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Advantages and Pitfalls in Experimental Models Of ALS

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that targets upper and lower motoneurons (MN) and leads to death in 2-5 years. 5–10% ALS cases are familial (fALS) with a Mendelian pattern of inheritance. The remaining 90% ALS cases are classified as having sporadic disease (sALS). Only in about 30% of fALS mutations in specific genes have been identified, whereas for the others the etiology is unknown. The clinical phenotype of fALS is usually indistinguishable from sALS.
An effective therapy is still lacking, even though many clinical trials have been already conducted. In order to perform preclinical studies to study the etiology and the molecular mechanisms, to design and to test new therapeutic targets and molecules, several *in vitro* and *in vivo* experimental models have been identified, to reproduce the hallmarks of the disease. Such models include transgenic or spontaneously mutated animals as well as *in vitro* preparations, i.e. MN/spinal cord organotypic cultures. Unfortunately, all these models fail to reproduce the complexity of the human disease, even though they represent a very useful tool to investigate several features of the disease.

2. Genetic animal models

To date, at least 13 genes and loci of major effect in fALS have been identified. The most frequent mutations found in fALS consist in mutations in the gene encoding copper/zinc superoxide dismutase 1 (Cu/Zn SOD1), a ubiquitously expressed enzyme that plays a key role in oxygen free radical scavenging. SOD1 catalyzes the dismutation of superoxide (O_2^-) to hydrogen peroxide (Scozzafava & Viezzoli, 1993; Tainer et al., 1982). On average, SOD1 mutations are responsible for about 20% of fALS cases (Deng et al., 1993; Rosen et al., 1993) and 5% of apparently sALS (Andersen et al., 2007). Over 70 different mutations at more than 60 residues throughout SOD1 (153 amino acids) have been linked to fALS (Andersen, 2000). The vast majority of mutations are amino acid substitutions, but a few cause C-terminal truncations of the protein. A spontaneous mutation in SOD1 has also been found in a canine degenerative myelopathy which resembles ALS (Awano et al., 2009).

Recently, mutations in the genes encoding the TAR DNA/RNA-binding protein 43 (TDP-43) (Corrado et al., 2009; Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008; Yokoseki et al., 2008) and Fused in sarcoma/Translocated in liposarcoma (FUS/TLS) (Corrado et al., 2010; Kwiatkowski et al., 2009; Vance et al., 2009) have also been identified in approximately 5%-10% typical fALS.
Other genetic causes of rare and/or atypical fALS include mutations in the genes encoding alsin (ALS2; Hadano et al., 2001) and senataxin (ALS4; Chen et al., 2004) in juvenile ALS, spatacsin (ALS5; Orlacchio et al., 2010), vescicle-associated membrane protein B (VAPB; ALS8; Nishimura et al., 2004), angiogenin (ALS9; Greenway et al., 2006), optineurin (ALS12; Maruyama et al., 2010) and dynactin (Puls et al., 2003). Looking for a genotype-phenotype correlation for the different genes, a significant correlation have been demonstrated only for single mutations and considerable intrafamilial phenotypic differences were observed in some families carrying various mutations in the SOD1, TARDBP and FUS genes (Millecamps et al., 2010).
Aiming to unravel the pathogenic mechanisms leading to ALS, several animal models based on genetic mutations in fALS have been created.

2.1 Typical ALS models

2.1.1 SOD1 mutations

The first transgenic mouse model of human fALS was realized by Gurney and colleagues (1994), consisting in overexpressing a dominant "gain-of-function" mutation of the human SOD1. The mutation is characterized by the amino acid substitution at position 93 from glycine to alanine (G93A), which has little effect on the activity of hSOD1. G93A mice express the largest amount of mutant SOD in the brain, and develop neurological changes reminiscent of human MN disorders: they become paralyzed in hindlimbs and die by 5 to 6 months of age. The severity of the disease, in terms of age of onset and death, depends on the number of copies of the transgene. Paralysis is due to spinal MN loss. The pre-symptomatic phase in these mice is characterized by vacuolar degeneration of MNs, leading to the atrophic, neuron-depleted appearance of the anterior horns at later time points (Dal Canto & Gurney, 1994). The end-stage pattern of disease most closely mimics the pathological changes which characterize human ALS, with filamentous inclusions in both neurons and axonal processes (Hirano, 1991). By and large, this is the most used experimental model of ALS. On the other hand, the expression level of mutated SOD1 can vary independently from the number of copies of the transgene, thus modifying the onset and progression of the disease. In fact, the appearance of the first symptoms varies among the animals, in a time window of 1/2 weeks. Afterwards, the progression of the disease is quite fast, and the animals die approximately in one month. In our experience, in order to determine the onset of the symptomatic phase in G93A mice and initiate experimental treatment, we use a battery of motor behavioral tests (Rotarod, Paw Grip Endurance and Neurological tests).
Alternatively, Ripps and coll. (1995) introduced a missense mutation in the mouse SOD1 gene, that switched the glycine at position 86 to an arginine (G86R). The same mutation has been observed in the corresponding amino acid residue (position 85) of some fALS patients (Deng et al., 1993; Rosen, 1993). High expression of this gene in the spinal cord, brain stem and neocortex of transgenic mice is associated with age-related progressive pathological changes of MNs. Moreover, biochemical studies indicate normal total SOD activity in

transgenic mice tissues, suggesting that also G86R mutation has no effects on SOD activity. The early symptoms are variable, consisting in either a flaccid or a spastic paralysis of forelimbs/hindlimbs, whereas the time of the onset is very predictable and the progression of the disease extremely rapid: in fact, the disorder progresses from a mild gait abnormality to total paralysis within a 5-days period: animals are healthy at the age of 3 months and dye by 4 months.

The original G85R mutation observed in fALS was introduced in a mouse background by Bruijn and coll. (1997). Low levels of accumulated human G85R are sufficient to cause severe MN disease without altering the protein and activity levels of endogenous SOD1. After an 8 month-latency period, MN loss proceeds nearly synchronously with 40% of large spinal MN axons degenerating in 2 weeks. Moreover, prominent SOD1-containing inclusions in astrocytes appear prior to clinical signs and increase markedly during disease progression, indicating astrocytes as first players in mutant-SOD1 mediated damage. Furthermore, in this mouse model the presence of glial glutamate transporter, the major glutamate transporter in spinal cord, decreases in end-stage G85R mice. This finding is strikingly similar to that observed in patients with sALS (Rothstein et al., 1995), implicating glutamate mediated excitotoxicity as a mechanism to account for the nearly synchronous degeneration of MNs.

Wong and coll. (1995) produced multiple lines of transgenic mice expressing the human G37R SOD1 mutant. These mice, expressing 5 to 14 times normal SOD1 activity levels, develop the clinical phenotype of MN disease. At lower levels of mutant protein accumulation, the pathology is restricted to lower MNs, whereas higher levels cause more severe abnormalities and affect a variety of other neuronal populations (striatum, thalamus, hypothalamus, pyriform cortex), with conspicuous vacuolar degeneration of axons and dendrites even in pre-symptomatic mice. However, vacuolar degeneration is not a well recognized component of MN pathology in human G37R fALS patients. Furthermore, in these mice neurofilamentous accumulations in cell bodies and proximal axons are rarely present, although they are a constant feature of the pathology in SOD1-linked fALS and in sALS (Hirano et al., 1984; Kato et al., 1991).

Wang and coll. (2002) engineered the human SOD1 gene, to encode mutations at the first two of the four histidine residues (46, 48, 63 and 120) that coordinately bind Cu^{2+} in the active site of the enzyme (Parge et al., 1992), as found in a Japanese and an English families, respectively (Aoki et al., 1995; Enayat et al., 1995). The experimental juxtaposition of disease-linked mutations at histidine 46 and 48 (H46R/H48Q) in transgenic mice creates a mutant enzyme with little or no superoxide scavenging ability that induced MN disease (Wang et al., 2003). These mice develop MN disease before 1 year of age, proportionally to the expression level of the transgene. The most prominent pathological feature in the spinal cord from paralyzed mice consists in the accumulation of high molecular weight SOD1 aggregates, and non-native, detergent-insoluble species of mutant protein. Combining the two disease-causing mutations at histidines 46 and 48 with two experimental mutations at histidines 63 and 120 (H63G and H120G), a stable but inactive protein (H46G/H48Q/H63G/H120G or Quad) is obtained: its expression in mice results in a MN disease clinically and pathologically similar to the H46R/H48Q mouse model (Wang et al., 2003).

Among the reported mutations, the hSOD1-D90A is the milder. Homozygous mice develop a fatal MN disease, but the progression is slow. Mice display bladder disturbances similar to those found in human fALS homozygous for this mutation (Jonsson et al., 2006). Transgenic

mice accumulate detergent-resistant hSOD1 aggregates in the spinal cord, and hSOD1 inclusions and vacuoles especially in the ventral horns.

In fALS Oki Family a 2 base pair deletion was found at codon 126 of SOD1 gene, causing a frame shift and a premature stop in the protein sequence at codon 131 (Leu126delTT; Nakashima et al., 1995; Pramatarova et al., 1994). The truncated protein lacks important parts for dimer contact and activity site loop (Watanabe et al., 1997). Transgenic mice expressing this mutation display loss of MNs, microglia activation and Lewy body-like hyaline (LBHIs) cytoplasmic inclusions, with a rapid progression to complete paralysis after initial hindlimb paraparesis (Watanabe et al., 2005).

Similarly, mice bearing other two mutations, consisting i) in the insertion of non-native sequence (TGGG) prior to premature termination (SOD1-G127X) (Jonsson et al., 2004) and ii) in a simple C-terminal deletion (SOD1-L126Z) (Wang et al., 2005), develop MN disease, and accumulation of relatively high levels of detergent-insoluble forms of the mutated variants in the spinal cord.

Taken together, transgenic mice bearing mutations on the SOD1 gene represent a good model for the study of human ALS, since they show several features of the disease: behaviorally, for the motor symptoms which can be assessed with standardized tests; morphologically, for the involvement of upper and lower MNs and the cellular alterations leading to death, together with the involvement of astroglia and microglia which make ALS a not cell-autonomous disease. Nevertheless, most clinical trials based on results obtained on SOD1 mice failed to obtain significant effects on disease (Benatar, 2007): it may be argued that SOD1 mice represent a good model for the only fALS, and even that most effective treatments are initiated in mice before the onset of the symptoms, which is not feasible in sALS.

Some experimental manipulations can be difficult to perform in mice for their innate size limitations, such as administration of compounds into the cerebrospinal fluid (CSF) or obtaining sufficient tissue to perform extensive biochemical analyses. Therefore, Nagai and coll. developed a rat model of ALS by expressing a human SOD1 transgene with two ALS-associated mutations: H46R and G93A (2001). Like the murine counterpart, these models reproduce the most common phenotypic features of human ALS. Rats with the highest transgene copy numbers and higher expression of the mutant protein develop a paralytic disorder characterized by MN death together with astrogliosis and microglia activation. In particular, H46R rat pathology is characterized by protein deposition and aggregation, as H46R/H48Q and G85R transgenic mice. Furthermore, H46R rats display a later onset of paralysis and a slower disease progression (24 days) than G93A rats (8 days). The rat model is ideal for drug administration via chronic intrathecal pumps, a strategy used in human ALS clinical trials. Rats can tolerate better than mice immunosuppressive therapy to allow survival of implanted cells. Moreover ALS rats allow CSF access, because their CSF volume is 10-20 fold greater than in the mouse (http://www.axon-neuroscience.at/rat_models_1.php).

ALS models have been engineered also in zebrafish (Lemmens et al., 2007) and in *Caenorhabditis elegans* (Wang et al., 2009). In particular, overexpression of hSOD1-G93A, hSOD1-G37R and hSOD1-A4V in zebrafish induces a very robust axonopathy in the embryos. The major advantages of this model in the zebrafish embryo are i) that spinal motor axons are easily identifiable, ii) that treatment of animals with small compound libraries is more feasible and iii) that drug testing can be performed within 2 days. The *C.*

elegans approach, instead, aims to reveal the primary target of the toxic effect of hSOD1 mutations. In fact, overexpression of the hSOD1-G85R mutant protein leads to severe locomotor defects, associated with macroscopic aggregation in neuronal cell bodies. The mutant protein is unable to fold properly producing neuronal dysfunction at the synaptic level and possibly causing a deficient trafficking of pre-synaptic vesicles.

2.1.2 TDP-43 mutations

Recently, great interest has been raised by studies on TDP-43, a protein implicated in regulation of alternative splicing of mRNA, mRNA stability and transcriptional control. Several different neurodegenerative diseases are characterized by TDP-43 proteinopathies (Wegorzewska et al., 2009; Zhou et al., 2010). For instance, abnormal levels of aggregated TDP-43 are detected in the majority of patients with ALS, frontotemporal lobar dementia (FTLD) and Alzheimer's disease (AD), with ALS and FTLD often associated. Mutations in the TARDBP gene result in neuronal aggregation of abnormal TDP-43 protein in patients dying of MN disease: TDP-43-positive inclusions are present in both sALS and in the syndrome affecting fALS patients with TARDBP mutations, although they are more frequent in the last ones (Sreedharan et al., 2008). The clinical course of patients with TARDBP mutations is similar to that of sALS. Elevated levels of TDP-43 has been found in ALS patients in which the lower MNs were mostly affected (Geser et al., 2011; Noto et al., 2011). Pathological TDP-43 seems to be a less consistent feature of other MN disease phenotypes, such as primary lateral sclerosis and progressive muscular atrophy (Dickson et al., 2007).

Therefore, TDP-43 has been targeted to create animal models of ALS in mice, rats and *Drosophila.* In *Drosophila melanogaster,* the depletion of the TDP homolog results in deficient locomotor activity and defects at neuromuscular junctions (NMJs) (Feiguin et al., 2009). On the other hand, wild-type (WT) TDP-43 overexpression exerts more severe effects on neuromuscular junction architecture, viability and MN vitality (Estes et al., 2011). The neurotoxicity is modulated by the proteasome, the chaperone Hsp70 and the apoptosis pathway (Estes et al., 2011).

Transgenic mice expressing the human mutated TDP-43 gene (A315T and M337V) or the human WT gene (hTDP-43), develop a severe early MN degeneration phenotype, that correlates with TDP-43 levels in spinal cord (Stallings et al., 2010). In particular, TDP-43-A315T mice develop later onset paralysis with cytoplasmic ubiquitin inclusions, gliosis and TDP-43 redistribution and fragmentation (Stallings et al., 2010). Moreover, *in vivo* and also *in vitro* the A315T mutant enhances neurotoxicity and the formation of aberrant TDP-43 species, including protease-resistant fragments (Guo et al., 2011).

Moderate overexpression of hTDP-43 results in TDP-43 truncation, increased cytoplasmic and nuclear ubiquitin levels, intranuclear and cytoplasmic aggregates immunopositive for phosphorylated TDP-43. Furthermore, abnormal juxtanuclear aggregates of mitochondria are observed, accompanied by enhanced levels of Fis1 and phosphorylated DLP1, the key components of the mitochondrial fission machinery. Conversely, a marked reduction in mitofusin 1 expression, which plays an essential role in mitochondrial fusion is observed. Furthermore, hTDP-43 mice show reactive gliosis, axonal and myelin degeneration, gait abnormalities, and early lethality (Xu et al., 2010). Wils and coworkers (2010) confirmed a dose dependent degeneration of cortical and spinal MNs and development of spastic quadriplegia, reminiscent of ALS.

Constitutive and conditional transgenic rat models, overexpressing the hTDP-43-M337V protein develop a widespread neurodegeneration, mostly affecting the motor system. Features of this proteinopathy include the formation of TDP-43 inclusions, cytoplasmic localization of phosphorylated TDP-43, and its fragmentation. The number of MNs is reduced, striated muscles are denervated, astrogliosis and microglial activation are present (Zhou et al., 2010).
Finally, depletion of TDP-43 with antisense oligonucleotide strategy in mouse adult brain alters the expression levels of 601 mRNA (including Fus, Tis, progranulin and other transcripts encoding neurodegenerative disease-associated proteins) and 965 splicing events (e.g. in sortilin, the receptor for progranulin) (Polymenidou et al., 2011). RNAs with the most depleted levels derive from genes with very long introns that encode proteins involved in synaptic activity. The same RNA are altered in ALS patients (Xiao et al., 2011).
Thus far, TDP-43 transgenic mice together with SOD1-G93A mice, represent the ALS model which more closely adheres to human ALS.
Another RNA binding protein, FUS, has been found mutated in both 5% fALS (Lagier-Tourenne & Cleveland, 2009) and 1% sALS (Mackenzie et al., 2010) patients: FUS interacts with TDP-43 (Strong & Volkening, 2011), as shown also in a model of FUS-related neuronal degeneration in *Drosophila* (Lanson et al., 2011). Knocking down either Tardbp or FUS in zebrafish causes a motor phenotype which can be rescued by wild type FUS expression (Kabashi et al., 2011). To our knowledge, there are no transgenic mouse models for studying FUS role in ALS, but studies on TDP-43 mice have shown that this protein and FUS are strictly correlated.

2.2 Rare ALS models

Sequence variants in a number of genes are reported to be associated with ALS. These variations are found in candidate genes or through genome wide association studies. In all cases, it is unclear whether these genes and/or variations play a role in ALS, and these associations remain to be confirmed.

2.2.1 Alsin mutation

Great expectancies have been raised by the discovery of ALS2/alsin, a guanine nucleotide exchange factor (GEF) for the small GTPase Rab5 (Rab5GEF). ALS2 is a novel Rac1 effector, involved in Rac1-activated macropinocytosis and in macropinocytosis-associated endosome fusion and trafficking, and in neurite outgrowth. Therefore, loss of ALS2 may perturb macropinocytosis and/or the following membrane trafficking, which gives rise to neuronal dysfunction in the ALS2-linked MN diseases (Devon et al., 2006; Kunita et al., 2007). In fact, ALS2 loss of function mutations in humans account for several juvenile recessive MN diseases: the juvenile ALS, the primary lateral sclerosis and the infantile-onset ascending hereditary spastic paralysis (Eymard-Pierre et al., 2006; Orban et al., 2007). Furthermore, alsin plays a role in AMPA receptor trafficking, thus providing a novel pathogenic link between ALS2-deficiency and MN degeneration, suggesting a protective role in maintaining the survival of MNs (Lai et al., 2006).
Knocking down of alsin by small interfering RNA in cultured MNs display a reduced apparent size of early endosomes and an increased intracellular accumulation of transferrin and L1 cell adhesion molecule. Moreover, it induces MN death and significantly inhibits axon growth in the surviving ones (Jacquier et al., 2006).

On the other hand, loss of ALS2 in mice does not have a dramatic effect on the survival or function of lower MNs (Cai et al., 2008). On the contrary, ALS2 deficiency causes an upper MN disease that closely resembles a severe form of hereditary spastic paralysis, that is quite distinct from ALS. The hereditary spastic paralysis is characterized by progressive axonal degeneration and slows movement without muscle weakness (Yamanaka et al., 2006). ALS2 -/- mice are more susceptible to oxidative stress compared to WT, although they fail to recapitulate clinical or neuropathological phenotypes consistent with MN disease by 20 months of age (Cai et al., 2005). In addition, the effects of ALS2 ablation strongly depend on the genetic background and sex (Hadano et al., 2010b). Loss of ALS2 in SOD1 mice exacerbates the pathology (Hadano et al., 2010a).

2.2.2 VAPB mutation

A mutation in the vesicle-associated membrane protein-associated protein-B (VAPB) is the cause of a fALS, ALS8 (Nishimura et al., 2004). This mutation is characterized by a proline to serine substitution at position 56 (P56S). The physiological function of VAPB is in part unclear, but it seems involved in bouton formation at the NMJ and in vesicle transport from the endoplasmic reticulum (ER) to the Golgi. Indeed, the protein mainly localizes to the ER compartment and to the junction between intracellular vesicles and cytoskeletal structures (Skehel et al., 2000; Soussan et al., 1999). Transgenic mice expressing the VAPBP56S mutation were created by Tudor and coworkers (2010). These mice develop cytoplasmic TDP-43 accumulations within spinal MNs at 18 months of age, pointing out a possible link between TDP-43 mislocalization and abnormalities in VAPB. Besides, both brain and spinal cord neurons, including MN, show an altered cellular distribution of VAPBP56S with ER-derived aggregates of this protein. Despite this pathological hallmark, the motor function and the MN number in spinal cord are not significantly affected by the mutation, indicating that these animals do not develop overt MN disease, in contrast to mutant SOD1 where low levels of expression can induce aggressive MN disease. The difference between these two ALS models could be related to the late-onset and slowly progressive atypical disease of the vast majority of ALS8 cases.

2.2.3 Cytoskeletal protein mutations: Dynactin, neurofilaments, peripherin, TAU

An increasing number of neurodegenerative diseases are being linked to mutations in genes encoding proteins required for axonal transport and intracellular trafficking (e.g., dynactin). Furthermore, one hallmark of MN degeneration in both affected ALS patients and transgenic mice expressing the SOD1 mutation consists in the accumulation of intermediate filaments (IFs), in particular, the neurofilaments (NFs, the major type of IFs), in the perikarya and axons of MNs.

A missense mutations in a dynactin gene (Dctn1) encoding the p150Glued subunit of dynactin complex has been associated to both fALS and sALS. The dynactin complex consists of at least 10 distinct components, including p150Glued that is the largest subunit: it binds directly to microtubules (MTs) and is required for coordinated anterograde and retrograde organelle movement (Haghnia et al., 2007). The missense mutation occurring in the glycine-rich cytoskeleton-associated protein (CAP-Gly) domain of the p150Glued polypeptide, results in the substitution of serine for glycine at position 59 (G59S), leading to a decreased binding to MTs (Puls et al., 2003). Other missense mutations in Dctn1 have been identified and are linked to ALS, but the specific role of

these mutations in the pathogenesis of the disease is unclear (Munch et al., 2005; Munch et al., 2004).

The mouse model expressing human mutant p150Glued dynactin (Thy-1; G59 $p150^{Glued}$) exhibits clinical and pathological hallmarks of MN disease and is characterized phenotypically by slowly progressive muscle weakness. MN degeneration in these mice is associated with abnormalities in intracellular vesicular trafficking, axonal swelling and axon-terminal degeneration. Evidence suggests that autophagic cell death is implicated in the pathogenesis of these mice (Laird et al., 2008). A similar animal model to study the pathogenic mechanism of the G59S mutation *in vivo* is represented by p150Glued G59S knock-in mice. These mice develop a late-onset, slowly progressive MN disease characterized by abnormal accumulation of NFs and synaptic vesicle proteins at the NMJs, loss of MNs, and gait abnormalities (Lai et al., 2007).

LaMonte and coworkers (2002) engineered a targeted disruption of the dynein-dynactin complex in MNs of transgenic mice by overexpression of a subunit of dynactin (dynamitin, p50) that causes the disassembly of the whole complex. Mice overexpressing dynamitin demonstrate a late-onset, slowly progressive MN degenerative disease characterized by muscle weakness, spontaneous trembling, abnormal posture and gait, and deficits in strength and endurance. These mice show MN degeneration and muscle denervation/atrophy. NF accumulations and retrograde transport inhibition observed in these mice confirm the critical role of the axonal transport in the pathogenesis of MN degenerative disease. Whether NF (or more generally IF) accumulations contribute to pathogenesis of human ALS remains unknown; it could simply be the consequence of neuronal dysfunction. However, emerging evidence suggests that sometimes IF aggregates can have detrimental as well as protective effects in MN disease. Transgenic mouse models are used to address whether cytoskeletal changes may contribute to MN disease.

NFs in adult MNs are made by the copolymerization of three proteins, the light (NF-L, 61 kDa), medium (NF-M, 90 kDa) and heavy (NF-H, 115 KDa) NFs. Mice knockout for any of the three NF genes do not develop MN disease, although these deficiencies have several harmful effects: the formation of perikaryal NF accumulations resembling those found in human (Elder et al., 1998a; Elder et al., 1998b; Rao et al., 1998; Zhu et al., 1997). Transgenic mice accumulating NF-L (4-fold the normal level) in the sciatic nerve, also display huge accumulations of NFs in spinal MNs, swollen perikarya, and eccentrically localized nuclei. NF accumulation is associated with some neurodegenerative hallmarks, such as an increased frequency of axonal degeneration, proximal axon swelling, and severe skeletal muscle atrophy (Xu et al., 1993). Also transgenic mice expressing the human NF-H gene (up to 2-fold the levels of the endogenous protein) progressively develop neurological defects and abnormal neurofilamentous swellings by 3-4 months of age (Cote et al., 1993). Transgenic mice expressing a fusion protein in which the carboxyl terminus of the NF-H is replaced by beta-galactosidase accumulates such protein as large filamentous aggregates in perikarya. Axons are not invested with NFs but develop only small calibers (Eyer & Peterson, 1994). Increased expression of NF subunits in all these transgenic mouse models leads to MN dysfunction, in absence of the widespread MN death typical of human ALS.

However, a more severe phenotype is produced by the expression of an assembly-disrupting NF-L mutant having a leucine to proline substitution (NF-L L394P): this mutation causes massive degeneration of spinal MNs accompanied by abnormal accumulations of NFs and severe neurogenic atrophy of skeletal muscles (Lee et al., 1994).

Abnormal NF aggregates are observed in both fALS patients and in SOD1 mice, pointing out that NFs may contribute to SOD1-mediated disease. Mating experiments between SOD1 mice and transgenic mice with deregulated levels of NF proteins were carried out. The overexpression of human NF-H proteins confers an effective protection against the SOD1 mutation, a phenomenon probably due to the ability of NF proteins to chelate calcium. Therefore, these studies show that disorganized NFs can sometimes have noxious effects resulting in neuronopathy. However, there is emerging evidence that in the context of MN disease caused by mutant SOD1, NF proteins may play a protective role (Eyer et al., 1998; Williamson et al., 1998).

Among the IF proteins, also peripherin overexpression in mice determines ALS. Peripherin is a type III neuronal IF protein of 57 kD, found within NF in the majority of IF inclusions (89%) in MNs of ALS patients (Corbo & Hays, 1992; Migheli et al., 1993). In adult rats, peripherin is mostly expressed in autonomic nerves and in peripheral sensory neurons while is barely detectable in spinal MNs (Escurat et al., 1990; Parysek & Goldman, 1988). However, peripherin gene expression is increased up to 300% in spinal MNs after injury of the sciatic nerve (Troy et al., 1990), whereas expression of the other NF proteins is reduced (Muma et al., 1990). Inflammation occurred in injured neurons could explain peripherin upregulation. In fact, peripherin gene expression is upregulated by the inflammatory cytokines interleukin-6 (IL-6) and leukemia inhibitory factor (Djabali et al., 1993; Sterneck et al., 1996). This suggests that peripherin may be part of a general response of MNs to noxious stress and may explain peripherin inclusion bodies in MNs of ALS patients. Beaulieu and coll. (1999) generated two transgenic mice to investigate the potential detrimental effects of peripherin overexpression in MNs, in which the endogenous peripherin gene, inserted with its own promoter or under the control of the Thy1 gene promoters, was overexpressed. Both mouse strains display a late-onset and selective MN disease characterized by the formation of IF inclusions similar to those found in human ALS and in SOD1 mice. Moreover, a deficiency in NF-L protein exacerbates the formation of IF inclusion bodies and the onset of disease precipitates, probably through a deleterious action of the large NF subunits on peripherin organization (Beaulieu et al., 1999).

Cytoskeletal abnormalities reported in ALS involve also MT-associated proteins such as the tau protein, predominantly expressed in neurons. Since tau is required for neurite elaboration in different cell types, a central role in neuronal process outgrowth and integrity is proposed. To date, 30 different intronic and exonic pathogenic mutations have been identified in the human tau gene and have been correlated to several pathological conditions recognized as tauopathies. Among these, exonic tau gene mutations (i.e., P301L and R406W) impair the ability of tau to bind and stabilize MTs as well as to promote their assembly (Hasegawa et al., 1998; Hong et al., 1998). These mutations contribute to the formation of fibrillary tau aggregates (Crowther & Goedert, 2000; Delobel et al., 2002; Vogelsberg-Ragaglia et al., 2000). Transgenic mice expressing the human tau containing the P301L mutation exhibit motor behavioral deficits, with age- and gene-dose-dependent development of neurofibrillary tangles (NFT). This phenotype occurs as early as 6.5 months in hemizygous and 4.5 months in homozygous animals. The spinal cord of both these animals display axonal spheroids, anterior horn cell loss and axonal degeneration in anterior spinal roots. Counts of spinal MNs show a reduction of approximately 48% compared to control animals. Moreover, the expression of P301L mutation results in peripheral neuropathy and skeletal muscle with neurogenic atrophy (Gotz et al., 2001; Lewis et al.,

2000). The specific role of tau in the ALS pathogenesis remains unsettled, but the phenotypical and histological observations in P301L mutant mice suggests that it could be a key molecule in neurodegeneration occurring in ALS.

2.2.4 VEGF

Studies on the vascular endothelial growth factor (VEGF) suggest a novel function for VEGF in the pathogenesis of MN degeneration. VEGF may be involved in spinal cord neuropathy mainly by affecting vascular growth or function. However, VEGF might also affect neural cells directly, but the *in vivo* relevance of such a mechanism has been poorly investigated. To study the potential role of VEGF in MN degeneration, Oosthuyse and coll. (2001) generated a 'knock-in' mouse, in which the hypoxia-response element sequence in the Vegf promoter was deleted. Vegf $^{-/-}$ mice appear healthy until 5 months of age, when they develop symptoms of MN disease, i.e. severe adult-onset muscle weakness due to degeneration of lower MNs, reminiscent of the clinical symptoms and neuropathological signs of ALS. Similarly, VEGF replacement has beneficial effects in SOD1 G93A mice (Lunn et al., 2009). These findings indicate that reduced neural perfusion and maybe also insufficient VEGF-dependent neuroprotection cause MN degeneration (Oosthuyse et al., 2001).

3. Spontaneous mutation models

Since many years, several mouse models bearing spontaneous mutations leading to MN death have been identified: for instance, Wobbler (Duchen & Strich, 1968), Nmd (Cook et al., 1995), Pmn (Schmalbruch et al., 1991), Wasted (Newbery et al., 2005), Loa (Rogers et al., 2001) and Cra (Hrabe de Angelis et al., 2000) mice.

3.1 Wobbler mouse

The Wobbler mouse mutation spontaneously occurred in the breeding stock of Falconer (1956) and was later mapped to the proximal mouse chromosome (Kaupmann et al., 1992). It is the oldest known model of spontaneous MN disease, following a spontaneous recessive mutation in the wobbler (wr) gene, which encodes for the vacuolar-vesicular protein sorting 54 (Vps54) with a missense mutation L967Q. Vps54 mutation has been rarely described in ALS patients (Corrado et al., 2011). The protein is a subunit of the Golgi-associated retrograde protein (GARP) complex, used for fusion of endosome-derived transport vesicles to the trans-Golgi network. This model is considered a good model of MN degeneration, although the molecular mechanisms of the pathology in these mice are still unclear. Duchen and coll. (1968) showed that the progression of disease can be divided into three phases. i) The first, presymptomatic one, during the first 3 weeks after birth, ii) followed by the evolutionary one where the mice show alterations in motor behavior such as fine tremor of the head and slightly unsteady gait. iii) The third phase consists in the stabilization of the disease with the end of the neurodegenerative process. The progression of the disease is variable and leads to progressive denervation of skeletal muscles, determining muscle atrophy especially in the head, neck, shoulders and forelimbs. Limb muscles display different levels of atrophy: distally, atrophy is more severe than proximally, although in this model the hindlimbs are less severely affected. Moreover, there is a final progressive weight loss partially due to nutritional deprivation following muscle atrophy of the face and forelimbs (Boillee et al., 2003). Mice usually live up to 3-4 months, but sometimes the stabilization of the disease can delay their death for longer.

3.2 NMD mouse

Cox and coll. (1998) identified mice carrying 2 independent mutations in the neuromuscular degeneration (Nmd) gene on two alleles: nmd^{j} and nmd^{2j}, leading to a progressive degeneration of spinal MNs and muscular atrophy. The nmd gene produces a ubiquitously expressed DNA helicase/ATPase protein previously described as immunoglobulin S-mu binding protein-2 (Smbp2; Fukita et al., 1993), glial factor 1 (Gf1; Kerr & Khalili, 1991), rat insulin enhancer binding protein 1 (rip1; Shieh et al., 1995) and cardiac transcription factor 1 (Catf1; Sebastiani et al., 1995).

Mutant mice are characterized by a progressive paralysis that initially begins with the hindlimbs. The forelimbs are affected to a variable degree with paralysis, generally evident only toward the latest stages of the disease. Homozygous Nmd mutants can be easily distinguished from their littermate controls at 2 weeks of age by their dorsally contracted hindlimbs and impaired movement. Affected mice rarely survive beyond 4 weeks of age, but the exact cause of death is unclear.

3.3 PMN mouse

Bommel and colleagues (2002) identified animals suffering of a progressive motor neuronopathy (pmn), caused by an autosomal recessive mutation in the tubulin-specific chaperone e gene (tbce). The mutation results from a tryptophan to glycine substitution at position 524 (T524G; Martin et al., 2002). This protein is one of the main cofactors involved in MT stability or polymerization dynamics. These mice between P14 and P20 display a progressive caudal-cranial degeneration of motor axons leading to muscular atrophy, hindlimb paralysis and death in 4 to 6 weeks after birth (Schmalbruch et al., 1991).

Although the pathophysiology of MN death in pmn mice is mainly due to loss of neuromuscular integrity by distal axonopathy rather than alteration of neuron soma (which is instead a common feature in ALS), this model can be considered a good one for MN neurodegeneration.

3.4 WASTED mouse

Wasted (wst) mice bear a spontaneous autosomal mutation that results in the loss of gene encoding the translation factor eEF1A2. From the 3rd week of age they exhibit immunodeficiency and neurological disorders in terms of tremors and disturbance of gait. Subsequently these mice present MN degeneration and hindlimb paralysis.

Moreover, Newbery and coll. (2005) observed reactive gliosis at P19 in the cervical spinal cord with a rostrocaudal gradient, and retraction of MN fibers. Mice develop a rapid neurodegeneration and die around P28. Finally, several studies demonstrated that this model bears increased chromosomal breakage and DNA damage (Shultz et al., 1982).

3.5 LOA and CRA mice

"Legs at odd angles" (Loa) and "Cramping 1" (Cra) mice arose as the result of two independent N-ethyl-N-nitrosourea-(ENU) induced mutagenenesis experiments (Hrabe de Angelis et al., 2000; Rogers et al., 2001).

Loa mouse presents a point mutation that changes phenylalanine to tyrosine at position 580 (F580Y) in the gene encoding the heavy chain of dynein. On the other hand, Cra mice have a substitution from tyrosine to cytosine at position 1055 (Y1055C) in the same gene. Dynein is

a motor protein which determines retrograde transport along MTs and this model has been partially used to evaluate the role of dynein in the neuronal migration. However, the disturbance in axonal transport leads to selective MN death, making these mice useful models in ALS studies. Particularly, Loa mice exhibit pathological features similar to human ones, as Lewy body-like inclusions containing SOD1, CDK5 and ubiquitin. However, Ilieva et al., (2008) found no α-MN loss at any age, but a sensory axon deficit of the large proprioceptive (type Ia) axons and the decrease of γ-motor axons innervating muscle spindles. Similarly, Cra mice do not show MN degeneration but rather peripheral sensory neuropathy due to loss of axons in dorsal roots and decrement of large proprioceptive axons (Dupuis et al., 2009).
In conclusion, the pathological features observed in both models do not properly correlate with hALS characteristics. Dynein mutation models will need further investigation, they better represent a model for sensory neuropathy rather than for MN disease.

3.6 Hereditary canine spinal muscular atrophy

Hereditary canine spinal muscular atrophy (HCSMA) is a lower motor neuron disease found in Brittany Spaniels. It shares clinical and pathological features with human ALS (Green et al., 2002). These animals show signs of oxidative stress (Green et al., 2001), but do not have mutations in the SOD1 gene (Green et al., 2002). From the histopathological point of view these animals are characterized by aberrant accumulation of extensively phosphorylated heavy (high molecular weight) neurofilament (NFH) and neurodegeneration (Green et al., 2005).

4. *In vitro* models

Despite *in vivo* models are extremely helpful for studying ALS, when the aim consists in studying the molecular mechanisms of MN death and the role and the involvement of the different cells in the pathological mechanisms, simplified *in vitro* models such as MN and organotypic cultures can be useful, allowing to control the experimental conditions.

4.1 Motoneuron and glia culture

Since the major feature of ALS consists in the progressive loss of upper and lower MNs, cultures of spinal MNs are a valuable tool for studying ALS pathomechanisms. Many authors have developed protocols to isolate MNs from newborn or embryonic murine spinal cord (Berg & Fischbach, 1978; Gingras et al., 2007; Schnaar & Schaffner, 1981), allowing to identify MNs for their size and their Choline Acetyl Transferase (ChAT) activity. However, more recently, Wiese and coll. (2010) have isolated embryonic spinal MNs from rodents using p75 neurotrophin receptor (NTR)-antibody panning step, a technique with low toxicity and high efficiency; in fact, at the embryonic stage, MNs are the only neurons expressing the p75 low-affinity nerve growth factor receptor (Camu & Henderson, 1992). Unlike most studies performed on cultured embryonic spinal MNs, those of cultures of spinal MNs from newborn rodents is less common. In 2004, Anderson and coworkers described a protocol for isolating and culturing neonatal spinal MNs positive to p75NTR and ChAT, in presence of a growth factor cocktail containing glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) (Anderson et al., 2004).

In vitro preparations are age-dependent, since cells or tissues are generally obtained from animals at late embryonic or neonatal stages, but ALS related-alterations or neurodegenerative defects are not yet evident at this early stage. Indeed Avossa and coll. (2006) characterized the alterations in WT and G93A embryonic spinal cord cells analyzing MNs, glial cells, interneurons, distribution of hSOD1, mitochondria, MTs, NFs and synapses. They did not find any substantial difference in all these parameters, excepted for a significant increase in inhibitory synapses compared to excitatory ones in mutant cells. Therefore, it is necessary to use specific molecules or drugs to induce cell death mechanisms in these apparently normal cells.
Since in ALS an imbalance in the glutamatergic system has been described, leading to an excitotoxic environment around MNs (Boillee et al., 2006), an *in vitro* approach can be represented by NMDA exposure. Rothstein and coll. (1993) were the first to use this model, demonstrating that MN degeneration is prevented by non-NMDA glutamate receptor antagonists, consequently used as neuroprotective agents.
Similarly, some authors have proposed that spinal MNs are more vulnerable to AMPA receptor agonists than to NMDA, particularly in spinal cord cultures (Carriedo et al., 1996; Van Damme et al., 2002). In fact, microdialysis perfusion of AMPA agonists in spinal cord reveals a consistent loss of spinal MNs and the consequently paralysis of the hindlimbs, probably due to an increased cytoplasmic Ca^{2+} concentration (Corona & Tapia, 2007).
Finally, it is well-known that also the glia (astrocytes and microglial cells) actively contributes to the death of MNs (Philips & Robberecht, 2011). Glia can be easily obtained and cultured both from embryos/newborns and adults; the first method has been described in 1972 by Booher and Sensenbrenner (1972). Now, concerning astroglia culture, "primary culture", "subculture" and "shaken culture (once or twice)" can be performed (Du et al., 2010), each one with advantages and pitfalls relative to purity of astrocytes, cell viability, expression of glial fibrillary acidic protein (GFAP) and bystin, a protein potentially involved in embryo implantation, which is markedly up-regulated in reactive astrocytes (Fang et al., 2008). Similarly, protocols to isolate microglial cells are easy and reproducible (Ni & Aschner, 2010; Yip et al., 2009), and they have been recently improved by the column-free magnetic separation technology: the cells can be labeled and isolated on the strength of their expression of CD11b, a specific microglial marker, thus allowing to isolate an high number of cells and significantly reducing the animals needed (Gordon et al., 2011).
Cocultures of healthy hMNs with human astrocytes carrying either the WT or mutated SOD1 cDNA have demonstrated the role of astrocytes in ALS disease, since MN number decreased about 50% in the presence of mutant SOD1-expressing astrocytes (Marchetto et al., 2008). This neurotoxicity is probably mediated by the release of soluble factors from astrocytes, finally involving the Bax-dependent death machinery within MNs (Nagai et al., 2007).

4.2 Cell line culture

Due to the technical difficulties in establishing a MN culture for their poor proliferation ability when differentiated, an alternative is represented by the use of a neural hybrid cell line named NSC-34 (neuroblastoma spinal cord), derived from the fusion of neuroblastoma cells with MN enriched spinal cord. These cells perfectly show morphological and physiological properties of primary MNs: extending processes, contacts with cultured myotubes, synthesis and storage of acetylcholine, action potentials and NF proteins (Cashman et al., 1992).

NSC-34 can be transfected with vectors containing mutant forms of hSOD1, allowing to study specific neurodegenerative aspects such as mitochondrial degeneration, NF accumulation or Golgi apparatus disruption (Gomes et al., 2008; Menzies et al., 2002a; Menzies et al., 2002b). This model can also enlighten on the implicated cell death pathways in ALS, i.e. the involvement of MAP kinases (Guo & Bhat, 2007), the interaction between bcl2-A1 and pro-caspase-3 (Iaccarino et al., 2011) or the involvement of kynurenine pathway implicated in the regulatory mechanisms of the immune response (Chen et al., 2011).
In addition, different therapeutic agents have been tested in culture, such as the vascular endothelial growth factor (VEGF) (Kulshreshtha et al., 2011) or the semi-synthetic tetracycline called minocycline (Guo & Bhat, 2007), known for their neuroprotective effects in neurodegeneration models (Orsucci et al., 2009; Storkebaum et al., 2004).
Similarly, other cell lines have been transfected with WT or mutant (G93A) hSOD1 and used for the same purpose: N18TG2 neuroblastoma, the non-neuronal Madin–Darby Canine Kidney (MDCK) (Raimondi et al., 2006) or the mouse embryonic fibroblast NIH3T3 cell lines (Shinder et al., 2001).
The recent finding of human induced pluripotent stem cells (iPSCs) present a novel opportunity for *in vitro* disease modeling. iPSCs can be generated from readily accessible tissue from patients. iPSCs show similar capacity for directed MN differentiation as ESCs (Boulting et al., 2011). iPSCs can be generated from patients, thus carrying the actual mutations associated with the disease and allowing to correlate, any cellular phenotype with the patient's clinical characteristics such as onset, duration, and severity of disease at the time of tissue collection. In 2008, iPSCs were generated from a skin sample from an elderly patient with fALS displaying a SOD1 mutation (Dimos et al., 2008).

4.3 Organotypic cultures

Since ALS is a complex disease originating from an important crosstalk among MNs, glia and muscles (Strong, 2003), a step forward is represented by using organotypic cultures, where the *in vivo* architecture is maintained and MNs as well as neighboring cells (interneurons and glial cells) are preserved. However, this system presents an important limitation, due to the lack of correlation between morphological results and behavioral impairment (as well as for all the *in vitro* preparations) and the isolated slices undergo a series of little traumas (axotomy, deafferentation…) that can unintentionally originate a neuron selection.
Preparations of spinal cord organotypic culture from newborn animals is actually done according the Stoppini's procedure (1991): MNs survive more than 2 months, maintaining their structural and metabolic characteristics and allowing both morphological and electrophysiological experiments. Moreover, these experimental conditions stimulate glial proliferation, reproducing the glial activation observed *in vivo* in ALS patients/animal models (Hall et al., 1998; Vercelli et al., 2008).
As already mentioned, such model requires tissue obtained from embryos or pups, when ALS alterations are not full-blown (Avossa et al., 2006). Therefore, it is possible to use slice cultures obtained from normal animals and to add a glutamate transporter inhibitor (as threohydroxyaspartate (THA) or D,L-threo-B-benzyloxyaspartate), able to induce a chronic glutamate neurotoxicity. This treatment causes selective MNs death, simulating the human ALS neurodegeneration (Bilak et al., 2004; Rothstein et al., 1993). Some authors prepare organotypic spinal slice cultures directly from G93A SOD1 mice and expose them to THA in order to induce loss of MNs (Kosuge et al., 2009).

This *in vitro* model is helpful either for studying specific pathways/organelles or for testing treatments. Particularly, Tolosa and coll. (2008) studied the effects of VEGF on the survival and vulnerability to excitotoxicity of spinal cord MNs treated with THA, demonstrating that this growth factor plays an important role in neuroprotection by activating the phosphatidylinositol 3-kinase/Akt signal transduction pathway. Similarly Kosuge and coworkers (2009) tested the effect of GDNF, showing that threo-hydroxyaspartate -induced MN death was significantly inhibited in G93A mice slice. The pathway of caspase-12 was among the causes of MNs death. Also the anti-convulsant drugs topiramate (Maragakis et al., 2003), valproic acid (Sugai et al., 2004) and lithium (Caldero et al., 2010) were found to significantly prevent MN degeneration in this model.

5. Conclusion

ALS is a devastating neurodegenerative disease the etiology of which is still unclear. Even though the clinical hallmarks are similar, several different genetic and sporadic causes have been found or hypothesized. Experimental models of ALS have been created in transgenic animals, and spontaneously mutated animals affected by MN disease have been identified. Due to the multifactorial causes of the disease, all these models, even though they show motor impairment and neuropathological aspects typical of the human disease, cannot fully address the complexity of the human disease. Nevertheless, they represent useful tools to study the early and late development of the disease from a neuropathological point of view, and the molecular mechanisms involved. The major pitfall of the use of animals models thus far consists in the failure to translate the positive therapeutic outcome in human patients. This might be ascribed to mistakes in both preclinical and clinical research strategy: on one hand, animals, unlike patients, tend to be treated in the early phases of the disease and in any case represent a restricted, uniform population in terms of age, sex and genetic characteristics; on the other hand, patients represent a much more variable population. Therefore, an increasing collaboration between preclinical researchers and clinicians is needed to favor translation from benchside to clinics back and forth.

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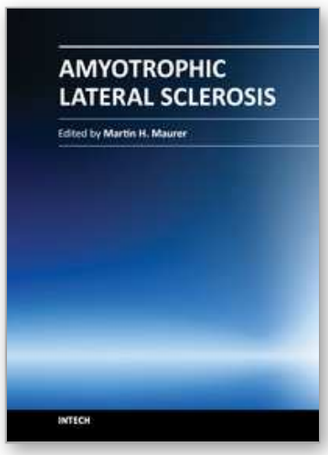

Amyotrophic Lateral Sclerosis

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Though considerable amount of research, both pre-clinical and clinical, has been conducted during recent years, Amyotrophic Lateral Sclerosis (ALS) remains one of the mysterious diseases of the 21st century. Great efforts have been made to develop pathophysiological models and to clarify the underlying pathology, and with novel instruments in genetics and transgenic techniques, the aim for finding a durable cure comes into scope. On the other hand, most pharmacological trials failed to show a benefit for ALS patients. In this book, the reader will find a compilation of state-of-the-art reviews about the etiology, epidemiology, and pathophysiology of ALS, the molecular basis of disease progression and clinical manifestations, the genetics familial ALS, as well as novel diagnostic criteria in the field of electrophysiology. An overview over all relevant pharmacological trials in ALS patients is also included, while the book concludes with a discussion on current advances and future trends in ALS research.

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