Summary of the project

TITLE: Animal and Clinical studies aiming at interfering with amyelotrophic lateral sclerosis (ALS)

a) Background and present state of the art in the research field

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting the motor nervous system. It causes progressive and cumulative physical disabilities in patients, and leads to eventual death due to respiratory muscle failure within 3 to 6 years.

The reported incidence varies from 0.2 to 2.5 cases per 100,000 per year, the overall rate being about 2 per 100,000. Although ALS is a rare condition, it places a tremendous socioeconomic burden on patients, family members, caregivers and health systems. The etiology of this debilitating disease, for which no pharmacotherapies and only minimal treatments are available, remains unclear. Sporadic ALS (SALS) is the most commonly diagnosed form of ALS as patients with familial ALS (FALS) only account for approximately 10% of all cases (1). The most frequent cause of FALS (25%) is currently attributed to mutations in the gene encoding cytosolic copper–zinc superoxide dismutase (SOD1), an enzyme important in the dismutation of superoxide anion to H2O2, but even the mechanism of mutant SOD1-induced motor neuron degeneration remains incompletely understood (2). In the spinal cord and brain of both patients with ALS and transgenic mice overexpressing a mutant human SOD1, marked microglial and astroglial proliferation and activation are present (3). Factors released by these cells may contribute to the pathogenesis of ALS, like MMPs. These matrix enzymes were shown to exert several beneficial effects in the central nervous system (CNS). One member of this family, gelatinase B, also called matrix metalloproteinase-9 (MMP-9), is of particular interest, as it is released by microglia, astrocytes and neurons (4) and it is also thought to play a role in the pathogenesis of several neurological disorders, such as multiple sclerosis, stroke, and Alzheimer's disease (5).

MMP-9 has been linked to ALS pathogenesis since increased MMP-9 immunostaining was observed in biopsies of atrophic muscles of ALS patients compared to normal muscle. High levels of MMP-9 activity were observed in the motor cortex and spinal cord of ALS patients compared to control (6). Increased expression levels of pro- and active forms of MMP-9 and increased MMP-9 activity were observed by ELISA in serum samples of ALS patients (7). Furthermore, Lorenzi et al. reported a slight elevation in proMMP-9 levels by ELISA in ALS plasma (8). MMP-9 levels in ALS cerebrospinal fluid (CSF) were not detected by zymography, thus studies have focused on circulating MMP-9 (9, 10). Oral administration of Ro 26–2853, an inhibitor of MMP, during the pre-symptomatic stage in transgenic SOD1G93A mice, improved motor function and significantly extended their survival time. A recent study revealed a significant increase in the life span of MMP-9 ''knock-out" transgenic SOD1G93A mice on a B6SJL background as well as a reduction of neuronal death in the spinal cord (12). These studies suggested that inhibition of MMP-9 could perhaps be a potential therapeutic approach for treating ALS. In contrast to the above, in another study, knockout of the MMP9 gene in TgSOD1G93A mice exhibited accelerated disease progression and reduced survival, indicating that MMP-9 possibly has a protective effect (13). Additionally Ilzecka et al. found decreased MMP-9 levels by ELISA in CSF from ALS patients compared to controls, and decreased MMP9 which correlated with clinical status deterioration (14). Hence, the discrepancy between the published studies indicates that an in depth study of MMP9 in ALS is warranted to determine a role of MMP9 in ALS.

Hence, we aim to develop and study a double-transgenic mouse model, over-expressing G93A mutation of SOD1 and MMP9 (Tg MMP9). We have already developed and characterized a TgMM9 mouse model, which expresses increased amounts of MMP9 in the CNS (see section b). The double-transgenic mouse model (TgSOD1 G93A/TgMMP9) will be evaluated with

regard to motor-related behaviour and the expression of MMP9 in each of the G93A, TgMMP9, and wild type mice. MMP9 levels will be determined in the plasma and CSF by ELISA, zymography, and western analysis. MMP9 will be also visualized in the CNS with advanced imaging approaches. Moreover, to compare the animal studies with the human disease, three categories of ALS patients will be subjected to CSF and plasma determination of MMP9 levels: ALS receiving standard, palliative treatment, patients undergoing intracranial stimulation of neurons, as well as patients having received transplantation of olfactory neural stem cell progenitors (olfNSCPs), at various stages following treatment.
The proposed studies will substantially contribute to a better understanding of the role of MMP9 in ALS and will also indicate possible targets for interfering with the onset and progression of this devastating disease

b) Description of the working program

In ALS mutations in the superoxide dismutase 1 (SOD1), account for ~25% of the familial forms. The mutant-mediated toxicity could be in part derived from the SOD1 aggregates found in ALS(16). Several common links were reported between ALS and Alzheimer's disease (AD), another neurodegenerative disease: AD is a progressive dementia with the pathological hallmark being Aβ peptide-containing plaques in the brain. This peptide is derived from the amyloid precursor protein (APP) by β – and γ-secretase cleavages (17). Aβ plays an important role in neurodegeneration and synaptic dysfunction in AD, since synthetic Aβ is toxic to neuronal cells in culture, and naturally secreted oligomers of Aβ adversely affect synaptic plasticity *in vivo* (18). Reports suggest that the Aβ peptide could also have a potential role in motor neuron disease. Aβ accumulation was observed in brain, skin and lumbar spinal cord of ALS patients (19-21) whereas increased amounts of Aβ were associated with acceleration of motor neuron impairment and SOD1 aggregation in an ALS mouse model (22). However, the potential role of Aβ in ALS remains poorly understood.
MMP-9 is ideally situated to regulate extracellular Aβ levels, because it is secreted and activated in the extracellular compartment**.** In the CNS MMP-9 is expressed at low levels under normal conditions but is induced under pathological conditions, such as cerebral ischemia, neurodegeneration and amyloid plaque load (5). It is possible then that under pathological conditions, MMP-9 may enhance Aβ clearance and/or accumulation: Based on our observations by our group and others MMP-9 degrades in vitro synthetic soluble Aβ(1-40) and Aβ fibrils, and also exerts α-secretase activity, thus lowering Aβ levels (23). Moreover**,** MMP9 has a pivotal role for sustained normal neuronal activity (24). To study the role of MMP9 in AD we have generated a transgenic mouse line overexpressing MMP9 in the brain which has improved cognitive abilities when tested by behavioural assays, and also demonstrates increased neuronal plasticity (longer sustained LTP, modulated spine formation: ***see Appendix Figs. 1-3***) compared to the control (manuscript under revisions). Moreover, we successfully crossed the TgMMP-9 mice with a mouse model for AD (5XFAD) and in pilot experiments we have detected lower amyloid plaque load in the brains of the double transgenic animals TgMMP9/5XFAD. Our objectives then are to use this unique TgMMP9 animal model, to study the role of MMP9 in ALS. The overall strategy consists of the generation of double transgenic mice over expressing MMP9 and SOD1 mutation G93A in order to examine the effect of MMP9 on Aβ elimination, SOD1 aggregation motor impairment, and ALS disease progression. Activation of MMP9 will be followed by applying fluorescence molecular tomography (FMT) imaging in vivo. Furthermore, a correlation between data from the double transgenic animal model and the human disease will be attempted, insofar as quantitative analysis of MMP9 is concerned: At the clinical level, MMP9 levels will be determined in the plasma and CSF of ALS patients receiving standard treatment, or following motor cortex stimulation with low frequency transcranial magnetic stimulation (TMS) of the brain, an alternative treatment of the disease. Finally, a quite unique treatment will be

included, that of olfactory neural stem cell progenitors (olfNSCPs) transplantation in ALS patients, following extended culture of olfactory neural stem cell progenitors (olfNSCPs: (collaboration with Dr. C. Lima, Portugal). In parallel, all patients of the study cohort will be scored for determination of disease progression*. We anticipate that modulation of MMP9 levels may correlate with delayed progression or sustained stability.
These studies will add substantial understanding about the role of MMP9 in ALS, and are anticipated to yield information leading to the development of innovative targets for interfering with the onset and progression of ALS, a devastating, lethal disease.

Experimental procedures:

1. Generation of double transgenic mice
Transgenic TgMMP9 mice expressing the wild-type sequence of MMP9 have been generated by pronuclear microinjection as previously described (25). Briefly, the cDNA encoding for the wild-type human MMP9 (hMMP9) sequence was subloned under the neuron specific Platelet-Derived Growth Factor β promoter (pPDGFβ) and the above transgene was used for the production of transgenic founders by pronuclear microinjection. Transgenic founders were identified by isolation of genomic DNA from tail tissue and Southern blot or slot blot using probes specific for hMMP9 or pPDGFβ sequence. The SOD1G93A (TgN[SOD1-G93A]1Gur) transgenic mice with a delayed phenotype will be obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and will be maintained by breeding hemizygous carriers to a C57BL6 (B6) mouse strain. This line carries a mutant form of human superoxide dismutase (SOD1G93A) that was originally made and characterized by Gurney *et al*. (1994) (26). Homozygous TgMMP9 mice will be crossed with hemizygous TgSOD1G93A mice. Progeny will be genotyped for the SOD1 gene by polymerase chain reaction (PCR) amplification of DNA extracted from the tails. All progeny littermates will be hemizygous for MMP9 with the SOD1G93A gene, named TgMMP9/SOD1G93A.
2. Determination of motor symptoms and function assessments
To study whether MMP9 influences the disease progression of (ALS), double transgenic mice TgMMP9/SOD1G93A will be assessed for symptoms and paralysis from pre-disease state (4 months) to the end-stage of disease in parallel with single transgenic mice TgSOD1 G93A and their non-transgenic littermates. Briefly, mice will be assessed monthly beginning at 4 months of age, then every 4–7 days from 7 months of age till the end-stage of disease for progression of symptoms as previously described (27). The age at which mice develop onset of motor symptoms, onset of paralysis, and progress to the end-stage of ALS will be recorded. Onset of motor symptoms is defined when mice developed a tremor and display loss of extension of hind limbs when picked up at the base of their tails. Onset of paralysis is defined when mice develop paralysis in one hind limb, and end-stage of paralysis is defined when mice developed complete paralysis in both hind limbs and can no longer reach for food or water. Equal numbers of female and male mice of both TgMMP-9/SOD1G93A and TgSOD1G93A will be used. Motor function will be assessed by measuring stride length monthly beginning at 4 months of age then every 4–7 days from 7 months of age until the end stage of disease for progression of disease as described (27).
3. Evaluation of Aβ levels and SOD aggregation in the brains and spinal cord of Tg mice
The brains and spinal cords of TgMMP9/SOD1G93A and TgSOD1G93A mice with no sign of paralysis and at end-stage disease will be measured using the double antibody capture sandwich ELISA and western blot analysis. Briefly, mice will be euthanized and transcardially perfused. Brains (divided into two hemispheres minus olfactory bulb, cerebellum) and spinal cord will be dissected. The half-hemispheres will be homogenized and separated in to soluble and membrane fractions. Soluble fraction will be used to measure Ab levels by ELISA and

western blot analysis using specific antibodies against Aβ, whereas membrane fractions will be used to quantitate SOD1.

4. Evaluation of neurodegeneration and gliosis in the spinal cords of Tg mice

The spinal cords prepared as above will be fixed and embedded in paraffin and will be examined by haematoxylin and eosin staining and by immunohistochemical staining for neuronal changes as previously described. For immunohistochemical staining antibodies against GFAP, ubiquitin, and SOD1 will be used.

5. Determination of MMP-9 activity in vivo by fluorescence molecular tomography (FMT) and Multi-spectral opto-acoustic tomography (MSOT)

To follow MMP9 activation during the disease progress we will perform FMT imaging in intact living animals. FMT imaging has considerable advantages over existing technologies in providing accurate three-dimensional information on the biodistribution and activation of fluorescent probes in intact living animals. For increased resolution the newly developed MSOT technique will be used, which can in addition resolve chromophores and fluorochromes with ultrasonic resolution through several centimeters of tissue. Imaging herein will utilize enzyme-activatable fluorochromes that can be detected throughout small animals and attain molecular sensitivity towards enzymes such as proteases, including MMP9. The activatable fluorochromes utilized are generally dark unless interaction with protease of interest, in which case they fluoresce and change their absorption spectrum so that they can be detected and quantified both by FMT and MSOT. We will follow well documented protocols (28, 29). Briefly, animals at different stage of the disease will be intravenously injected with the MMP9 imaging probe. Immediately prior to imaging, animals will be anesthetized and FMT / MSOT imaging will be performed 16 to 24 h after probe administration, based upon timing parameters optimization. The FMT acquisition time will last approximately 5 min, whereas MSOT lasts approximately 1 second per slice.

6. Evaluation of MMP9 levels in plasma and CSF of ALS patients upon motor cortex stimulation

Glutamate-mediated excitotoxicity has been proposed as a possible cause of cell death in ALS (30). Transcranial magnetic stimulation (TMS) of the brain can activate non-invasively glutamatergic circuits of the human motor cortex (31). Repetitive transcranial magnetic stimulation (rTMS) is a non invasive approach that modulates cortical excitability and has been safely employed to treat several neurological and psychiatric disorders (32). The effects of rTMS on cortical excitability vary according to the frequency and the pattern of stimulation.Motor cortex excitability studies in humans and experimental studies on cell cultures suggest that rTMS can modulate glutamatergic activity of the human cerebral cortex.

Repetitive TMS could also interfere with other molecular determinants of ALS such us oxidative stress and reduced bioavailability and/or release of neurotrophins. TMS has been found, in fact, to reduce oxidative damage in a rat model of Huntington's disease and in patients with spinocerebellar degeneration (33-34). Moreover, rTMS has been found to modulate plasma levels of brain-derived neurotrophic factor (BDNF), a potent survival factor for MNs (35). In recent years a remarkable number of papers about the potential effects of rTMS in several neurological disorders including ALS has been published (33-34). Preliminary studies have shown that rTMS of the motor cortex, at frequencies that decrease cortical excitability, causes a slight slowing in the progression rate of ALS, suggesting that these effects might be related to a diminution of glutamate-driven excitotoxicity (30). Taking in to consideration that MMP-9 it was shown by in situ zymography to co-localize with synaptic NMDA and AMPA-type glutamate receptors (15), we are aiming to determine the levels of MMP-9 in the plasma and CSF of ALS patients upon motor cortex stimulation by treating patients with low frequency rTMS as previously described (36).

7. Evaluation of MMP-9 levels in plasma and CSF of ALS patients upon symptomatic treatment

The management of ALS patients is still supportive and symptoms-based and, actually, riluzole is the only compound that demonstrated a beneficial effect on ALS patients, but with only modest increase in survival. Neurodegenerative diseases could occur as a result of pathological expression of different proteins. For example, Aβ for Alzheimer's disease, phosphorylated tau for Frontotemporal Dementia and Supranuclear Palcy, synuclein for Parkinson's disease and Lewy Body dementia and huntigtin for Huntington Disease. MMP9 could represent a biological marker and also contribute to new treatments for this dramatic disease.

8. Evaluation of MMP-9 levels in plasma and CSF of ALS patients following transplantation of neural stem cell progenitors (olfNSCPs)

Neural stem cells (olfNSCPs) will be isolated from small amount of tissue obtained from the olfactory mucosa (~1-2mm^2). The cells will be grown under appropriate conditions in culture and will be sub-cultured until adequate number of neural stem cells is obtained (2-3 months). The neural stem cells will in sequence be transplanted (autologous transplantation: injected into the intrathecal space through a simple lumbar puncture). Several patients have already undergone this procedure according to the European regulations for human transplantation. Disease status of patients will be analyzed with ALS functional rating scale-revised (ALSFRS-R)* for primary outcome measure and additional clinical findings after treatment will be collected for secondary outcome measure and safety. Check-points for each patient will be performed every 3 months for ALSFRS-Rand 6 months for the other assessments during a one year period.

*Neurological history and examination

ALS functional rating scale-revised (ALSFRS-R)
Videorecording of the initial general and motor status
MRI of the brain and spinal cord, cortical motor spectroscopy and tMRI (tractography)
Electrophysiological studies: EMG
Spasticity scale (Ashworth),
Otolaryngological and olfactory assessment, CT scan of the nose and sinus
Pneumological functional assessments (Vital capacity)
Psychological assessment
Adverse effects monitoring

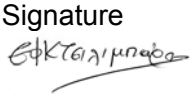
c) Potential health impact and exploitation/dissemination of expected project results

The proposed study will provide novel insights towards interfering with the devastating disease of ALS. The combination of translational research making use of transgenic animals to derive information about a potential role of MMP9 with clinical studies, will unravel correlations between the two approaches with pivotal importance for generating new means to combat ALS. The results could then be applied towards new protocols foe determining the efficacy of different ways of treating ALS.

d) Added value of the proposed transnational collaboration

The collaboration between researchers using different approaches to better understand the role of MMP9 in novel transgenic animal models in parallel with samples from ALS patients treated with different means is only available by taking advantage of the different expertise and therapies used by participating scientists. Hence, novel models of transgenic ALS animals will be investigated with novel approaches involving in vivo imaging of active MMP9, and the obtained data will be compared with conventional and also novel approaches (transcranial stimulation, neural stem cell transplantation) for interfering with ALS and its progression. This collaboration will make use of the expertise of participating scientists from Greece (also collaborating with Portugal), Germany and Italy.

Signature



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Appendix

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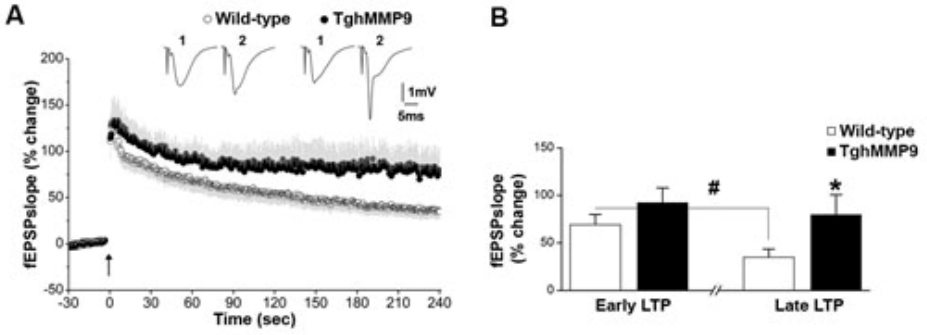

Figure 1. Increased hippocampal late LTP in TghMMP9 mice compared to their wild-type littermates. (A) The effect of TBS (arrow) in the fEPSP slope in wild-type and TghMMP9 mice. Means (± SEM) of the percent increase of fEPSP slope are shown. Insets show examples of fEPSPs collected 5 min pre- (1) and 4 h post-TBS (2) from a wild-type (*left*) and a TghMMP9 (*right*) hippocampal slice. (B) TBS induced early LTP (60 min) of similar magnitude in both animal groups, but significantly greater late LTP (240 min) in TghMMP9 mice compared to their wild-type littermates (* P<0.05). Potentiation of fEPSP four hours post-TBS was greatly reduced only in wild-type hippocampal slices (#, P<0.001).

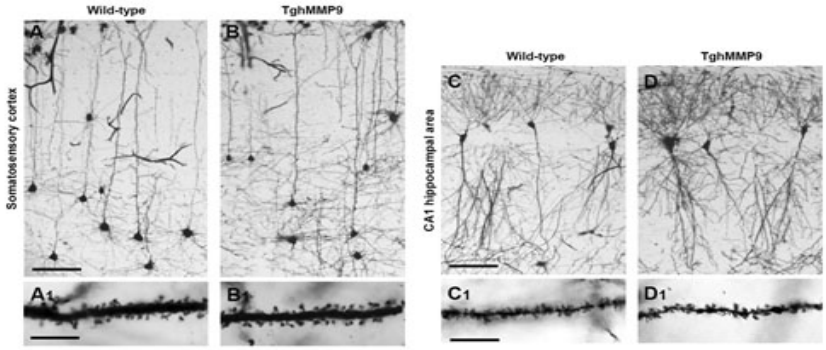

Figure2. Dendritic spine density in hippocampal and cortical pyramidal neurons is increased in TghMMP9 mice. (A-D**)**Photomicrographs of Golgi-stained cells in the somatosensory cortex (A,B) and CA1 area (C,D) of a wild type (A,C) and a TghMMP9 (B,D) animal. Scale bars:100μm. (A1-D1) A higher magnification of dendritic segments: panels A1 and B1 correspond to apical dendrite of a layer V pyramidal neuron from the wild-type (A1) and TghMMP9 (B1) animal, whereas panels C1 and D1 correspond to basal dendrite of a CA1 pyramidal neuron from the wild-type and the TghMMP9 animal, respectively. Scale bars:10μm.

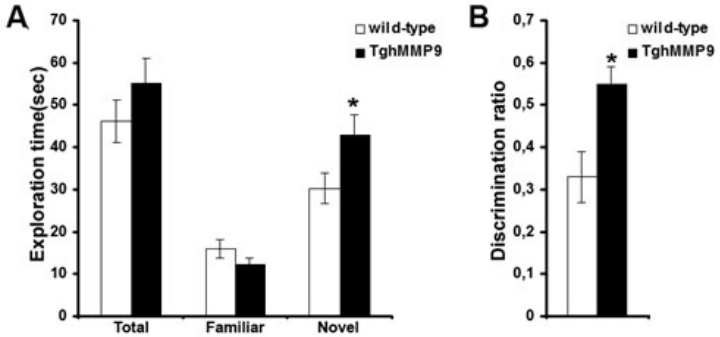

Figure 3. TghMMP9 mice display an enhanced performance in cognitive tasks. (A) Mean time (±SEM) exploring the objects during the probe trial of the task. No statistically significant difference was observed . (B) Mean discrimination ratio (±SEM): all animals were able to discriminate between the novel and the familiar object; nevertheless, TghMMP9 mice performed better in this task.