Examining Regeneration Capacity and Innervation of NMJs by iPSC-Derived Motor Neurons

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Background and Hypothesis

Distal axon degeneration and denervation of neuromuscular junctions (NMJs) is a hallmark of ALS.[1] While motor neurons derived from human iPSCs (hMNs) hold promise for advancing ALS research, the length of axons, regenerative capacity, and mutant-specific innervation of NMJs by these human neurons is not well-characterized.

There is evidence that motor axon outgrowth and regeneration is impaired in ALS. Mis-localization of TDP-43 into cytoplasmic inclusions is present in the majority of ALS cases.[2] Recently, it was demonstrated that loss of nuclear TDP-43 leads to the production of non-functional, truncated of stathmin-2 transcripts.[3,4] Axonal regeneration was impaired in TDP-43 knock-down hMNs,[3,4] which could be rescued by transduction of stathmin-2.[3] Though TDP-43 pathology is absent in ALS patients with mutations in SOD1, SOD1^{A4V} mutant hMNs had fewer axons and shorter outgrowth compared to controls.[5,6]

We hypothesized motor axon regeneration in SOD1^{A4V} mutant hMNs would be impaired compared to isogenic control hMNs. We investigated motor axon outgrowth in microfluidic devices, which allow for specific interrogation of distal axons by separating neuronal cell-bodies from their axons. In addition, microfluidic devices allow for compartmentalized coculture of hMNs and myofibers, which may be used to examine mutation-specific innervation of NMJs. We hypothesized that co-culture of hMNs with primary human myoblast-derived myofibers (hMFs) would lead to *in vitro* formation of NMJs.

Methods

iPSC-derived Motor Neurons (hMNs): hMNs of spinal motor neuron identity were differentiated from iPSCs according to standard protocols from the ALS Stem Cell Core at JHU.[7] Following treatment with Ara-C to reduce glial progenitor cells, neurons were plated into microfluidic devices. hMN experiments were initiated between 55DIV and 60DIV. Axons were severed at the juncture between microchannels and axonal compartments using vigorous vacuum aspiration.

iPSC-derived Myofibers (hMFs): hMFs were differentiated from primary human myoblasts according to established protocols.[8] Cultured myoblasts in growth medium were seeded into one axonal compartment following hMN plating and axon extension. Once 95% confluent, myoblasts were differentiated into hMFs with horse-serum containing- medium.

PDMS Microfluidic Devices: PDMS and cross-linker and cast onto silane-coated stainless steel wafer-molds, and hardened in a 80℃ oven. Holes for cell-culture were punched into the molded PDMS. Clean devices were assembled by plasma-bonding PDMS to round glass-bottomed dishes, washing with ddH20 and coating with poly-L ornithine and laminin.

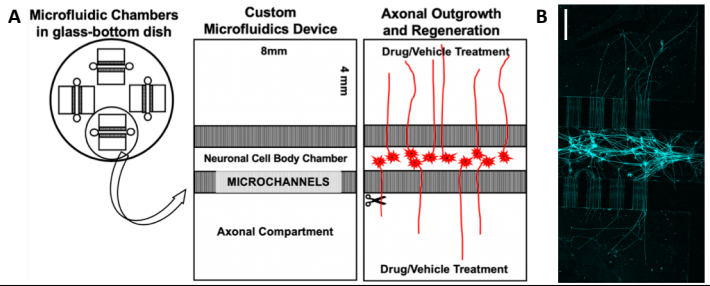

Figure 1: Microfluidic device that separates neuronal cell bodies from axons. (**A**) Compartmentalized co-culture systems have the potential to elucidate the effects of drugs, disease-causing mutations, and cell types on distal axons. Axons can grow into either axonal compartment. (**B**) Axonal outgrowth of mature hMNs in a custom microfluidic device stained for β3-Tubulin. Scale bar= 500μm

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Methods

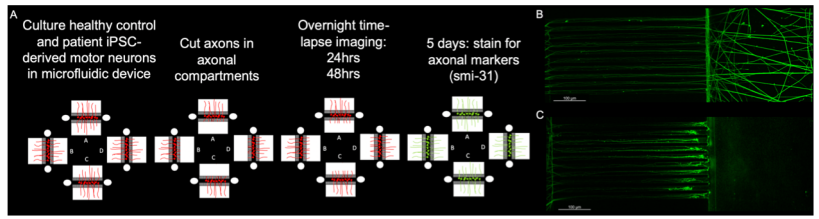

Figure 2: Axotomy of hMNs in microfluidic devices. (**A**) Experimental design to assess axonal regeneration of hMNs. (**B**) Uncut axons exiting microchannels (left) stained for smi-31. (**C**) Cut axons in microchannels following vacuum aspiration of axonal compartment stained for smi-31. Scale bar: 100 μm.

Results

Accelerated regeneration in SOD1^{A4V} hMNs

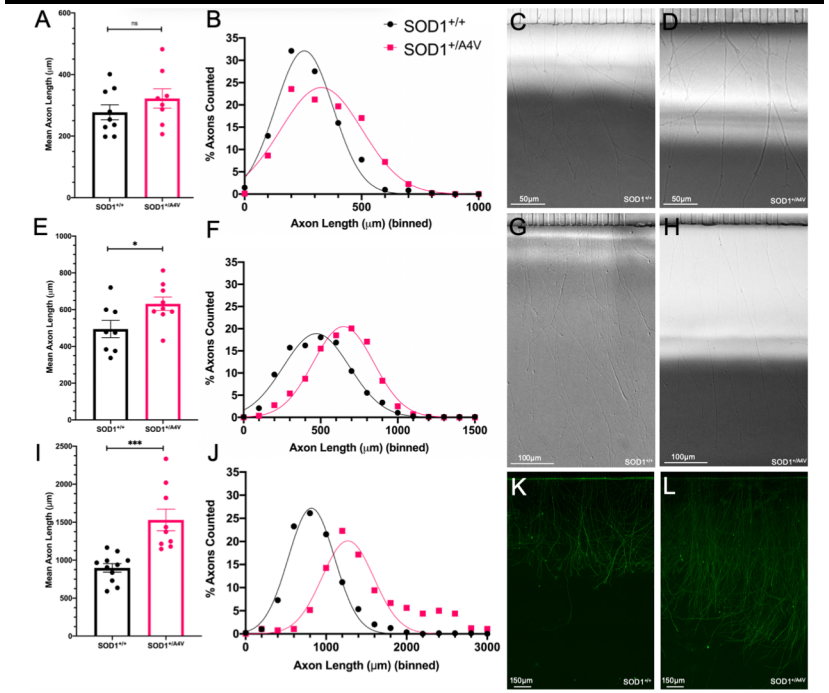

Figure 3. SOD1$^{+/A4V}$ mutant hMNs regenerate more quickly than SOD1$^{+/+}$ isogenic hMNs. (**A**) Mean axon length 24 hours post-axotomy. (**B**) Frequency distribution of axon length 24 hrs. (**C-D**) Representative images of regenerating axons 24 hours post axotomy SOD1$^{+/+}$ (**C**) SOD1$^{+/A4V}$ (**D**). (**E**) Mean axon length 48 hours post-axotomy. (**F**) Frequency distribution of axon length 48 hours post axotomy. (**G-H**) Representative images of regenerating axons 48 hours post axotomy SOD1$^{+/+}$ (**G**) SOD1$^{+/A4V}$ (**H**). (**I**) Mean axon length 5 days post-axotomy. (**J**) Frequency distribution of axon length 5 days post axotomy. (**K-L**) Representative images of regenerating axons 5 days post axotomy SOD1$^{+/+}$ (**K**) SOD1$^{+/A4V}$ (**L**). For plots of mean axon length, each data point represents 80-100 axons from 1 individual axonal compartment.

Results

Stathmin-2 expression in regenerating hMNs

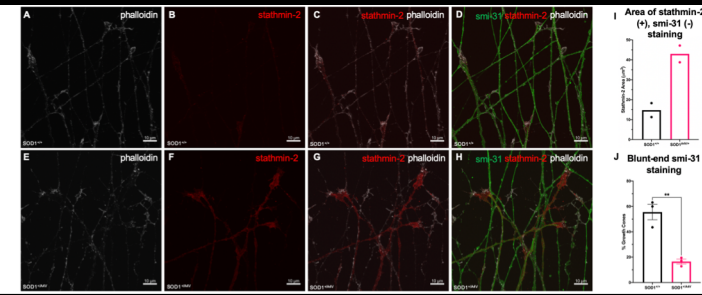

Figure 4: Growth cone of regenerating axons 24 hours post axotomy. (**A-D**) SOD$^{+/+}$ hMN regenerating axons 24 hours post axotomy. (**E-H**) SOD$^{+/A4V}$ hMN regenerating axons 24 hours post axotomy. (**I-J**) Preliminary quantification of (**I**) stathmin-2 area in the growth cone (**J**) smi-31 staining pattern in near growth cone.

Co-culture of hMNs and hMFs

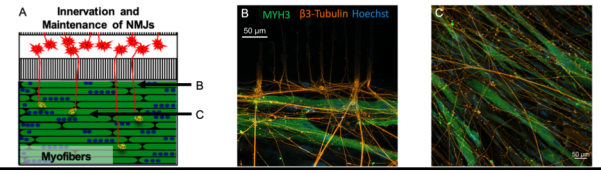

Figure 5: hMNs and hMF co-culture in microfluidic devices. (**A**) Diagram of neuron and muscle co-culture. (**B**) hMN axons (stained for β3-tubulin) exiting microchannels (top) and entering axonal compartment with hMFs (stained for myosin heavy chain, MYH3). (**C**) Low-mag image of axonal compartment containing hMN axons and multinucleated hMFs.

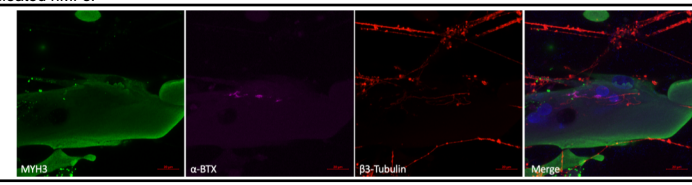

Figure 6: α-bungarotoxin staining of hMNs and hMFs co-cultures demonstrate potential formation of neuromuscular junctions. (**A**) Myosin heavy chain (MYH3) staining of hMFs. (**B**) α-bungarotoxin staining for post-synaptic NMJ (**C**) hMN axons stained for β3-tubulin. (**D**) Merge.

Conclusions

Unexpectedly, SOD1$^{+/A4V}$ mutant axons regenerated more quickly following axotomy in microfluidic devices. Co-culture of hMNs and hMFs resulted in neuromuscular contact, forming possible NMJs *in vitro*.

References

1. Fischer LR, Culver DG, Tennant P, et al. Amyotrophic lateral sclerosis is a distal axonopathy: Evidence in mice and man. Exp Neurol. 2004;185(2):232-240.
2. Prasad A, Bharathi V, Sivalingam V, Girdhar A, Patel BK. Molecular mechanisms of TDP-43 misfolding and pathology in amyotrophic lateral sclerosis. Front Mol Neurosci. 2019.
3. Melamed Z, López-Erauskin J, Baughn MW, et al. Premature polyadenylation-mediated loss of stathmin-2 is a hallmark of TDP-43-dependent neurodegeneration. Nat Neurosci. 2019.
4. Klim JR, Williams LA, Limone F, et al. ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair. Nat Neurosci. 2019.
5. Kiskinis E, Sandoe J, Williams LA, et al. Pathways disrupted in human ALS motor neurons identified through genetic correction of mutant SOD1. Cell Stem Cell. 2014;14(6):781-795.
6. Egawa N, Kitaoka S, Tsukita K, et al. Drug screening for ALS using patient-specific induced pluripotent stem cells. Sci Transl Med. 2012.
7. Taga A, Dastgheyb R, Habela C, et al. Role of Human-Induced Pluripotent Stem Cell-Derived Spinal Cord Astrocytes in the Functional Maturation of Motor Neurons in a Multielectrode Array System. Stem Cells Transl Med. October 2019.
8. Sun C, Serra C, Lee G, Wagner KR. Stem cell-based therapies for Duchenne muscular dystrophy. Exp Neurol. 2020.