Studying the effects of NF subunit abnormalities in neuromuscular junction morphology and function

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Introduction

Neurofilament (NF) subunit dysregulation has been identified as an early pathological event in ALS using monolayer cultures of induced pluripotent stem cell (iPSC)-derived motor neurons (MNs) (1). However, it remains unclear if this NF subunit imbalance has implications on neuromuscular junction (NMJ) formation, maintenance and/or function. Recent advances in organ-in-a-chip technology and 3D *in vitro* modelling allow the culture of cellular structures like the NMJ in a more physiological environment to study disease relevant phenotypes.

Methods

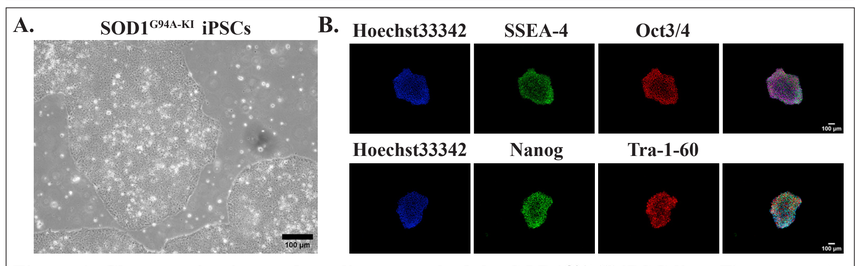
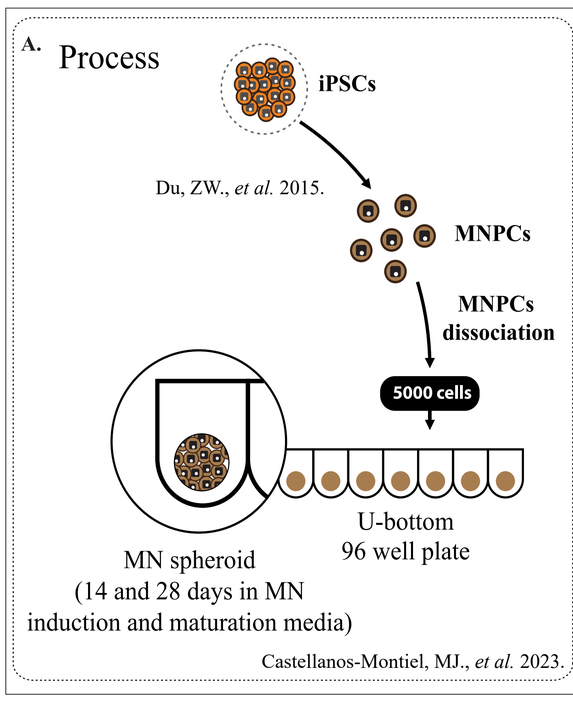


Figure 1. Characterization and quality control of the SOD1$^{G94A-KI}$ iPSC line. The noncarrier AIW002-02 iPSC line reprogrammed as previously described (2) was used to engineer a homozygous knock-in iPSC line carrying the G94A mutation in SOD1 (3). **A.** Representative phase-contrast image of SOD1$^{G94A-KI}$ iPSCs. **B.** Representative images of immunocytochemistry against pluripotency-associated markers.



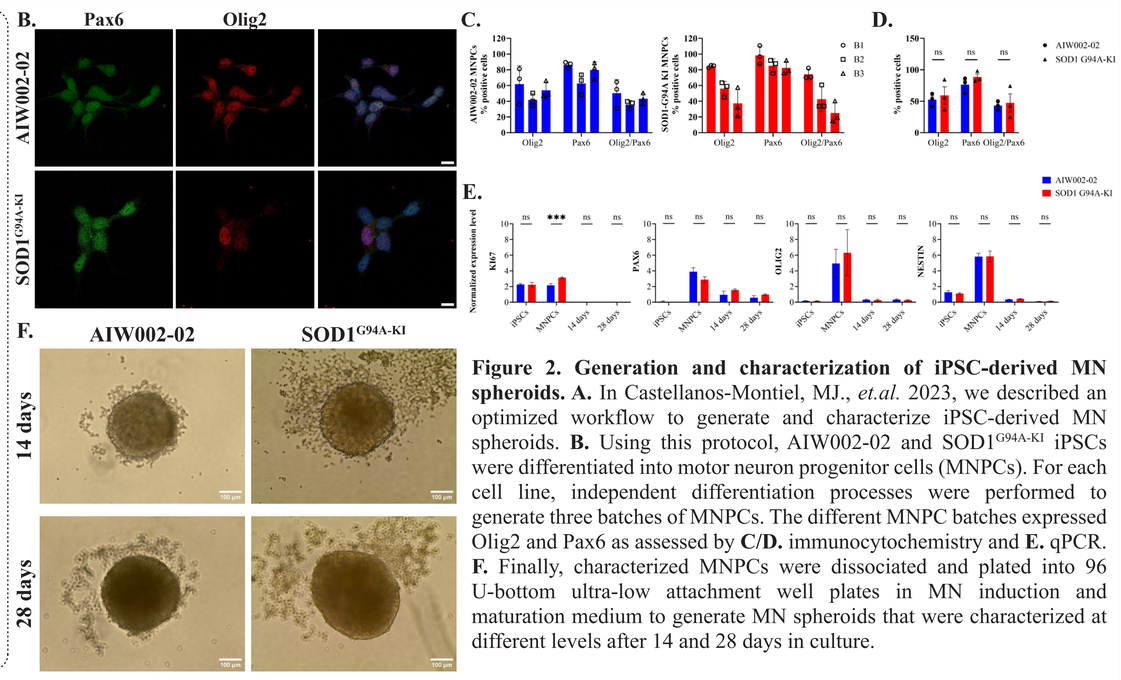


Figure 2. Generation and characterization of iPSC-derived MN spheroids. A. In Castellanos-Montiel, MJ., *et.al.* 2023, we described an optimized workflow to generate and characterize iPSC-derived MN spheroids. **B.** Using this protocol, AIW002-02 and SOD1$^{G94A-KI}$ iPSCs were differentiated into motor neuron progenitor cells (MNPCs). For each cell line, independent differentiation processes were performed to generate three batches of MNPCs. The different MNPC batches expressed Olig2 and Pax6 as assessed by **C/D.** immunocytochemistry and **E.** qPCR. **F.** Finally, characterized MNPCs were dissociated and plated into 96 U-bottom ultra-low attachment well plates in MN induction and maturation medium to generate MN spheroids that were characterized at different levels after 14 and 28 days in culture.

Results

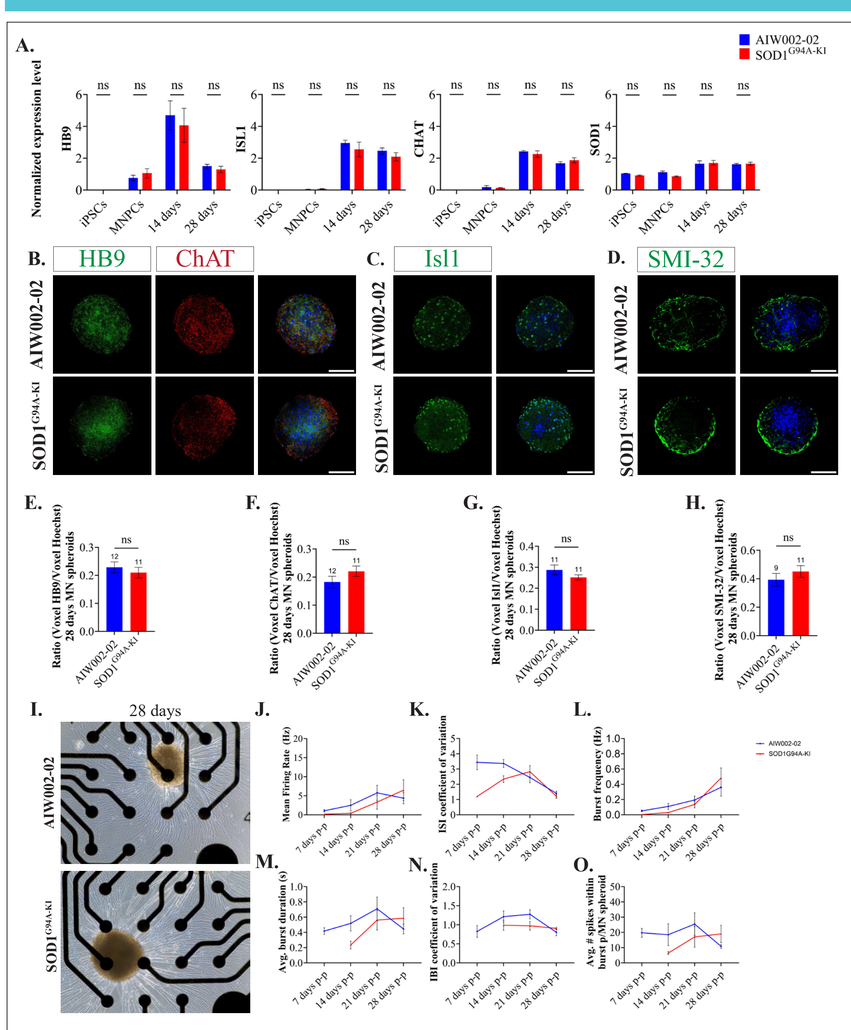


Figure 3. Characterization of 28 days old MN spheroids. Characterization of MN spheroids by **A.** qPCR and **B-H.** immunocytochemistry showed that MN spheroids generated from AIW002-02 and SOD1$^{G94A-KI}$ iPSC lines express specific MN markers. Additionally, the recording of spontaneous activity over time using a **I.** microelectrode array (MEA) system shows no differences in the assessed **J-O.** activity features between cell lines.

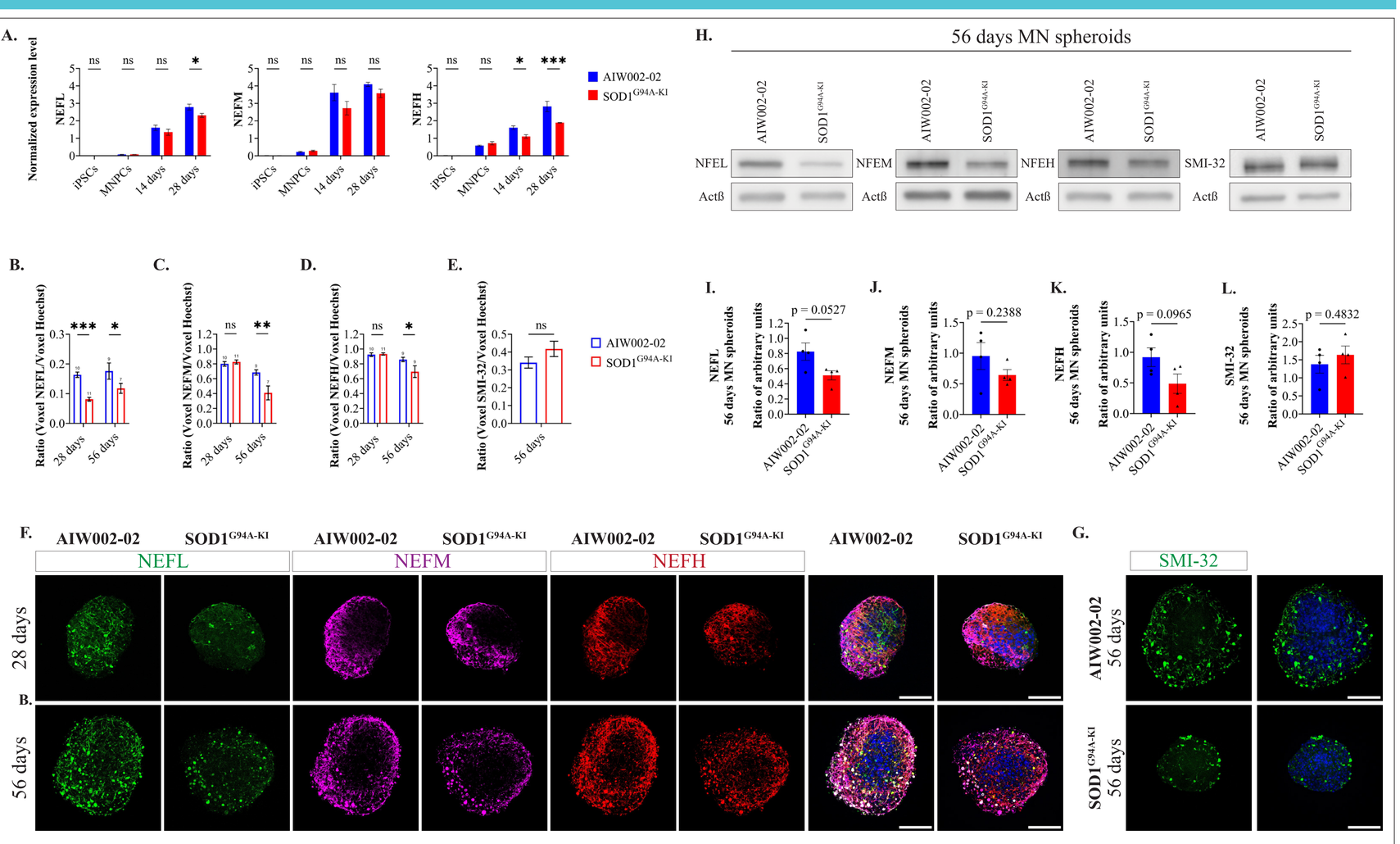
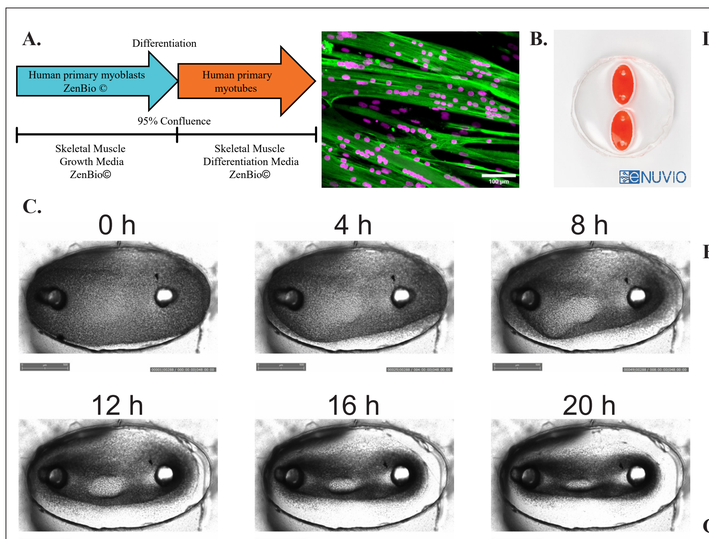


Figure 4. Neurofilament subunit imbalances in SOD1$^{G94A-KI}$ MN spheroids. Characterization of neurofilament (NF) subunits light (NEFL), medium (NEFM), and heavy (NEFH) at the **A.** transcriptional level showed downregulation of NEFL and NEFH at 28 days. Further characterization of NF subunits at the protein level by **B-G.** immunocytochemistry and **H-L.** Western Blot confirmed the downregulation of NF subunits in SOD1$^{G94A-KI}$ MN spheroids after 28 and 56 days in culture.

Discussion



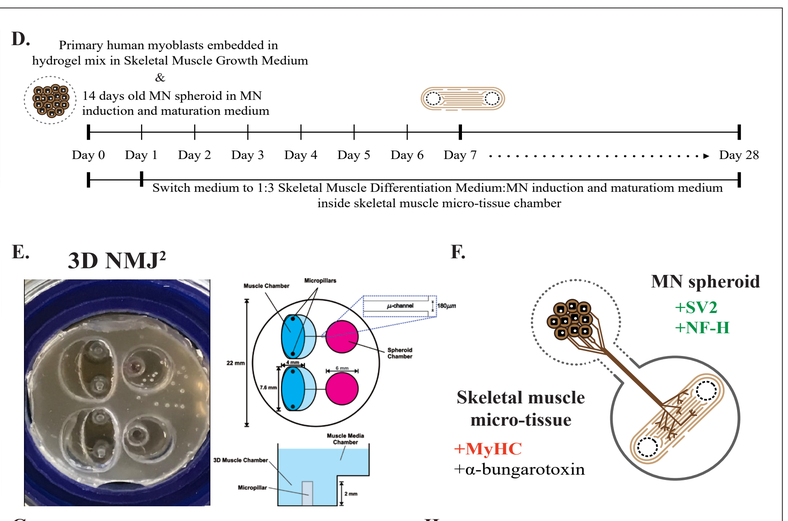


Figure 5. Generation of human 3D neuromuscular junction (NMJ) *in vitro* to study NF imbalances. A. Commercial human primary myoblasts (ZenBio) can be plated inside **B.** PDMS devices that promote their differentiation in 3D by enabling them to **C.** organize around the anchor points of the device. **D.** An optimized pipeline allows the co-culture of 3D skeletal muscle with MN spheroids inside a **E.** customized PDMS microfluidic device which **F.** only allows the contact of the axons of the MNs with the 3D skeletal muscle. **G-H.** After 21 to 28 days of co-culture, the MN spheroid has grown axons (NF-H/SV2 staining) that make contact with the acetylcholine receptors (α-bungarotoxin) in the membrane of 3D skeletal muscle (MyHC staining).

Conclusion

Following our optimized workflow to generate and characterize iPSC-derived MN spheroids (5), we successfully generated MN spheroids from a homozygous knock-in iPSC line carrying the G94A mutation in SOD1 (3) and conducted phenotypic comparisons with its isogenic control (AIW002-02) (2). Characterizing MN spheroids generated from both iPSC lines revealed that the *SOD1* mutation does not impact MN development after 14 or 28 days in culture. Also, both cell lines expressed similar *SOD1* mRNA levels at both time points. Nevertheless, characterization for neurofilament subunits light (NEFL), medium (NEFM), and heavy (NEFH) at 28 and 56 days showed that SOD1$^{G94A-KI}$ MN spheroids exhibit a significant downregulation of NF subunits. These results are consistent with previous observations made on 2D iPSC-derived MNs generated through a different differentiation protocol and using an alternative gene editing technique to generate the isogenic control (1). However, it remains to be elucidated if NF imbalances will persist or worsen when MNs are innervating their target tissue, skeletal muscle. To address this, we developed a pipeline to co-culture our MN spheroids with 3D skeletal muscle to build a model of the neuromuscular junction (NMJ) in a dish. Given their 3D nature, these co-cultures are expected to closely recapitulate human physiology and give us insights into NMJ morphology and function.

Acknowledgements

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