EXCITOTOXIC MECHANISMS, NEUROINFLAMMATORY RESPONSE AND SERUM FACTORS IN EXPERIMENTAL AND HUMAN AMYOTROPHIC LATERAL SCLEROSIS

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Duration: 3 years
1. Summary of the project

Amyotrophic lateral sclerosis (ALS) is a dramatic neurodegenerative disease. Neurologists do not have any satisfactory treatment able to appreciably improve its fatal course. From the pathogenetic point of view, besides the research efforts and the important progress made in the last few years, the disease is still enigmatic in many aspects. There are several theories for ALS pathogenesis, none of which are mutually exclusive. The aim of this project was to join the expertise of five different labs in order to contribute to the understanding of some mechanisms involved in motoneuron (MN) degeneration and to identify new targets for ALS therapy. Each partner is from a lab experienced in a distinct but complementary facet that we consider relevant in the context of the present knowledge of ALS pathogenesis. We think that the current status of ALS research is in a particularly exciting period. It can be expected that the new concepts emerging from cellular and molecular neuroscience as well as from immunology, together with the availability of animal models of the disease, will produce, in the next years, great advances in the understanding of the complexity of the disease as a biological entity. We expect that these joint efforts will help in providing the basis for a future ALS therapy. It is under this scope that we propose the research that we would like to develop in the present project, in which some unsolved aspects about neurotoxicity by glutamate and neuroimmunological phenomena will be addressed according to the following main objectives:

1.1 Exploration of the cell-specific mechanisms involved in MN degeneration by chronic glutamate toxicity. As a hypothesis it is assumed that excitotoxicity by glutamate exerts an important role in MN damage in ALS. It is proposed: first, to elucidate how a sublethal excitotoxic stimulus selectively alters protein processing at the endoplasmic reticulum, intracellular calcium homeostasis, and vesicular membrane transport in MNs, in a model of chronic sublethal excitotoxicity; second, to evaluate the neuroprotective effects of agents interfering excitotoxic signaling in MNs in “in vivo” and “in vitro” systems; and third, to analyze the plastic changes of neuromuscular connections by MNs under chronic excitotoxicity.
1.2 To analyze the influences of mutated SOD1 (mSOD1) expression on the impact of glial cells on MN survival. We hypothesize that the expression of mSOD1 leads to functional changes in glial cells and that some of these changes may potentially exert negative effects on neuronal survival. To verify our hypothesis we will use mixed glial cultures (astrocytes and microglia), enriched astrocyte cultures and enriched microglia cultures from wild-type and mSOD1 mice. These cells will be obtained from newborn, 30-day-old and 60-day-old mice to simulate the different stages of illness. The first objective will be to characterize the microglial response after LPS treatment in order to analyse whether mutant SOD1 mice have exacerbated glial activation in comparison with wild-type mouse microglia. To this end, we will determine the release of nitric oxide (NO) and tumour necrosis factor alpha (TNF-α), the nuclear translocation of nuclear factor kappa B (NF-κB), the increase of the transcription factor C/EBPβ and the increase of p21Cip1. All these compounds take part in the glial neuroinflammatory response. The second objective will consist in studying the effect of culture medium conditioned by LPS treated glial cells from mSOD1 and wild-type on MN viability. The third objective will be to inhibit, pharmacologically or through siRNA, the above mentioned factors and to study, in each case, the effect of such inhibition on neuronal viability.

1.3 Study of the interactions between excitotoxicity and neuroinflammation. Elucidating the mechanisms by which cytokines influence neuronal function is important for understanding both normal function and pathological conditions in the nervous system. We hypothesize that high levels of cytokines such as TNF-α, released by activated microglia make MNs more vulnerable to excitotoxicity. TNF-α would have a facilitator role in glutamate mediated excitotoxicity, directly potentiating glutamate transmission (increasing expression of AMPA receptors on synapses) and indirectly inhibiting glial glutamate transporters on astrocytes). We aim to study the relationship between TNF-α receptors and glutamate receptors and transporters, because we think the link between inflammation in ALS and MN excitotoxicity could lie in these interconnected pathways. We propose to use “in vitro” models of cultured spinal cord in order to study: 1) the direct effects of cytokines on MNs, focusing on cell viability, cytoskeleton alterations, etc., 2) the possible exacerbation of excitotoxicity induced by high levels of cytokines, 3) the alterations in trafficking
of glutamate receptors (AMPAR, NMDAR, mGluR) and transporters induced by high levels of TNF-α.

**1.4 Identification of circulating autoantibodies in sera from ALS patients.** The diagnosis of ALS is based on clinical and electromyographic data. A specific laboratory marker for diagnostic or monitoring purposes is greatly desired by neurologists. Autoantibodies against calcium channels have been identified in ALS patients sera but neither its origin nor its pathogenetic role in the disease has been defined. Our objective is the application of new methods for detecting and characterizing circulating antibodies in ALS patients in order to evaluate their specificity or relevance. Xenopus oocytes transfected with different channel membrane protein subunits will be used for analysis of currents evoked by ALS autoantibodies. The effects of these antibodies on chick embryos developing MNs and the neural antigens recognized will be also analyzed.

**2. Results**

**2. Results team Dr Josep E. Esquerda Colell**

Team members: Jordi Calderó Pardo, Anna Casanovas Llorens, Dolors Ciutat Falcó, Olga Tarabal Moztazo. PhD students: Núria Brunet García, Sara Hernandez Estañol

The excitotoxic mechanisms were investigated in spinal cord MNs by using organotypic cultures of chick embryo spinal cord. Glutamatergic agonists and mitochondrial toxins were used to induce neuronal damage and to determine the alteration in intracellular calcium homeostasis. We also tested in this system the putative neuroprotective effects of lithium; these experiments were performed on the basis of the controversy recently generated about the lithium treatment in ALS. Our results pointed out that although lithium can exert an anti-excitotoxic effect, it does not prevent the appearance of degenerative changes in MNs submitted to an excitotoxic stimulus.
We have also described the normal development of microglia and its activation after excitotoxicity in the chick embryo spinal cord.

We have shown that degenerating neurons in transgenic rodents overexpressing SOD1G93A displayed a strong immunoreactivity to antibodies against the purinergic receptor P2X4. Neurons displaying this property are associated with activated and neuronophagic microglia. By means of proteomics we discovered that P2X4-immunoreactive material corresponds to neurotoxic mSOD1 conformers that exposed an epitope normally hidden in normally folded SOD1. We think that the neutralization of the neurotoxic activity of misfolded SOD1 conformers may have a therapeutic effect in ALS. Based on this hypothesis we have developed new anti-SOD1 antibodies with conformational specificity and we have initiated also an immunotherapeutic trial in transgenic animal models of ALS displaying several SOD1 mutations.

In collaboration with groups 4 and 5 we have shown that sera from some ALS patients contain anti-semaphorin 3A antibodies. Further studies are necessary to evaluate the clinical relevance of this finding.

2. Results team Dr Joan Serratosa Serdà

Team members: Carme Solà Subirana, Josep Saura Martí, Josep Maria Tusell Puigbert, Pilar Mancera Aroca, Tony Valente i Guido Dentesano

Neuroinflammation is thought to play a pathogenic role in many neurodegenerative disorders including amyotrophic lateral sclerosis (ALS). In this study we demonstrate that expression of NO synthase-2 (NOS2) and cyclooxygenase-2 (COX2) induced by lipopolysaccharide (LPS) + interferon-γ was higher in microglial-enriched cultures from G93A-SOD1 mice, an ALS animal model, than from wild-type mice. The levels of CCAAT/enhancer binding protein β (C/EBPβ), a transcription factor that regulates proinflammatory gene expression, were also up regulated in activated G93A-SOD1 microglial cells. In vivo, systemic LPS also induced an exacerbated neuroinflammatory response in G93A-SOD1 mice vs. wild-type mice, with increased expression of GFAP, CD11b, NOS2, COX-2, proinflammatory cytokines and C/EBPβ. Finally, we report that
C/EBPβ is expressed by microglia in the spinal cord of ALS patients. This is the first demonstration to our knowledge of microglial C/EBPβ expression in human disease. Altogether these findings indicate that G93A-SOD1 expression results in an exacerbated pattern of neuroinflammation and suggest that C/EBPβ is a candidate to regulate the expression of potentially neurotoxic genes in microglial cells in ALS.

2. Results team Dr Jerònia Lladó Vich

Team members: Gabriel Olmos Bonafé, Francesc Xavier Miralles Morell, Víctor J. Asensio Landa. PhD students: Laia Tolosa Pardo, Margalida Mir Mas, Víctor Caraballo Miralles

In addition to glutamatergic excitotoxicity and oxidative stress, neuroinflammation has recently emerged as a relevant contributor to motoneuron damage in ALS. The accumulation of reactive microglia in the degenerating areas of ALS tissue is a key cellular event creating a chronic inflammatory environment that results in motoneuron death. The proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) have been proposed as being involved in ALS-linked microglial activation. In fact, high levels of circulating proinflammatory cytokines have been shown both in human patients and in animal models of ALS.

The first aim of our study was to elucidate the effects of IFN-γ and TNF-α on iNOS expression and NO production in microglial cells. In the BV-2 microglial cell line we showed that IFN-γ and TNF-α have complementary roles in iNOS expression and this might be relevant to understand the molecular mechanisms of microglial activation associated with the pathogenesis of several neuroinflammatory disorders in which increased levels of IFN-γ and TNF-α have been reported.

The second objective was to study the effects of these cytokines on a motoneuron culture in the presence of glial cells in a new culture system developed in our laboratory. In these cultures, the combined exposure to the above cytokines resulted in an increased expression of prooxidative enzymes as
compared to each cytokine alone. TNF-α and IFN-γ also cooperated to promote protein oxidation and nitration, thus increasing the percentage of motoneurons immunoreactive for nitrotyrosine. In addition, apoptotic motoneuron death was cooperatively induced by TNF-α and IFN-γ. Interestingly, these cytokines did not affect the viability of purified spinal cord motoneurons in the absence of glial cells. These results suggested that the proinflammatory cytokines TNF-α and IFN-γ have cooperative/complementary roles in inflammation-induced motoneuron death.

The third objective was to study whether the above cytokines had a facilitator role in glutamate-induced motoneuron neurotoxicity. In rat spinal cord organotypic cultures, chronic glutamate excitotoxicity induced by the glutamate uptake inhibitor, threohydroxyaspartate (THA), resulted in motoneuron loss that was associated with a neuroinflammatory response. In the presence of TNF-α, THA-induced excitotoxic motoneuron death was potentiated. Co-exposure to TNF-α and THA also resulted in down-regulation of the astroglial glutamate transporter 1 (GLT-1) and in increased extracellular glutamate levels, which were prevented by nuclear factor-kappaB (NF-κB) inhibition. Furthermore, TNF-α and THA also cooperated in the induction of oxidative stress in a mechanism involving the NF-κB signalling pathway as well. The inhibition of this pathway abrogated the exacerbation of glutamate-mediated motoneuron death induced by TNF-α. To our knowledge, this is the first time that TNF-α-induced NF-κB activation has been reported to potentiate glutamate excitotoxicity on motoneurons. These data link two important pathogenic mechanisms, excitotoxicity and neuroinflammation, suggested to play a role in ALS and support the hypothesis of an intricate interplay between these mechanisms, important in the pathology of neurological diseases including ALS. This interplay has to be considered for the design of new therapies for ALS.

2. Results team Dr. Carles Solsona Sancho

Team members: Jordi Marsal, Joan Blasi, Mireia Marti-Satué, Artur Llobet, Mònica Povedano
PhD students: Laura Teixidó
The familial form of ALA is associated with mutations in the gene encoding Cu/Zn superoxide dismutase 1 (SOD-1) enzyme, but it accounts for fewer than 10% of cases; the rest, more than 90%, correspond to the sporadic form of ALS. Although many proposals have been suggested over the years, the mechanisms underlying the characteristic selective killing of MN in ALS remain unknown. In this study we tested the effect of sera from sporadic ALS patients on NMDA receptors (NMDAR). We hypothesize that an endogenous serum factor is implicated in neuronal death in ALS, mediated by the modulation of NMDAR.

Sera from ALS patients and from healthy subjects were pretreated to inactivate complement pathways and dialyzed to remove glutamate and glycine. IgGs from ALS patients and healthy subjects were obtained by affinity chromatography and dialyzed against phosphate-buffered saline. Human NMDAR (NR1 and NR2A subunits) were expressed in *Xenopus laevis* oocytes, and ionic currents were recorded using the two-electrode voltage clamp technique.

Sera from sporadic ALS patients induced transient oscillatory currents in oocytes expressing NMDAR with a significantly higher total electrical charge than that induced by sera from healthy subjects. Sera from patients with other neuromuscular diseases did not exert this effect. The oscillatory currents recorded were due to internal calcium mobilization. The currents were inhibited by MK-801, a non-competitive blocker of NMDAR. To obtain the response the co-expression of NR1 and NR2A subunits is needed. In addition, the response to glutamate of the oocytes expressing NMDRA was potentiated after a challenge of sera from control and patients. Isolated IgGs from ALS patients significantly affected the activity of oocytes injected with NMDAR, causing a 2-fold increase over the response recorded for IgGs from healthy subjects.

Our data support the notion that ALS sera contain soluble factors that mobilize intracellular calcium, not opening directly the ionic conductance, but through the non-canonical activation of NMDAR, probably interacting with Gq protein.
2. Results team Dr Ronald William Oppenheim

Team members: Carol Milligan

The experiments outlined in the proposal are designed to investigate whether specific toxic and/or trophic substances are present in the serum or cerebrospinal fluid of ALS patients. Results showed a MN survival promoting activity in assays performed in the chick embryo.

With collaboration with team 1 by using proteomic technology we have detected anti-semaphorin 3A antibodies in ALS patients’ sera. These antibodies promote the branching of intramuscular nerves which may explain the survival-promoting activity of ALS sera. Some ALS patients have elevated titers of anti-semaphorin 3 antibodies. Further studies are necessary to evaluate the clinical significance of these findings.

3. Relevance and possible clinical implications of the final results

ALS, or motor neuron disease remains one of the most devastating, and incurable, neurological diseases. Riluzole®, the only drug that has been approved by the FDA for treatment of ALS, has shown a slight benefit in modestly increasing survival time. Every 90 minutes, an American dies of ALS (data from ALS and MND associations). On the biological point of view the disease is still enigmatic but during the past several years an increasing number of researchers have devoted their efforts to unravelling its mysteries. Only the joint efforts of researchers around the world with the new potent analytical techniques at the cellular and molecular levels will change the field of ALS management in the near future. On the ALS association’s web page one can read “Despite the mysterious nature of ALS, breathtaking advances in science, medicine and technology are shaping a future of unparalleled hope for those with ALS. The ALS Association is at the forefront in this new world, encouraging young scientists to combine new thinking with these advances to unlock the mysteries of ALS – to push the envelope in therapy and scientific research. The ALS Association is waging
the war against this killer 24/7. Every 90 minutes, an American dies of ALS.
Time isn’t on the side of those afflicted”.

The aim of this project was to join the expertise coming from five different laboratories in order to contribute to the understanding of some mechanisms involved in MN degeneration and to identify new targets for ALS therapy. We think that the current status of ALS research is in a particularly exciting period. It can be expected that the new concepts emerging from cellular and molecular neuroscience as well as from immunology, together with the availability of animal models of the disease, will produce, in the next years, great advances in the understanding of the complexity of the disease as a biological entity. We expect that these joint efforts will help providing the basis for a future ALS therapy. It is under this scope that we have proposed the research that we have developed in the project. Our contributions have been published in a worldwide scientific journals for this field and they will contribute in the future advances in ALS research and therapy. For example, on the basis of data obtained in this project, a therapeutic vaccine directed to neutralizing the neurotoxic activity of mutant SOD1 is now under development and preclinical testing. A new young person (Sara Hernández), who has obtained her PhD under this project, is now working as a postdoctoral fellow in one of the world’s most reputable laboratories involved in ALS research (Dr Wim Robberecht at the Department of Neurology, University of Leuven). It is expected that after this period she will start an independent career as a new ALS researcher in Catalonia increasing our potential in this field.

4. Publications

*Strong P2X4 purinergic receptor-like immunoreactivity is selectively associated with degenerating neurons in transgenic rodent models of amyotrophic lateral sclerosis.*
J Comp Neurol. 506:75-92. (IF: 3.93)

*Development of microglia in the chick embryo spinal cord: implications in the regulation of motoneuronal survival and death*

*Journal of Neuroscience Research* 87:2447-2466. (IF: 3.19)


*Excitotoxic motoneuron degeneration induced by glutamate receptor agonists and mitochondrial toxins in organotypic cultures of chick embryo spinal cord by* *Journal of Comparative Neurology* 516:277-90. (IF: 3.93)


*Neurotoxic species of misfolded SOD1G93A recognized by antibodies against the P2X4 subunit of the ATP receptor accumulate in damaged neurons of transgenic animal models of amyotrophic lateral sclerosis.*


*Lithium prevents excitotoxic cell death of motoneurons in organotypic slice cultures of spinal cord.*

*Neuroscience* 165:1353-1369. (IF: 3.52)


*Increased intramuscular nerve branching and inhibition of programmed cell death of chick embryo motoneurons by immunoglobulins from patients with motoneuron disease.*


*Upregulation of p21Cip1 in Activated Glial Cells.*

*Glia,* 57: 524-534. 2009. (IF: 5.09)
*C/EBPβ expression in activated microglia in amyotrophic lateral sclerosis.*
Neurobiology of Aging (submitted)

*Vascular endothelial growth factor protects spinal motoneurons against glutamate-induced excitotoxicity via phosphatidylinositol 3-kinase.*
Journal of Neurochemistry 105:1080-1090. (IF: 4.20)

*Complementary roles of tumor necrosis factor alpha and interferon gamma in inducible microglial nitric oxide generation.*
Journal of Neuroimmunology 204:101-109 (IF: 2.83)

*Vascular endothelial growth factor protects motoneurons from serum deprivation-induced cell death through phosphatidylinositol 3-kinase-mediated p38MAPK inhibition.*
Neuroscience 158:1348-1355. (IF: 3.52)

*Tumor necrosis factor alpha and interferon gamma cooperatively induce oxidative stress and motoneuron death in rat spinal cord embryonic explants.*
Neuroscience 162:959-971. (IF: 3.52).

Tolosa, L.; Caraballo-Miralles, V.; Olmos, G.; Lladó, J. (2011)
*TNF-α potentiates glutamate-induced spinal cord motoneuron death via NF-κB.*
Molecular and Cellular Neuroscience 46:176-186. (IF: 3.88)

*Sera from amyotrophic lateral sclerosis patients induce the non-canonical activation of NMDA receptors "in vitro".*
Neurochemistry International, (submitted)
Texidó L., Martín-Satué M., Alberdi E., Solsona C., Matute C (2011)
*Amyloid-β peptide oligomers directly activate NMDA receptors* Abbreviated title: *Amyloid-β activates NMDA receptors.*

The rescue of developing avian motoneurons from programmed cell death by a selective inhibitor of the fetal muscle-specific nicotinic acetylcholine receptor.
Developmental Neurobiology. 68:972-980. (IF: 2.79)