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The metastability of the proteome of spinal motor neurons underlies their selective vulnerability in ALS

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Abstract

Amyotrophic lateral sclerosis (ALS) is a heterogeneous motor neuron disease with familial forms linked to numerous mutations in a range of genes. The resulting variant proteins, including SOD1, TDP-43, and FUS, disturb protein homeostasis in a variety of ways and lead to the formation of intracellular inclusion bodies that are characteristic of different neuropathological subtypes of the disease. These inclusions are made up of scores of proteins that do not appear at first to share obvious characteristics other than coaggregation. Recent evidence, however, suggests that these aggregating proteins can be characterized as being supersaturated in spinal motor neurons, as they exhibit cellular concentrations exceeding their solubilities. Here, we show that the average supersaturation of the entire spinal motor neuron proteome is greater than that of the ALS-resistant oculomotor neurons, suggesting that the vulnerability of spinal motor neurons is linked to the overall metastability of their proteome against aggregation. Consistently, ALS expression data suggest that affected neurons respond to pathology by transcriptional downregulation of supersaturated proteins, including specifically ion channels. These results identify a mechanism by which protein homeostasis imbalance leads to inclusion body formation in ALS, and to a disruption of other processes dependent on proteins that are supersaturated, thereby resulting in the dysfunctional excitability alterations observed in vivo.

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The metastability of the proteome of spinal motor neurons underlies their selective vulnerability in ALS

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a heterogeneous motor neuron disease with familial forms linked to numerous mutations in a range of genes. The resulting variant proteins, including SOD1, TDP-43, and FUS, disturb protein homeostasis in a variety of ways and lead to the formation of intracellular inclusion bodies that are characteristic of different neuropathological subtypes of the disease. These inclusions are made up of scores of proteins that do not appear at first to share obvious characteristics other than coaggregation. Recent evidence, however, suggests that these aggregating proteins can be characterized as being supersaturated in spinal motor neurons, as they exhibit cellular concentrations exceeding their solubilities. Here, we show that the average supersaturation of the entire spinal motor neuron proteome is greater than that of the ALS-resistant oculomotor neurons, suggesting that the vulnerability of spinal motor neurons is linked to the overall metastability of their proteome against aggregation. Consistently, ALS expression data suggest that affected neurons respond to pathology by transcriptional downregulation of supersaturated proteins, including specifically ion channels. These results identify a mechanism by which protein homeostasis imbalance leads to inclusion body formation in ALS, and to a disruption of other processes dependent on proteins that are supersaturated, thereby resulting in the dysfunctional excitability alterations observed *in vivo.*

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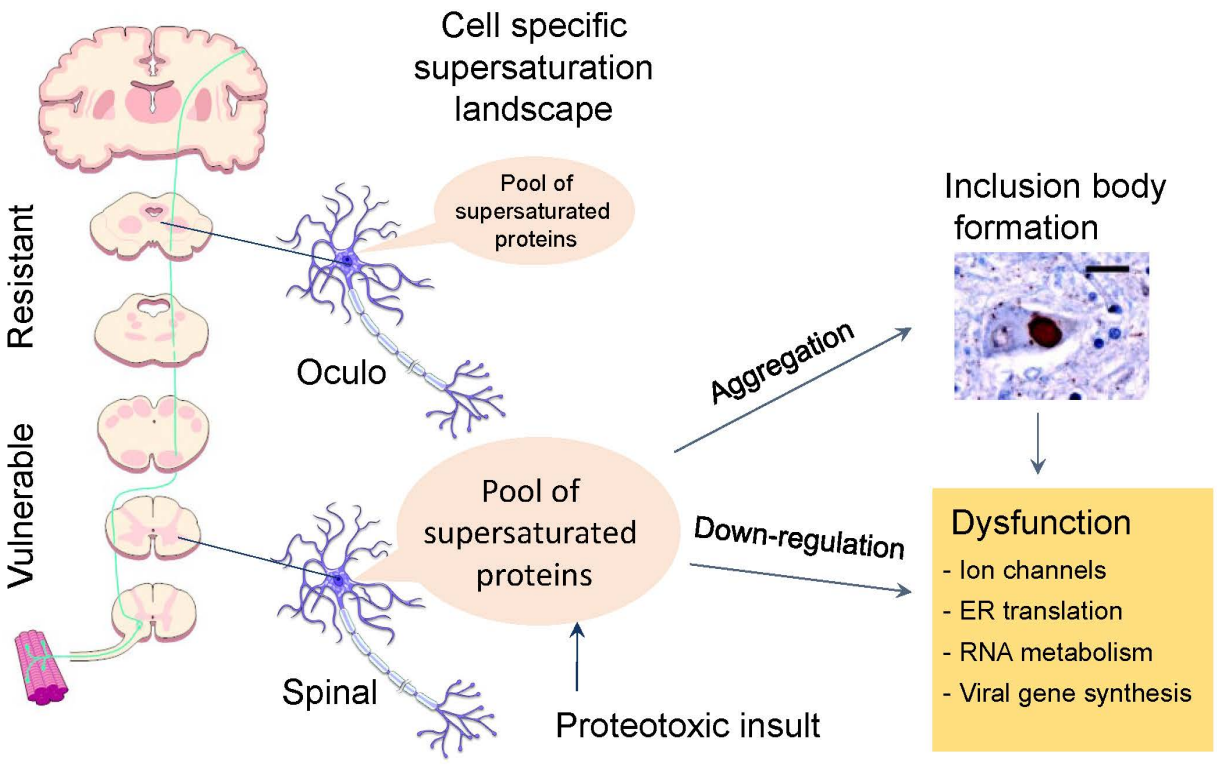
Keywords: Protein aggregation, protein misfolding, protein homeostasis, supersaturation, SOD1, TDP-43, FUS.



Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disorder in which the selective loss of upper and lower motor neurons in the motor cortex and spinal cord leads to impairment of muscle control, paralysis and eventually death. Protein aggregation and inclusion formation is associated with all forms of ALS, suggesting that protein misfolding is a common feature of the various forms of ALS (1-3). In this respect, ALS is similar to other neurodegenerative disorders, such as Alzheimer's, Parkinson's and Huntington's diseases (4-6), which are also characterised by the formation of aberrant protein deposits.

While ~90-95% of ALS cases are sporadic (sALS) and of unclear cause, the remainder of cases are inherited (familial ALS, or fALS) and can be linked to specific genetic mutations. Mutations in one or more of at least a dozen genes give rise to fALS, with most resulting in the aggregation of TDP-43, while in forms where TDP-43 pathology is absent FUS or SOD1 aggregates are present. In the context of ALS, the protein aggregate load correlates with areas of neuronal loss in the spinal cord (2, 7-10), and with cell death in culture (11), consistent with the idea that protein aggregates are intimately linked with motor neuron cell death. Recent work also suggests that disease progression may be a result of a prion-like propagation of protein misfolding and aggregation throughout the nervous system (12-14). While the precise reason for inclusion formation to be associated with most ALS cases is unclear, it is apparent that protein homeostasis is perturbed (15).

Protein aggregates consisting of a wide range of proteins are increasingly recognized as being common to a range of neurodegenerative diseases, an observation attributable to the fact that even in their native states many proteins can be unstable towards aggregation (5, 16-18). To understand why some proteins aggregate in

disease states whereas others remain soluble, we recently observed that many proteins in the proteome exceed their solubilities at the level at which they are expressed (19), that is, they are supersaturated (20, 21). In the specific context of ALS, we found that the combination of a spinal motor neuron expression profile and a high supersaturation score can explain many key features of the disease-specific protein inclusion fingerprint (19). In addition, we recently showed that the mutant SOD1 induced alterations in ubiquitin homeostasis were partly explained by an increase in ubiquitylation of supersaturated proteins (22). Previous work has found that across neurodegenerative disorders more generally, including Alzheimer's and Parkinson's diseases, proteins in major disease-associated pathways, as well as those that coaggregate within inclusion bodies, tend to be supersaturated (21). It has also been recently shown, in the case of Alzheimer's disease, that the characteristic progression of pathology across brain tissues is recapitulated by a protein expression signature in healthy brains of aggregation-prone proteins (23), which is also responsible for the selective vulnerability of specific neuron types (24). Collectively these data are consistent with the notion that protein homeostasis breakdown and inability of the cell to deal with supersaturated proteins is associated with neurological disorders (5, 20, 21, 23, 24).

Recently, it has been proposed that the downregulation of supersaturated proteins in Alzheimer's disease may limit their aggregation in response to compromised protein homeostasis (25). In the present study, we examined experimental information acquired from expression analysis of vulnerable motor neurons in healthy and diseased tissue (26-28). We aimed specifically to determine the relationship between protein supersaturation, cell-specific vulnerability and the transcriptional changes that occur during ALS. We found distinct differences in supersaturation between resistant oculomotor neurons and vulnerable spinal motor neurons. Moreover, genes downregulated in ALS generally correspond to metastable proteins at risk of aggregation, as they are supersaturated, while those that are upregulated correspond to proteins that are within their solubility limits. In the long term, however, while the downregulation of supersaturated proteins may represent a mechanism to limit aggregation, the chronic decrease of vital proteins such as ion channels may in turn lead to neuronal dysfunction and ultimately death.

Methods

Identification of co-aggregating proteins. Co-aggregating proteins in ALS were identified as in (19). Proteins were only included if published data clearly showed co-localisation in human post-mortem tissue.

Identification of axonal channels and transporters. Previous work using proteomics identified axonal proteins from rat neuronal primary cultures (29). A list of all axonal channels, pumps and transporters was generated from that of all identified axonal proteins.

Calculation of Zyggregator scores. Zyggregator scores were calculated as described in (21).

Calculation of supersaturation scores. Motor neuron specific supersaturation scores were calculated for unfolded states from transcriptomic data (denoted σ^u) as described in (19) using (28) (GSE20589) based on the original method outlined in (21). For the calculation of spinal motor neuron and comparable oculomotor neuron specific supersaturation scores, microarray mRNA expression levels were obtained from (26) (GSE40438). Up and downregulated genes were previously identified in microdissected anterior horn spinal cord material (27) and supersaturation scores were generated for the corresponding proteins from healthy motor neurons (GSE20589) as above. To eliminate the dominance of a small number of proteins on the overall supersaturation score we compared median scores in our analysis rather than mean scores. To compare median values between two independent groups we used the Mann Whitney U test.

Calculation of fold changes. Fold changes were calculated as described in (21) as the linear difference between the logarithmic medians of two sets. The linear fold difference d between the medians of the supersaturation scores of the control set C and experiment set E being tested was defined as:

$$d = 10^{median(E) - median(C)} \qquad (S1)$$

Results

Vulnerable spinal motor neurons have a metastable proteome

We have shown previously that ALS inclusions are formed by proteins that tend to be supersaturated under physiological conditions (19). These particular proteins were found to be distinguished from the proteins that form the functional network of normal interaction partners of the ALS-associated proteins SOD1, TDP-43 and FUS by their supersaturation levels when calculated using expression values in motor neurons, but not when averaged over several tissues.

Here we investigated whether the supersaturation levels of proteins observed to form aggregates in ALS (ALS aggregators) could explain why spinal motor neurons are lost while oculomotor neurons are spared in the disease. To perform this analysis, we calculated the supersaturation scores using mRNA expression levels from non-diseased microdissected motor neurons and oculomotor neurons (GSE40438). We found that the supersaturation scores of the list of all known proteins (co-aggregators; Supplementary Table 1) associated with ALS inclusions (19), while greater in spinal (n = 64) compared to oculomotor neurons (n = 64), are not significantly different (σ_u, p=0.57; **Figure 1A**).

Shifting attention from the aggregating proteins to their protein homeostasis regulation we next asked if, rather than focusing on a small subset, analysis of the entire transcriptome would distinguish vulnerable from resistant neurons. Consistent with the fact that motor neurons are selectively vulnerable in ALS, we found that the supersaturation scores of non-diseased spinal motor neurons (n = 16,571) are significantly elevated relative to those of oculomotor neurons (**σ_u**: oculomotor neuron proteome on average 0.85-fold relative to spinal motor

neurons, $p<0.0001$, n = 16,571, U = $1.29x10^8$; **Figure 1B, Supplementary Table 2**). This difference is statistically significant and its relatively small value is consistent with its possible role as a subtle but persistent driving force behind the slow progression of the disease. To discover which pathways were most vulnerable in spinal motor neurons we first identified the most supersaturated proteins (top 2%, Supplementary Table 3) and then ranked these in terms of supersaturation differences ($\boldsymbol{\sigma}_u$ spinal motor neuron – $\boldsymbol{\sigma}_u$ oculomotor neuron). This procedure provided a list of 95 most supersaturated proteins in spinal motor neurons relative to oculomotor neurons. Next, a gene ontology analysis indicated that these proteins are enriched in processes such as endoplasmic reticulum (ER) co-translation, mRNA metabolism, viral metabolism and cytosolic translation (Supplementary Table 4).

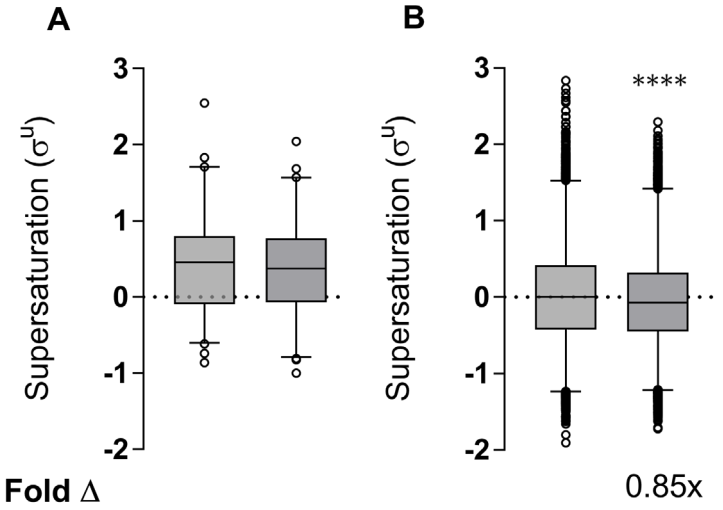


Figure 1. Vulnerable motor neurons have a supersaturated proteome. (**A)** The median supersaturation scores calculated for unfolded ($\boldsymbol{\sigma}_u$) states of proteins are shown for the combined set of co-aggregators associated inclusions, calculated from spinal (light) and oculomotor neurons (dark). (**B)** Supersaturation scores when unfolded states of proteins were calculated using the entire set of mRNA expression levels derived from non-diseased oculomotor (dark) and spinal motor neurons (light) (GSE40438). Fold **Δ** refers to the change in supersaturation score from spinal motor neurons. Boxplots extend from the lower to the upper quartiles, with the internal lines referring to the median values. Proteins identified in the literature as co-localised to all ALS inclusions are from (19). Statistical significance was assessed by the one-sided Wilcoxon/Mann-Whitney U test (****$p < 0.0001$).

Genes transcriptionally downregulated in ALS encode metastable proteins

Previous work showed that many transcriptional changes identified in aging and in Alzheimer's disease are associated with protein aggregation (25), consistent with the idea that the protein homeostasis system responds to maintain metastable proteins in solution by reducing their concentration. To test whether ALS co-aggregators are associated with downregulated genes we compared the expression levels of the genes altered in the proteome in ALS (583/25,272 transcripts; Supplementary Table 5) to those encoding co-aggregators. We found that downregulated genes were over-represented among co-aggregators (**Figure 2A**; 7/64). In contrast, only a small number of genes whose proteins are found in inclusions were found to be upregulated in ALS (**Figure 2A**; 1/64 compared to 561/25,272 in the whole transcriptome, Supplementary Table 6).

We reasoned that proteins at risk of aggregation in motor neurons might be downregulated during the proteome stress induced by ALS pathology. To test this hypothesis, we calculated the metastability to aggregation of proteins, either down or upregulated in ALS, in terms of their supersaturation scores (σ^u; calculated using non-diseased mRNA levels), which represent their risk of aggregation at the concentrations at which they are normally expressed (21). We found proteins corresponding to genes downregulated in ALS (n = 553) to be 1.7-fold (P <0.0001, U = 3.25×10^6) more supersaturated than those for the proteome as a whole (n = 17,835) (**Figure 2B**). In contrast, we found proteins encoded by genes upregulated in ALS (n = 528) to be less supersaturated than those downregulated (0.6-fold compared to downregulated genes, P < 0.0001, U = 8.4×10^4) and to the proteome as a whole (0.9-fold, P = 0.0033, U = 4.38×10^6) (**Figure 2B**).

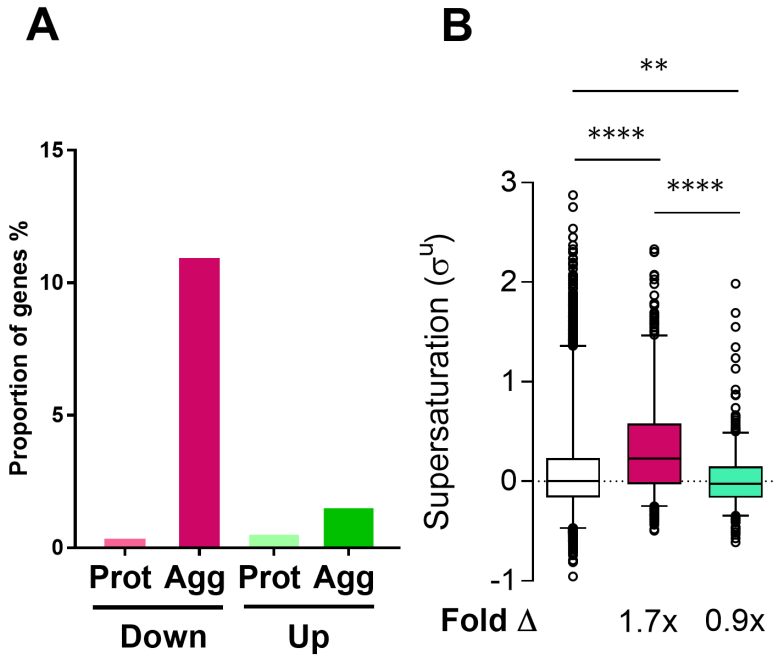


Figure 2. In ALS, the metastability of proteins to aggregation is correlated with the downregulation of the corresponding genes. **(A)** Proportion of genes transcriptionally down or upregulated in ALS in the whole proteome (Prot) or for the co-aggregators (Agg). **(B)** Metastability levels, assessed by supersaturation scores, for proteins associated with differentially expressed genes in the whole proteome (white), downregulated in ALS (red), and upregulated in ALS (green). Median fold difference in supersaturation from the proteome is indicated by Fold Δ. ****P ≤ 0.0001, one-sided Wilcoxon/Mann–Whitney U test.

Axonal ion channels and transporters are metastable to aggregation and transcriptionally downregulated in ALS

Altered excitability has been observed in ALS patients (30, 31), and in animal and cell models (reviewed in (32)), and recent work suggests that this altered excitability is consistent with a widespread decrease in the number of Na^+ and K^+ ion channels (33). Given that dysfunction occurs in a distal to proximal fashion (34), we hypothesised that axonal ion channels might be at particular risk of aggregation in ALS. Using a list of axonal ion channels and transporters (Supplementary Table 7) generated from proteomic analysis of axons from primary neuronal cultures (29), we examined their supersaturation scores generated from healthy motor

neuron expression data. We found that the supersaturation scores of the axonal ion channels and transporters (n = 68) were indeed significantly supersaturated when compared to the proteome (n = 17,835) as a whole (2-fold, $P < 0.0001$, $U = 2.6 \times 10^5$; **Figure 3A**).

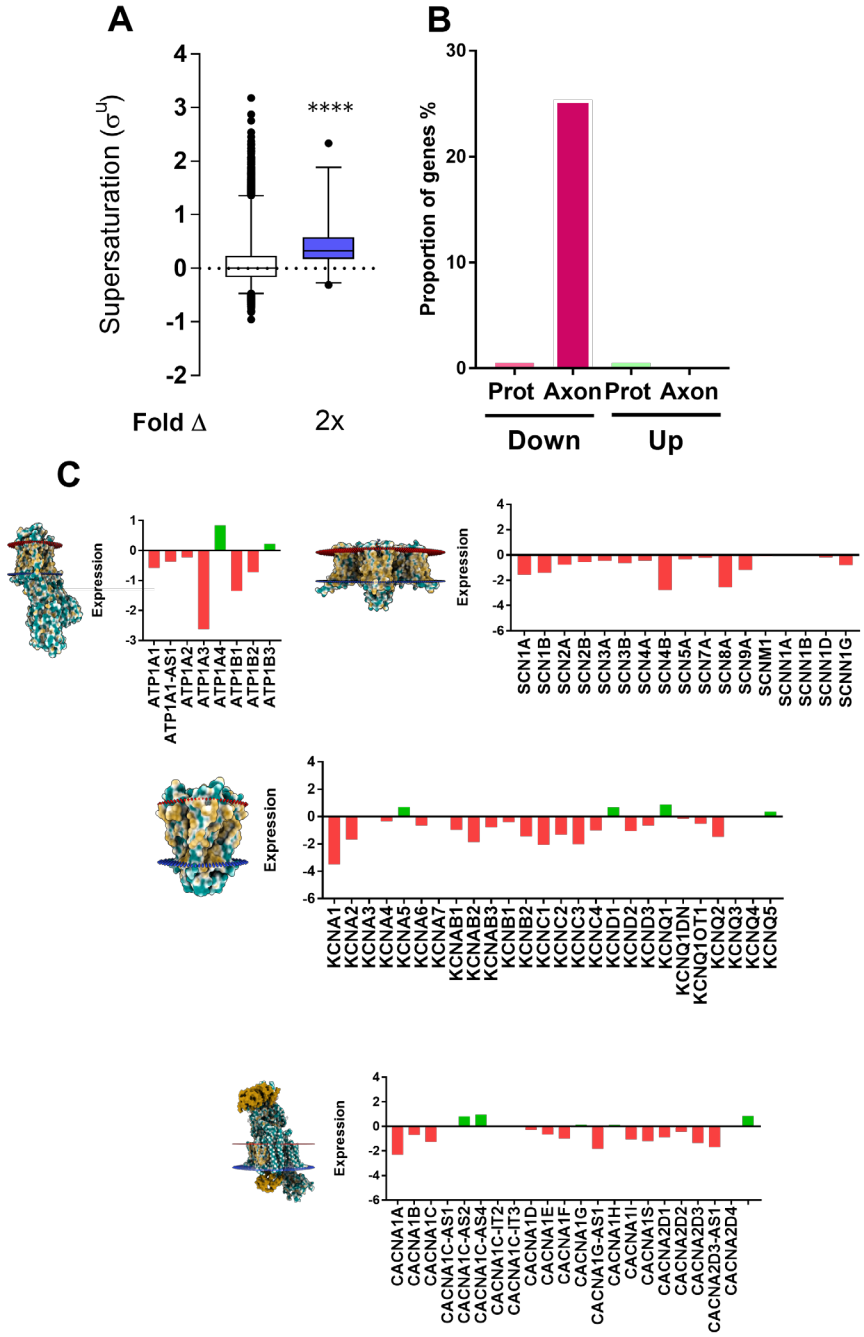


Figure 3. In ALS, the metastability to aggregation of axonal ion channels and transporters is correlated with their downregulation. (A) Metastability levels, assessed by supersaturation scores, for the proteome (white) and axonal channels and transporter proteins (blue). The median fold difference in supersaturation is indicated by Fold Δ. ****$P \leq 0.0001$, one-sided Wilcoxon/Mann–Whitney U test. (B) Proportion of genes transcriptionally down or upregulated in ALS in the whole proteome (Prot) or axonal ion channels and transporters (Axon). (C) Relative levels of ATP dependent pumps, voltage gated Ca2+ channels, K+ channels and Na+ channels that were detected in microdissected ALS spinal tissue (19).

Given our observation that proteins at risk of aggregation in motor neurons are downregulated in ALS, we reasoned that axonal ion channels and transporters might be downregulated compared to the proteome as a whole. We found that transcriptionally downregulated genes were overrepresented among axonal ion channels and transporters with ~25% of this set (17/67) of proteins being significantly downregulated. In contrast, there were no genes encoding channel and transporter proteins that were transcriptionally upregulated in ALS (**Figure 3B**). To expand these findings further we examined the complete sets of ATP dependent Na^+/K^+ pumps, voltage gated Ca^{2+} channels, K^+ channels and Na^+ channels, not just those restricted to axonal proteins. These sets of transcripts were predominantly downregulated, consistent with a widespread lowering of the expression levels of channels, pumps and transporters in the ALS condition (**Figure 3C,** Supplementary Table 8).

Discussion

It has been recently reported that the proteins associated with ALS inclusions tend to be metastable to aggregation because they are supersaturated, specifically in motor neurons (19). Here we have found that the supersaturation level of the entire proteome differentiates vulnerable from resistant motor neurons, and observed that a cellular response to the intrinsic metastability of the proteome is the transcriptional downregulation of supersaturated genes. In support of these conclusions, we have observed a relationship between the genes up and downregulated in ALS with their metastability, and the enrichment of downregulated genes in those proteins that co-aggregate in inclusions. Analysis of the transcriptional response to the collapse of protein homeostasis associated with ALS has allowed us to address a central question regarding the physical symptoms of ALS, specifically the way in which protein homeostasis imbalance affects the alterations in motor neuron excitability measured in patients.

In our previous work two different supersaturation scores were used to evaluate the risk of proteins to aggregate from two pools - the unfolded states and the native states. The risk of aggregation is different in these two states because in the folded state the most aggregation-prone regions tend to be buried in the core of the structure, and in the unfolded state the core is exposed (35). Due to availability of datasets, here we have used only the unfolded score, which does not take into account protein levels and so may underestimate the supersaturation of proteins with long half-lives. However, while the two calculations represent different aspects of the proteome, our previous work suggests that similar trends appear regardless of the score used, consistent with the idea that proteins should be resistant to aggregation in all the states that they populate.

Our analysis identified several pathways that were most supersaturated in spinal motor neurons compared to oculomotor neurons, suggesting that these pathways are particularly at risk to proteotoxic stress in motor neurons. Most significantly enriched amongst the most supersaturated were proteins associated with ER

protein synthesis, mRNA metabolism and viral gene expression. Strikingly, the pathways identified are well-established features of ALS pathology, in particular ER stress and mRNA metabolism dysfunction. Further, our data that suggests viral gene expression pathways are at risk may predict that an emerging aspect of ALS pathology, that is the activation of human endogenous retroviruses, also induces protein homeostasis imbalance. Together, these pathways are particularly at risk in motor neurons and could be potential therapeutic targets.

The findings reported previously along with those reported here suggest that the widespread transcriptional downregulation of genes encoding metastable proteins at risk of aggregation may represent a cellular strategy to combat disruptions in protein homeostasis. However, prolonged downregulation of important genes may lead to disruption of pathways at risk of aggregation, and result in a loss of certain functional processes.

Of particular interest is the fact that altered axon excitability has been observed in ALS patients (30, 31), and recent data predicts that this altered excitability is associated with a decrease in both Na^+ and K^+ channels (33). Supporting the notion that axons are particularly vulnerable is the fact that the distance from the cell body is an important factor in this context, as dysfunction occurs in a distal to proximal fashion (34). Mounting evidence suggests that the changes that are occurring distally in the axons are amongst the earliest pre-symptomatic functional and pathological changes (reviewed in (36)). In mouse models these changes precede, and can be independent of, the loss of cell bodies (reviewed in (37)). Channel alterations are also measured in motor neurons derived from human induced pluripotent stem cells (iPSCs) generated from fibroblasts obtained from ALS patients with *TARDBP* or *C9ORF72* ALS mutations (38). A recent analysis has indicated that ALS patient iPSC-derived motor neurons possess an initial hyperexcitability, with a subsequent and progressive loss in action potential firing (38). The authors concluded that loss of ion channels may contribute to the initiation of downstream degenerative pathways that ultimately lead to motor neuron loss in ALS. What causes the apparent loss of these ion channels in the motor neurons has, however, remained to be investigated. This question is particularly relevant considering that while it is clear that electrophysiological changes are intimately linked with ALS pathology, the underlying molecular alterations that result in such physiological outcomes remains unknown. Here we have presented an analysis that shows that the proteome of spinal motor neurons are particularly at risk to protein homeostasis imbalance, and that these neurons respond to such stress by downregulating proteins at risk of aggregation. One potential consequence of this response is, however, the loss of axonal channels resulting in electrophysiological dysfunction.

The present work provides support for the view that a progressive impairment of protein homeostasis is associated with the development of ALS pathology. This system-level impairment could be the result of a variety of causes, including expression of one or more aggregation-prone proteins, genetic lesions to key components of the protein homeostasis network or changes due to aging such as downregulation of protein homeostasis networks. Such dysfunction puts the solubility of the motor neuron proteome at risk and could result in widespread aggregation. Our analysis suggests that, in order to limit protein aggregation, motor

neurons respond by downregulating specific metastable proteins. This response strategy, however, is not sustainable over long periods of time, as the prolonged downregulation of supersaturated proteins may lead to cellular dysfunction, including the downregulation of ion channels and subsequent excitability changes observed in ALS patients.

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