Neuromuscular junction mitochondrial enrichment: a "double-edged sword" underlying the selective motor neuron vulnerability in amyotrophic lateral sclerosis

Topaz Altman, Eran Perlson*

Motor neurons are highly polarized cells, with long axons that extend to more than 1 m in the adult human. The axons further arborize into a specialized synaptic compartment, the motor unit, containing up to 2000 neuromuscular junctions (NMJs). While the size of other neuronal synapses can be up to 1 µm, the NMJ is much larger and can reach 10–30 µm (Jones et al., 2017). The vast size of the motor unit requires motor neurons to evolutionally adapt and supply this distal portion with a sufficient amount of ATP, as well as to replenish the axonal protein pool in order to maintain their synapses. To address its substantial energetic needs, the NMJ is enriched with a vast network of mitochondria. This is supported by ultrastructural studies using electron and confocal microscopy, which revealed that only ~50% of active synapses in the adult rodent central nervous system (CNS) contain any mitochondria, whereas all NMJs are enriched with a tightly packed mitochondrial network (Misgeld and Schwarz, 2017; Altman et al., 2019). The mechanisms leading to this distinct enrichment, as well as its implications and functional meaning in the context of neurodegeneration, remain poorly understood. Here, we discuss and suggest a possible explanation for how the mitochondrial enrichment of the NMJ can be relevant to the selective vulnerability of motor neurons in motor neuron diseases, and in particular amyotrophic lateral sclerosis (ALS).

To better understand why mitochondrial enrichment can be a source of vulnerability rather than to lead to natural resistance, we first need to acknowledge the unique role mitochondria play in NMJ function and homeostasis. Mitochondrial function and activity are known to be important for various neuronal functions, such as calcium buffering, neurotransmitter release, and ATP provision (Misgeld and Schwarz, 2017). Despite the fact that several metabolic processes in the axon, such as axonal transport (Zala et al., 2013) and synaptic vesicular fusion, were shown to heavily depend on glycolysis, the uniqueness of the NMJ regarding its mitochondrial enrichment was not directly addressed. In motor neurons, as we recently showed using an *in vitro* co-culture system, there is a bias towards distal accumulation of mitochondria, compared with other neurons such as sympathetic neurons (Altman et al., 2019). This accumulation seems to occur in both axons and specifically in synapses. Furthermore, mitochondrial enrichment in the NMJ is not random and provides a functional meaning, since the ATP in the NMJ was found to be largely derived from mitochondrial respiration, whereas a substantial amount of the ATP in the sympathetic synapse was derived from glycolysis (Altman et al., 2019). The higher dependency of the NMJ on mitochondrial respiration (Carrasco et al., 2012; Altman et al., 2019) is in contrast to other CNS synapses, which were shown to largely depend on glycolysis to sustain synaptic release and neurotransmitter recycling. Taken together, this suggests that mitochondria may play a more regulatory role in other synapses, providing ATP at a time of need, whereas in the NMJ, mitochondrial respiration is constitutively active.

Other than the metabolic role of ATP generation, the enrichment of mitochondria in the NMJ is also associated with cellular processes important for synaptic maintenance, such as protein synthesis and degradation (Misgeld and Schwarz, 2017). As previously mentioned, most of the motor neuron cytoplasm is found in the distal arborized axonal compartment and its multiple NMJs (Misgeld and Schwarz, 2017). How can a single neuronal nucleus supply the proteins necessary for this extended axon and synapses? This fundamental question in the field of axonal biology has been debated for the last two decades; however, recent evidence might provide a decisive answer. The old hypothesis, claiming for lack of axonal protein synthesis has been refuted by numerous papers, proving the vital role of local protein synthesis in axons. First, papers described the presence of mRNA in axons, and later, papers reported the active synthesis of this mRNA to proteins in growing axons, injured axons, and as a response to environmental cues (Misgeld and Schwarz, 2017; Cioni et al., 2019; Hafner et al., 2019). Recently, various works have also shown the presence of local protein synthesis in axons and synapses in healthy adult animals (Hafner et al., 2019). This supports a regulatory role of local synthesis for neuronal maintenance as well as a response to stress. However, currently, little is known about how axonal protein synthesis contributes to mitochondrial health, which might be crucial in long and complex neurons such as motor neurons. Nevertheless, some evidence supports the finding of nuclear encoded mitochondrial mRNAs in axons, and even that those mRNAs are enriched in motor neuron axons (Maciel et al., 2018). Furthermore, some mRNAs are translated in proximity to axonal mitochondria (Cioni et al., 2019). The mechanism governing the axonal transport of these mRNAs remains elusive, since some of them were found to be directly bound by endosomes; however, other alternative mechanisms are possible such as direct mitochondrial binding. In any case, local protein synthesis contributes to mitochondrial health, since nuclear encoded mitochondrial genes are the most abundant mRNAs in the axonal transcriptome of human motor neurons (Maciel et al., 2018). The exact mechanism by which nuclear encoded mitochondrial mRNAs reach the synapse, and their synaptic translation process are still under investigation; still, there is no doubt about their presence and importance.

Mitochondria's role in maintaining healthy motor neuron axons and NMJs can also be vital to better understand important aspects of motor neuron diseases, such as ALS. ALS specifically targets and leads to the degeneration of the upper and lower motor neurons, which results in muscle wasting and death from respiratory failure within 3–5 years of diagnosis (Cook and Petrucelli, 2019). The sequence of events leading to axonal degeneration and motor neuron death is still under intense investigation, but a key finding described by many researchers is the vulnerability of the NMJs, which are considered to be a primary site of onset following the "dying-back" hypothesis. This hypothesis is supported by several works which found that NMJs degenerate before the death of the spinal cord motor neurons. This suggests that an unknown mechanism sensitizes the NMJs to be more vulnerable to the cellular toxicity implicated in the disease, and specifically to mitochondrial stress (Carrasco et al., 2012).

Several mechanisms were suggested to play a key role in ALS pathogenesis, some of which are glutamate excitotoxicity, defects in axonal transport, protein aggregation, nuclear pore complex abnormalities, and aberrant RNA metabolism (Cook and Petrucelli, 2019). Substantial evidence supports the role of each of these cellular mechanisms and without doubt, they all play a key role in the disease. However, they fail to explain why other neurons with increased activity (such as some neurons in the CNS) or with extended length (e.g., sensory and sympathetic neurons) are spared, whereas motor neurons and the NMJs are particularly vulnerable. Another key mechanism of ALS, which was debated (to some extent), is mitochondrial toxicity (Smith et al., 2017). This mechanism is probably the oldest, since it was known already after the first ALS mutation in the *SOD1* gene was discovered in 1993. *SOD1* mutation causes a toxic gain of function, leading to defects in mitochondrial transport and the accumulation of toxic protein aggregates in the mitochondria (Carrasco et al., 2012). Later, as other ALS mutations were found, most if not all of them were also found to directly disrupt mitochondrial function. The predominantly nuclear RNA binding protein TAR DNA-binding protein 43 (TDP-43), whose cytoplasmic aggregation is a hallmark of the disease, since it is found in ~95% of patients (Hergesheimer et al., 2019), can be localized to the mitochondria and drives neuronal toxicity when mutated. Fused in Sarcoma, another nuclear RNA binding protein, was shown to induce mitochondrial fission and to reduce mitochondrial activity. The *C9ORF-72* gene, the most common genetic cause of ALS and the leading genetic mutation in ALS sporadic cases, was shown to induce the formation of toxic dipeptides that can bind a mitochondrial complex V protein and induce mitochondrial damage in ALS patient brains and in mouse neurons (Choi et al., 2019). Taken together, accumulating evidence directly links ALS to mitochondrial defects, thus suggesting that mitochondrial toxicity can lead to motor neuron degeneration in the disease (Smith et al., 2017; Cook and Petrucelli, 2019).

It is important to mention that many neurodegenerative diseases, other than ALS, were also linked to mitochondrial alterations.

To date, Alzheimer's, Parkinson's, and Huntington's diseases were all shown to include mitochondrial toxicity as a hallmark of the disease. So how can the mitochondria serve as a source of specific motor neuron vulnerability, if neurodegenerative diseases, which harm other neuronal populations, stem from the same phenomena?

Other than the previously mentioned spatial enrichment of mitochondria in the NMJs, an additional answer might be related to another hallmark of ALS mentioned earlier, the fundamental mechanism underlying TDP-43 cytoplasmic mis-localization in ALS. TDP-43 is primarily a nuclear DNA and RNA binding protein; it plays key roles in transcription, RNA splicing, mRNA transport, and translational regulation (Hergesheimer et al., 2019). In ALS, TDP-43 was shown to mis-localize to the cytoplasm in 95% of human patients. Once in the cytoplasm, TDP-43 can form pathological amyloid-fiber-like aggregates, and it can cause defects in the formation of membranelles organelles, namely, stress granules. This dangerous function of TDP-43 is induced by the existence of a low-complexity domain in the protein sequence, leading to a pathological phase-separation of the protein and the formation of cytoplasmic granules that cannot be dissolved by cell protein degradation mechanisms (Hergesheimer et al., 2019). Not surprisingly, almost all of the genetic TDP-43 mutations that can lead to familial ALS are found in the protein low-complexity domain (Hergesheimer et al., 2019). This phenomenon of TDP-43 aggregation is not unique to ALS; it is also found in cases of frontotemporal dementia and Alzheimer's disease. However, unlike those diseases, TDP-43 aggregation is much more common in ALS, and it occurs in almost every ALS case that does not originate from SOD-1 mutation (Cook and Petrucelli, 2019; Hergesheimer et al., 2019). TDP-43 toxicity is also attributed to some extent to a loss of nuclear function of the protein, but this does not contradict gain-of-function mechanisms to be present as well. The implications of TDP-43 cytoplasmic aggregation and loss of function are not fully understood; however, recent evidence suggests that it can lead to defects in RNA metabolism, and subsequently, to disrupted mRNA splicing, the formation of aberrant RNA species in the cytoplasm, and ultimately to altered protein translation and degradation.

TDP-43 cytoplasmic aggregation and mitochondrial defects are both known drivers of ALS pathogenicity. However, it has not yet been suggested to combine these key findings to investigate the selective vulnerability of motor neurons in ALS. Future studies should focus on the connection between these mechanisms and their unique influence over motor neurons. The probable dependency of NMJ mitochondria on local protein translation, described earlier, can make motor neurons extremely vulnerable to defects in the transport, splicing, or degradation of axonal mRNA. Furthermore, the enrichment of nuclear encoded mitochondrial transcripts in motor neuron axons, together with the accumulation of mitochondria in the NMJ, might lead to synaptic mitochondrial dysfunction which serves as a source of cellular vulnerability, leading ultimately to NMJ disruption and axonal degeneration (**Figure 1**). In summary, this conjoined process might explain for the first time the mechanism underlying the selective vulnerability of motor neurons in ALS.

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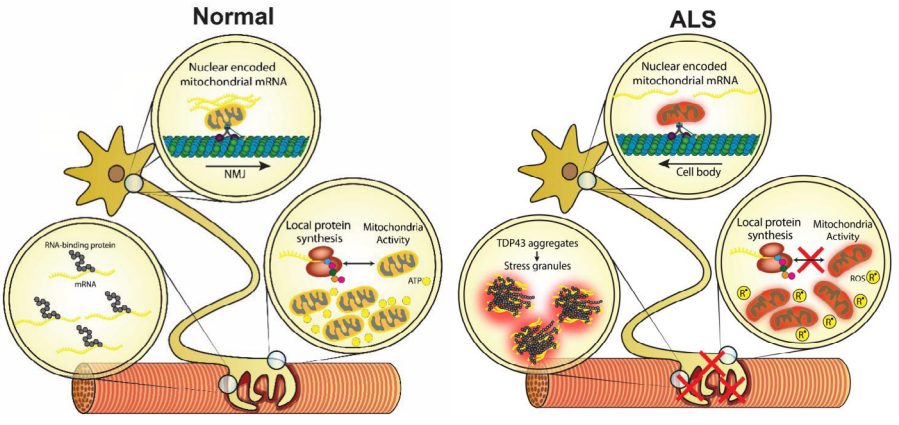

Figure 1 | **Hypothesized model for how TDP aggregation hinders axonal translation of nuclear encoded mitochondrial proteins, leading to NMJ disruption.**
Under normal conditions, motor neuron mitochondria accumulate at the NMJ, and are dependent on the transport of nuclear encoded mitochondrial mRNA from the nucleus to the synapse, carried by RNA binding proteins, endosomes, or by the mitochondria itself. The local translation of nuclear encoded mitochondrial transcripts in the NMJ is vital for synaptic mitochondrial function and for ATP generation, which is highly dependent on mitochondrial function in the NMJ. In ALS, cytoplasmic and possibly axonal aggregates of RNA binding proteins (especially TDP-43) can form RNA granules that encapsulate mRNAs, making them unavailable for translation. This process can lead to decreased local protein synthesis in the NMJ and to mitochondrial toxicity through decreased translation of nuclear encoded mitochondria proteins. Finally, transport of damaged mitochondria retrogradely to the cell body occurs, further decreasing the anterograde transport of nuclear encoded mitochondrial mRNA to the NMJ, which exacerbates the degeneration process. ALS: Amyotrophic lateral sclerosis; NMJs: neuromuscular junctions; TDP-43: TAR DNA-binding protein 43.

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