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Muscle and Its Neuromuscular Synapse – Players in the Pathogenesis of Motor Neuron Disease

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Commentary

Motor Neuron Disease (MND) of which Amyotrophic lateral sclerosis (ALS) is the most common form, is a devastating disorder where approximately 80% of patients die within 3-5 years of diagnosis. The highly variable clinical presentation, course, and prognosis between individuals suggests that a variety of factors underlie the pathogenesis of the disease [1,2]. A central event in cases of MND with lower motor neuron involvement is the withdrawal of the motor nerve terminal from its target muscle cells [1,3]. The resulting decline in the integrity of neuromuscular connections leads to progressive muscle paralysis and death, suggesting that disrupted communication between skeletal muscle and its innervating motor neuron is a key driver of MND/ALS pathology. While this idea is at odds with the proposed pathogenic mechanisms originating within the CNS [4-6], and the majority consensus that MND is driven by motor neurons, it is becoming clear from several MND research groups that skeletal muscle is a key contributor to MND [7-10]. Indeed, evidence from our group and others highlight defective signaling at, and maintenance of nerve-muscle interactions in MND. Whether the destruction of nerve-muscle connections is driven by disturbed retrograde feedback to the motor neuron due to its altered physiological properties, or by perturbed bidirectional synaptic signaling at the neuromuscular synapse remains an area of investigation [7-15]. The latter possibility has recently been investigated by our group [16] using muscle obtained from MND and non-MND donors and is the subject of this commentary.

The study sought an understanding of the cellular and molecular mechanisms that contribute to the loss of neuromuscular connections in MND patients. To achieve this goal, we collected muscle biopsies that were donated with informed consent from 17 MND and 16 non-MND donors. These biopsies were used to examine neuromuscular synapses and muscle pathology, using confocal, widefield-light and electron microscopy. In addition, we isolated muscle stem (satellite) cells and developed two different cell culture assays to test the ability of MND muscle cells to form postsynaptic specializations in response to either human motor axons, or to the pro-synaptic molecule neural-agrin that is released from the motor nerve ending [17].

In biopsies of MND muscle, microscopy revealed evidence of type I muscle fiber grouping within the *vastus lateralis*, consistent with denervation/reinnervation activity [18,19]. In brief, ~40% of surface muscle fibers in non-MND samples were type I (slow twitch) and 60% type II (fast twitch; *n*=3). By contrast in MND muscle, slow twitch muscle fibers had increased to ~60% with corresponding loss of fast twitch

muscle fibers (down to 40%; *n*=6). The fiber type grouping seen is consistent with clinical observations of expanding motor units (i.e., sprouting of axons from one motor neuron to substitute for loss of neighboring motor axons; [20,21]). There was also a small amount of muscle atrophy (presence of small angular muscle fibers), but this was not widespread, consistent with early-stage disease.

Next, we conducted cell and molecular morphological analyses of neuromuscular synapses from MND and non-MND donors. Intact bundles of muscle fibers were studied by immunolabelling and 3D confocal microscope reconstruction to reveal details of neuromuscular synapses. This approach permitted quantitative comparison of the pre- and post-synaptic changes occurring at these synapses between MND cases and controls. Importantly, we were able to sample neuromuscular synapses from an approximately equal number of MND and non-MND muscle samples, the first time such a comparison has been made. These analyses revealed several synaptic differences in MND muscle that arose above the general heterogeneity of synaptic morphology. These included the following: 1) terminal axonal thinning; 2) dispersal of postsynaptic acetylcholine receptor (AChRs) clusters around the motor nerve terminal, which could be due to flattening of the post synaptic membrane from its normal "cup" shape, or a true loss of AChRs from the muscle membrane; 3) nerve terminal axonal sprouting; 4) a 50% drop in the extent to which motor nerve terminals covered the specialized post-synaptic membrane, consistent with progressive withdrawal of the motor nerve terminal from muscle (i.e., denervation); and 5) a delocalization of muscle specific kinase (MuSK) from the postsynaptic region of the muscle membrane. Qualitative electron microscopic examination of MND neuromuscular synapses revealed evidence of flattened postsynaptic membrane not occupied by a motor nerve terminal and nerve terminal mitochondria with clear vacuoles, structural alterations that have also been reported by other researchers [11,22,23]. By contrast, the synaptic basal lamina that overlies the post-synaptic membrane including its junctional infolds appeared intact at MND neuromuscular junctions.

Since MuSK is essential for neuromuscular synapse formation, we wanted to know whether the delocalization of MuSK away from the post-synaptic region might help explain the apparent loss of AChRs at MND neuromuscular synapses. To investigate this issue under controlled conditions, two *in vitro* bioassays were developed. The first assay involved a human motor neuron primary muscle co-culture system whereby motor neurons derived from healthy human stem cells were cultured in chamber one of a microfluidic device and allowed to send their motor axons into a second chamber where they could interact with multinucleated muscle fibers (myotubes) derived from MND or non-MND muscle biopsies. The muscle cells were generated from isolated muscle satellite cells from biopsies of MND or non-MND donors. In the second *in vitro* bioassay, MND and non-MND myotubes were exposed to neural-agrin, which signals via MuSK to induce AChR clustering. The assay readout for each assay was the degree to which the myotubes responded to form large clusters of AChRs (described below).

Examination of motor neuron muscle microfluidic co-cultures revealed that muscle cells sourced from non-MND donors displayed large AChR clusters near ingrowing axons, as expected (*n*=4). By contrast, 3 out of the 4 of the MND muscle cultures failed to form large AChRs clusters in the presence of the motor axons. They displayed only small AChRs. The one exception was for muscle cells derived from MND patient 8, a patient with no known MND mutation, which did produce large AChR clusters in close proximity to NF-SV2 motor axons. At this early stage the phenomenon of MND muscle not responding to the presence of motor axons has only been observed with muscle cells sourced from MND patients that did not carry a known MND mutation. Cleary more of these co-cultures will need to be done to reveal if there are particular subsets of MND patients whose muscle cells do respond to the presence of motor axons versus those that do not. In particular, the testing of motor axon induced AChR clustering response in satellite cells derived from MND patients that carry known MND mutations would be an interesting avenue to pursue, particularly as it was not assessed in this study.

These preliminary observations suggest that muscle cells generated from MND patients may have lost their ability to respond to pro-synaptic signaling from the motor nerve. Motor nerve-induced AChR clustering depends upon the neural-agrin MuSK signaling system which plays a vital role in the development of neuromuscular connections, and their maintenance throughout life [17,24,25]. Neural-agrin released from the motor nerve binds to a receptor made up of the low-density lipoprotein receptor-related protein 4 (LRP4) – MuSK receptor complex in the postsynaptic muscle membrane. This binding drives a tyrosine kinase signaling cascade. Dok7, binding to the cytoplasmic domain of MuSK enhances its activation (phosphorylation) [26]. A phosphorylation cascade involving Abl and Src kinases and phosphorylation of the acetylcholine receptor (AChR) recruits rapsyn, a cytoplasmic scaffolding protein, to stabilize and cluster postsynaptic AChRs [17]. Through this pathway, neural-agrin drives the high-density clustering of postsynaptic AChRs beneath the overlying motor nerve terminal – a feature needed for effective muscle contraction. The details of this pathway have, in part, been revealed through the well-established neural-agrin AChR clustering bioassay, where satellite cells from several species are used to create newly formed muscle cells in culture. Recombinant neural-agrin is then added to these cultures and via the above pathway results in the formation of large clusters of AChRs in the muscle membrane [27-31].

This neural-agrin AChR clustering bioassay was used to determine if there might be a fault in the neural-agrin signaling pathway in muscle cells derived from MND patients. Treatment of non-MND muscle cells with neural-agrin produced an

increased number of large AChRs, confirming what other researchers have shown for satellite derived muscle cell in several other vertebrate species [27-31]. By contrast, MND muscle cells treated with neural-agrin failed to grow large clusters of AChRs. This failure occurred with muscle cultures derived from both sporadic (*n*=7), and familial (*n*=2, one carrying a SOD1 mutation and the other carrying a C9orf72 mutation) MND patients. Other studies in both animal models and in human derived stem muscle cells carrying SOD1-MND mutation have shown that, the mutant SOD1 in the muscle does indeed contribute to MND disease [10,32,33]. Clearly it will be important to study the neural-agrin responsiveness of muscle cells derived from a larger sample of MND patients carrying MND mutations to substantiate this interesting finding.

So, what might be the basis of this non-responsiveness by MND muscle cells to neural-agrin? To begin to investigate this issue, the expression levels of the key protein components of neural-agrin pathway in muscle cells cultured from MND was compared to non-MND muscle cells by Western blotting. Higher levels of MuSK and Caveolin-3 were found in MND muscle cells compared to non-MND muscle cells. By contrast, neural-agrin's receptor LRP4 and MuSK's key regulator Dok7 were decreased in MND muscle cells compared to non-MND muscle cells. Normally MuSK, LRP4 and Dok7 all increase after muscle is denervated. The finding that LRP4 and Dok7 expressions were instead reduced in cultured muscle cells from MND patients might explain the poor response of these cells to neural-agrin. If true, then intervening to enhance expression of either Dok7 or LRP4 in MND muscle cells might restore responsiveness to neural-agrin to MND muscle cells.

These new findings [16] and those from other researchers support the idea that there might be an intrinsic fault within MND muscle cells [13] or, more precisely put - an intrinsic fault in the satellite cells sourced from MND patients [7,8,12]. This possibility is distinct from any immediate effects of muscle denervation. The muscle cells that we studied in culture were derived from satellite cells (MND and non-MND) and had never been innervated, let alone denervated. Moreover, the non-MND (control) muscle cells (myotubes) did indeed respond to neural-agrin producing large AChRs clusters, in a similar manner to what has been demonstrated in several other species (see above).

So how might such a fault within myotubes derived from MND satellite cells lead to a poor responsiveness to neural-agrin? We examined the growth, fusion, and early morphology of muscle satellite cells through to formed multinucleated myotubes (newly formed muscle fibers). These analyses were combined with mRNA sequencing from single nuclei from MND and non-MND myotube cultures. While no clear mechanism has been identified from these experiments, some interesting findings were observed, some of which support previous MND satellite cell research (e.g., [7]). First, the proliferation capacity of muscle satellite cells from non-MND and MND biopsies were no different - an observation supported by others [7]. Second, the rates at which desmin-positive myoblasts fused to form multi-nucleated myotubes (expressing myosin heavy chain; MHC) were likewise similar between MND and non-MND cultures. Thus, MND and non-MND satellite cells seemed to have similar myogenic potential. However, all three non-MND cultures had a higher percentage of MHC-positive myotubes per visual field compared to MND muscle cultures. On average, MND myotubes appeared thinner. These observations raised the possibility that thinner MND myotubes might contribute the impaired neural-agrin-induced AChR cluster formation. To control this confounding variable, we restricted our analysis of AChR clusters to myotubes that were of similar maturity; namely between 300 to 350 µm long and between 25-30 µm in average diameter (measured at 3 intervals).

We next conducted a pilot gene expression comparison of myotubes from 2 MND and 2 non-MND cultures using single nuclei RNA sequencing (snRNAseq). The aim was to identify possible changes in muscle gene expression including genes that encode for proteins involved in the neural-agrin signaling cascade that might help explain the lower density and slower maturity of MND myotubes, along with their poor responsiveness to neural-agrin. The limited transcript data obtained revealed that there were no differences in the expression of myogenic genes or genes of the n-agrin-MuSK signaling cascade between MND and non-MND myotubes. This finding suggests that the above altered levels of MuSK, Dok7, LRP4 and caveolin-3 protein in MND muscle cultures was likely due to post-translational mechanisms. An important caveat to this conclusion is that the nuclei sequenced were from just 2 MND and 2 non-MND cultures and myotubes from the patient samples in question had not been assessed for their responsiveness to neural-agrin. They had been sourced from MND patients who carried either a SOD1 or a C9orf72 mutation. It will be imperative to expand the single nucleus RNA sequence analysis to a greater biological sample size and include samples that have also be tested in the neural-agrin AChR cluster growth assays. These most intriguing discoveries point to the need to conduct such gene and protein expression experiments alongside protein phospho-omics analyses in the presence and absence of neural-agrin.

This commentary has focused on the impaired response of muscle from MND patients to the motor neuron derived synapse-induction factor neural-agrin [16]. However, sigma-1 receptor signaling can influence development of motor neuron degeneration in animal models of ALS, including the loss of nerve-muscle connections [34,35]. Sigma-1 receptors have been shown to play a variety of roles modulating neuron activity, synaptic stability and trans-synaptic signaling (reviewed by [36]). For example, sigma-1 receptors are enriched in the postsynaptic region of cholinergic C-terminal bouton synapses on motor neurons that regulate neuronal activity [36,37]. This raises the possibility that sigma-1 receptors might also be enriched in postsynaptic region of cholinergic neuromuscular junctions. Sigma-1 activation

can induce the expression-stabilization of synaptic adhesion molecules (e.g., neurexin [38]); enhance local production of motor neuron trophic factors such as BDNF and GDNF [39], and reduce cellular stress [36]. The relationship between neural-agrin signaling and sigma-1 receptor signaling in preserving NMJ function and structural integrity in MND/ALS, remains to be investigated.

The potential of physiotherapeutic interventions to modify the above biochemical cascades within muscle of MND patients (so as to improve muscle performance) is not known (see [40]). So far, studies on traumatic brain injury patients and Alzheimer's Disease animal models have suggested that acupuncture therapy can improve neuromotor performance via an increase in neurotrophic factors such as BDNF, along with a reduction in neural inflammation and oxidative stress [40]. It will be of interest to see if such interventions can help slow muscle weakness in MND/ALS.

The work conducted by us, and the related work by other researchers, cement the notion that skeletal muscle is a key peripheral contributor to the pathogenesis of MND/ALS. While the central mechanism by which NMJs are lost remains to be wholly defined, our work highlights dysregulation in key components of the agrin-MuSK signaling pathway that are likely to contribute. This is not to say that other biological pathways that play independent roles at the nerve-muscle interface are not involved. Rather, it is highly likely that alterations in a combination of pathways might converge onto the pathological feature of NMJ dismantling. Complementary studies that address the range of potential biological players that could influence the maintenance of NMJs are needed to identify pathways that could be targeted skeletal muscle-directed therapeutics. In this regard, the use of therapeutic compounds in combination with physiotherapeutic approaches may be helpful is maximizing the sustaining of muscle function in MND – this approach presents an interesting avenue for future investigation.

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