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany). Representative sections of all specimens were processed for haematoxylin and eosin, Klüver-Barrera and Nissl stains. To define different cell populations we used serial sections stained with Nissl, astroglial and microglial markers (GFAP and HLA-DR, see below).

3.8. Immunohistochemistry in ALS patients

GFAP (rabbit, 1:4000, DAKO, Glostrup, Denmark; monoclonal mouse, 1:50, DAKO), vimentin (mouse clone V9, 1:400, DAKO), neuronal nuclear protein (NeuN) (mouse, clone MAB377, IgG1; 1:1000, Chemicon, Temecula, CA, USA), major histocompatibility complex class II antigen (HLA)-DP, DQ, DR (HLA-DR; mouse clone CR3/43; 1:400, DAKO), and CD68 (mouse, clone PG-M1, 1:200, DAKO) were used in the routine immunohistochemical analysis of ALS specimens. For the detection of ET-1 we used a rabbit polyclonal antibody (1:500,

Bachem). Single-label immunohistochemistry was performed as previously described (Aronica et al., 2001; Aronica et al., 2003) with the Powervision kit (Immunologic, Duiven, The Netherlands) and 3,3-diaminobenzidine (DAB) as chromogen. For double-label immunohistochemistry with anti-GFAP and HLA-DR, the chromogens used were Vector Blue- and Nova RED-substrate (Vector Laboratories). Immuno-double stained slides were analyzed with the use of Nuance 3.0 Spectral Imaging (Caliper Life Sciences, Hopkinton, MA, USA).

3.9. Immunohistochemistry in G93A mice

Transgenic mice at different ages (60, 90 and 120 days) overexpressing the human G93A SOD1 and control wild type mice were used. Animals were perfused transcardially, the spinal cords were removed, stored in 4% PFA for one day and then transferred in 30% sucrose solution for three days. Frozen sections (40 μm thick) were incubated with TBS containing 0.4% Triton for 30 min at RT, then with the blocking solution [TBS containing 4% normal goat serum (Vector), 5% bovine serum albumin (Sigma) and 0,2% Triton] for 30 min at RT and

subsequently with the anti-ET-1 antibody (rabbit, 1:4000, Bachem) o.n. at 4°C. After washing, sections were incubated with the biotinylated secondary antibody. The avidin−biotin−peroxidase/DAB method was used to develop the colour reaction. Sections were then mounted on gelatine-coated slides and analyzed with the use of a Zeiss microscope (Imager.D2). Double-labelling experiments aimed at studying the expression of ET-1 in reactive astrocytes were performed by co-incubating slices in primary antibodies anti-ET-1 (rabbit, 1:4000, Bachem) and anti-GFAP (mouse, 1:1000, Millipore); signal was revealed with anti-rabbit and anti-mouse secondary antibodies conjugated with Alexa Fluor 488 and Cy3, respectively. For experiments aimed at evaluating localization of ET-1 in microglia, sections were first incubated with ET-1 antibody o.n. at 4°C, washed, incubated with anti-rabbit fluorescent secondary antibody (Alexa Fluor 488) for 1 hour at RT, then fixed in 4% PFA for 20 min, and then incubated with anti-Iba1 antibody for 4 hours (rabbit, 1:500 Wako), washed and then incubated with anti-rabbit fluorescent secondary antibody (Cy3) for 45 min at RT. Sections were then mounted with gelatine on slides and analyzed

with the use of Zeiss confocal laser-scanning microscope (LSM 510 Meta).

4. RESULTS

4.1. Endothelin-1 is up-regulated in reactive astrocytes in G93A mice and ALS patients

Immunohistochemistry and Western blotting experiments revealed that ET-1 is up-regulated in the spinal cords of G93A mice (**Fig. 6**). ET-1 was detected in G93A mice at an early stage of disease (30 days) and further increased at 100 or 130 days of age, suggesting that its expression may correlate with disease progression (**Fig. 6 C**). ET-1 positive cells were clearly identified as stellate astrocytes in the spinal cord white matter and ventral horn in single labelling experiments (**Fig. 6 A-B, D-E**). Double-labelling experiments with anti-GFAP antibodies confirmed the expression of ET-1 in reactive astrocytes (**Fig. 6 F**). In contrast, ET-1 immunoreactivity was not localized in the vast majority of Iba-1 positive microglial cells (**Fig. 6 G**).

ET-1 immunoreactivity (IR) was increased in the astrocytes of ALS patients as well. In all ALS cases analyzed, the intensity of ET-1 appeared to be increased in cells with typical

glial morphology in both white matter (lateral corticospinal tract) and gray matter (ventral horn) (**Fig. 7 A-B**). As observed in G93A mice, double-labeling revealed ET-1 expression in reactive astrocytes (GFAP positive cells; **Fig. 7 D**), but not in cells of the microglial/macrophage lineage (HLA-DR positive cells) (**Fig. 7 E**). No difference in the ET-1 IR total score (see material and methods) was detected between ALS patients having a short-term (< 18 month, ALS.st) and long-term survival (ALS-lt, > 48 months) (**Fig. 7 C**).

4.2. Endothelin-1 exerts a toxic effect on motor neurons

We used MSCs obtained from rat embryos (13-15 day old) to assess the effect of ET-1 on MN survival. Our cultures, as previously established (D'Antoni et al., 2011), are enriched of reactive astrocytes (90-95% of total cell number) and contain only a small percentage of MNs (0.7-1.5 % of total cell number; 8-15% of MAP-2 positive neurons). Cells were treated for different lengths of time and with different concentrations of the

peptide. An exposure to 100 nM ET-1 for 8, 24, 48 and 72 hours revealed a time-dependent toxic effect to MNs reaching a plateau at 48 hours (**Fig. 8 A-E**). Degenerated MNs appeared as SMI32 positive cells with small somata and without an extensive dendritic arborization (**Fig. 8 A-D**).

A 48 hours treatment with increasing concentrations of ET-1 (1, 10, 100, 200, 500 nM and 1 μM) showed the highest levels of MN death (about 45%) at relatively low concentrations (100 nM), with no further MN degeneration at higher concentrations (**Fig. 8 F**). To validate the specificity of ET-1 toxic effect the peptide was denatured by heating at 80°C for 4 hours and then added to the cells (100 nM) for 48 hours. As expected, the toxic effect was completely abolished after the peptide degradation (data not shown).

To evaluate the selectivity of ET-1 toxic effect on MNs, we examined cell survival of the total number of neurons after 48-hours of ET-1 exposure by immunocytochemistry and Western blotting analysis using a MAP2 antibody. ET-1 exposure was toxic for all neurons (MAP2 positive cells) (**Fig. 9 A-D**), although its effect was lower (about 20%) than that exerted

on MNs (SMI32 positive cells) (**Fig. 9 C**). ET-1 did not exert any toxic effect on astrocytes, as revealed by GFAP expression using Western blotting analysis (**Fig. 9 D**) and immunocytochemistry (not shown).

4.3. The toxic effect of Endothelin-1 is mediated by reactive astrocytes

To assess the role of astrocytes in the toxic effect of ET-1 we used experimental conditions which promote or halt glial cells proliferation and differentiation. The toxic effect of ET-1 was invariably found in NB-G5 medium (**Fig. 10 A, B, C, D**), which is believed to mimic an *in vitro* state of reactive gliosis (Pollenz and McCarthy, 1986; Shafit-Zagardo et al., 1988; Tokita et al., 2001; Rohl et al., 2003; Vermeiren et al., 2005; D'Antoni et al., 2011). NB-G5 medium contains a high percentage of serum, which stimulates glial proliferation, growth factors (such as bFGF and EGF) and agents that regulate cAMP and cGMP kinase (such as forskolin and IBMX) and induce changes in the morphology and gene expression of astrocytes. Interestingly, no

toxic effect was observed on cell cultures grown in NB-G5 medium and treated at 3 DIV with AraC 5 μM (**Fig. 10 A, B, C, D**) and in a serum-free medium rich in antioxidants and substances that support neuronal survival (NB-B27), but did not favour the proliferation of astrocytes (**Fig. 10 A, B, C, D**). Our results suggest that the toxic effect of ET-1 is associated with the presence of a high percentage of astrocytes and can thus be mediated by these cells. We also observed that in experimental conditions that did not favour glial proliferation there is a reduction of expression of ET_A and ET_B receptors suggesting that they are mainly expressed in glial cells (**Fig. 11 A**). However the reduction of ET_B receptor in cells grown in NB-G5 supplement and treated with ET-1 for 48 hours (**Fig. 11 A**) suggests that this receptor is also expressed in neurons. Double-labelling immunocytochemistry experiments performed with SMI32 and anti-GFAP antibodies confirmed that both receptors are expressed in astrocytes (**Fig. 11 B-C**) whilst ET_B receptors are also present in motor neurons, although at low levels (**Fig. 11 D-E**).

4.4. Astrocytic ET_A and ET_B receptors are both implicated in MN cell death

ET-1 acts on both ET_A and ET_B receptors. Exposure to ET-3 (100 nM) and Sarafotoxin S6C (10 nM), which are selective agonists of ET-B receptors, for 48 hours had a toxic effect quantitatively similar to that exerted by ET-1 (**Fig. 12 A**). A treatment with each agonist together with ET-1 did not further increase MN death (**Fig. 12 A**) suggesting the involvement of ET_B receptors. ET-3 and Sarafotoxin S6C did not exert any toxic effect in cultures grown in NB-B27 without serum (data not shown), suggesting the involvement of ET_B receptors expressed in astrocytes. In contrast, the ET-1 effect was only slightly reversed by the ET_A competitive receptor antagonist BQ123 (2 μM) and was not affected by the ET_B competitive receptor antagonist BQ788 (2 μM) (**Fig. 12 B**). Note that BQ788 (2 μM) added to cultures for 48 hours in the absence of ET-1 reduced MN survival (-15%).

Similar results were obtained by using a lower concentration of BQ788 (200 nM) (not shown). Overall these

data suggest that the astrocytic ET receptors responsible for the observed ET-1/ET-3 toxicity has an atypical pharmacological profile (see discussion).

4.5. Endothelin-1 exposure increases the susceptibility of MNs to AMPA-induced toxicity

MNs grown in NB-G5 supplement are particularly susceptible to AMPA toxicity (D'Antoni et al., 2011). ET-1 has been shown to increase glutamate levels following blockade of glutamate transporter in astrocytes. We tested whether the effect of ET-1 might be ascribed to the activation of AMPA receptors by endogenously released glutamate by exposing cell cultures to ET-1 in the presence of the selective AMPA receptor antagonist GYKI 52466 (20 μM). Interestingly, a prolonged exposure to GYKI 52466 for 48 hours reduced MN survival, but did not prevent ET-1 mediated MN degeneration (**Fig. 13 A**). Furthermore, in cultures pre-treated with ET-1 and subsequently challenged with AMPA (50 μM) for 15 min, excitotoxicity was significantly higher (**Fig. 13 B**), suggesting that ET-1 mediated

MN death is not dependent either directly or indirectly on AMPA receptor activation.

5. DISCUSSION

ALS is regarded as a MN specific disorder, but increasing evidences indicate that a dysfunctional cross-talk between glial and neuronal cells might be crucial for its pathogenesis (Van Den Bosch and Robberecht, 2008). Astrocytes regulate GluA2 subunit expression in MNs and their vulnerability to AMPA toxicity (Van Damme et al., 2007) and stimulate MN death by diffusible toxic factors in culture models of ALS (Di Giorgio et al., 2007; Nagai et al., 2007). Factors secreted by activated astrocytes might contribute to MN degeneration and disease progression. The identification of glia-secreted factors and their possible role in MN survival and degeneration is one of the main goals in ALS research. The pharmacological targeting of these factors might be useful for slowing down disease progression and increasing survival of ALS patients.

Activated astrocytes and microglia express endothelins and ET-1 is implicated in reactive gliosis observed in many pathological conditions and in regulation of glial inflammatory

mediators. However the role of this peptide in neurodegeneration and its involvement in reactive gliosis in ALS has not been investigated. A study on the expression of an entire genome showed that ET-1 mRNA is over-expressed in the cortex of ALS patients (Lederer et al., 2007). Here, we report that there is an up-regulation of ET-1 expression in the spinal cords of G93A mice and in ALS patients and that ET-1 is *per se* toxic for MNs. In both animal models and human specimens ET-1 was expressed in reactive astrocytes. The toxic effect of ET-1 is present in MSCs under an experimental condition of enrichment in astrocytes bearing a stellate phenotype that mimics the reactive gliosis observed in ALS (see Vermeiren et al., 2005; D'Antoni et al., 2011). This effect is critically dependent on the presence of astrocytes, because it was not observed when a drastic reduction of astrocytes was achieved by using NB-27 serum free medium or AraC. ET-1 and their receptors are present in astrocytes (Schinelli, 2006; Gadea et al., 2008) and the activation of ET receptors in astrocytes might trigger a vicious ET-1 release as well as release of other factors potentially detrimental for MNs. This finding may have an important significance for the

pathophysiology of ALS. Interestingly, we observed that AMPA toxicity is increased in ET-1 treated cultures. Excitotoxic death of spinal cord MNs is largely mediated by AMPA receptors, which in MNs form calcium-permeable ion channels because of a low expression of the GluA2 subunit. Our data indicates that AMPA- and ET-1 toxicity do not converge on a common pathway since an additive effect was observed when AMPA exposure was carried out on ET-1 treated MNs. Further evidence that AMPA receptors are not involved in the ET-1 toxic effect is the lack of protection by the selective AMPA receptor antagonist GYKI 52466. An ET-1 embedded microenvironment, as it is possible to occur *in vivo* in ALS patients, may render MNs more susceptible to excitotoxicity induced by increased glutamate levels.

It is difficult to define which receptor mediates the ET-1 toxic effect observed in our cultures because of controversial results obtained with agonist and antagonists. ET-1 is active on both ET_A and ET_B receptors, while ET-3 and Sarafotoxin S6C are selective for ET_B receptors. We show that ET-3 and Sarafotoxin induced MN cell death and that ET-1 and ET-3 (or Sarafotoxin

S6C) together did not have an additive effect, suggesting that ET_B receptors are responsible for ET-1 induced MN death. Thus, we expected a reversal of ET-1 toxic effect with the specific ET_B receptor antagonist BQ788, which did not occur. In contrast, ET-1 mediated MN death was partially blocked by ET_A receptor antagonist BQ-123, suggesting an involvement of ET_A receptors.

Our Western blotting and immunofluorescence experiments suggest the presence of both ET_A and ET_B receptors in astrocytes. Interestingly while ET_A receptors appeared to be exclusively expressed in astrocytes, ET_B receptors are also expressed in MNs. It is possible that the activation of neuronal ET_B receptors by endogenous ETs sustain MN survival, since we observed a small reduction of MN survival after a 48 hours treatment with BQ-788. This is in line with evidences suggesting that ET_B receptors sustain neuronal survival both in *in vitro* and *in vivo* models (Ehrenreich et al., 1999; Yagami et al., 2002, 2005; Vidovic et al., 2008; Laziz et al., 2011). The involvement of astrocytic ET_B receptors in mediating MN degeneration is also sustained by the evidence that Sarafotoxin S6C and ET-3 did not exert any toxic effect in B27 medium without serum, a condition

which hampers astrocytic proliferation. The observed lack of an inhibitory effect by selective antagonists in our system might be explained by a compensation mediated by the presence of the other unblocked receptor as shown (Jensen et al., 1998; Rapoport and Zuccarello, 2011). However, when both receptors were blocked in the presence of BQ-123 and BQ-788 no protection was observed. This is more consistent with astrocytic receptors insensitive to the canonical antagonists used in our study. Accordingly, the existence of ET-1 binding sites insensitive to BQ-123 and BQ-788 has been described in cultured astrocytes (Jensen et al., 1998). ET receptors present in cultured cortical astrocytes exhibit an atypical binding perhaps based on the formation of ET_A/ET_B heterodimers which do not respond canonically to antagonists (Hasselblatt et al., 1998). This is consistent with the presence of both ET_A and ET_B receptors in astrocytes, and ET_B only in neurons and should be taken into account when studying the effect of astrocytic ET receptors. Future experiments using RNA interference techniques might better clarify which receptor is responsible for ET-1 toxicity.

Our results may represent a starting point for future investigations in ALS and can open perspectives for new therapeutic approaches in this devastating disease. ET receptors blockade has been shown to have therapeutic potential in experimental and clinical studies of hypertension, pulmonary disease and atherosclerosis (Luscher and Barton, 2000; Higuchi et al., 2013); future studies aimed at developing new ET blockers specific for astrocytes and testing the effects of ET blockers in *in vivo* ALS models will clarify whether these drugs might be proposed for ALS therapy.

6. FIGURES

Figure 1

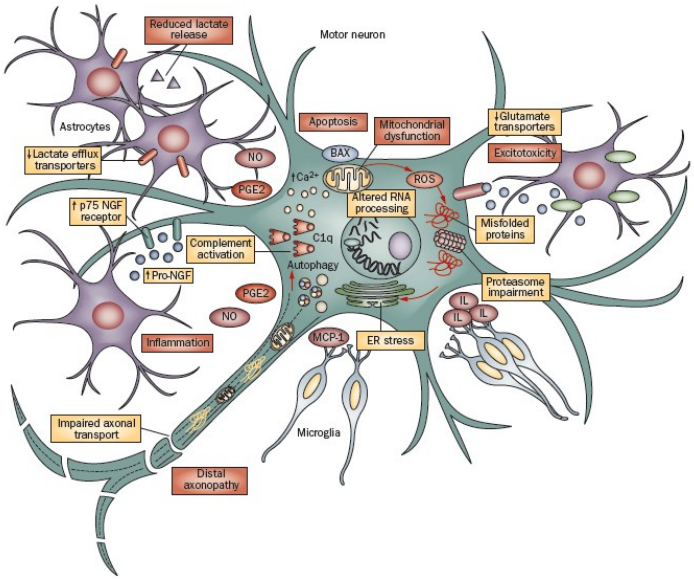


Figure 2

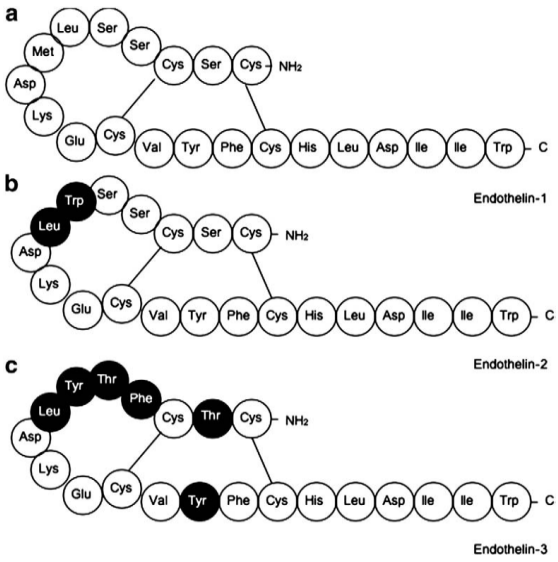


Figure 3

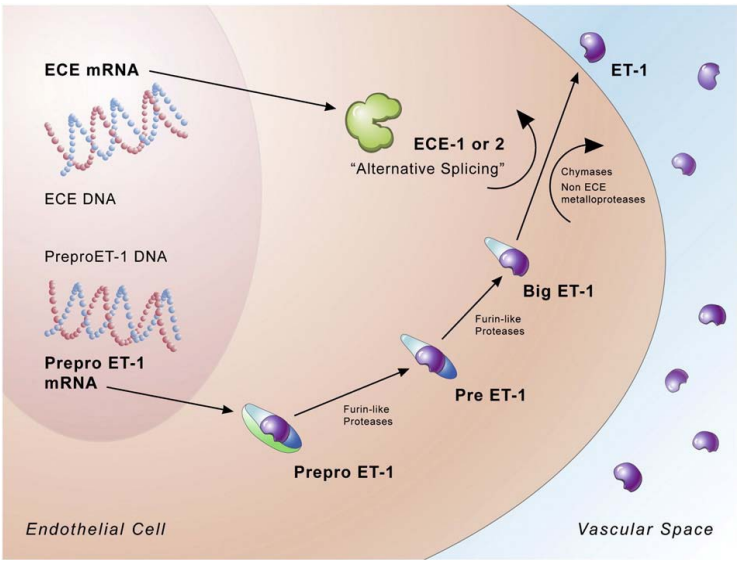


Figure 4

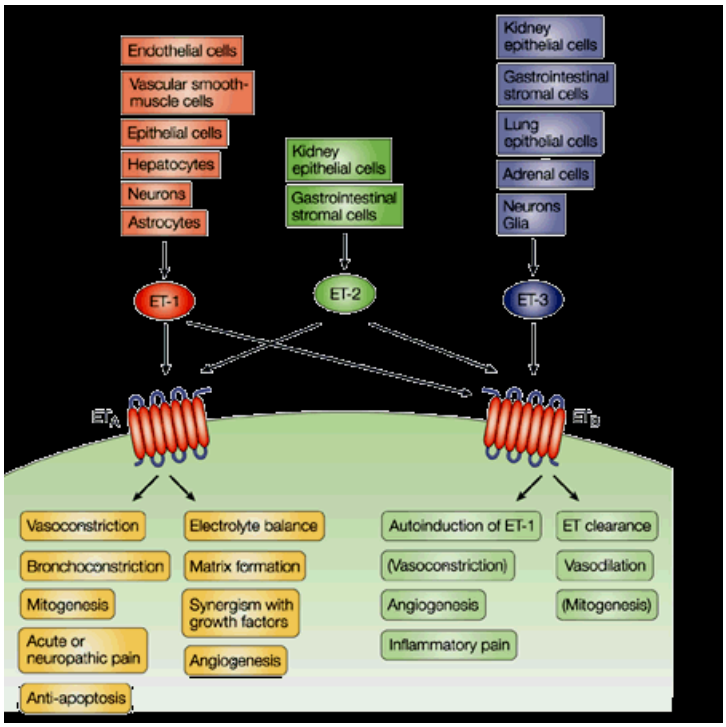


Figure 5

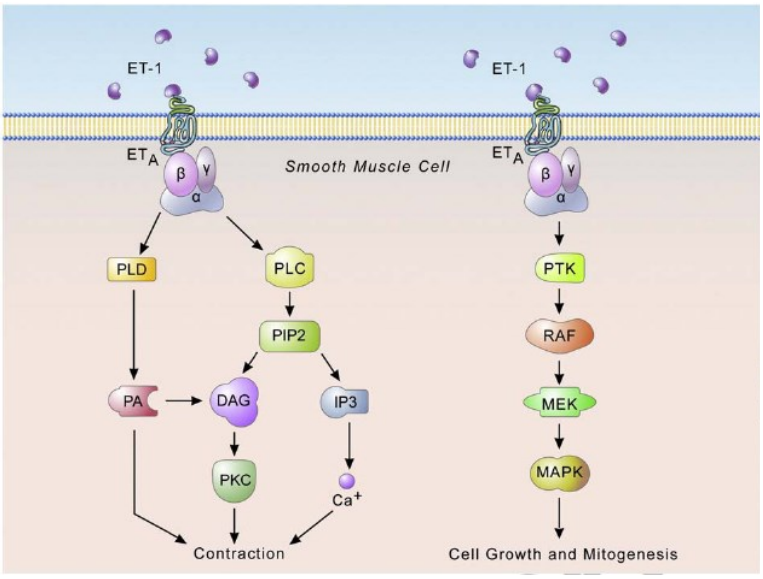

ET-1
ET-1
ET$_A$
ET$_A$
β
γ
α
β
γ
α
Smooth Muscle Cell
PLD
PLC
PIP2
PA
DAG
IP3
PKC
Ca$^+$
Contraction
PTK
RAF
MEK
MAPK
Cell Growth and Mitogenesis

Figure 6

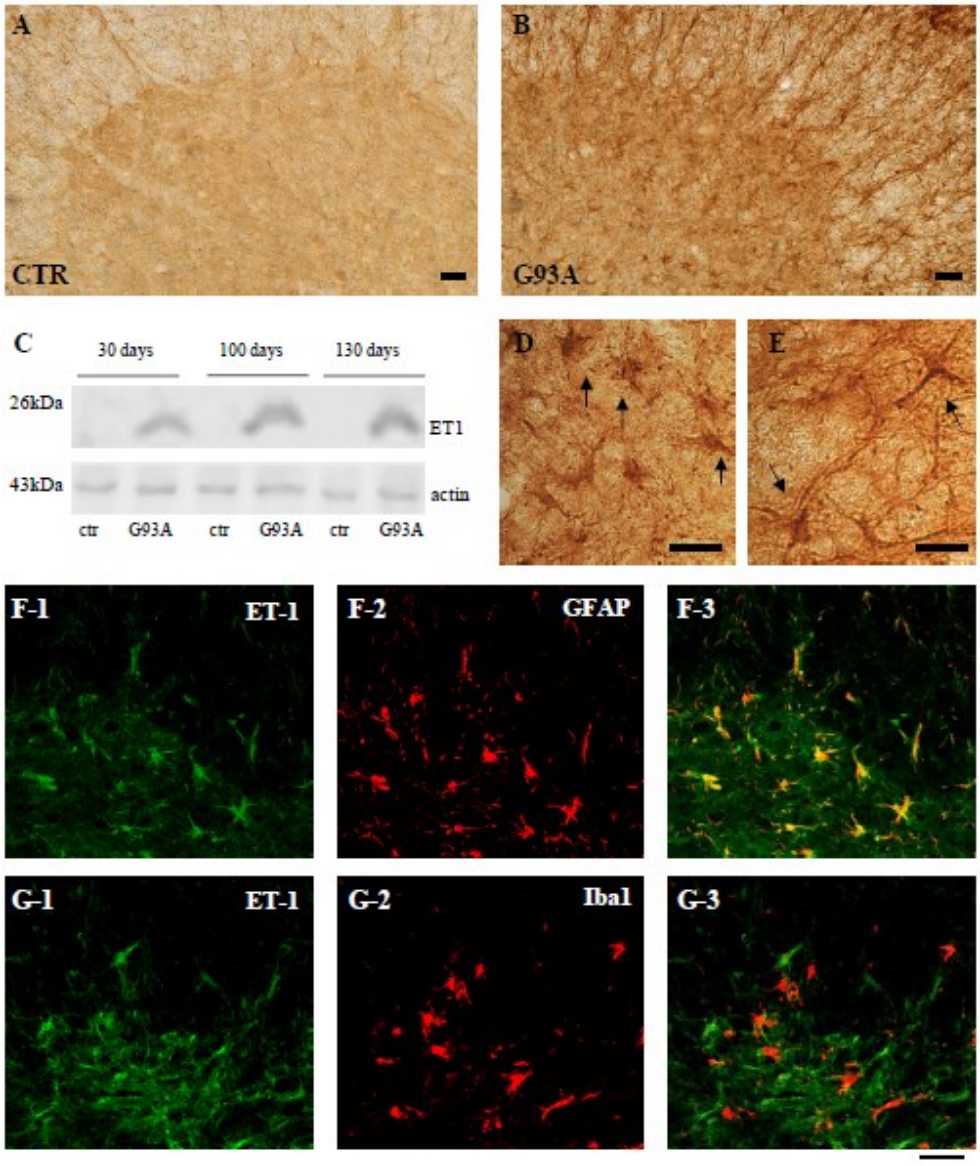

A
CTR
B
G93A
C
30 days
100 days
130 days
26kDa
ET1
43kDa
actin
ctr
G93A
ctr
G93A
ctr
G93A
D
E
F-1
ET-1
F-2
GFAP
F-3
G-1
ET-1
G-2
Iba1
G-3

Figure 7

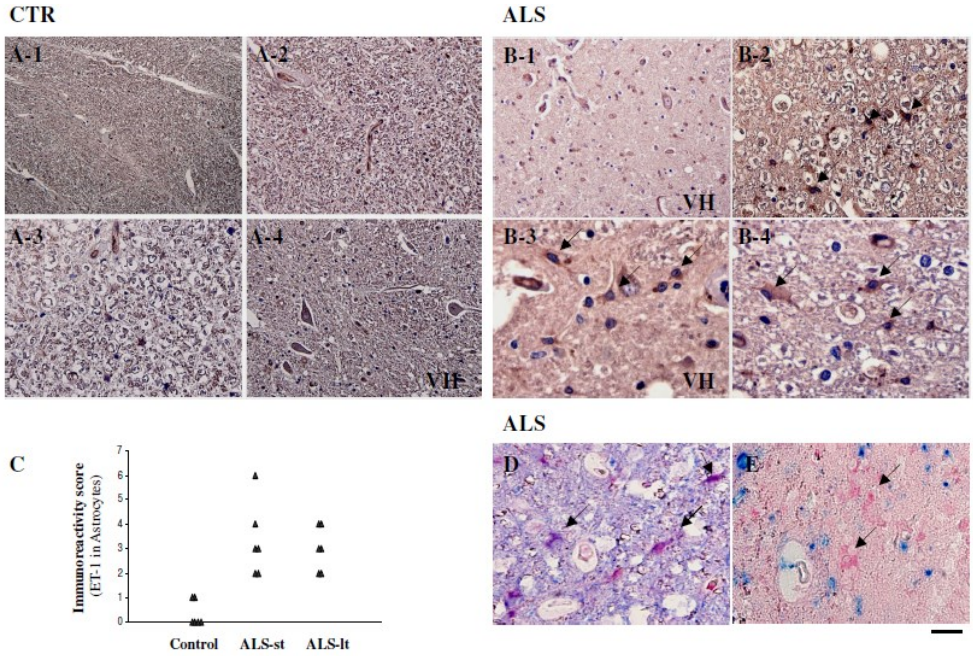
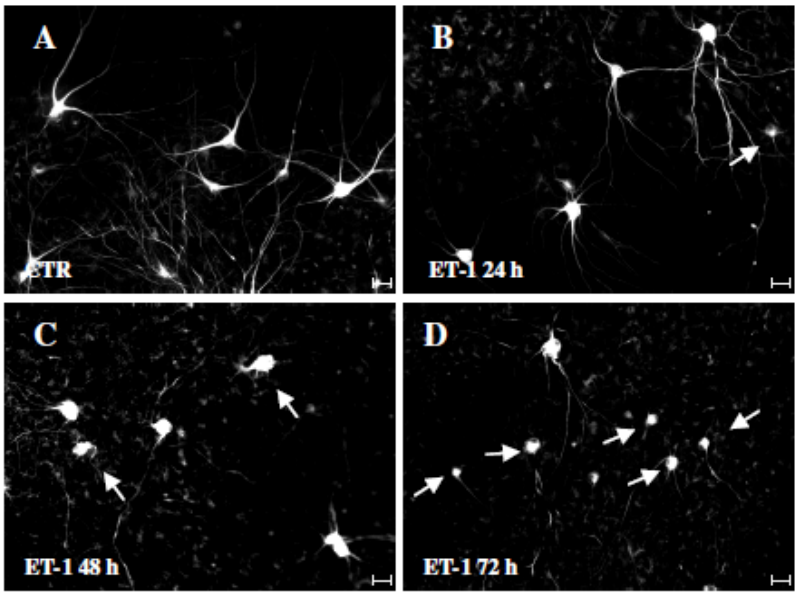
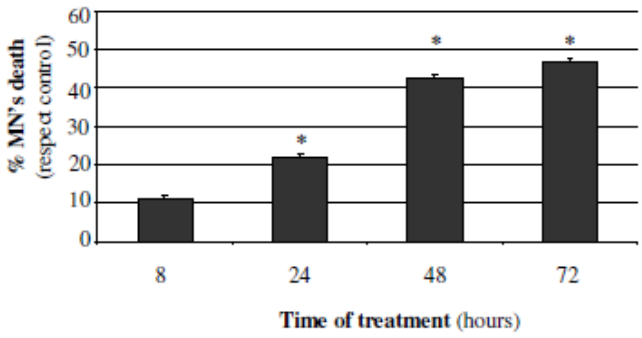

CTR
ALS
A-1
A-2
A-3
A-4
VH
B-1
B-2
B-3
B-4
VH
C
Immunoreactivity score
(ET-1 in Astrocytes)
0
1
2
3
4
5
6
7
Control
ALS-st
ALS-lt
ALS
D
E

Figure 8


A
CTR
B
ET-1 24 h
C
ET-1 48 h
D
ET-1 72 h

E


% MN's death
(respect control)
60
50
40
30
20
10
0
*
*
*
*
8
24
48
72
Time of treatment (hours)

F

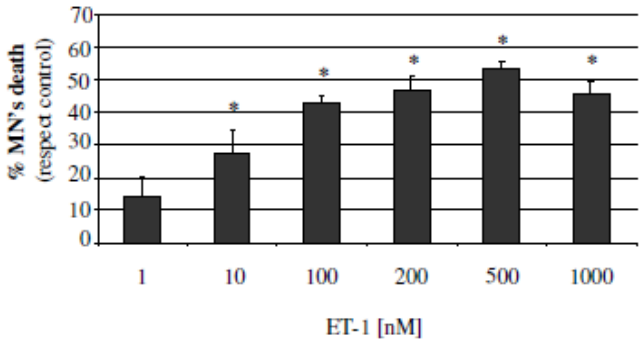

% MN's death
(respect control)
70
60
50
40
30
20
10
0
*
*
*
*
*
*
1
10
100
200
500
1000
ET-1 [nM]

Figure 9

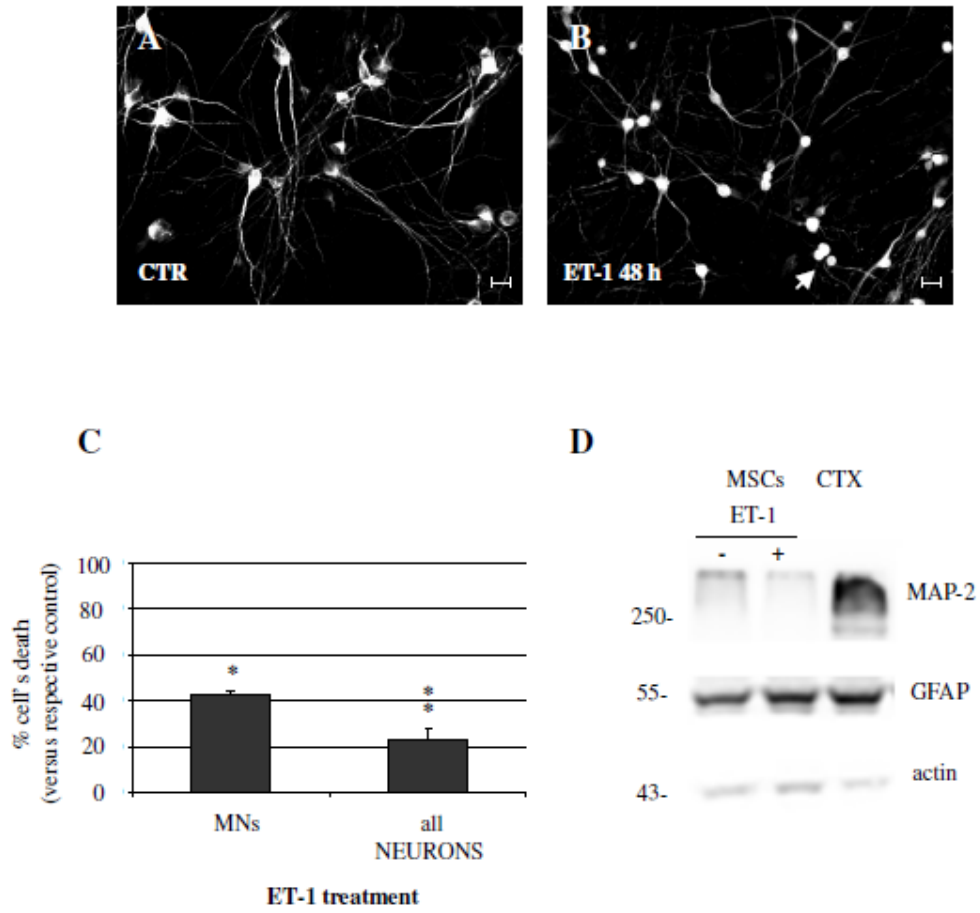

A
CTR
B
ET-1 48 h
C
% cell's death
(versus respective control)
100
80
60
40
20
0
*
**
MNs
all
NEURONS
ET-1 treatment
D
MSCs
CTX
ET-1
-
+
250-
MAP-2
55-
GFAP
43-
actin

Figure 10

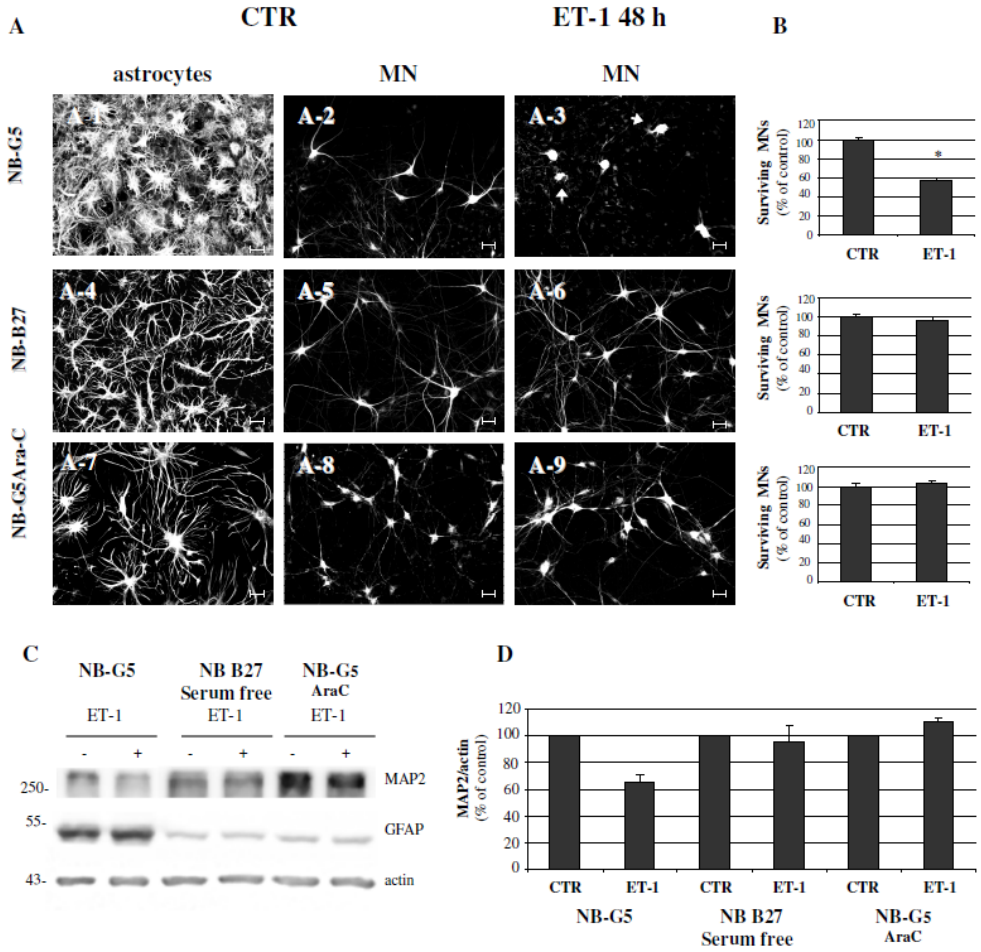

A
CTR
ET-1 48 h
astrocytes
MN
MN
NB-G5
NB-B27
NB-G5Ara-C
A-1
A-2
A-3
A-4
A-5
A-6
A-7
A-8
A-9
B
Surviving MNs (% of control)
CTR
ET-1
C
NB-G5
NB B27 Serum free
NB-G5 AraC
ET-1
250-
55-
43-
MAP2
GFAP
actin
D
MAP2/actin (% of control)
NB-G5
NB B27 Serum free
NB-G5 AraC

Figure 11

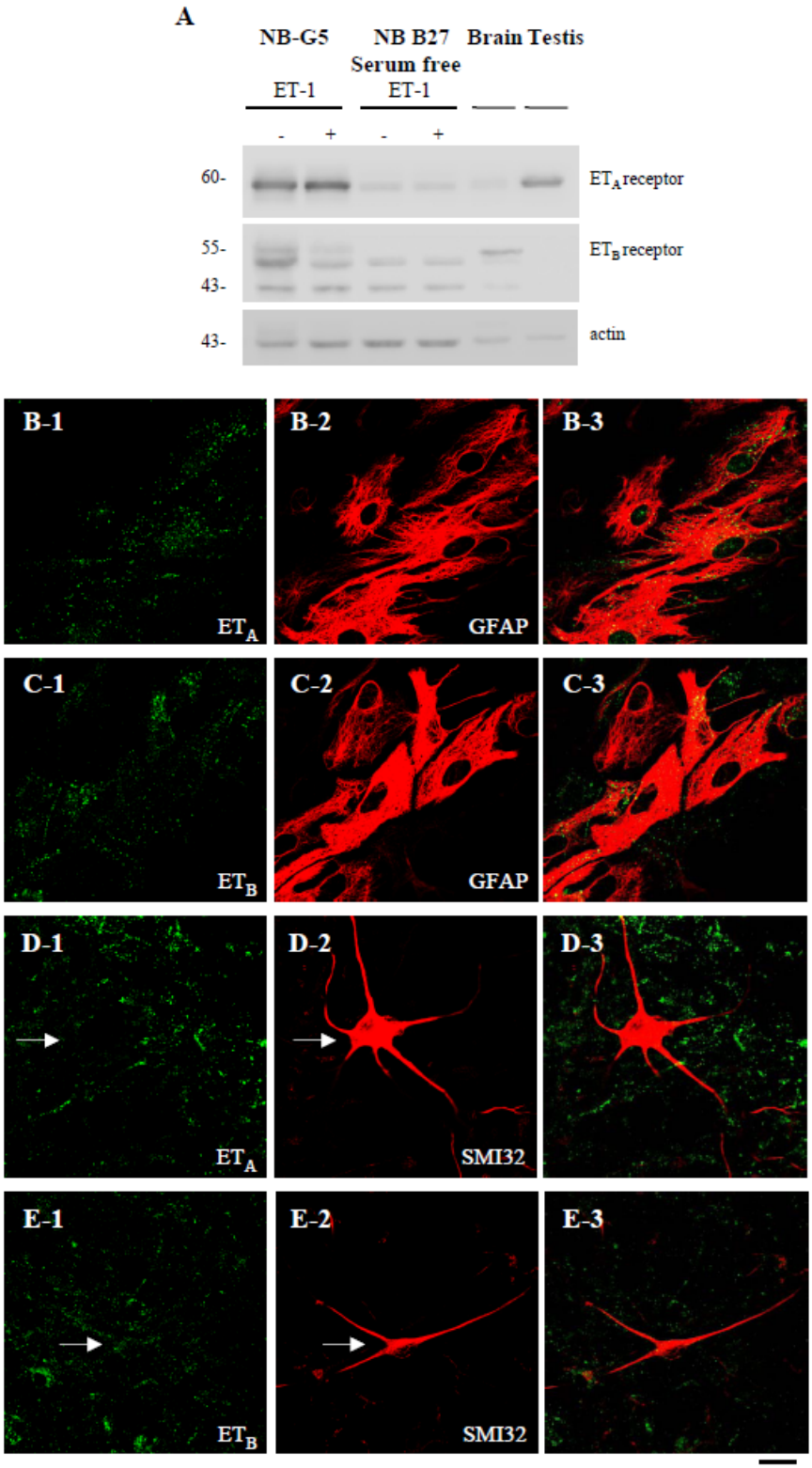

A
NB-G5
NB B27
Serum free
Brain Testis
ET-1
ET-1
- + - +
60-
55-
43-
43-
ETA receptor
ETB receptor
actin
B-1
ETA
B-2
GFAP
B-3
C-1
ETB
C-2
GFAP
C-3
D-1
ETA
D-2
SMI32
D-3
E-1
ETB
E-2
SMI32
E-3

Figure 12

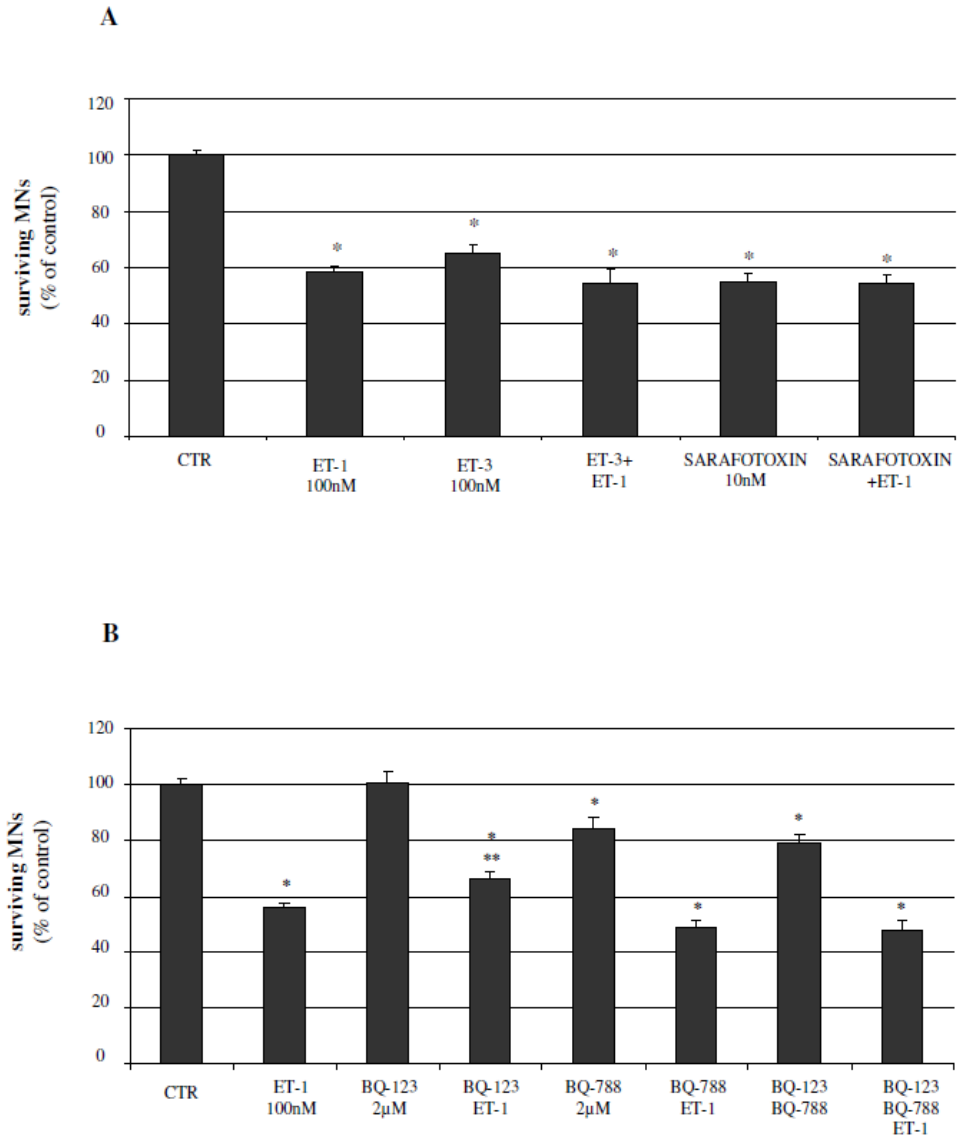

A
surviving MNs
(% of control)
120
100
80
60
40
20
0
CTR
ET-1
100nM
ET-3
100nM
ET-3+
ET-1
SARAFOTOXIN
10nM
SARAFOTOXIN
+ET-1
B
surviving MNs
(% of control)
120
100
80
60
40
20
0
CTR
ET-1
100nM
BQ-123
2μM
BQ-123
ET-1
BQ-788
2μM
BQ-788
ET-1
BQ-123
BQ-788
BQ-123
BQ-788
ET-1

Figure 13

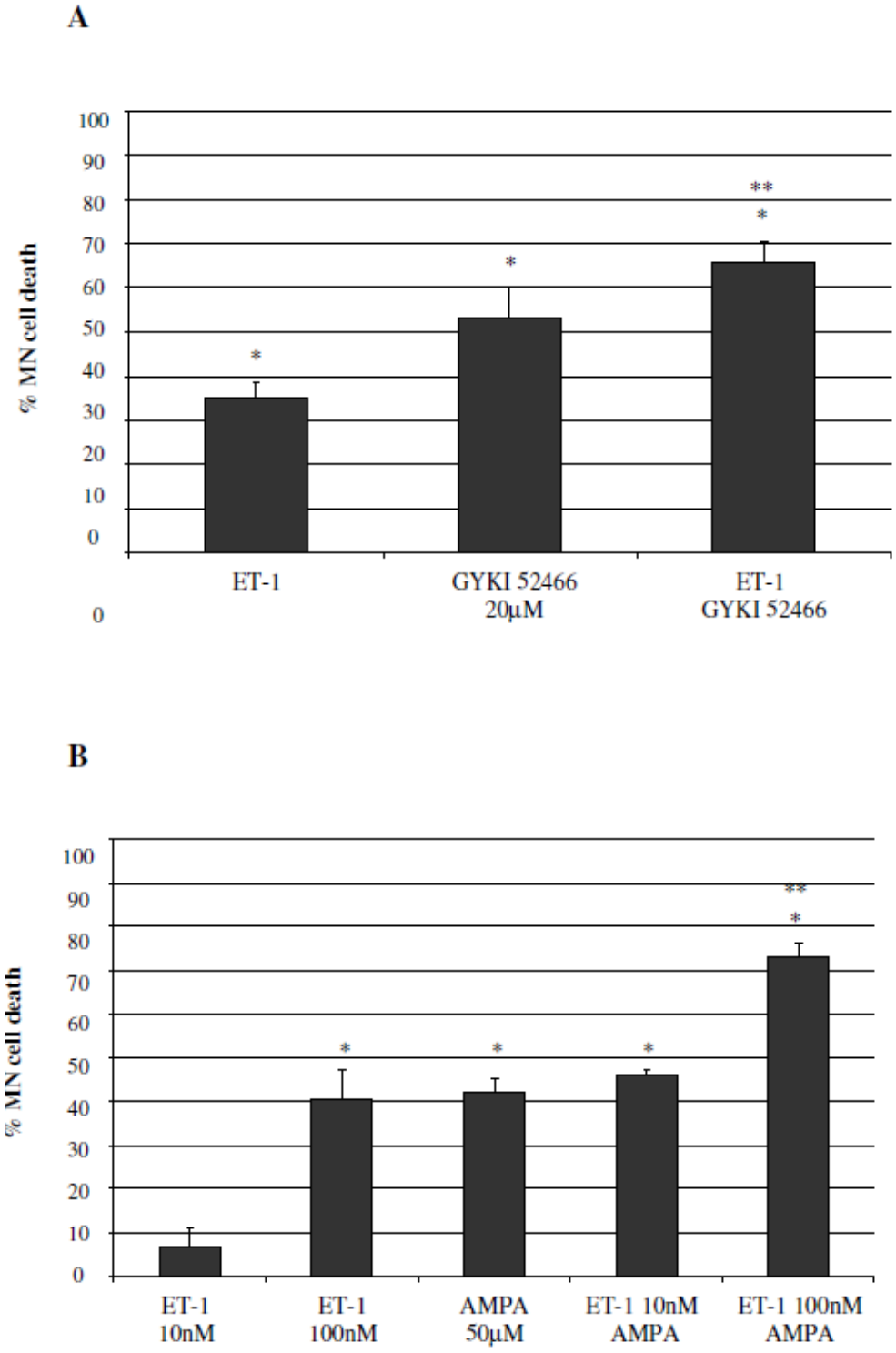

A
% MN cell death
100
90
80
70
60
50
40
30
20
10
0
0
*
*
**
*
ET-1
GYKI 52466
20µM
ET-1
GYKI 52466
B
% MN cell death
100
90
80
70
60
50
40
30
20
10
0
*
*
*
**
*
ET-1
10nM
ET-1
100nM
AMPA
50µM
ET-1 10nM
AMPA
ET-1 100nM
AMPA

Table 1

ALS type	Onset	Inheritance	Locus	Gene	Protein
ALS1	Adult	AD[1]	21q22.1	*SOD1*	Cu/Zn superoxide dismutase
ALS2	Juvenile	AR	2q33-35	*ALS2*	Alsin
ALS3	Adult	AD	18q21	unknown	
ALS4	Juvenile	AD	9q34	*SETX*	Senataxin
ALS5	Juvenile	AR	15q15-21	*SPG11*	Spatacsin
ALS6	Adult	AD[2]	16p11.2	*FUS*	Fused in sarcoma
ALS7	Adult	AD	20p13	unknown	
ALS8	Adult	AD	20q13.33	*VAPB*	VAMP-associated protein B
ALS9	Adult	AD	14q11	*ANG*	Angiogenin
ALS10	Adult	AD	1q36	*TARDBP*	TAR DNA-binding protein
ALS11	Adult	AD	6q21	*FIG4*	PI(3,5)P(2)5-phosphatase
ALS12	Adult	AR/AD	10p15-p14	*OPTN*	Optineurin
ALS-FTD1	Adult	AD	9q21-22	unknown	
ALS-FTD2	Juvenile	AD	9p13.2-21.3	unknown	

Table 2

Patiens	Sex	Age	Duration of disease (months)	Clinical diagnosis
1	F	57	7	sALS
2	F	70	7	sALS
3	M	59	8	sALS
4	M	51	13	sALS
5	M	60	16	sALS
6	M	55	18	sALS
7	M	81	48	sALS
8	M	69	52	sALS
9	F	64	72	sALS
10	M	67	90	sALS
11	F	40	96	sALS
12	M	41	96	sALS

Age (mean ± SEM):59.5 ± 3.4
Duration of disease (mean ± SEM):43.5 ± 10.6

1	M	57	-	NC
2	F	60	-	NC
3	M	73	-	NC
4	M	71	-	NC
5	F	73	-	NC
6	M	53	-	NC

Age (mean ± SEM): 64.5 ± 3.6

NC: normal controls; sALS: sporadic ALS.

Figure Legends

Figure 1: Molecular mechanisms of motor neuron injury in ALS, by Ferraiuolo et al., Nat. Rev. Neurol. 7, 616-630 2011

Figure 2: Endothelin (ET) structure, by Khimji et al., Cell Signal. 22(11):1615-25, 2010

Figure 3: Endothelin-1 biosynthetic pathway, by Khimji et al., Cell Signal. 22(11):1615-25, 2010

Figure 4: Endothelin expression and effects, by Nelson et al., 2003

Figure 5: Endothelin signalling, by Khimji et al., Cell Signal. 22(11):1615-25, 2010

Figure 6: ET-1 is up-regulated in the spinal cord of G93A mice (A-E). ET-1 is expressed in astrocytes and not in microglia (F-

G). (A-B) Immunohistochemistry with anti-ET-1 antibody performed in spinal cord of four months old control (A) and G93A mice (B). Scale bar=20 μm. (D-E) High magnification of ET-1 immunohistochemistry in spinal cord ventral horn grey matter (D) and white matter (E) of four month old G93A mice; arrows indicate strong immunoreativity in stellate astrocytes. Scale bar=20 μm. (C) Western blot of spinal cords of control and G93A mice of different ages with anti-ET-1 and anti-actin antibodies. 100 μg of proteins were loaded. Identical results were obtained in two different preparations each performed in triplicate. (F-G) Confocal images of double-labeling immunohistochemistry with anti-ET-1 together with anti-GFAP (F-1, ET-1 green, F-2 GFAP red, F-3 merge) or anti-Iba1 (G-1, ET-1 green, G-2, Iba-1 red, G-3, merge) antibodies performed in spinal cord of four months old G93A mice. Scale bar=10 μm

Figure 7: ET-1 is up-regulated in the spinal cord of ALS patients (A-B). ET-1 is expressed in astrocytes and not in microglia (D, E). Evaluation of ET-1 astroglial immunoreactivity (IR) in control and ALS spinal cord (C). (A 1-4, B 1-4)

Immunohistochemistry with anti-ET-1 antibody performed in spinal cord of control (A 1-4; A-1, A-2 and A-3: white matter/lateral corticospinal tract; A-4, ventral horn, VH) and ALS patients (B1-4; B-1 and B-3:ventral horn, VH; B-2 and B-4 white matter/lateral corticospinal tract). Arrows show ET-1 positive astrocytes. (C) Plots showing the distribution of ET-1 astroglial IR in controls and ALS spinal cord. The IR score represents the total score, which was taken as the product of the intensity score and the frequency score (for details see methods section). ALS-st: patients with rapid disease progression and short-term survival < 18 month; ALS-lt: patients with slow disease progression and long-term survival > 48 months. (D, E) Double-labeling immunohistochemistry for ET-1 (red) with GFAP (D; astrocytes; blue) and-HLA-DR (E; microglia; blue) performed in spinal cord of ALS patients. Double stained cells are purple. Arrows show ET-1 positive astrocytes. Scale bar: A-1: 320 µm; A-2, B-1: 160 µm; A-3, A-4, B-2: 80 µm; B-3, B-4, D-E: 40 µm

Figure 8: Exposure to ET-1 is toxic for MNs in a time and concentration dependent manner (A-F). (A-D). Immunocytochemical stainings with SMI32 antibody performed on MSCs at 14 DIV treated with ET-1 (100 nM) at different times. Arrows indicate degenerated MNs. Scale bar=20 μm. (E) Percentage of MN cell death 8, 24, 48 and 72 hours after ET-1 (100 nM) treatment. Data represent mean ± SEM of three experiments, each performed in quadruplicate. *$p < 0.001$ versus control by one-way ANOVA followed by post hoc Holm-Sidak method. (F) Percentage of MN cell death 48 hours after ET-1 treatment at different concentration (1, 10, 100, 200, 500 nM and 1μM). Data represent mean ± SEM of three experiments, each performed in quadruplicate. *$p < 0.001$ versus control by one-way ANOVA followed by post hoc Holm-Sidak method

Figure 9: MNs are more susceptible than the overall number of neurons to the toxic effect of ET-1. (A-D). (A, B) Immunocytochemical stainings with an anti-MAP2 antibody carried out on MSCs at 14 DIV treated with ET-1 (100 nM) for 48 hours. Arrows indicates degenerated neurons. Scale bar=20

μm (C) Percentage of MN (SMI-32 positive cells) and total neurons cell death (MAP-2 positive cells) 48 hours after ET-1 treatment (100 nM). Data represent mean ± SEM of two experiments, each performed in quadruplicate. *$p < 0.001$ versus respective control by t-test, **$p < 0.05$ versus respective control by t-test. (D) Western blotting of lysates of control and ET-1-treated cells at 15 DIV. 60 μg of proteins and 40 μg of positive control [total lysates from adult rat cortex (CTX)] were loaded. Identical results were obtained in three independent cultures

Figure 10: The toxic effect of ET-1 is mediated by reactive astrocytes (A, B). (A) Immunocytochemical stainings with anti-GFAP (A-1, A-4 and A-7) and SMI32 (A-2, A-3, A-5, A-6, A-8 and A-9) antibodies on mixed spinal cord cultures treated with ET-1 (100 nM) for 48 hours in NB-G5, NB-G5 with Ara-C and NB-B27 serum free medium and untreated control. Arrows indicate degenerated MNs. Scale bar=20 μm (B) Percentage of surviving MNs 48 hours after ET-1 treatment (100 nM) as in (A). Data represent mean ± SEM of three experiments, each performed in quadruplicate. *$p < 0.001$ versus respective control

by t-test. (C) Western blot of lysates of control and ET-1-treated cells as indicated in (A) with anti-MAP2, anti-GFAP and anti-actin antibodies. 30 µg of proteins were loaded. Identical results were obtained in two different preparations each performed in triplicate. (D) Semi-quantitative analysis of MAP2 expression levels in Western blots as in (C)

Figure 11: ET_A and ET_B receptors are both expressed in astrocytes whilst ET_B receptors are also present in motor neurons (A-E). (A) Western blot of lysates of control and ET-1-treated MSCs (13-15 DIV) grown in NB-G5 and NB-B27 serum free medium using anti-ET_A and ET_B receptors antibodies. 60 µg of proteins of MSC and positive controls [total lysates from adult mouse testis and brain] were loaded. (B-E) Confocal images of double-labeling immunocytochemistry for anti-ET_A (B, D) and ET_B (C, E) receptors antibodies together with anti-GFAP (B-1 ET_A green, B-2 GFAP red, B-3 merge; C-1 ET_B green, C-, GFAP red, C-3 merge) and SMI32 (D-1 ET_A green, D-2 SMI32 red, D-3 merge; E-1 ET_B green, E-2 SMI32 red, E-3 merge) antibodies on mixed spinal cord cultures. Scale bar= 20 µm

Figure 12: ET_A and ET_B receptors are both implicated in MN cell death (A-B). (A) Percentage of surviving MNs 48 hours after a treatment with ET-1 (100 nM), ET-3 (100 nM) and Sarafotoxin S6C (10 nM) either alone or in combination. Data represent mean ± SEM of four experiments, each performed in triplicate. *$p < 0.001$ versus control; (B) Percentage of surviving MNs 48 hours after a treatment with ET-1 (100 nM) in the presence of BQ123 (2 μM) and BQ788 (2 μM). Data represent mean ± SEM of five experiments, each performed in quadruplicate. *$p < 0.001$ versus control. **$p < 0.001$ versus ET-1 by one-way ANOVA followed by post hoc Holm-Sidak method

Figure 13: A prolonged exposure to GYKI 52466 for 48 hours reduced MN survival, but did not prevent ET-1 mediated MN degeneration (A). Exposure to ET-1 for 48 hours increases the susceptibility of MNs to acute AMPA-mediate excitotoxicity (B). (A) Percentage of MN cell death 48 hours after a treatment with ET-1 (100 nM) and GYKI 52466 (20 μM), alone and in combination. *$p < 0.001$ versus control; **$p < 0.001$ versus ET-1

by one-way ANOVA followed by post hoc Holm-Sidak method. Data represent mean ± SEM of three experiments, each performed in triplicate. (B) Percentage of MN cell death 20 hours after AMPA toxicity (50 μM for 15 min) in cultures untreated or pre-treated with ET-1 (10 and 100 nM) for 48 hours. Data represent mean ± SEM of four experiments, each performed in triplicate. *$p < 0.001$ versus control **$p < 0.001$ versus ET-1 by one-way ANOVA followed by post hoc Holm-Sidak method

Table 1: Different subtypes of FALS and their genetic determinants, by Ticozzi et al., Archives Italiennes de Biologie, 149: 65-82, 2011

Table 2: Summary of clinical and neuropathological data of ALS and control patient

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