INTRODUCTION

Motor neuron Diseases (MND) is a term used to cover a spectrum of neurodegenerative syndromes with fatal outcome characterized by a progressive and selective degeneration of motor neurons. In addition to classical Amyotrophic Lateral Sclerosis (ALS), this group includes Spinal Muscular Atrophy (SMA), adult-onset spinal muscular atrophies (Kennedy’s syndromes) and Primary Lateral Sclerosis.

ALS is the most frequent adult-onset MND whose hallmark is the selective death of motor neurons in the primary motor cortex, brain stem and spinal cord, leading to paralysis of voluntary muscles. "Amyotrophy" refers to the atrophy of muscle fibers, which are denervated as their corresponding anterior horn cells of the spinal cord degenerate, leading to weakness of affected muscles. "Lateral sclerosis" refers to hardening of the anterior and lateral corticospinal tracts as motor neurons in these areas degenerate and are replaced by gliosis. The mean age of onset for ALS is about 60 years, with a slight male prevalence. Due to its uniform lethality, ALS appears as a rare disease (prevalence of 4-6 per 100,000 each year), although its incidence is of 1-2 per 100,000 each year. Unfortunately, at present the clinician has no effective therapy to modify the course of this terrible disease, and diagnostic instruments are limited and insufficient. Given the public health and economic-social impact of ALS, its characterization and early diagnosis, as well as the introduction of effective therapies for the management of this disorder, are important priorities.

The slow progress towards a therapy for ALS is due to the paucity of knowledge on its molecular basis and the etiopathogenic heterogeneity of this disease. Thus, the molecular pathway causing motor neuron degeneration in ALS is likely to be the result of a complex interplay between multiple pathogenic cellular mechanisms.

Most cases (90%) of ALS are classified as sporadic (SALS), as they are not associated with a documented family history. The remainder 10% are inherited and referred to as familial ALS. Six Mendelian genes defects have been reported to cause ALS. The protein products of these mutated genes are cytosolic Cu/Zn superoxide dismutase (SOD1), alsin, senataxin (SETX), VAMP (vesicle associated membrane protein)-associated protein B (VAPB), TAR DNA binding protein (TDP-43) and dynactin. 20% of familial ALS have a mutation in the SOD1 gene and about 2-5% have mutations in the TDP-43 gene. Two percent of apparently sporadic patients have SOD1 mutations and TDP-43 mutations also occur in sporadic cases. Sporadic and familial forms are clinically and pathologically similar, suggesting at least some common pathogenetic pathways. Unraveling the disease mechanisms of FALS-linked genes will be key to understanding the causes underlying the pathogenesis of the much more frequent sporadic form of ALS. In our Institute, we are investigating the cellular mechanisms of the pathogenicity of two of the known gene products that cause FALS: SOD1 and VAP-B.
4a. MOLECULAR MECHANISMS OF MUTANT SOD1 TOXICITY IN AMYOTROPHIC LATERAL SCLEROSIS CELLULAR MODELS

CURRENT STAFF:
- Grazia Pietrini
- Maura Francolini
- Marco Righi

PhD student
- Valeria Padovano

Graduate students
- Valentina Alari
- Arianna Crespi

Characterization of the effect of mutant SOD1 (SOD1G93A) on secretory pathways in microglial models of familial ALS (FALS)

Evidence indicates that ALS is not cell autonomous and that toxicity of mutant SOD1 in astrocytes and microglia contribute to the motor neuron degeneration in familial ALS. The role of astrocytes in the disease has been documented, whereas the role of microglia activation is still controversial. In astrocytes, mutant SOD1 expression causes GLT1 astroglial glutamate transporter loss, and, in a cell model of ALS, we have shown that transporter loss is due to increased internalization and degradation of surface GLT1 (Vanoni et al, J Cell Sci 2004). To investigate the role of microglia in the disease's progression, we have generated microglial cell models of familial ALS by immortalization of embryonic primary cortical cultures from murine SOD1 G93A transgenic mice. In these cells, our preliminary data indicate that mutant SOD1 stimulates the release of cytosolic proteins through a non conventional route. We are now characterizing the secretory pathway(s) affected by mutant SOD1 and the composition of the microglia-G93A conditioned medium.

The toxicity of the microglia-G93A medium will be tested on muscle cell lines and motoneurons (cell line NSC-34). By comparing the effects of SOD1 G93A overexpression in non neuronal and motoneuronal cell lines, we have previously demonstrated an exclusive alterations in the mitochondrial network of motoneuronal NSC-34 cells (Raimondi et al., Eur J Neurosci 2006). With this in mind, we will study the effect of the microglia-conditioned medium on the mitochondrial network of NSC-34 cells (untransfected or transfected with mutant or wild-type SOD1).

Characterization of the effect of mutant SOD1 on neuromuscular junctions

ALS is an axonopathy, and one of the earliest findings in human ALS patients and in mouse ALS models is the denervation of the muscular junction long before motoneuron death. Although evidence indicates that also muscles are primary sites of action of mutant SOD1 toxicity, the contribution of skeletal muscle to initiating and/or propagating denervation has been so far largely underestimated. However, it is known that the skeletal muscle system may contribute to neuron degeneration by generating negative signals and/or by decreasing the expression of positive factors. To study the role of muscles in ALS we are analyzing neuromuscular junctions (NMJs) of skeletal muscle systems in the SOD1 G93A transgenic mice. By electron and confocal microscopy and by immunocytochemistry using antibodies against specific markers of innervated and denervated NMJs, we are investigating the NMJs in mice exposed to inflammatory stimuli, anabolic steroids and strenuous physical exercise and whether these treatments regulate the expression of the muscle-derived growth factor IGF-1, an activator of axonal outgrowth that has been found to be decreased in ALS, and other factors associated with muscle denervation and ALS severity (e.g. Nogo-A and Nogo-C).

Figure 1 – A: Postsynaptic membrane (Butx); B: Presynaptic; terminal (synapsin I); C: Merge

SELECTED PUBLICATIONS


COLLABORATIONS
- C. Bendotti, V. Bonetto, M. Tortarolo, Istituto Ricerche Farmacologiche “Mario Negri”, Milan, Italy
- M. Bentivoglio, R. Mariotti, Department of of Morphological and Biomedical Sciences, University of Verona, Verona, Italy

GRANTS
- Italian Ministry of University and Research, PRIN
4b. ROLE OF THE VAP-B GENE IN THE PATHOGENESIS OF ALS

CURRENT STAFF

Nica Borgese
Francesca Navone
Maura Francolini

Graduate students
Elisa Fasana
Matteo Fossati
Annamaria Ruggiano

The VAP-B gene was recently linked to MNDs in 7 Brazilian families of common Portuguese origin and defects in this gene cause adult-onset, autosomal dominant ALS. VAPB has six exons that encode a ubiquitously expressed 27.2 kDa homodimer, which belongs to a family of vesicle-associated/membrane bound associated proteins characterized by the so called Major Sperm Protein (MSP) domain and involved in intracellular trafficking.

VAP-B is an Endoplasmic Reticulum (ER) resident protein, with a hydrophobic domain close to the C-terminus and a cytosolic N-terminal portion containing the MSP domain. This mode of insertion in the membrane is typical of the so called "tail-anchored proteins". The dominant VAP-B mutation identified in the Brazilian family is a nucleotide substitution that results in the substitution of S for a P within the MSP domain (at position 56) of the protein (P56S). It is generally believed that VAPB functions as a receptor for cytosolic proteins, recruiting them to the ER. The association between these proteins and VAPB is mediated by the so called FFAT motif (2 phenylalanines in an acidic tract) in the ligands and a binding pocket in VAP-B. VAP-B ligands have been implicated in non- vesicular transport of lipids, in transcriptional regulation, and in organization of the microtubule cytoskeleton.

In view of the complex variety of VAP-B functions, the mechanism of action of the P56S mutation may depend either on the loss of function of a protein with key roles in intracellular traffic and stress response or to a gain of function disease mechanism due to the toxicity of the mutant form of protein. In this respect, VAP-B P56S has been previously reported to form intracellular inclusions, whose relation to the ER was poorly understood, and which have been implicated as the cause of motor neuron toxicity. To elucidate the molecular mechanisms underlying the pathogenic effect of mutant VAPB, we characterized the formation and localization of intracellular VAP-B P56S inclusions in cells transiently expressing this mutant protein.

After expression of VAP-B in cultured cells, we have demonstrated that: (i) Except at very early time points after inducing its expression (up to 2 hours after microinjection of the cDNA), VAP-B P56S is exclusively contained in inclusions, in contrast to the wild-type protein, which localizes to the ER; (ii) the inclusions colocalize to different extents with different ER markers. Transmission EM analysis of mutant VAP-B expressing transiently transfected cells show a unique ER morphology, consisting most commonly of pairs of ER cisternae united by an electron-dense layer of cytosol approximately 30 nm thick; these apparent cisternal pairs are most likely due to indentations within single cisternae. Immunelectron microscopy reveals that VAP-B P56S is highly enriched on juxtaposed ER membranes. FRAP and FLIP analysis show that a GFP-tail anchored reporter freely diffuses between the aggregates and the surrounding ER and that the VAP-B P56S inclusions are continuous with the entire ER compartment. These observations indicate that the previously identified inclusions are formed via a profound remodeling of ER membranes, probably as a consequence of high amounts of oligomerizing mutant protein residing in the membranes. Indeed, we find that VAP-B P56S inserts into membranes in the same way as the wild-type protein. Further immunocitochemical analysis with Golgi apparatus markers shows the close proximity between the VAP-B P56S-induced ER structures and the cis-medial Golgi subcompartment.

To investigate the effects of different levels of VAP-B P56S on ER morphology, we generated stable Tet-Off cell lines expressing the mutant protein in a doxycycline-dependent fashion. Both immunofluorescence and western blot analysis show that a progressive accumulation of VAP-B P56S can be achieved by different time and dose exposures to doxycycline. By modulating the expression of mutant VAP-B it will be possible to correlate protein levels with the appearance of ER membrane inclusions. Finally, by comparing the effects of VAP-B P56S on the functional viability of non neuronal and neuronal cells, we expect to obtain important information on the pathogenic mechanism of this mutant protein in the development of ALS.

SELECTED PUBLICATIONS

Fasana E, Fossati M, Brambillasca S, Francolini M, Borgese N. The early steps in the biogenesis of VAP-B and the mutant P56S ALS-linked form. Manuscript in preparation

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