TOPICAL REVIEW

Motoneuron excitability dysfunction in ALS: Pseudo-mystery or authentic conundrum?

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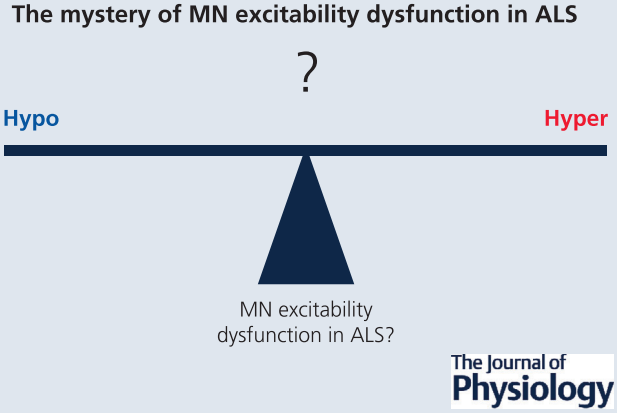

Abstract In amyotrophic lateral sclerosis (ALS), abnormalities in motoneuronal excitability are seen in early pathogenesis and throughout disease progression. Fully understanding motoneuron excitability dysfunction may lead to more effective treatments. Yet decades of research have not produced consensus on the nature, role or underlying mechanisms of motoneuron excitability dysfunction in ALS. For example, contrary to Ca excitotoxicity theory, predictions of motoneuronal hyper-excitability, normal and hypo-excitability have also been seen at various disease stages and in multiple ALS lines. Accordingly, motoneuron excitability dysfunction in ALS is a disputed topic in the field. Specifically, the form (hyper, hypo or unchanged) and what role excitability dysfunction plays in the disease (pathogenic or downstream of other pathologies; neuroprotective or detrimental) are currently unclear. Although several motoneuron properties

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that determine cellular excitability change in the disease, some of these changes are pro-excitable, whereas others are anti-excitable, making dynamic fluctuations in overall 'net' excitability highly probable. Because various studies assess excitability via differing methods and at differing disease stages, the conflicting reports in the literature are not surprising. Hence, the overarching process of excitability degradation and motoneuron degeneration is not fully understood. Consequently, the discrepancies on motoneuron excitability dysfunction in the literature represent a substantial barrier to our understanding of the disease. Emerging studies suggest that biological variables, variations in experimental protocols, issues of rigor and sampling/analysis strategies are key factors that may underlie conflicting data in the literature. This review highlights potential confounding factors for researchers to consider and also offers ideas on avoiding pitfalls and improving robustness of data.

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Abstract figure legend A diagram illustrating the mysterious nature of motoneuron (MN) excitability dysfunction in amyotrophic lateral sclerosis (ALS) with conflicting data ranging between hyper and hypoexcitability.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal, adult-onset, neurodegenerative disease. ALS is classically defined as progressive and selective degeneration of motoneurons in the cortex, brainstem and spinal cord (MNs, Charcot, 1874; Cleveland & Rothstein, 2001). ALS is a heterogeneous disease in which symptoms may manifest as weakness in one limb (some muscles may present as normal), or as bulbar or spinal onset, before paralysis spreads to upper and lower extremities. The majority of patients die within 3–4 years of diagnosis because the death of respiratory MNs impedes breathing. ALS pathogenesis may well be multifarious, and several pathogenic models are under investigation. Given their key role in motor control as the final common pathway, as well as their central role in the disease, MNs have been the focus of decades of investigations into ALS pathogenesis. Fully defining and understanding MN excitability dysfunction, which has been predicted and reported in the ALS literature, is therefore a promising strategy for the development of effective ALS treatments.

Several pieces of evidence suggest that abnormalities in neuronal excitability could be linked to disease pathogenesis. For example, early MN abnormalities at the embryonic (Kuo *et al.*, 2005; Martin *et al.*, 2013; Pieri *et al.*, 2003) and neonatal (Quinlan *et al.*, 2011) stages impact regulation of cell excitability. Prior to paralysis, ALS patients suffer muscle fasciculations, which are consistent with abnormal excitability and spontaneous firing of MN cell bodies and/or motor axons. Notably, the severity of these fasciculations correlates with shortened survival of patients (Shimizu *et al.*, 2014). Riluzole, the earliest FDA-approved drug for ALS treatment, but with modest extension in survival (Abe *et al.*, 2014; Bellingham, 2011; Okada *et al.*, 2018), suppresses MN excitability directly. Taken together, this suggests that there is a critical need to understand excitability dysfunction in ALS, an effort that may well identify new drug targets. In the absence of such knowledge, the development of effective ALS therapies will remain challenging. However, MN excitability dysfunction in ALS is currently a mysterious and oft-disputed topic in the field because of the conflicting data reported in the literature. These discrepancies on MN excitability dysfunction represent a substantial barrier to our understanding of the disease. Although both upper and lower MNs degenerate in the disease, with their interplay influencing excitability dysfunction, this review focuses on lower MNs. We critically discuss the conflicting information on their excitability dysfunction in ALS and propose steps for unravelling the causes underlying these discrepancies.

ALS: one disease but many pathologies

ALS is considered to be a multifactorial disease involving both autonomous and non-autonomous cell mechanisms. Determining primary *vs*. secondary aetiology is arduous as a result of the multidirectional interactions among pathologies. MNs are especially susceptible in ALS because: (1) MNs must maintain extremely large somas and processes; (2) MNs have increased expression of voltage-gated Ca channels and Ca-permeable AMPA receptors that produce large influxes of Ca during synaptic activation; and (3) MNs have low Ca buffering capabilities because of the absence of the Ca buffers calbindin and parvalbumin (Alexianu *et al.*, 1994; Ince *et al.*, 1993).

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This creates a precarious physiological environment in which energy demands are high, Ca influxes are large and Ca buffering capacity is low, with mitochondria having to accommodate all of this. Any pathology that increases internal Ca concentration or disrupts energy production could cause devastating effects. In addition to excitability dysfunction and potential upstream triggers of excitotoxicity, other ALS pathologies include mitochondria dysfunction, axon transport dysfunction, accumulation of misfolded proteins, RNA processing deficits and glial activation. There is also potential for system homeostasis within the MN pool, such as sprouting of living MNs to reinnervate muscle fibres. Although MN excitability dysfunction might be secondary to other pathologies in ALS, the current lack of therapies with long-term effect renders the potential to extend MN life and/or ameliorate disease symptoms by stabilizing their excitability a valuable direction to pursue. One particular challenge to developing therapies is that ALS is diagnosed in patients long after pathology begins. Although animal models provide important information on early stages of disease, there are no equivalent data from humans to compare to early animal data. Thus, extrapolating from animal models to human pathology is fraught with pitfalls. Accordingly, the present review focuses on preclinical data, with select examples of human studies, aiming to assess the quandaries of the excitotoxicity theory, and discusses the controversy over the involvement of MN excitability dysfunction in ALS.

MN excitability

MN excitability refers to the firing capacity of the cell and its sensitivity to inputs. Although several methods are available to assess MN excitability, the gold-standard method is intracellular electrophysiological recordings in which the cell is activated via somatic current injection of triangular ramps or long pulses via a micropipette or synaptic activation via muscle stretch (Granit *et al.*, 1966) or tendon vibration (Heckman & Binder, 1988) at the same time as measuring the cell spiking activity and firing rate directly. The outcome is the frequency-current ($F-I$) relationship, for which slope (aka 'cell gain') is a good measure of the cell 'net' excitability resulting from interacting membrane properties. Thus, a cell with lower $F-I$ gain would fire less for a given input *vs.* a cell with a higher $F-I$ gain. Although injected and synaptic $F-I$ relationships assess the MN excitability, they differ in the way the cell is driven (somatically *vs.* dendritically, respectively) and how ion channels are activated (synaptic inputs activate dendritic before somatic ion channels). Given that muscle stretch and tendon vibration could be infeasible in some preparations (e.g. cell culture and slice preparations), recording the $F-I$ relationship via somatic current injection is more common in the literature. To assess MN excitability in animal models of ALS, mutant MNs are compared with controls (cells from age- and sex-matched non-diseased transgenic animals). Mutant MNs are considered hyper (or hypo) excitable when their FI gain is higher (or lower) relative to that of control MNs. Another complementary measure of cell net excitability is rheobase (i.e. the minimum current needed to evoke a single action potential). However, although rheobase assesses the capacity of the cell to initiate cell firing, it might not capture the effects of slow ion channels only active during repetitive firing (e.g. Cav1.3 and Kv2.1 channels), which are captured by the FI gain.

Excitotoxicity theory: unfulfilled predictions on MN excitability dysfunction

Classic excitotoxicity theory predicts that MN death in ALS results from excessive MN activation by glutamate, leading to high levels of Ca entering the cell and triggering apoptosis (Bellingham, 2011; Kuo *et al.*, 2005). This theory can explain the benefits of Riluzole because it suppresses MN excitability (Bellingham, 2011), thereby reducing Ca currents. However, contrary to excitotoxicity theory predictions, smaller Ca transients were found in MNs of the superoxide dismutase (SOD) mouse model of ALS (Quinlan *et al.*, 2015). Excitotoxicity theory also predicts that MNs would become *hyper*excitable, yet both normal (Delestree *et al.*, 2014) and *hypo*excitability (Marchand-Pauvert *et al.*, 2019; Martinez-Silva *et al.*, 2018) have also been observed in ALS patients and animal models. Additionally, hyper-excitability is viewed as detrimental by some (Kanai *et al.*, 2006; Pieri *et al.*, 2009; van Zundert *et al.*, 2008; Wainger *et al.*, 2014), but neuroprotective by others (Leroy & Zytnicki, 2015; Leroy *et al.*, 2014; Saxena *et al.*, 2013). In sum, there is disagreement on the form of excitability dysfunction expected in ALS (hyper *vs.* hypo), as well as on the role of these excitability changes in the disease (neuroprotective *vs.* detrimental). Additionally, excitotoxicity theory would suggest that slow-twitch MNs (S-MNs) should die before fast-twitch MNs (F-MNs), given that S-MNs are more excitable, are recruited first and more often, fire for longer durations, and have more sustained persistent inward currents (PICs) that introduce more Ca into the cell, compared to F-MNs. However, this is contrary to what happens in ALS, in which F-MNs die before S-MNs (Pun *et al.*, 2006). Also, drugs that increase Ca PIC shortened survival in neonatal, but not adult, G93A SOD mice, whereas drugs that decrease Ca PIC did not prolong survival (Koschnitzky *et al.*, 2014). Together, these inconsistencies between predictions and how MNs actually die indicate that current excitotoxicity theory is incomplete, raising questions on the form and role of MN

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excitability dysfunction in the disease. Elucidating this mystery would ideally involve analysing a rich array of data on MN excitability dysfunction published by the ALS field. Unfortunately, much data from the field is contradictory. Thus, our next step, and the focus of this review, is to analyse potential causes for these discrepancies.

Biological variables influencing MN excitability dysfunction in ALS

The complexity of studying MN excitability dysfunction in ALS is compounded by several factors, which probably contribute to the conflicting literature on the disease. First, the 'net' excitability of a given MN is influenced by many interacting membrane properties (termed excitability 'subcomponents' in this review). In ALS, each excitability subcomponent appears to be individually affected, in that some disease changes would increase, whereas others would decrease, net excitability. This suggests a dynamic, but silent, disease progression, in which countervailing subcomponents are impacting net MN excitability, and in which cell death is progressing in absence of symptoms (subcomponent changes in the G93A high expressor line) (Fig. 1). Examples of the interacting excitability subcomponents are: (1) cell size: a larger cell has greater membrane area and input conductance (G_{in}), which lowers cell excitability; (2) passive membrane properties: lower membrane conductivity (G_m) increases cell excitability; (3) voltage-gated PICs: larger PICs increase cell excitability; (4) voltage-gated outward currents: larger outward currents lower cell excitability; and (5) synaptic inputs: stronger excitatory or weaker inhibitory, synaptic current increases cell excitability. Together, these and other excitability subcomponents interact to determine the cell's net excitability, which is best assessed using the frequency-current ($F-I$) relationship gain. In ALS, MNs experience concurrent pathological changes in all the excitability subcomponents listed above throughout disease progression. Specifically, the ALS literature has reported: increased cell size (Dukkipati *et al.*, 2018; Shoenfeld *et al.*, 2014), increased dendritic complexity (Amendola & Durand, 2008; Filipchuk & Durand, 2012), alterations in membrane passive properties (Bories *et al.*, 2007; Pambo-Pambo *et al.*, 2009; Quinlan *et al.*, 2011), upregulation in NaPIC and CaPIC (Kuo *et al.*, 2005; Quinlan *et al.*, 2011), downregulation in Kv1.2 channels (Shibuya *et al.*, 2011) and alteration in synaptic potentials (Bories *et al.*, 2007), as well as dendritic processing of synaptic inputs (Elbasiouny *et al.*, 2010). Strikingly, these changes appear early, long before MN death and symptom onset in multiple ALS lines, such as high- and low-copy G93A and G85R SOD mice. Three challenges have arisen from these many excitability subcomponent changes in the disease. First, many studies characterized mutant MNs as hypo- or hyper-excitable based on changes in excitability subcomponents, even when their net excitability ($F-I$ gain) was unchanged (we term this state 'pseudo-normal' excitability) (Fig. 2) (Huh *et al.*, 2021; Meehan *et al.*, 2010; Quinlan *et al.*, 2011). Contributing to this first challenge, these subcomponent changes have opposing effects on cell net excitability. Thus, the MN is continuously impacted, and probably stressed, by countervailing excitability effects. These countervailing effects probably represent disease and compensatory changes. Further, the relative magnitudes of these pro- and anti-excitability changes also dynamically change during the disease. Thus, excitability subcomponent changes of opposing effects would be expected to be seen throughout the disease. This dynamic, ongoing push–pull process leads to fluctuating cell net excitability, which could explain the conflicting reports in the ALS literature. Thus, using excitability subcomponent changes, as opposed to net excitability measurements, to characterize MN excitability in the disease could be misleading and would probably contribute to conflicting conclusions. The hypothesis of dynamic, competing, disease *vs.* compensatory excitability subcomponent changes is supported by the fact that as many as ∼70–80% of MNs may have already died by the time of symptom onset (Hegedus *et al.*, 2007; Hegedus *et al.*, 2008). Specifically, this indicates that strong homeostatic mechanisms within individual MNs and/or the larger system are acting to obviate cell death and/or sustain motor function despite loss of MNs. Also, time course changes in a number of excitability subcomponents, such as soma size, input conductance, resting membrane potential and PIC amplitude, support fluctuations in cell properties throughout disease progression (Dukkipati *et al.*, 2018; Huh *et al.*, 2021). Importantly, some studies showed that the pseudo-normal MN net excitability falls apart at symptom onset and after. For example, Draper *et al.* (2019) reported reduced $F-I$ gain (i.e. hypoexcitability), whereas Jensen *et al.* (2020) reported increased $F-I$ gain (i.e. hyperexcitability). Hyperexcitability of symptomatic MNs was attributed to increased PICs (Jensen *et al.*, 2020), a finding that has been disputed by other studies (Huh *et al.*, 2021). However, PICs were estimated in the study by Jensen *et al.* (2020) from the firing behaviours of mutant MNs (which could be influenced by many factors other than PICs), whereas the study by Huh *et al.* (2021) measured PICs in voltage clamp (which is a direct and more accurate assessment).

Second, MNs comprise different types that respond differently to ALS. For example, large, F-MNs die before small, S-MNs (Pun *et al.*, 2006), a degeneration differential indicating that S-MNs are less vulnerable to ALS than F-MNs. This suggests that these MN subtypes could be responding differently in the disease, contributing to the conflicting ALS literature, especially in studies when MN

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types are mixed together. Furthermore, F-MNs have been shown to undergo differential excitability changes not experienced by S-MNs, such as soma size changes or inability to fire repetitively (Dukkipati *et al.*, 2018; Huh *et al.*, 2021).

Third, sex appears to be another biological variable in the disease because males are more commonly and aggressively affected than females (McCombe & Henderson, 2010). This suggests that MNs from male diseased animals are more vulnerable to ALS and therefore respond differently in the disease *vs.* those from female diseased animals. Thus, when sex differences are not accounted for (i.e. data of both sexes are pooled together), this contributes to the conflicting ALS literature. This sex differential hypothesis is supported by data showing that the soma size of male, but not female, MNs is enlarged (Dukkipati *et al.*, 2018; Shoenfeld *et al.*, 2014). Additionally, sex and cell type differences have been

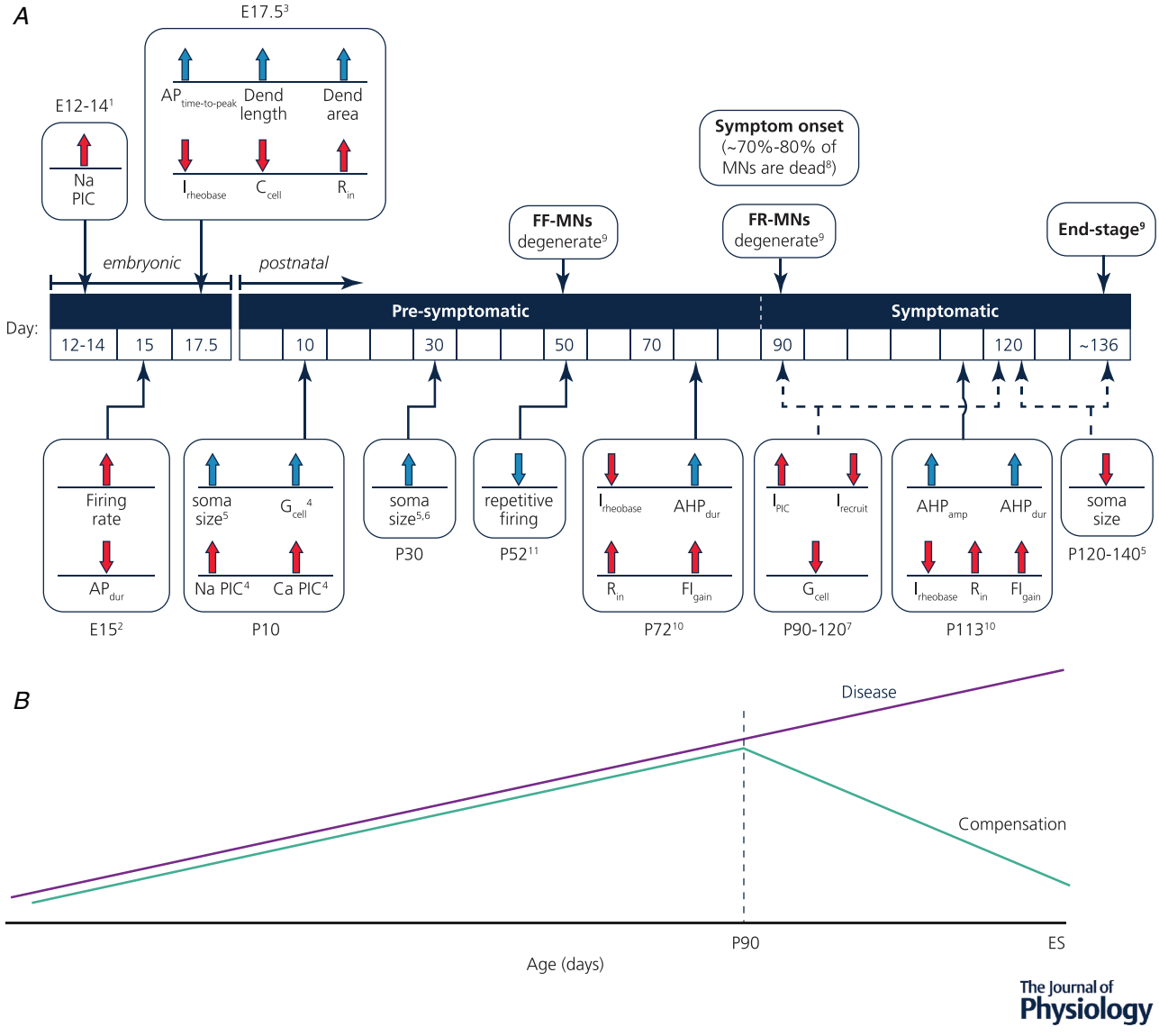

Figure 1. MN excitability dysfunction in ALS
A, illustration of several MN excitability changes reported throughout the life span of G93A high expressor mice. The directions of arrows show whether there is an increase (upward arrow) or decrease (downward arrow) in each parameter. Pro (i.e. hyper) excitability changes are shown in red, whereas anti- (i.e. hypo) excitability changes are shown in blue. The cited references are: (1) Kuo *et al.* (2005); (2) Pieri *et al.* (2003); (3) Martin *et al.* (2013); (4) Quinlan *et al.* (2011); (5) Dukkipati *et al.* (2018); (6) Shoenfeld *et al.* (2014); (7) Huh *et al.* (2021) (8) Hegedus *et al.* (2007); (9) Pun *et al.* (2006); (10) Jensen *et al.* (2020); and (11) Martinez-Silva *et al.* (2018). *B*, the presence of concurrent disease and compensatory mechanisms mask symptoms, leading to silent MN death during the pre-symptomatic phase. Symptoms emerge when compensation starts to fail.

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shown to be important biological variables in studying normal MN electrophysiological properties (Allen & Elbasiouny, 2018; Highlander *et al.*, 2020). A summary of biological variables is provided in Fig. 3.

Potential experimental design variables influencing MN excitability dysfunction in ALS

A number of experimental design variables also appear to contribute to the discrepancy in the ALS literature on MN excitability dysfunction (Fig. 3). For example, although several animal preparations have been used to examine MN excitability in ALS (such as cell culture, slices, whole-cord *in vitro* and *in vivo*), hypo-excitability was mostly seen in *in vitro* preparations (Amendola & Durand, 2008; Amendola *et al.*, 2007; Filipchuk *et al.*, 2021) and *in vivo* preparations (Huh *et al.*, 2021; Maglemose *et al.*, 2017; Martinez-Silva *et al.*, 2018; Meehan *et al.*, 2010). It should be noted that MN-supporting cells are present in these two preparations. On the other hand, hyper-excitability was always seen in cultured preparations (Kuo *et al.*, 2004; Kuo *et al.*, 2005) and sliced MN preparations (Leroy *et al.*, 2014; Pambo-Pambo *et al.*, 2009; Quinlan *et al.*, 2011), with the exception of the G85R data in Pambo-Pambo *et al.* (2009), which reported hypoexcitability. It should be noted that, in these preparations, MNs are isolated. In other words, hypo-excitability is seldomly seen in reduced preparations. This suggests that surrounding environment may influence MN excitability mechanisms and thus suggests that cell preparations are an experimental variable that should be considered (Boillee *et al.*, 2006). Additionally, MN dendrites enlarge and overbranch in the disease (Amendola & Durand, 2008; Filipchuk & Durand, 2012); these dendrites, including mid and distal branches, are shown to contain many active channels (Ballou *et al.*, 2006; Carlin *et al.*, 2000; Elbasiouny *et al.*, 2005; Mousa & Elbasiouny, 2020; Zhang *et al.*, 2008). Because MN dendrites are usually not fully developed in cell cultures, and are truncated in slices, the reduction of dendrites and their active conductances in these preparations could miss or eliminate disease changes and induce errors in measured cell properties. This in turn could contribute to differences in observed excitability changes.

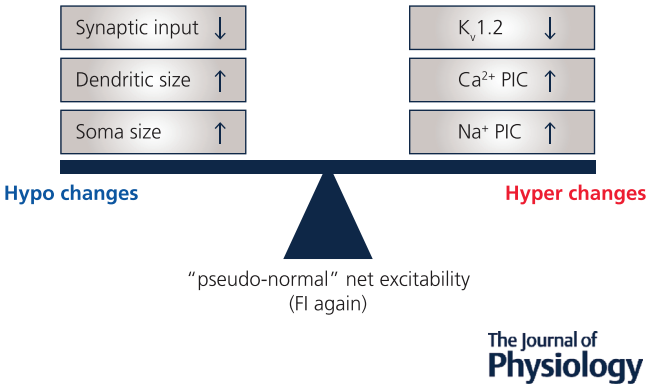

Figure 2. MN excitability dysregulation at P10
An illustration of how the opposing effects of pro- (i.e. hyper) and anti-excitability (i.e. hypo) changes could result in 'pseudo-normal' net excitability of mutant MNs.

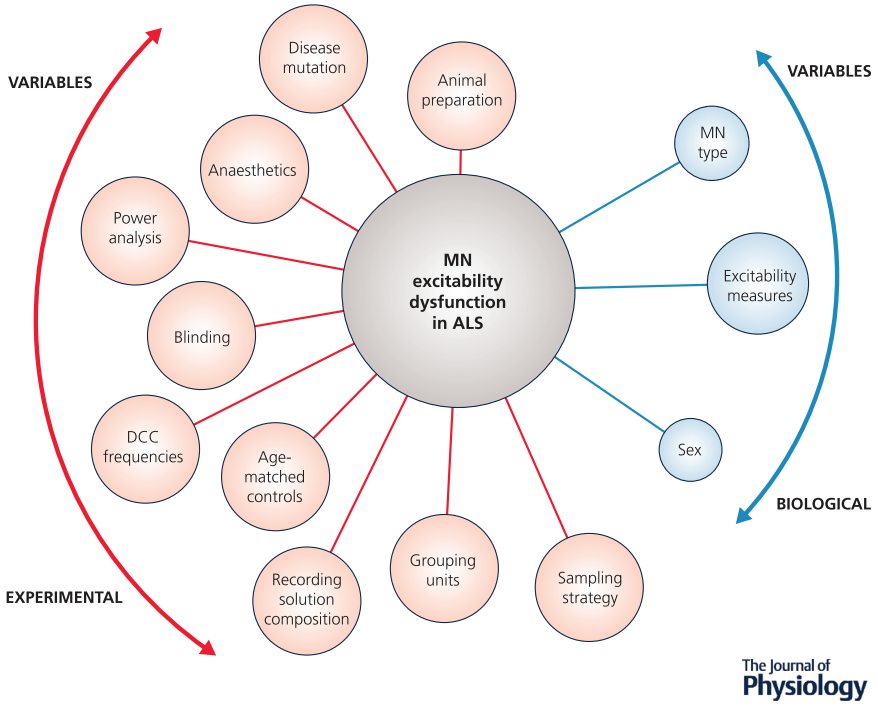

Figure 3. Biological and experimental variables
Illustration of the biological and experimental variables contributing to the conflicting literature on MN excitability dysfunction in ALS.

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Importantly, a recent modelling study by Mousa & Elbasiouny (2021) showed that the slice preparation with thin slices ($\leq$350 μm) (as performed by Pambo-Pambo *et al.*, 2009; Quinlan *et al.*, 2011; Hadzipasic *et al.*, 2014) or thick but asymmetric soma position within the slice (as performed by Leroy *et al.*, 2014) would induce large errors in measured electrical properties, and would therefore not distinguish between ALS-resistant (i.e. slow) and ALS-vulnerable (i.e. fast) MNs. This is because the slice preparation, which truncates the large MN dendrites, eliminates the morphological differences between slow and fast cells under these conditions. Thus, the use of slice preparations in ALS studies may well contribute to the conflicting literature on MN excitability dysfunction in the disease.

An additional experimental design factor that may contribute to contradictory data on MN excitability dysfunction in the ALS literature involves the multiple animal models and mutations used to study ALS. This diversity of animal models is very useful because they mimic the heterogeneity of the disease in humans, although their biological differences need to be considered during data comparison and interpretation. A notable issue is the differing timelines of disease onset and progression among models. Yet, when data are compared across different mutations, not all comparisons account for these differing timelines. Such studies may be comparing snap shots of excitability changes at the same age, but at a different disease stage, which is not truly comparable. Thus, it is important to compare mechanisms across models by key ALS disease progression milestones (i.e. start of cellular changes, start of MN death, measurable motor impairment, etc.). This cross-model comparison will improve interpretation of how these key events in pre-clinical models relate to similar events in ALS patients with different genetic mutations.

Indeed, when analysing the ALS literature, it becomes clear that there is great diversity in the experimental designs and techniques used (Fig. 3). This makes comparison of data among independent study groups difficult. There are also studies that do not detail methods sufficiently to reproduce them or to incorporate their results into field-wide analysis. This state of affairs is compounded by the use of suboptimal techniques. Perrin (2014) called for improved rigor in the ALS field. To that end, we have included a brief list of additional experimental factors, drawn from the ALS literature (Scott *et al.*, 2008), which should be examined for their contributions to discrepancies in the literature on MN excitability dysfunction in ALS: (1) There is variation in which anaesthetics are used; (2) power analysis and blinding of data collection are often not reported, which makes it difficult to know if these practices were followed; (3) suboptimal discontinuous current clamp (DCC) frequencies are sometimes used, which can give incorrect measurements; (4) some studies use controls that are not age-matched, which reduces rigor; (5) variations in recording solution can lead to variations in results; (6) the use of improper grouping units (such as reporting biological replicates as independent samples) reduces rigor; and (7) sampling strategies that (a) collect small samples or samples based on human judgment and thus could allow bias, (b) mix mice of unknown sexes and (c) pool MNs of different types for analysis, all reduce the rigor of results (Fig. 3).

Some consequences of suboptimal experimental design and technique were illustrated in a meta-analysis on excitability subcomponent changes in Dukkipati *et al.* (2017). It was shown that different and sometimes opposing results could be concluded from the same dataset, depending on the grouping unit used in data analysis (by animal *vs.* by cell *vs.* by cluster). Additionally, results from the same dataset also differed depending on (1) whether or not the experimenter was blinded in collecting the data and (2) which strategy was used for sampling immunohistochemistry clusters for analysis (i.e. a few *vs.* many clusters per cell).

There are additional instances in the literature that suggest variations in experimental design may contribute to contradictory results: In Jensen *et al.* (2020), although pre-symptomatic mice were compared to age-matched controls, symptomatic mice were not. Also, Bories *et al.* (2007) used an unusually high Ca concentration in their recording solution (4 mM of $CaCl_2$ *vs.* the 2–2.5 mM range used in most electrophysiology studies). Herron & Miles (2012) used small sample sizes, mixed mice of unknown sexes at some time points and pooled MNs of different types together. In a more complex example, Delestree *et al.* (2014), Martinez-Silva *et al.* (2018) and Jensen *et al.* (2020) studied the same mouse model (G93A, high copy) using the same preparation (*in vivo*) and using the same anaesthetics (sodium barbiturate) in mice of the same sex (males) at the same disease stage (pre-symptomatic); however, opposing forms of MN excitability dysfunction were reported. Delestree *et al.* (2014) reported no change, Martinez-Silva *et al.* (2018) reported hypoexcitability and Jensen *et al.* (2020) reported hyperexcitability. In their studies, both Delestree *et al.* (2014) and Jensen *et al.* (2020) did not consider MN type differences (i.e. MNs of different types were analysed together). Also, Jensen *et al.* (2020) used abnormally low DCC frequencies (which evokes artificial cell firing, deforms the action potential shape and overestimates cell excitability; Manuel, 2021). Finally, Martinez-Silva *et al.* (2018) based their excitability assessment solely on the inability of mutant MNs to fire repetitively, a property that is sensitive to technical factors; this finding has also been challenged by Jorgensen *et al.* (2020) and Jensen *et al.* (2020). It should be noted that some mutant MNs in the study by

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Martinez-Silva *et al.* (2018) did fire repetitively and appeared to have normal *F–I* gain.

Excitability dysfunction is implicated in ALS from the earliest pre-symptomatic stages in mouse models and is implicated throughout disease progression in pre-clinical and human studies. Yet, the mechanisms and meaning of these phenomena will remain difficult to decipher as long as there remain so many differences in experimental design and techniques and the inconsistent results they produce. It may be useful for the field to co-operatively assess the strengths, weaknesses and accuracy of the experimental techniques that we use. A consensus on multiple, independent and rigorous techniques for assessing excitability dysfunction in ALS (i.e. the use of $F - I$ gain to measure and compare cell excitability) may allow the field to generate more cohesive, robust, and fruitful results.

Concluding remarks

The discrepancy in the literature on MN excitability dysfunction in ALS is currently hindering progress in our understanding of the disease. Many biological and experimental variables appear to contribute to the inconsistencies in the ALS literature. MN cell type and sex are key biological variables that need to be accounted for in ALS studies. Animal preparations need to study the MN in its surrounding environment, without truncating its dendrites and removing critical active conductances. Also, an adequate DCC frequency is needed for improved accuracy in ALS studies. Given that every experimental technique has technical limitations, confirming research findings on MN excitability dysfunction in ALS via multiple, independent and rigorous techniques would provide higher confidence in the validity of our results. In conclusion, and echoing earlier concerns (Perrin, 2014), enhancing experimental rigor in ALS studies appears to be the most needed consideration to clarify and resolve disputed questions about excitability dysfunction in ALS, which will help to advance this field.

References

Abe, K., Itoyama, Y., Sobue, G., Tsuji, S., Aoki, M., Doyu, M., Hamada, C., Kondo, K., Yoneoka, T., Akimoto, M., & Yoshino, H. (2014). Confirmatory double-blind, parallel-group, placebo-controlled study of efficacy and safety of edaravone (MCI-186) in amyotrophic lateral sclerosis patients. *Amyotroph Lateral Scler Frontotemporal Degener*, **15**(7–8), 610–617.

Alexianu, M. E., Ho, B. K., Mohamed, A. H., La Bella, V., Smith, R. G., & Appel, S. H. (1994). The role of calcium-binding proteins in selective motoneuron vulnerability in amyotrophic lateral sclerosis. *Annals of Neurology*, **36**(6), 846–858.

Allen, J. M., & Elbasiouny, S. M. (2018). The effects of model composition design choices on high-fidelity simulations of motoneuron recruitment and firing behaviors. *Journal of Neural Engineering*, **15**(3), 036024.

Amendola, J., & Durand, J. (2008). Morphological differences between wild-type and transgenic superoxide dismutase 1 lumbar motoneurons in postnatal mice. *Journal of Comparative Neurology*, **511**(3), 329–341.

Amendola, J., Gueritaud, J. P., Lamotte d'Incamps, B., Bories, C., Liabeuf, S., Allene, C., Pambo-Pambo, A., & Durand, J. (2007). Postnatal electrical and morphological abnormalities in lumbar motoneurons from transgenic mouse models of amyotrophic lateral sclerosis. *Archives Italiennes De Biologie*, **145**, 311–323.

Ballou, E. W., Smith, W. B., Anelli, R., & Heckman, C. J. (2006). Measuring dendritic distribution of membrane proteins. *Journal of Neuroscience Methods*, **156**(1–2), 257–266.

Bellingham, M. C. (2011). A review of the neural mechanisms of action and clinical efficiency of riluzole in treating amyotrophic lateral sclerosis: What have we learned in the last decade? *CNS Neuroscience & Therapeutics*, **17**, 4–31.

Boillee, S., Vande Velde, C., & Cleveland, D. W. (2006). ALS: A disease of motor neurons and their nonneuronal neighbors. *Neuron*, **52**(1), 39–59.

Bories, C., Amendola, J., Lamotte d'Incamps, B., & Durand, J. (2007). Early electrophysiological abnormalities in lumbar motoneurons in a transgenic mouse model of amyotrophic lateral sclerosis. *European Journal of Neuroscience*, **25**(2), 451–459.

Carlin, K. P., Jones, K. E., Jiang, Z., Jordan, L. M., & Brownstone, R. M. (2000). Dendritic L-type calcium currents in mouse spinal motoneurons: Implications for bistability. *European Journal of Neuroscience*, **12**(5), 1635–1646.

Charcot, J. (1874). De la sclerose laterale amyotrophique. *Progress in Medicine*, **2**, 325–455.

Cleveland, D. W., & Rothstein, J. D. (2001). From Charcot to Lou Gehrig: Deciphering selective motor neuron death in ALS. *Nature Reviews Neuroscience*, **2**(11), 806–819.

Delestree, N., Manuel, M., Iglesias, C., Elbasiouny, S. M., Heckman, C. J., & Zytnicki, D. (2014). Adult spinal motoneurones are not hyperexcitable in a mouse model of inherited amyotrophic lateral sclerosis. *Journal of Physiology*, **592**(7), 1687–1703.

Draper, C., Mahrous, A. A., & Elbasiouny, S. M. (2019). Hypo-excitability and hyperexcitability in sacral motorneurons of SOD1G93A high copy ALS mice: Disease versus compensation. In *The 2019 annual meeting of the Society for Neuroscience*. Chicago, IL, USA.

Dukkipati, S. S., Chihi, A., Wang, Y., & Elbasiouny, S. M. (2017). Experimental design and data analysis issues contribute to inconsistent results of C-Bouton changes in amyotrophic lateral sclerosis. *eNeuro*, **4**(1), ENEURO.0281-16.2016.

Dukkipati, S. S., Garrett, T. L., & Elbasiouny, S. M. (2018). The vulnerability of spinal motoneurons and soma size plasticity in a mouse model of amyotrophic lateral sclerosis. *Journal of Physiology*, **596**(9), 1723–1745.

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Elbasiouny, S. M., Amendola, J., Durand, J., & Heckman, C. J. (2010). Evidence from computer simulations for alterations in the membrane biophysical properties and dendritic processing of synaptic inputs in mutant superoxide dismutase-1 motoneurons. *Journal of Neuroscience*, **30**(16), 5544–5558.

Elbasiouny, S. M., Bennett, D. J., & Mushahwar, V. K. (2005). Simulation of dendritic Cav1.3 channels in cat lumbar motoneurons: spatial distribution. *Journal of Neurophysiology*, **94**(6), 3961–3974.

Filipchuk, A., Pambo-Pambo, A., Gaudel, F., Liabeuf, S., Brocard, C., Patrick Gueritaud, J., & Durand, J. (2021). Early hypoexcitability in a subgroup of spinal motoneurons in superoxide dismutase 1 transgenic mice, a model of amyotrophic lateral sclerosis. *Neuroscience*, **463**, 337–353.

Filipchuk, A. A., & Durand, J. (2012). Postnatal dendritic development in lumbar motoneurons in mutant superoxide dismutase 1 mouse model of amyotrophic lateral sclerosis. *Neuroscience*, **209**, 144–154.

Granit, R., Kernell, D., & Lamarre, Y. (1966). Algebraical summation in synaptic activation of motoneurones firing within the 'primary range' to injected currents. *Journal of Physiology*, **187**(2), 379–399.

Hadzipasic, M., Tahvildari, B., Nagy, M., Bian, M., Horwich, A. L., & McCormick, D. A. (2014). Selective degeneration of a physiological subtype of spinal motor neuron in mice with SOD1-linked ALS. *PNAS*, **111**(47), 16883–16888.

Heckman, C., & Binder, M. D. (1988). Analysis of effective synaptic currents generated by homonymous Ia afferent fibers in motoneurons of the cat. *Journal of Neurophysiology*, **60**(6), 1946–1966.

Hegedus, J., Putman, C. T., & Gordon, T. (2007). Time course of preferential motor unit loss in the SOD1G93A mouse model of amyotrophic lateral sclerosis. *Neurobiology of Disease*, **28**(2), 154–164.

Hegedus, J., Putman, C. T., Tyreman, N., & Gordon, T. (2008). Preferential motor unit loss in the SOD1G93A transgenic mouse model of amyotrophic lateral sclerosis. *Journal of Physiology*, **586**(14), 3337–3351.

Herron, L. R., & Miles, G. B. (2012). Gender-specific perturbations in modulatory inputs to motoneurons in a mouse model of amyotrophic lateral sclerosis. *Neuroscience*, **226**, 313–323.

Highlander, M. M., Allen, J. M., & Elbasiouny, S. M. (2020). Meta-analysis of biological variables' impact on spinal motoneuron electrophysiology data. *Journal of Neurophysiology*, **123**(4), 1380–1391.

Huh, S., Heckman, C. J., & Manuel, M. (2021). Time course of alterations in adult spinal motoneuron properties in the SOD1(G93A) mouse model of ALS. *Eneuro*, **8**(2), ENEURO.0378-20.2021.

Ince, P., Stout, N., Shaw, P., Slade, J., Hunziker, W., Heizmann, C. W., & Baimbridge, K. G. (1993). Parvalbumin and calbindin D-28k in the human motor system and in motor neuron disease. *Neuropathology and Applied Neurobiology*, **19**(4), 291–299.

Jensen, D. B., Kadlecova, M., Allodi, I., & Meehan, C. F. (2020). Spinal motoneurones are intrinsically more responsive in the adult G93A SOD1 mouse model of amyotrophic lateral sclerosis. *Journal of Physiology*, **598**(19), 4385–4403.

Jorgensen, H. S., Jensen, D. B., Dimintiyanova, K. P., Bonnevie, V. S., Hedegaard, A., Lehnhoff, J., Moldovan, M., Grondahl, L., & Meehan, C. F. (2020). Increased axon initial segment length results in increased Na(+) currents in spinal motoneurones at symptom onset in the G127X SOD1 mouse model of amyotrophic lateral sclerosis. *Neuroscience*, **468**, 247–264.

Kanai, K., Kuwabara, S., Misawa, S., Tamura, N., Ogawara, K., Nakata, M., Sawai, S., Hattori, T., & Bostock, H. (2006). Altered axonal excitability properties in amyotrophic lateral sclerosis: Impaired potassium channel function related to disease stage. *Brain*, **129**(4), 953–962.

Koschnitzky, J. E., Quinlan, K. A., Lukas, T. J., Kajtaz, E., Kocevar, E. J., Mayers, W. F., Siddique, T., & Heckman, C. J. (2014). The effect of fluoxetine on disease progression in a mouse model of ALS. *Journal of Neurophysiology*, **111**(11), 2164–2176.

Kuo, J. J., Schonewille, M., Siddique, T., Schults, A. N. A., Fu, R., Bar, P. R., Anelli, R., Heckman, C. J., & Kroese, A. B. A. (2004). Hyperexcitability of cultured spinal motoneurons from presymptomatic ALS mice. *Journal of Neurophysiology*, **91**(1), 571–575.

Kuo, J. J., Siddique, T., Fu, R., & Heckman, C. J. (2005). Increased persistent Na+ current and its effect on excitability in motoneurones cultured from mutant SOD1 mice. *The Journal of Physiology*, **563**(3), 843–854.

Leroy, F., Lamotte d'Incamps, B., Imhoff-Manuel, R. D., & Zytnicki, D. (2014). Early intrinsic hyperexcitability does not contribute to motoneuron degeneration in amyotrophic lateral sclerosis. *Elife*, **3**, e04046.

Leroy, F., & Zytnicki, D. (2015). Is hyperexcitability really guilty in amyotrophic lateral sclerosis? *Neural Regeneration Research*, **10**(9), 1413–1415.

Maglemose, R., Hedegaard, A., Lehnhoff, J., Dimintiyanova, K. P., Moldovan, M., Grondahl, L., & Meehan, C. F. (2017). Potassium channel abnormalities are consistent with early axon degeneration of motor axons in the G127X SOD1 mouse model of amyotrophic lateral sclerosis. *Experimental Neurology*, **292**, 154–167.

Manuel, M. (2021). Suboptimal discontinuous current-clamp switching rates lead to deceptive mouse neuronal firing. *eNeuro*, **8**(1), ENEURO.0461-20.2020.

Marchand-Pauvert, V., Peyre, I., Lackmy-Vallee, A., Querin, G., Bede, P., Lacomblez, L., Debs, R., & Pradat, P. F. (2019). Absence of hyperexcitability of spinal motoneurons in patients with amyotrophic lateral sclerosis. *Journal of Physiology*, **597**(22), 5445–5467.

Martin, E., Cazenave, W., Cattaert, D., & Branchereau, P. (2013). Embryonic alteration of motoneuronal morphology induces hyperexcitability in the mouse model of amyotrophic lateral sclerosis. *Neurobiology of Disease*, **54**, 116–126.

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Martinez-Silva, M. L., Imhoff-Manuel, R. D., Sharma, A., Heckman, C. J., Shneider, N. A., Roselli, F., Zytnicki, D., & Manuel, M. (2018). Hypoexcitability precedes denervation in the large fast-contracting motor units in two unrelated mouse models of ALS. *Elife*, **7**, e30955.

McCombe, P. A., & Henderson, R. D. (2010). Effects of gender in amyotrophic lateral sclerosis. *Gender Medicine*, **7**(6), 557–570.

Meehan, C. F., Moldovan, M., Marklund, S. L., Graffmo, K. S., Nielsen, J. B., & Hultborn, H. (2010). Intrinsic properties of lumbar motor neurones in the adult G127insTGGG superoxide dismutase-1 mutant mouse in vivo: evidence for increased persistent inward currents. *Acta physiologica (Oxford, England)*, **200**(4), 361–376.

Mousa, M. H., & Elbasiouny, S. M. (2020). Dendritic distributions of L-type Ca(2+) and SKL channels in spinal motoneurons: a simulation study. *Journal of Neurophysiology*, **124**(4), 1285–1307.

Mousa, M. H., & Elbasiouny, S. M. (2021). Estimating the effects of slicing on the electrophysiological properties of spinal motoneurons under normal and disease conditions. *Journal of Neurophysiology*, **125**(4), 1450–1467.

Okada, M., Yamashita, S., Ueyama, H., Ishizaki, M., Maeda, Y., & Ando, Y. (2018). Long-term effects of edaravone on survival of patients with amyotrophic lateral sclerosis. *eNeurologicalScience*, **11**, 11–14.

Pambo-Pambo, A. B., Durand, J., & Gueritaud, J. P. (2009). Early excitability changes in lumbar motoneurons of transgenic SOD1G85R and SOD1G93A-Low mice. *Journal of Neurophysiology*, **102**(6), 3627–3642.

Perrin, S. (2014). Preclinical research: Make mouse studies work. *Nature*, **507**(7493), 423–425.

Pieri, M., Albo, F., Gaetti, C., Spalloni, A., Bengtson, C. P., Longone, P., Cavalcanti, S., & Zona, C. (2003). Altered excitability of motor neurons in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Neuroscience Letters*, **351**(3), 153–156.

Pieri, M., Carunchio, I., Curcio, L., Mercuri, N. B., & Zona, C. (2009). Increased persistent sodium current determines cortical hyperexcitability in a genetic model of amyotrophic lateral sclerosis. *Experimental Neurology*, **215**(2), 368–379.

Pun, S., Santos, A. F., Saxena, S., Xu, L., & Caroni, P. (2006). Selective vulnerability and pruning of phasic motoneuron axons in motoneuron disease alleviated by CNTF. *Nature Neuroscience*, **9**(3), 408–419.

Quinlan, K. A., Lamano, J. B., Samuels, J., & Heckman, C. J. (2015). Comparison of dendritic calcium transients in juvenile wild type and SOD1(G93A) mouse lumbar motoneurons. *Frontiers in Cellular Neuroscience*, **9**, 139.

Quinlan, K. A., Schuster, J. E., Fu, R., Siddique, T., & Heckman, C. J. (2011). Altered postnatal maturation of electrical properties in spinal motoneurons in a mouse model of amyotrophic lateral sclerosis. *Journal of Physiology*, **589**(9), 2245–2260.

Saxena, S., Roselli, F., Singh, K., Leptien, K., Julien, J. P., Gros-Louis, F., & Caroni, P. (2013). Neuroprotection through excitability and mTOR required in ALS motoneurons to delay disease and extend survival. *Neuron*, **80**(1), 80–96.

Scott, S., Kranz, J. E., Cole, J., Lincecum, J. M., Thompson, K., Kelly, N., Bostrom, A., Theodoss, J., Al-Nakhala, B. M., Vieira, F. G., Ramasubbu, J., & Heywood, J. A. (2008). Design, power, and interpretation of studies in the standard murine model of ALS. *Amyotroph Lateral Scler*, **9**(1), 4–15.

Shibuya, K., Misawa, S., Arai, K., Nakata, M., Kanai, K., Yoshiyama, Y., Ito, K., Isose, S., Noto, Y., Nasu, S., Sekiguchi, Y., Fujimaki, Y., Ohmori, S., Kitamura, H., Sato, Y., & Kuwabara, S. (2011). Markedly reduced axonal potassium channel expression in human sporadic amyotrophic lateral sclerosis: An immunohistochemical study. *Experimental Neurology*, **232**(2), 149–153.

Shimizu, T., Fujimaki, Y., Nakatani-Enomoto, S., Matsubara, S., Watabe, K., Rossini, P. M., & Ugawa, Y. (2014). Complex fasciculation potentials and survival in amyotrophic lateral sclerosis. *Clinical Neurophysiology*, **125**(5), 1059–1064.

Shoenfeld, L., Westenbroek, R. E., Fisher, E., Quinlan, K. A., Tysseling, V. M., Powers, R. K., Heckman, C. J., & Binder, M. D. (2014). Soma size and Cav1.3 channel expression in vulnerable and resistant motoneuron populations of the SOD1G93A mouse model of ALS. *Physiological Reports*, **2**(8), e12113.

van Zundert, B., Peuscher, M. H., Hynynen, M., Chen, A., Neve, R. L., Brown, R. H., Jr., Constantine-Paton, M., & Bellingham, M. C. (2008). Neonatal neuronal circuitry shows hyperexcitable disturbance in a mouse model of the adult-onset neurodegenerative disease amyotrophic lateral sclerosis. *Journal of Neuroscience*, **28**(43), 10864–10874.

Wainger, B. J., Kiskinis, E., Mellin, C., Wiskow, O., Han, S. S., Sandoe, J., Perez, N. P., Williams, L. A., Lee, S., Boulting, G., Berry, J. D., Brown, R. H., Jr., Cudkowicz, M. E., Bean, B. P., Eggan, K., & Woolf, C. J. (2014). Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. *Cell Reports*, **7**(1), 1–11.

Zhang, M., Moller, M., Broman, J., Sukiasyan, N., Wienecke, J., & Hultborn, H. (2008). Expression of calcium channel CaV1.3 in cat spinal cord: Light and electron microscopic immunohistochemical study. *Journal of Comparative Neurology*, **507**(1), 1109–1127.

Additional information

Competing interests

The author declares that he has no competing interests. The content is solely the responsibility of the author and does not necessarily represent the official views of the National Institutes of Health.

Author contributions

SE was responsible for the conception and design of the work, the analysis and interpretation of data , and drafting and revising the article. SE approved the final version of the manuscript submitted for publication and agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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