Working with ALS Mice

Guidelines for preclinical testing & colony management

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Introduction

Amyotrophic Lateral Sclerosis (ALS) is a devastating and rapidly fatal disease with currently only one available, FDA-approved, modestly effective treatment. There is therefore an urgent need for new therapies. With the development of the first genetically based mouse model of ALS in 1994, the field of preclinical testing was energized, but there have been a number of unforeseen complexities along the way.

This document is designed to 1) summarize the current best practices and recommendations available for designing and conducting preclinical studies using currently available SOD1-based mouse models of ALS, and 2) summarize the current best practices and most current information regarding breeding and maintaining SOD1 mutant mouse colonies.

For the purposes of the materials covered in this document, the recommendations to follow focus specifically on preclinical studies, meaning those experimental studies whose primary goal is to develop a therapy for human use. While we believe the following breeding and design recommendations may also benefit general proof-of-concept studies, designed to examine fundamental mechanisms and elucidate new biological targets of ALS, this is not the primary purpose of these materials.

Overview of Various SOD1 Animal Models

As first reported in (Rosen *et al.*, 1993) mutations in the Cu/Zn Superoxide Dismutase 1 gene (SOD1) account for ~20% of Familial ALS (FALS) cases, corresponding to 2-3% of all ALS cases. Transgenic mutant SOD1 mice are the only ALS mouse models currently available that exhibit all of the histopathological hallmarks observed clinically in sporadic and familial ALS.

SOD1 is a ubiquitous, mostly cytosolic, 153 amino acid protein that catalyzes the dismutation of superoxide anion radicals leading to the formation of hydrogen peroxide. The enzyme functions as a homodimer, in which each monomer binds one zinc and one copper atom. Copper binding is thought to be important for catalytic activity while zinc binding is believed to be critical for structural stability.

Over 146 mutations scattered throughout SOD1 have been identified in FALS patients, the majority of these being point mutations of highly conserved amino acids (Cleveland and Rothstein 2001). A continuously updated list of human mutations can be found on the ALSOD online database, alsod.iop.kcl.ac.uk. Intriguingly, all mutations, with the exception of D90A, seem to be inherited in an autosomal dominant manner.

Because there is no obvious mutational hotspot and no clear correlation between the level of enzymatic activity of the mutant SOD1 protein and the observed disease phenotype or clinical progression (refer to Table 1), SOD1 is thought to act primarily via a toxic gain of function in ALS (Pasinelli and Brown 2004, Bruijn *et al.*, 2004), although loss of function may also contribute to disease pathophysiology (Fischer *et al.*, 2007). It is generally thought that the different mutant SOD1 proteins are likely to cause ALS by a similar mechanism. Several hypotheses for SOD1 mutant mediated neuronal loss have been advanced including excitotoxicity, oxidative damage, impaired energy metabolism, inflammation, and insufficient growth factor signaling.

Several transgenic mouse models have been generated that model mutations found in FALS patients (see Table 1 below for comparisons of key characteristics), including the G93A (Gurney *et al.*, 1994), G37R (Wong *et al.*, 1995), G85R (Bruijn *et al.*, 1997), G127X (Jonsson *et al.*, 2004), D90A (Jonsson *et al.*, 2006b), and H46R mutations (Sasaki *et al.*, 2007). In all of these mouse models, massive death of motor neurons in the ventral horn of the spinal cord and loss of myelinated axons in ventral motor roots ultimately leads to paralysis and muscle atrophy. A limited number of other neuronal populations have also been shown to be affected in various SOD1 mutant mouse models, including upper corticospinal motor neurons in

G93A mice (unpublished data presented by P. H. Ozdinler in Istanbul, Turkey, July 2009), sensory neurons in dorsal root ganglia in G85R (Bruijn *et al.*, 1997), and neurons of brainstem cranial nuclei in G37R mice (Wong *et al.*, 1995).

All of these mouse models have been reported to exhibit the same histopathological hallmarks associated with ALS in humans: progressive accumulation of detergent–resistant aggregates containing SOD1 and ubiquitin and aberrant neurofilament accumulations in degenerating motor neurons. In addition to neuronal degeneration, reactive astroglia and microglia have also been detected in diseased tissue in the mice, similar to that observed in humans.

Despite these histopathological similarities, the timing of onset and rate of disease progression differ (often dramatically) among the various SOD1 transgenic mouse models. To date, researchers have not be able to account for these differences in onset or progression by looking at particular characteristics of the mutant protein, as disease onset and progression do not appear to correlate with the presence or absence of enzyme activity, or with the stability of the various mutant SOD1 proteins (refer to Table 1 below for summary comparison). However disease progression, but not disease onset, may correlate with aggregation propensity (Wang *et al.*, 2008).

Table 1: Characteristics of Commonly Used ALS SOD1 Mutant Mouse Models

Characteristic	G93A	G85R	G37R	D90A	G127X	H46R
	Line Gur1; (high Tg copy #); B6SJL Hybrid	**Line 148**	**Multiple lines were analyzed**	**Line 134**	**Line 716**	**Line 70**
Inheritance	Dominant	Dominant	Dominant	Recessive	Dominant	Dominant
Protein Aggregation Propensity	High[7]	High[7]	Moderate[7]	High[7]	High [4]	Low[10]
Enzyme Activity	Active[3]	Inactive[1,5]	Active[1]	Inactive	Inactive[5]	Inactive[8]
Protein Stability	Stable[5]	Reduced[5]	Reduced[1]	Stable[5,6]	Unstable[5]	Stable[8]
Disease Onset	Early (3- 4 mo)[3]	Late (7.5 mo)[2]	Moderate (4-6m o)[11]	Late (12mo)[6]	Late (8-9 mo)[4]	Moderate (5 mo)[9]
Disease Progression	Moderate (3 wks)[3]	Fast (2 wks)[2]	Slow (4-6 wks)[12]	Slow (4 wks)[6]	Fast (7-10 dys)[4]	Slow (4 weeks)[9]
Original Publication	Gurney *et al.* *Science* 1994	Bruijn *et al.* *Neuron* 1997	Wong *et al.* *Neuron* 1995	Jonsson *et al.* *J Neuropathol Exp Neurol* 2006b	Jonsson *et al.* *Brain* 2004	Sasaki *et al.* *J Neuropathol Exp Neurol* 2007

References

(1) Borchelt *et al.*, 1994 (2) Bruijn *et al.*, 1997 (3) Gurney *et al.*, 1994 (4) Jonsson *et al.*, 2004 (5) Jonsson *et al.*, 2006c (6) Jonsson *et al.*, 2006b (7) Prudencio *et al.*, 2009 (8) Ratovitski *et al.*, 1999 (9) Sasaki *et al.*, 2007 (10) Strom *et al.*, 2008 (11) Wong *et al.*, 1995 (12) JAX, personal communication, 2009

Part I. Considerations for Preclinical Studies Using SOD1 Mice

Several recent workshops, webinars, and publications have focused on issues relating to the most rigorous and interpretable design of SOD1 mutant mouse-based preclinical studies. In preparing the following materials, we have in particular drawn from the recommendations of the ALS Therapy Development Institute, the first report of the ENMC Group for the Establishment of Guidelines for the Conduct of Preclinical and Proof of Concept Studies in ALS/MND Models, and the recent Second Workshop on Guidelines for the Preclinical Evaluation of Potential Therapeutics in ALS/MND, held in Reisensburg, Germany. In addition, these materials have been reviewed and valuable feedback provided by an esteemed list of expert stakeholders, enumerated in Appendix D.

As you review the following materials, you will notice that in general the recommendations presented and the data summarized are based on the use of the G93A-SOD1 B6SJL hybrid background mutant mouse model. The reason for this is quite simply because this was the first genetically based ALS animal model developed, and so it has become the most widely used and well-characterized mouse model of ALS, not because of any inherent scientific superiority or patient applicability of this model vs. any of the other mutant SOD1 models. We believe that many of the recommendations to follow have general applicability for preclinical studies using other ALS mouse models, but certainly the specifics of timing of disease onset, lifespan/disease duration, cohort numbers required for suitable confidence/statistical power, and so forth will be model and strain/background dependent. Throughout the document we have tried to highlight where the recommendations have general applicability across the various models and where they are specific to the G93A hybrid model.

Consideration 1: Gender

Recommendation: It is necessary to use equal numbers of males and females in all cohorts that will be compared for preclinical studies.

The SOD1 mutant model currently with the most detailed gender-based data is the G93A SOD1 mutant on a mixed B6SJL hybrid background. This model shows clear gender differences in survival, with female animals living on average 4-7 days longer than males, depending on the specific colony (Heiman-Patterson *et al.*, 2005, Scott *et al.*, 2007, JAX internal communications, 2009) so it is critical to always use a gender balanced study design with these animals**.** In practice, this entails ensuring equal numbers of males and females in all cohorts that will be compared for preclinical studies. Congenic C57BL/6J animals carrying a G93A SOD1 mutation also show gender differences in survival (Heiman-Patterson *et al.*, 2005, Cat Lutz, personal observations, 2009).

Consideration 2: Litter

Recommendation: When using animal models on non-congenic backgrounds, it is necessary to balance littermates across experimental cohorts

As has been demonstrated conclusively for the widely used G93A mutant SOD1 model on a mixed B6SJL hybrid background (Scott *et al.*, 2008), it is critical when using hybrid animal models to use matched littermates across experimental cohorts. In their 2008 publication, ALSTDI showed that across their cohort of over 5000 animals, siblings from the same litter were more likely to have similar ages of

onset and death than non-siblings (Scott *et al.*, 2008). Litter is therefore an important factor related to observed disease onset and survival in ALS mouse studies using non-congenic/hybrid animals. Litter is far less of a potentially confounding factor when using ALS models on "pure" congenic backgrounds, such as animals backcrossed for 10 generations onto a C57BL/6J background, as currently exist for G93A, G37R, G85R, D90A, and G127X (Zetterstrom *et al.*, 2007). Please see Part II of this manual, which discusses colony management recommendations, for more information on the potential impact of strain background and genetic drift.

Consideration 3: Transgene Copy Number

Recommendation: It is imperative to quantitatively assess transgene copy number for all animals used in preclinical studies

Over time it has become apparent that the mutant G93A transgene undergoes a background level of copy loss, due to meiotic rearrangement of the transgene array. Decrease in transgene copy number has been clearly correlated with extension of lifespan not only in the G93A animals (Alexander *et al.*, 2004) but in other highly overexpressing transgenic mouse models as well, such as several of the Huntington's Disease transgenic mouse models. Given this background level of copy number loss, it is critical that users of G93A and other highly over-expressing ALS mouse models obtain quantitative data regarding transgene copy number either via quantitative PCR or quantitative Southern blotting.

The animals provided through the Prize4Life mouse colony have all been checked for copy number loss via qPCR but this may not be the case for animals obtained through other sources. Therefore, researchers interested in conducting preclinical studies are cautioned as to the need to obtain this information for each and every transgenic animal used (in both control and treatment cohorts), as historically, numerous studies have been confounded by undetected copy number drop, wasting precious time, money, and leading to erroneous findings.

Consideration 4: Exclusion Criteria

Recommendation: Any animal which fails to undergo the predicted disease progression should be systematically excluded from treatment analysis and the reason for exclusion should be recorded and reported

In the course of conducting ALS preclinical studies, it is occasionally the case that an animal will die of a cause unrelated to the progress of the disease. Death by infection, death resulting from damage incurred in the process of delivering the therapeutic intervention of interest, or other non-disease-related deaths should be tracked and these animals excluded accordingly. If an animal dies before showing typical and predicted disease progression, for example as assessed by increasing neurological score, this animal should be excluded from the treatment analysis and the reason for exclusion should be reported. It is also important to exclude the animal's gender matched littermate from the comparison cohort so as to maintain litter-matched balance in preclinical studies using animals on mixed backgrounds.

It is critical to be systematic about tracking and accounting for these early deaths, as they can confound interpretation of the true effect of a therapeutic intervention. An analysis by ALSTDI (Scott *et al.*, 2008) indicated that failing to account for non-ALS related deaths within preclinical studies is likely historically to be the largest potential source of noise and spurious results. This recommendation applies to all preclinical studies, regardless of which line or mutation model is being used.

Consideration 5: Onset/Timing of Treatment

Recommendation: The combination of peak body weight followed by decreasing neurological score is a reasonable measure to determine disease onset in ALS SOD1 mouse models

This is a challenging issue and one on which there is little consensus in the ALS preclinical research field. There are strong arguments to be made for requiring therapeutic delivery at disease onset. As ALS is only diagnosed in patients post-onset, there is a very real concern that potential therapies that have been shown to be efficacious in animals prior to onset will fail to show any benefit in humans. However others have argued that given the highly aggressive nature of the existing ALS mouse models, particularly the G93A and other high copy number expressing mutation models, waiting until disease onset to test potential therapies may lead researchers to miss/throw-out potentially promising therapies. In practice, many labs have opted for a pre-onset delivery when conducting their preclinical studies.

According to the ENMC Review (Ludolph *et al.*, 2007) the vast majority of previously conducted preclinical studies using the G93A animal have tested compounds between day 40 and day 70. The recommendation of the ENMC is to conduct proof of concept studies (exploring basic questions of therapeutic efficacy) between day 50 and day 70 (in the G93A B6SJL animal model) and, if a drug has a robust effect during this pre-symptomatic window, to then re-test the compound in later symptomatic phases (at or post onset).

As to when exactly disease onset occurs in ALS mouse models, the greatest consensus seems to currently exist that peak body weight is a reasonable and consistent determinant of onset (Ludolph *et al.*, 2007) particularly in combination with a measure of neurological score. Use of neurological scores alone are also common and well accepted, although subject to greater potential for user error and user bias if studies are not conducted in a suitably blinded fashion. Some example of commonly used neurological scoring systems include: measurements of splay (or other measures of paralysis) and beam walk. Please request accompanying video material if you are interested in viewing a demonstration of a commonly used neurological scoring system.

As highlighted by ALSTDI (Scott *et al.*, 2008) a combination of use of weight and neurological score may be the most reliable and gentle (*i.e.* not introducing additional stressors) way to both measure onset as well as to identify non-ALS mediated deaths in animals that don't demonstrate the typical progression of weight loss in combination with increasing neurological score. For the G93A B6SJL mixed background animals onset, as defined based on first signs of a decrease in body weight, is typically around day 100. Please refer to the ALS mouse model comparison chart found in Table 1 for estimates of disease onset for the other SOD1 mutation-based models.

Consideration 6: Endpoint

Recommendation: Although there is a lack of consensus in the field, the most commonly used disease endpoint in preclinical studies involving ALS SOD1 mouse models, is the inability of an animal to right itself within 15-30 seconds if laid on either side

Just as there is debate in the field regarding the best way to measure disease onset in ALS mouse models, there is likewise debate as to whether it is preferable to use a functional or a survival-based outcome measure (endpoint) or a combined measure reflecting both. Survival (or rather "death") in preclinical studies is typically measured as the inability of an animal to right itself within 15-30 seconds if laid on either side (Ludolph *et al.*, 2007and Scott *et al.*, 2008). Common functional measures used include rotarod, grip strength, running wheel activity, and gait analysis-but there is far less agreement as to which,

if any, of these is preferable to any other, nor is there any standardization between/among them (*i.e.* way to convert or directly compare time to a given rotarod score to time to a given grip strength score). For the purposes of these recommendations, we support the use of survival endpoints, given the humane, relatively straightforward, and widespread use of the 15-30 sec/side righting test, although we believe that functional measures may provide a more sensitive gauge of therapeutic efficacy.

Consideration 7: Proving Access of Treatment to Target Tissue

Recommendation: Before undertaking thorough preclinical studies in large cohorts of animals, ensure that the therapeutic intervention of interest has the intended effect on the target tissue of interest using a reasonable biological correlate

The ability of a therapeutic intervention to affect the target should be determined before preclinical efficacy studies are begun. In the case of ALS, this often (although not always) means showing that a treatment is able to cross the blood brain barrier (in the case of treatments applied systemically) and have the intended effect in the brain and/or spinal cord. Because these target tissue confirmation studies are really proof-of-concept studies (prior to initiation of a preclinical study) it is possible to use smaller numbers of animals for this type of study (although keeping the considerations enumerated above in mind) to establish this issue of proper drug distribution.

In addition to this basic penetration/access question, it is also essential before embarking on a preclinical study to have evidence of some direct measure of efficacy/biological correlate *i.e.* in the case of a treatment which is believed to act via increased proliferation of mitochondria, provide direct measure indicating such (mitochondrial counts in drug vs. controls) or if a proposed survival explanation involves an increase in autophagy, provide direct measure indicating such. Providing data on biological correlates adds considerable weight to the interpretation of preclinical studies and also can be an early warning sign that a preclinical study is not warranted if the treatment of interest is unable to reproduce *in vivo* the biological effect predicted or shown *in vitro*.

Finally, histopathological measurements are highly recommended as an independent measure of treatment efficacy. Stereologically appropriate motor neuron cell counts, somal measurements, and other immunohistochemical observations of glial activation or immune system responses can provide valuable insights into treatment efficacy and mechanism of action at the micro-level. These types of analyses are complimentary to the survival and behavioral measurements that are usually the major focus of preclinical studies. It is reasonable (and cost effective) to collect tissues of interest during the course of a preclinical study for future histopathological examination should a therapeutic effect be detected.

Consideration 8: Dose response

Recommendation: Although demonstrating a full dose response curve is not a necessary component of ALS preclinical studies, demonstration that the effects of a treatment of interest show some correlation with dose is an important and powerful confirmation of efficacy

For purposes of translation from preclinical testing results into human trials, it is necessary to demonstrate a dose-response curve. While it is clear that mice are not humans and there are many factors which must be considered when trying to convert effective doses from mouse to man, the simple demonstration that within a particular dose range, increasing or decreasing dose changes the therapeutic effect of a treatment is a critical proof-of-concept for any proposed therapy. Single dose studies are difficult to interpret, particularly in the absence of pharmacokinetic data, and are insufficient for moving a compound forward into human-based studies. For this type of study (as with the target access confirmation studies discussed above), once a therapeutic effect has been detected, it is possible to use smaller animal cohorts (fewer animals/dose) to determine dose response curves.

Consideration 9: Pharmacokinetics

Recommendation: Once therapeutic efficacy has been shown, pharmacokinetic analysis of the therapy of interest is an essential step in the effort to translate preclinical findings into human treatments

Pharmacokinetics is the study of what happens to a drug once it enters the body. Also referred to as ADME analysis (Absorption, Distribution, Metabolism, and Excretion), understanding the pharmacokinetic properties of a compound is critical to establishing a treatment as a viable therapy (as, in combination with obtaining a thorough understanding of a given intervention's toxicity profile, ADME analysis is an essential regulatory step before moving into human trials).

Absorption: the process of a substance entering into the body

Distribution: the dispersion of a substance throughout the various compartments/tissues of the body

Metabolism: the breakdown of a substance into its metabolites and/or component parts

Excretion: the removal of a substance (and its metabolites) and redistribution to outside of a body

These types of studies (also see section above on providing that compound of interest is brain/spinal cord penetrant) to determine the mechanism(s) of absorption and distribution of a given intervention, the rate at which a treatment's action begins and the duration of the treatment effect, the changes that happen to a given substance in the body (*e.g.* the effect of enzymes or pH), and the effects and routes of excretion of any metabolites of the compound, are critical for interpreting the outcome of a preclinical study.

Consideration 10: Statistics

Recommendation: Use conservative statistical analyses, given the potential for uncontrolled variables to influence study outcomes

Given the various challenges and limitations in translating preclinical studies into clinical effects (particularly in the field of neurodegeneration), we recommend that researchers use fairly conservative statistical analyses when analyzing their preclinical data. The ENMC group has recommended use of a Cox proportional hazards analysis (Ludolph *et al.*, 2007) for experiments where the outcome is influenced by multiple variables (*e.g.* experiments on mixed backgrounds where both gender and litter may influence outcomes). Other statistical analyses may also be useful, but researchers should be careful to consider the number of major variables that should be taken into consideration when selecting which statistical calculations to perform. It is essential to use blinded observers when taking animal measurements throughout a preclinical experiment.

Consideration 11: Environmental Factors

Recommendation: SOD1 mouse models of disease, especially on congenic backgrounds, appear to be quite sensitive to environmental factors, therefore it is important to standardize environmental conditions as much as possible as these factors may influence measured lifespan

Environmental factors are commonly acknowledged to influence the health and survival of laboratory mice in general and there is evidence to suggest that SOD1 mutant mice may be more susceptible to these

potential stressors than wildtype animals. Discussions of exercise and housing-related factors in particular are included below and should be considered when designing SOD1 mouse preclinical studies.

Exercise is the primary environmental factor that has been studied to date with respect to ALS SOD1 mouse models. There is a fair bit of data to suggest that there is an interaction between exercise and disease progression and potentially between exercise and survival in both ALS mice as well as ALS patients. The effects of exercise in ALS are not straightforward and appear to depend on the type and intensity of physical activity. Intense, high endurance exercise regimes have been shown to exacerbate motor defects and shorten lifespan (Mahoney *et al.*, 2004). According to ALS-TDI, multiple sequential rotarod trials given twice weekly, along with grip strength and stride length tests, appear to accelerate disease progression in SOD1 mice (Gill *et al.*, 2009). However, others have shown that moderate levels of low endurance exercise appear to be beneficial to SOD1 mice as well as ALS patients (McCrate and Kaspar, 2008). While the precise role that exercise plays in disease progression and survival remain poorly defined, the impact of exercise, and use of outcome measures requiring exercise (such as rotarod or exercise wheels) should be carefully considered when designing drug testing studies.

In addition to exercise, both stressful and enriched housing conditions can cause physiological and behavioral consequences in laboratory mice (Olsson and Dahlborn 2002) and may impact the measured survival of SOD1 mutant transgenic mice. Potential housing-related stressors (or conversely enrichments) include excessive handling, crowded cages, presence/absence of nesting material, toys, and cage inserts (such as houses, tubes, and platforms), noisy environments, and erratic changes in light/dark cycle. Due to the mundane nature of these concerns, they are often ignored as factors influencing outcomes of animal testing. However differential treatment of mice could unintentionally introduce variability in the experimental design and confound interpretation of results. Therefore it is critically important to standardize mouse handling and housing conditions as much as possible for experiments using SOD1 mutant mice (particularly those on pure/congenic backgrounds).

Consideration 12: Multi-drug Treatment

Multi-drug treatments (polytherapy) are complex from both a scientific and a regulatory perspective. A discussion of these complexities is beyond the scope of these materials but at a bare minimum, it is necessary to either show that each component of a multi-drug treatment has efficacy on its own and/or to show an additive or synergistic effect of components on the relevant biological correlate(s).

Part II. Colony Management Considerations

Many labs and companies have already chosen to breed and maintain their own colonies of ALS mouse models rather than obtain these commercially; therefore in addition to considering specific questions of preclinical study design using ALS mouse models, it is also important to consider the current best practices and information regarding breeding and maintaining SOD1 mutant mouse colonies. The following sections are designed to provide background and highlight issues to consider when either developing one's own colony or when obtaining animals from non-commercial sources.

Consideration 1: Strain Background

Inbred/Straight Lines

As a bit of background, there are currently well over 400 "straight" (not genetically modified) inbred strains of mice (excluding the various congenic and recombinant lines made using these strains), with extensive documented genealogies. Inbred strains of mice are nearly identical to each other in genotype as a result of at least twenty generations of brother x sister mating. Examples of a few of the work-horses of these inbred strains include C57BL/6J, C3H/HeJ, and FVB/NJ.

Each of these inbred strains is genetically unique with distinct phenotypic characteristics. Sometimes these characteristics are useful in research, while at other times they may preclude the use of a particular strain for a given research project. For example, strain AKR has a high background incidence of leukemia while C57BL/6 mice have relatively low levels of cancer, and tend to be relatively resistant to carcinogens.

Congenic Lines

While there is a multiplicity of existing mouse lines, we will confine the majority of our discussions to congenic lines and F1 hybrids using the C57BL/6J and SJL strains, as these are the most relevant strains/backgrounds for the currently existing mouse models of ALS.

In the creation of a wide variety of disease models, targeted mutations and transgenes are frequently transferred into inbred backgrounds to minimize genetic variability within experiments and to ensure reproducibility of conclusions. The resulting mutant/transgenic line is referred to as a congenic line and is produced by repeated backcrosses to an inbred strain, with selection for a particular marker from the donor strain. Figure 1 demonstrates the estimated statistical dilution of the donor strain genome with the new host genome with each generation. After 10 generations, the genetic background is statistically overwhelmingly that of the host (inbred) strain (and can be considered a fully congenic line.

Hybrid Lines

In the specific case of the SOD1-G93A (or G93A-SOD1) transgene, the original transgenic animals were on a non-uniform background consisting of a mixture of SJL and C57BL/6J genetic backgrounds. These mixed B6SJL animals, sometimes mistakenly referred to as "Gurney" mice after the original paper announcing their creation, are still widely used in ALS research. These mice are maintained by breeding transgenic males back to a wildtype B6SJL F1 female (see the next section for more extensive discussion of F1 hybrids). This approach is often embraced as a method of maintaining genetic diversity, since constant inbreeding (*i.e.* brother sister mating) over time can result in the fixation of undesirable alleles.

While these hybrid animals are widely used in the ALS research field, many ALS experiments are also currently conducted on a congenic line where the transgene has been transferred (backcrossed for 10+ generations) to a C57BL/6J background. These animals are referred to as B6.Cg-Tg(SOD1*G93A)1Gur/J mice or often by the shorthand "B6 G93A mice". The nomenclature of this congenic strain (indicated by the "Cg"), denotes the transfer of the SOD1-G93A transgene from a complex background to the host C57BL/6J strain (abbreviated B6) with the punctuation mark of a period indicating that the mutation has been backcrossed onto the B6 genetic background for greater than 10 generations.

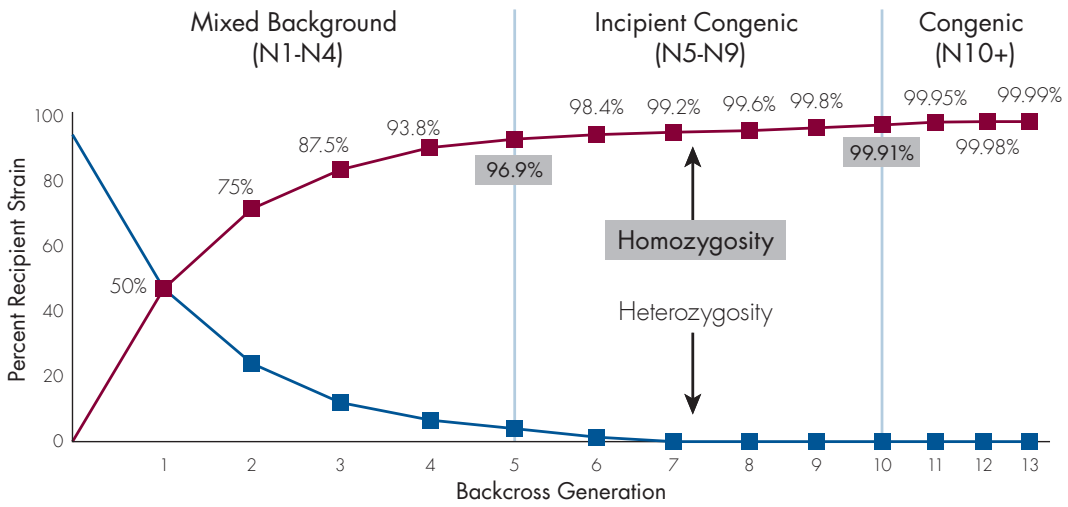


Figure 1. In the making of congenic strains, donor strain A (indicated in blue) is mated with recipient strain B (indicated in red). The progeny of this cross has a 50% genetic contribution from the host and 50% percent genetic contribution from the donor strain. With every passing generation of backcrossing to the host strain, approximately 50% of the donor genome (green) is replaced with the new recipient genome (red). After 10 generations, the residual amount of unlinked donor genome in the strain is likely to be less than 0.01 percent.

Mixed Genetic (Hybrid) Background: Advantages and Disadvantages

As mentioned above, breeding transgenic males back to a B6SJL F1 hybrid female ensures randomization of alleles inherited from either strain within a population of alleles. In general, breeding back to an F1 offers other advantages in that progeny tend to be more robust, tend to live longer, exhibit fewer idiosyncrasies of the parental strains, and are less sensitive to adverse environmental conditions than inbred strains. This is the concept of hybrid vigor. When mating mice back to an F1 hybrid, the resulting offspring will be genetically different from one another, but proportionately homozygous or heterozygous at either parental locus. This breeding scheme ensures randomization of alleles inherited from either strain within a population of alleles. The major experimental advantage of having the G93A transgene on the mixed B6SJL background is that the phenotypic onset of the disease is significantly earlier than on the inbred background.

A disadvantage of mating animals back to an F1 hybrid (thereby maintaining the transgene on a mixed background) is that each animal resulting from this mating has a unique genetic background, so there is no information on the genotypes of individuals unless each is specifically genotyped. The evidence of segregating alleles between C57BL/6J and SJL, for example, is immediately evident in the wide variety of coat colors manifest in the resultant offspring, which may be White Bellied Agouti, Black, Albino, or Tan w/pink eyes. Phenotypic variation is usually greater than is found with congenic strains, as individuals differ due to both genetic and non-genetic factors. This means that more animals are usually needed in a given experiment to achieve a given level of statistical precision. It is because of this increased variability that litter-mate matching in preclinical studies is so critical.

Another potential disadvantage with using mixed background/hybrid animals is the increased risk of incorrect mating in this breeding scheme. Animal husbandry personnel may not fully understand the necessity to mate transgenic animals back to the pure strain hybrid F1 generation, and may accidentally intercross mice, drastically changing the genetic background of the resulting colony. This genetic drift and the creation of mouse sub-colonies with differing characteristics can cause significant problems with interpretability of preclinical studies and colony-to-colony reproducibility.

Inbred Lines: Advantages and Disadvantages

There are several major advantages of using inbred strains. First, congenic strains are co-isogenic, meaning that all animals within a strain are virtually genetically identical. This lack of genetic variation promotes phenotypic uniformity within the strain such that the only variation between individuals is likely to be due to non-genetic causes. One consequence of this increased uniformity is that fewer inbred animals will be needed to achieve a given level of statistical precision than if hybrid/mixed background animals had been used (need for littermate matching is reduced/eliminated).

Second, as a broad generalization, inbred strains tend to be more sensitive to environmental influences than F1 hybrids. This increased environmental sensitivity may present an advantage in that congenic animals tend to be more sensitive to experimental treatments than other types of animals. Third, because congenic strains normally stay genetically constant for long periods, accidental genetic contamination is more easily identified than when using a mixed background. A wide range of existing DNA genetic markers makes genetic quality control relatively simple when using congenic animals (refer to quality control section).

Regarding disadvantages to using congenic animals, as mentioned above, congenic animals show increased sensitivity to environmental factors versus hybrid animals. While this may be advantageous in some instances, in some experimental contexts it may actually present a serious disadvantage because the impact of any uncontrolled/unexamined environmental variables will be magnified and animals will tend to show greater variability in their measured responses due to causes outside of the focus of the experiment. Because of this enhanced sensitivity, when using congenic animals extra care is required to ensure that such animals have highly controlled and similar environments so as not to confound experimental results and interpretations.

Another disadvantage of transferring mutations onto inbred backgrounds is that litter sizes are usually significantly smaller than maintaining a mutation on an F1 background. This often translates into significant increases in animal husbandry costs depending on the litter size or breeding performance of the inbred line. In spite of this potential disadvantage, with respect to ALS research the pure C57BL/6J strain (the strain used in the creation of the majority of congenic ALS mutant transgenic lines) is considered a good breeding strain with a relatively good litter size ranging from 4-8 pups per litter.

Consideration 2: Genetic Backgrounds and Breeding Strategies of Existing G93A-SOD1 Mutant Lines

The Hybrid B6SJL Line

The SOD1-G93A (or G93A-SOD1) transgene was designed with a mutant human *SOD1* gene (harboring a single amino acid substitution of glycine to alanine at codon 93) driven by its endogenous human *SOD1* promoter. This transgene was injected into fertilized B6/SJL F1 mouse eggs and founder animals were obtained. Transgenic mice on a mixed B6/SJL genetic background were sent to The Jackson Laboratory and are currently distributed as (Stock No. 002726, www.jax.org/jaxmice/strain/002726).

The hybrid strain is maintained by breeding hemizygous carrier males to B6SJL F1 hybrid females at each generation. Transgenic mice on this background have a decreased life span compared to congenic animals (on a pure C57BL/6J background), with 50% survival observed at 128.9+/-9.1 days in the mixed background versus 50% survival at 157.1+/-9.3 days for the pure congenic background. Although female transgenic mice with the mixed B6SJL background occasionally produce litters, as with the congenic C57BL/6J line, there is a very high incidence of non-productive matings. In addition to the transgenic line itself, researchers who choose to maintain their own colonies by breeding this line in their own vivarium must also house independent colonies of C57BL/6J and SJL/J colonies to produce the (C57BL/6J X SJL/J) F1 females necessary for mating. Alternatively, the F1 females can be obtained from The Jackson Laboratory (Stock No. 100012, www.jax.org/jaxmice/strain/100012).

The Congenic C57BL/6J Line

Upon receipt of the original hybrid B6SJL mutant SOD1-G93A animals, some of these mice were backcrossed to C57BL/6J for at least 10 generations to generate a congenic strain (Stock No. 004435, www.jax.org/jaxmice/strain/004435). The backcross was completed in July 2002 by Dr. Greg Cox.

These animals can be maintained by simply breeding hemizygous transgenic males to C57BL/6J females (the transgenic females are not very efficient breeders).

The G93A-SOD1 Mutant Transgene on Other Strain Backgrounds

Since genetic background is known to affect disease onset and progression, a number of different genetic backgrounds have been used in exploring modifier alleles in ALS. When choosing a genetic background one should consider 1) if disease onset and progression is conducive to the experiment, and 2) the strain characteristics of the genetic background being considered.

The transfer of the SOD1-G93A transgene onto different genetic backgrounds has identified two groups of inbred strains; early onset and late onset strains. Late onset strains include the C57BL/6J congenic mice 161+/-10 days, BALB/cByJ congenic mice 148+/-11 days, and the DBA/2J congenic mice 169+/-10 days. The early onset strains include the ALR/LtJ congenic mice 116+/-11 days, NOD-Rag1 null congenic mice 111+/-12 days and (from Terry Heiman-Patterson at Drexel) SJL congenic mice 119+/-10 days. (Dr. Greg Cox, JAX communication, 2009).

The characteristics of individual inbred strains are important factors to consider when selecting a particular line to work with and/or interpreting experimental results. For example, SJL mice are noted for extreme aggression in males, and have also been shown to have an increased rate of muscle regeneration after injury when compared to BALB/c mice. ALR/Lt mice have a genetic basis for resistance to free radical mediated stressors, as well as to immune system mediated stress. ALR is also homozygous for *Cdh23*^{ahl}, the age related hearing loss 1 mutation, which on this background results in progressive hearing loss with onset prior to three months of age. There are various other strain differences too numerous to mention here, the general characteristics of mouse strains can be viewed in Dr. Michael Festing's *Inbred Strains of Mice* accessible on line at www.informatics.jax.org/external/festing/search_form.cgi

Consideration 3. Quality Assurance Measures for SOD1-G93A Colonies

Copy Number and Pedigree Analysis

The number of tandem SOD1-G93A transgenes that integrated into the genome in the original Gurney line is estimated to be upwards of 28 copies (Gurney *et al.*, 1994) and is susceptible to spontaneous drops in copy number at an incidence ranging from 2-6% (Alexander *et al.*, 2004; Scott *et al.*, 2008). The most sensitive way to monitor copy number is through QPCR (see attached protocol Appendix A). It is imperative that every animal be monitored for copy number loss against standardized controls, especially the breeder males. The longer lived male breeder (with copy number drop) will produce many more progeny than the shorter lived high copy number SOD1-G93A male and the reduced copy number genotype will quickly contaminate/monopolize a small breeding colony. Any animal exhibiting a reproducible drop in copy number should be discarded and siblings/progeny monitored closely. Related to this, any animal displaying a significant delay in disease onset or moribund status should be eliminated from the breeding colony, along with any descendants from that animal (as the most likely explanation for this delay is copy number drop). Likewise, siblings of an animal with a delay in onset should also be monitored closely. Retesting for copy number in these animals is recommended, although it is not necessarily the case that all copy number drops will be detectable.

Although QPCR detects large drops in copy number very well, copy number loss of less than 25-30% can fall below the threshold of sensitivity of this assay. **Thus, it is imperative to couple copy number analysis with disease onset and moribund analysis.** Animals with disease onset are readily identifiable by visual inspection and all breeders and experimental cohorts should be examined weekly during regular

cage changing. Symptoms of onset in animals include an unsteady wobbly gait with the development of a scruffy-looking coat and reduced limb splay (refer to Appendix B and accompanying video). In addition to visual onset, animals should be weighed at least weekly, as disease onset strongly correlates with maximum weight gain and thus weight loss is an excellent indicator of disease onset. Moribund analysis is performed by placing an animal on its side (refer to accompanying video). Failure of the animal to right itself on all four limbs within 15-30 seconds is an indicator that the animal is no longer capable of reaching the food hopper or water source and should thus be euthanized.

It is particularly important for researchers maintaining their own colony of mice to check the copy number in all of their breeders and progeny (Appendix A) and to monitor disease onset and moribund status. This information should be tracked in a detailed pedigree ledger. See Appendix C for an example of a pedigree ledger and explanation of use.

Genetic Quality Control

The aim of genetic quality control programs is to detect genetic contamination of one strain through an inadvertent mating with another strain. Genetic contamination is often uncovered by noting changes in breeding performance, physical appearance (such as coat color or body size) or deviations in Mendelian ratios. Given the variability of phenotypes observed particularly for the B6SJL mixed background animals, genetic contamination is more difficult to quickly detect. Therefore, we strongly recommend that researchers maintaining their own SOD1 colonies routinely monitor their colonies approximately 2-4 times per year as a precaution.

Monitoring to detect genetic contamination is quite straightforward; we recommend using a genotype-based approach to assay single-nucleotide polymorphism (SNP) markers positioned throughout the genome. A panel of 28 SNP assays was determined to be sufficient to identify strain contamination (Petkov *et al.*, 2004). This approach provides a quick and cost-effective way to genetically monitor mouse colonies using only a small tissue sample and is easily performed in individual research laboratories.

In addition to monitoring colonies for gross contamination through accidental inter-breeding, researchers also need to ensure that the genetic backgrounds of their mice do not drift. Genetic drift is the ability of established inbred strains to genetically diverge when bred at different facilities over time if proper genetic control measures are not taken. It is true that new mutations are relatively rare, and only a quarter of these are likely to be fixed even with continuous full sibling mating, so inbred strains tend to stay genetically constant for quite long periods of time. However, sublines can and have occurred when strains have been separated and not "refreshed" with founder stocks for too many generations. Unfortunately, many of the mutations that do occur will show no obvious phenotype except in unusual circumstances. For example, at least seven major substrains of the C57BL/6 line have emerged and been established over the years as a result of isolated breeding at various institutions. Very recently it was discovered that a deletion in a gene called Nnt1, which regulates metabolism, exists in some C57BL/6 substrains but not others (Mekada *et al.*, 2009).

Strains can also drift as a result of selective breeding. For example, strains that have severe neurological phenotypes are often subject to selection from animal husbandry personnel, who may inadvertently select animals from a litter with a milder phenotype for breeding. Sharing of colonies among independent research labs greatly adds to the propagation of both naturally occurring mutations and accidental contamination.

Genetic drift in ALS SOD1 colonies is extremely undesirable as it has the potential to confound experimental results between and among different research labs. In order to prevent genetic drift between individual mouse colonies, breeders from foundation stocks must be obtained *from the original pedigreed stock* at a minimum of every 10 generations.

Conclusions

The G93A mouse line is currently the most widely used experimental model in ALS research and drug testing. This transgene currently exists on two genetic backgrounds, the B6SJL mixed hybrid background and the C57BL/6J congenic background, and these two groups of animals differ significantly in their disease onset and survival. Both backgrounds present experimental advantages and disadvantages and careful consideration should be taken in choosing a genetic background based on the researcher's experimental objectives. Regardless of genetic background, the G93A transgene has been shown to spontaneously drop copy number which can greatly confound experimental results. Proper colony management in the form of copy number analysis, phenotypic assessment, and genetic quality controls, as outlined above, is essential for achieving validity and consistency in experimental results.

Appendices

Appendix A. QPCR Protocol for Determining Copy Number

Introduction

The principle behind the QPCR technique is that the gene of interest and an internal control gene can be amplified by PCR simultaneously, and the PCR products can be monitored after each cycle of amplification, based on reporter fluorescence specific for either the gene of interest or the internal control. The earliest cycle (CT, cycle threshold), at which an increase of reporter fluorescence above a baseline signal is measured, should be recorded for the gene of interest and an internal control, and the difference between these, called ΔCt, must be calculated. The higher the copy number, the fewer cycles needed to amplify sufficient product for detection. Therefore cycle number is very useful for monitoring change in transgene copy numbers.

Half a cycle drop (ΔCt value) translates to a 33% drop in the transgene copy number, which is currently accepted as the detection threshold of qPCR sensitivity for changes in copy number (reference: Liu D, Schmidt C, Billings T, Davisson M. 2003. Quantitative PCR genotyping assay for the Ts65Dn mouse model of Down syndrome. *BioTechniques* 35(6):1170-1180). Any animal exhibiting a reproducible drop in copy number should be discarded and the animal's siblings/progeny should be monitored closely.

Materials and Methods

Taqman QPCR protocols are run on an ABI 7500, 7700, 7900 or the Roche Light Cycler 480. It is important to use an appropriate instrument-specific Fluorophore/Quencher. The transgene zygosity is determined by comparing ΔCt values of each unknown sample against a standard high copy control (HC) and low copy control (LC), using appropriate endogenous references.

Protocol Primers

Primer	5′ Label	Sequence 5′ --> 3′	3′ Label
IMR1544	**none**	CAC GTG GGC TCC AGC ATT	**none**
IMR3580	**none**	TCA CCA GTC ATT TCT GCC TTT G	**none**
IMR9665	**none**	GGG AAG CTG TTG TCC CAA G	**none**
IMR9666	**none**	CAA GGG GAG GTA AAA GAG AGC	**none**
TmoIMR0105	Cy5	CCA ATG GTC GGG CAC TGC TCA A	Black Hole Quencher 2
TmoIMR0147	6-FAM	CTG CAT CTG GTT CTT GCA AAA CAC CA	Black Hole Quencher 1

Reaction Component	Volume (µl)	Final Concentration	Total Volume (µl)
2 X TaqMan® Universal Master Mix	6.25	1.00 X	6.25
40 uM oIMR9665	0.13	0.42 uM	0.13
40 uM oIMR9666	0.13	0.42 uM	0.13
40 uM oIMR1544	0.13	0.42 uM	0.13
40 uM oIMR3580	0.13	0.42 uM	0.13
5 uM TmoIMR0105	0.38	0.15 uM	0.38
5 uM TmoIMR0147	0.38	0.15 uM	0.38
DNA	5.00	-	5.00

Representative Data

Sample Name	Cycle Threshold (Ct)	Delta Cycle Threshold dCT (internal control - gene of interest)
Gene of interest	28.79	
Internal control	24.98	24.98 - 28.79 = -3.81

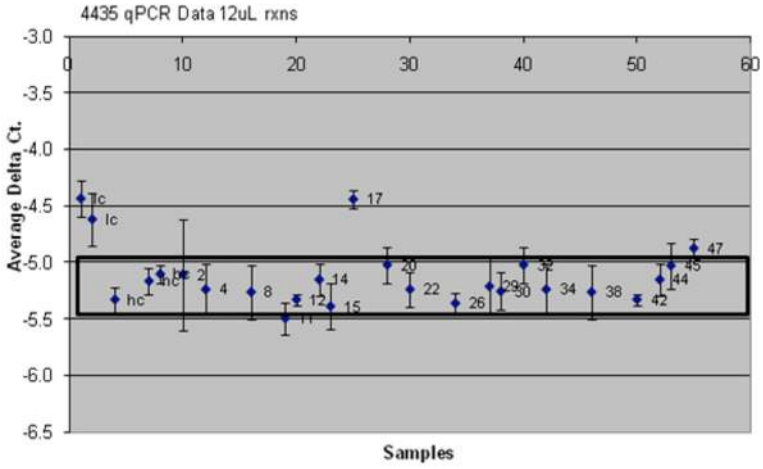
1. Run samples in triplicate
2. Obtain delta Ct for each sample: **dCt=int. control - gene of interest**
3. Obtain average and standard deviation for each triplicate set
4. Plot dCt values of sample set with *known* high copy and low copy controls

Control Data

Average Delta Cycle Threshold	Standard Deviation	Sample Name
-4.436666667	0.155349069	low copy control
-5.33	0.112694277	high copy control

Watch for:

- Large standard errors
- Low copy number animals that don't group with others and align with low copy controls (*e.g.*, sample 17 at right)



Any animal displaying a ΔCt value greater than a half cycle difference, or whose standard deviation places that animal at more than a half cycle difference as illustrated above, should be re-sampled or discarded. Copy number drops are estimated to occur at a rate of ~2-6%. Copy number analysis should always be coupled with disease onset and moribund analysis.

Appendix B. Sample Neurological Scoring System

Neurological Score

Regardless of which neurological scoring system is used, scores should be assessed for both hind legs. The example neurological scoring system below employs a scale of zero to four developed by ALSTDI. Example criteria used to assign each score under this system (see accompanying video for visual demonstration of scoring system) are:

Score Criteria

Score of 0: Full extension of hind legs away from lateral midline when mouse is suspended by its tail, and mouse can hold this for two seconds, suspended two to three times.

Score of 1: Collapse or partial collapse of leg extension towards lateral midline (weakness) or trembling of hind legs during tail suspension.

Score of 2: Toes curl under at least twice during walking of 12 inches, or any part of foot is dragging along cage bottom/table*.

Score of 3: Rigid paralysis or minimal joint movement, foot not being used for generating forward motion.

Score of 4: Mouse cannot right itself within 30 seconds after being placed on either side.

*If one hind leg is scored as 2, food pellets are left on bedding. If both hind legs are scored as 2, Nutra-Gel® (Bio-Serve #S4798) is provided as food in addition to food pellets on bedding and a long sipper tube is placed on the water bottle.

Appendix C. Template Pedigree Ledger

A pedigree ledger is used to track the information on a strain over the entire history of a colony and is useful in recording a variety of information, such as breeding generation, litter size, dam/sire information *etc*. The recording of information in a pedigree ledger can be extremely detailed to include many observations or it can be customized to capture only the information immediately required by an investigator. Below is an example of a simplified pedigree ledger designed in Microsoft Excel on a G93A mouse colony.

Strain Name	For example	B6.Cg-Tg(SOD1*G93A)1Gur/J					
A	B	C	D	E	F	G	H
Parents#/Ped#	Sex/Geno	Birth date	Date Mated	Gen#	euthanized	QPCR REF #	comments
796x102							
808	F B6	9/15/08	10/31/2008	N28			
104	M HEMI	9/20/08			148 days	36859	non productive
799x103							
844	F B6	9/17/08		N28			
105	M HEMI	9/30/09	11/6/2008		155 days	40433	
844x105							
1037	F B6	12/19/08	1/27/2008	N29			
1038	F B6	12/19/08					
1039	F B6	12/19/08					
106	M HEMI	12/25/09			158 days	45983	

Note that pedigree numbers are assigned sequentially and are unique within a strain. Column A contains two sets of pedigree numbers, one for the existing parents (#796 for the C57BL/6J dam and 102 for the G93A transgenic male), and the pedigree numbers assigned to a new C57BL/6J female (#808) and the offspring from mating 796x102 (male 104). Note that male 106 from mating 844x105 was used to propagate the line, but mating 808x104 was noted as non productive in the comments section.

A variety of columns can be added to this spreadsheet so as to capture as much (or as little) information as desired by the investigator; for example onset of wobbly gait or the week where weight loss was noted. This example shows birth dates, mating dates, backcross generation number, and the days of age a given male was euthanized. One column should contain the QPCR data for each animal so that one can easily reference that data in the event the animal lives longer than expected.

Pedigree ledgers are necessary tracking tools for any colony, but are particularly useful for the G93A colony. For example, if a given male survived to 200 days, one could eliminate any subsequent mating of animals sired by this male and still maintain the overall integrity of the colony. In addition, any data already derived from animals resulting from that mating would immediately be called into question.

Appendix D. List of Reviewers

Expert Reviewers who Provided Feedback

1. Dave Borchelt, Ph.D. (University of Florida)
2. Bob Brown Jr., M.D. Ph.D. (University of Massachusetts)
3. Lucie Bruijn, Ph.D. (ALS Association)
4. Greg Cox, Ph.D. (The Jackson Laboratory)
5. Merit Cudkowicz, M.D., M.Sc. (Northeast ALS Consortium (NEALS))
6. Sharon Hesterlee, Ph.D. (Muscular Dystrophy Association Venture Philanthropy)
7. Terry Heiman-Patterson, M.D. (Drexel University College of Medicine)
8. Brian Kaspar, Ph.D. (Ohio State University)
9. Jean-Pierre Julien, Ph.D. (Laval University)
10. Jonathan Matthews, Ph.D. (University of Massachusetts)
11. Steve Perrin, Ph.D. (ALS Therapy Development Institute)
12. Mercedes Prudencio, Ph.D. (University of Florida)
13. Jeffrey Rothstein, M.D., Ph.D. (Johns Hopkins University)
14. Fernando Vieira, M.D. (ALS Therapy Development Institute)

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