Human induced pluripotent stem cells (iPSC) hold promise for modeling diseases in individual human genetic backgrounds and thus for developing precision medicine. Here, we generate sensorimotor organoids containing physiologically functional neuromuscular junctions (NMJs) and apply the model to different subgroups of amyotrophic lateral sclerosis (ALS). Using a range of molecular, genomic, and physiological techniques, we identify and characterize motor neurons and skeletal muscle, along with sensory neurons, astrocytes, microglia, and vasculature. Organoid cultures derived from multiple human iPSC lines generated from individuals with ALS and isogenic lines edited to harbor familial ALS mutations show impairment at the level of the NMJ, as detected by both contraction and immunocytochemical measurements. The physiological resolution of the human NMJ synapse, combined with the generation of major cellular cohorts exerting autonomous and non-cell autonomous effects in motor and sensory diseases, may prove valuable to understand the pathophysiological mechanisms of ALS.
The neuromuscular junction (NMJ) is the core synapse in the neuromuscular nervous system, with broad diagnostic and therapeutic relevance to motor neuron diseases, neuropathies, junction disorders, and myopathies. In amyotrophic lateral sclerosis (ALS), a devastating and rapidly fatal degenerative disease of the motor nervous system, degeneration of the NMJ occurs as the first pathological feature. Some 90% of cases are apparently sporadic while 10% are familial and result from mutations in one of over 30 individual genes, including superoxide dismutase 1 (SOD1), fused-in-sarcoma (FUS), TAR DNA-binding protein 43 (TARDBP, which encodes TDP-43), profilin 1 (PFN1), and intronic hexanucleotide repeat expansion in the C9orf72 gene. Familial ALS genes may be broadly grouped into two familial ALS lines (19f and MGH5b harboring C9orf72 repeat expansion differentiated from a single control iPSC line using a from ALS iPSC-derived motor neurons co-cultured with myo- as Brachyury) lineage markers (Fig. 1b). Characterization of the potency of individual spheres for generating myogenic (enriched in TBXT), neurogenic (enriched in SOX2), or neuromesodermal (equal TBXT and SOX2) lineages showed that all five lines had strong neuromesodermal potential, as measured either on the level of individual spheres (Fig. 1c) or whole-well staining (Fig. 1d). Representation of strictly myogenic or neurogenic spheres was substantially lower in all lines (Supplementary Fig. 1a, b).

After the week of patterning in suspension, the number of spheres across the five lines was consistent (one-way ANOVA, F = 0.758, P = 0.632, Supplementary Fig. 1c). Two to three days later, after plating the spheres at fixed density in matrigel-coated plates, cells migrated outward (Supplementary Video 1) to form a confluent, adherent organoid culture comprised of the plated spheres and outgrowing structures. Within the next 2 weeks, both ectodermal and mesodermal lineages were evident: scattered TUJ1-positive neurons, as early as day 8 (Supplementary Fig. 1d) and more densely at 4 weeks (Fig. 1e), as well as cells that expressed the transcription factor Pax7 in proximity to sarcomeric α-actinin-positive myocytes at 2 weeks (Fig. 1f and Supplementary Fig. 1e). The organoid cultures contained neurons and muscle distributed in discrete areas (Fig. 1g), and we next used single-cell sequencing of the early organoids to confirm the developing cell identities in an unbiased manner.

Single-cell RNA-seq confirms multiple mesodermal and ectodermal cell types. We generated organoid cultures from three independent differentiations of a control iPSC line (11a) and dissociated them at 2 weeks, the earliest time at which we consistently observed both ectoderm and mesoderm derivatives. Using inDrop single-cell RNA-seq, we sequenced libraries from 27,000 cells (9000 cells/differentiation) at an average read depth of 35,000 reads/cell covering an average of 2,555 unique genes/cell. Cells were clustered into 25 independent groups (Fig. 2a) using the Seurat package in R17, and cluster identity was assessed using differentially-expressed cluster marker genes (Fig. 2b, c). Neural crest cells (clusters 0, 11, 12, 13, 14, 17, 20) were identified by expression of FOXD3 and SOX10, critical transcription factors implicated in cell fate decisions of nascent neural crest cells, as well as SNAI2 and SOX2. Cells expressing MEST, MAPB, BMP7, SOX2, and OLIG3 (2, 3, 6, 8, 9, 15, 16, 18), which are important for early lineage specification of neural crest derivatives, were classified as intermediate progenitors. Neuronal progenitors (5, 21) were identified by overlapping expression of intermediate progenitor markers OLIG3, SOX2, and DLL1 in combination with expression of neuronal markers MAP1B and TUBB3. Maturing neurons (10, 22, 24) were readily identified by strong expression of pan-neuronal markers TUBB3 and MAP1B. Cells in clusters 7 and 19 were classified as mesenchymal progenitors based on the expression of CXCL12, CD164, TWIST1, and PRRX1. The clear separation of a neuronal cluster from the remaining neural crest derivatives at an early point (Fig. 2b) is consistent with single-cell RNA-Seq data from developing mouse neural crest showing an initial specification of a neuron fate.

To examine the effects of variable maturation state and to validate our Seurat analysis using an orthogonal approach, we used SPRING software, which infers developmental connections

Results
Neuromesodermal progenitors give rise to ectodermal and mesodermal derivatives in organoid cultures. We differentiated iPSCs in suspension to form spheres, which were patterned for a week, plated at a density of 46 spheres/cm², and cultured under adherent conditions for up to 15 weeks total (Fig. 1a). Initial experiments used five iPSC lines: two controls (11a, FA0000011), two familial ALS lines (19f and MGH5b harboring C9orf72 repeat expansion and FUS mutations, respectively), and one sporadic ALS line (FA0000012) (see “Methods” for line details). In order to generate both motor neuron and muscle, patterning was designed to produce neuromesodermal progenitors via FGF and WNT agonists as well as forskolin7,15. Two day-old spheres contained neuromesodermal progenitors, identified by expression of both ectodermal (SOX2) and mesodermal (TBXT, also known as Brachyury) lineage markers (Fig. 1b). Characterization of the potency of individual spheres for generating myogenic (enriched in TBXT), neurogenic (enriched in SOX2), or neuromesodermal (equal TBXT and SOX2) lineages showed that all five lines had strong neuromesodermal potential, as measured either on the level of individual spheres (Fig. 1c) or whole-well staining (Fig. 1d). Representation of strictly myogenic or neurogenic spheres was substantially lower in all lines (Supplementary Fig. 1a, b).

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To examine the effects of variable maturation state and to validate our Seurat analysis using an orthogonal approach, we used SPRING software, which infers developmental connections
among cells\textsuperscript{29}. SPRING analysis revealed 22 clusters (Supplementary Fig. 2) validated by gene ontology (GO) analysis, including neuronal (5, 8, 12), maturing skeletal muscle (7, 17, 19), vasculature development (0), and hematopoiesis (4), in general agreement with the Seurat analysis.

Organoid cultures generate neuroectodermal derivatives including neurons and astrocytes. The single-cell RNA-seq analysis identified three distinct clusters that expressed the pan-neuronal genes TUBB3 and NCAM1 as well as NEUROG1 and NEUROG2, which demarcate early neuronal development (Fig. 3a)\textsuperscript{30–32}. Other expressed genes suggested specification of neuronal type: for example, ISL (ISLI) marks primarily motor and nociceptor neurons but also select interneuron populations\textsuperscript{6,33}. In motor neurons, enrichment of STMN2 expression supports microtubule assembly for axonal outgrowth\textsuperscript{3,4}. In sensory neurons, the transcription factor encoding POU4F1 (which encodes BRN3A; Seurat clusters 10 and 24) mediates essential TrkA signaling for nociceptor development\textsuperscript{34} and remains expressed in post-natal nociceptors (Allen Brain Atlas, http://portal.brain-map.org/). Similarly, the gene encoding for the neurofilament protein peripherin (PRPH) delineates unmyelinated nociceptive C-fibers and autonomic fibers in the peripheral nervous system\textsuperscript{35}.

To validate the single-cell RNA-seq data and the expression of ISL in TUJ1-positive neurons of more mature organoids, we first immunostained whole-organoid cultures after 4 weeks and found groups of ISL-positive neurons (Fig. 3b). To assess multiple subpopulations within the same organoid culture, we then used a two-step process of fluorescence-activated cell sorting (FACS)
followed by immunocytochemistry immediately after plating the purified cells to quantify neuronal subtypes. We found that NCAM-based FACS selected 23.0 ± 7.1% (mean ± SEM) of cells (Fig. 3c and Supplementary Fig. 3), and subsequent staining for TUJ1 (Fig. 3d) revealed that 38.0 ± 1.3% of the NCAM-purified cells (mean ± SEM, Fig. 3d) were neuronal. NCAM, which has been useful for prior FACS-based neuronal purification3, is also expressed in some non-neuronal cells— including embryonic skeletal muscle36, consistent with neuronal and non-neuronal NCAM expression in our single-cell RNA-seq data (Fig. 3a; Supplementary Dataset 1 for Fig. 2). Combining the FACS purification and staining, 8.7 ± 2.7% of total cells were NCAM- and TUJ1-positive neurons (mean ± SEM), in agreement with the 8.5% of cells in neuronal clusters identified by single-cell RNA-seq (Supplementary Table 1).

In the NCAM-purified and TUJ1-positive neurons, we identified a large percentage of ISL-positive cells (81.5 ± 5.5%, mean ± SEM) (Fig. 3e). To distinguish among ISL-expressing neuronal subtypes, we stained and quantified BRN3A-positive sensory neurons (30.5 ± 4.2%, mean ± SEM) and choline O-acetyltransferase (ChAT)-positive motor neurons (54.3 ± 3.5%, mean ± SEM) (Fig. 3e). The cumulative proportion of sensory and motor neurons (86%) was concordant with the overall expression of ISL (81.5 ± 5.5%, mean ± SEM, Fig. 3e) and consistent with the specificity of ISL for these two neuronal subtypes. Additionally, labeling of whole organoid cultures at 4 weeks confirmed the presence of ChAT+ neurons expressing the motor neuron marker HB9 (MNX1, also known as HoxB9) (Fig. 3f Supplementary Fig. 4), an established profile demarcating spinal motor neurons37. Quantitative RT-PCR at 10 weeks showed persistent expression of sensory (POU4F1 and PRPH, Supplementary Fig. 5a) as well as motor neuron (LHX3, CHAT, Supplementary Fig. 5b) markers.
Based on the expression of the sensory neuron markers, we looked for structures in the organoid cultures that contained sensory neurons and resembled dorsal root ganglia38. BRN3A-positive sensory neurons formed ganglia-like clusters (Fig. 3g) expressing PRPH-positive neuronal processes (Fig. 3h), similar to their *bona fide* counterparts; in contrast, neurons outside the ganglia did not express PRPH. We then investigated whether the candidate sensory neurons expressed functional nociceptor-specific channels and ionotropic receptors that serve key roles in pain sensory transduction. We first tested for the functional expression of TRPV1 receptors using capsaicin, which is a pungent compound from hot chili peppers. Using calcium imaging of individual cells in ganglia-containing fields with (red) and without (gray) response to capsaicin at 6–7 weeks. Capsaicin sensitivity was confirmed in three independent biological differentiation replicates of two lines (11a, MGH5b).

Quantification of peak sodium (middle) and potassium (right) current amplitudes in the absence (gray) or presence of TTX (cyan, cell without TTX-resistant sodium currents; magenta, the cell with TTX-resistant sodium currents). Expression within neuronal clusters from single-cell RNA-seq data. Color intensity indicates expression level; dot size indicates the proportion of cells expressing each gene. ISLR (+), TTX-R-lacking cells: 4 pA [5–8], n = 4/14 cells; median peak sodium amplitude [IQR]; Mann–Whitney test *U* = 0, two-tailed, *P* = 0.002. We observed no difference in voltage-gated potassium currents in the same cells (TTX-R-containing cells: 492 [429–613] pA, n = 10/14 cells; TTX-R-lacking cells: 484 pA [438–572], n = 10/14 cells; median steady-state potassium amplitude [IQR]; Mann–Whitney test *U* = 19, two-tailed, *P* = 0.945).

Consistent with the overlapping developmental pathways for neurons and astrocytes, as well as prior studies showing astrocytes in brain organoids42, we anticipated finding astrocytes in the sensorimotor organoids. Indeed, we identified GFAP-positive cells by immunocytochemistry as early as 4 weeks (Fig. 3k–m). Early GFAP-positive cells migrated out of the spherical structures but did not show an obvious astrocyte morphology until 8 weeks in culture (Fig. 3i and Supplementary Fig. 5c), and quantification of GFAP-positive staining from whole organoid cultures by confocal imaging showed no significant differences among the lines (n = 5–7 organoid cultures obtained from two independent biological differentiation replicates for each of five indicated lines; Kruskal–Wallis, *P* = 0.388). Scale bars 25 (f), (g), 50 (e), (k), (l), and 100 (b), (h) µm. Bars indicate mean and SEM.

Organoid cultures yield mesodermal derivatives including microglia, endothelial cells, and skeletal muscle. During embryonic development, microglia originate from the extra-embryonic yolk sac, migrate to the developing spinal cord, and remain after the formation of the blood-brain barrier43,44. The
The presence of microglia suggested the existence of primary vasculature, as the two share a common embryonic origin. Staining with tomato isolectin B4 (IB4), which labels endothelial cells in microvasculature, showed a characteristic reticulated pattern in proximity to neurons (Fig. 4d) and in some cases coursing adjacent to IBA1-positive microglia (Fig. 4e). Although IB4 also marks non-peptidergic nociceptor neurons, the observed labeling pattern was not typical for neurons. To confirm the presence of vasculature, we detected expression of the vascular endothelial cadherin CDH5 by qPCR at 10 weeks (Supplementary Fig. 6b), and electron microscopy revealed the paradigmatic feature of blood vessels, namely endothelial cells lining a lumen (Fig. 4f).

Finally, given the presence of myocytes early in organoid culture (Fig. 1e), as well as the myogenic clusters in the RNA-seq data, we asked whether further muscle development and maturation occurred. We observed elongated, thin, and fused striated cells with peripheral nuclei, indicative of myotube maturity in skeletal muscle. Electron microscopy highlighted the archetypal ultrastructural features of skeletal muscle, including Z-lines within I-bands and H-bands with M-lines (Fig. 4h). The total area of skeletal muscle occupied a consistent percentage of the total well area at 6 weeks in culture (n = 5–6 organoid cultures from two independent biological differentiation replicates of each of five lines; Kruskal–Wallis, P = 0.642). Control lines: 11a, FA0000011. ALS lines: MGH5b, 19f, and FA0000012.
skeletal muscle regulators MYF5 and MYOG\(^7\) was also detected by qPCR at 10 weeks in culture (Supplementary Fig. 6d).

**NMJs produce neuron-dependent skeletal muscle contractions, which are reduced in ALS-derived organoid cultures.** To assess the structural and functional integration of motor neurons and skeletal muscle, we looked for NMJs, the critical synapse in neuromuscular disease. The organoids generated a complex network of neurons and muscle punctated by α-bungarotoxin (α-BTX)-binding nicotinic acetylcholine receptors (Fig. 5a), which can form clusters before muscle innervation but are reorganized by synaptic initiation and activity at the NMJ\(^50\). We observed clusters of postsynaptic α-BTX staining opposite the presynaptic neuronal marker bassoon (BSN, Fig. 5b)\(^31\), and electron microscopy confirmed the presence of synaptic densities flanked by presynaptic vesicles and longitudinally-cut postsynaptic muscle fibers (Fig. 5c)\(^48\).

Beginning at 8 weeks in culture, we observed the onset of spontaneous muscle contractions (Supplementary Video 2), as expected given the incipient motor neuron network activity in both purified primary rodent and purified human stem cell-derived motor neurons\(^32\) as well as the spontaneous transmitter release from motor neurons yielding muscle contractions during in vivo development\(^35\). Contractions persisted for up to a month in culture and occurred consistently in all lines studied. To confirm that motor neuron activity was sufficient to elicit contractions, we expressed the blue-shifted channelrhodopsin TsChR2 under the neuronal-specific SYN1 promoter\(^34\), and illumination elicited immediate and robust muscle contraction (Fig. 5d and Supplementary Video 3).

The pharmacology of the mammalian NMJ is well-characterized and serves as the basis for paralytics used broadly in clinical anesthesia\(^35\). To assess whether motor neuron signaling was necessary for the spontaneous muscle contractions, we applied the presynaptic and postsynaptic blockers botulinum toxin and curare, respectively, to organoid cultures. We selected individual sites that exhibited large, spontaneous muscle contractions from the five iPSC lines, including control and ALS lines, at 8–9 weeks in culture and quantified contractions using a Fiji (ImageJ 1.52p) optic flow plug-in\(^36\) to quantify pixel movement associated with each contraction (Supplementary Video 4). The application of curare, an antagonist of postsynaptic skeletal muscle nicotinic acetylcholine receptors, quickly abolished muscle contractions (Fig. 5e and Supplementary Fig. 7a). To verify that the spontaneous muscle contractions resulted from presynaptic motor neuron activation, we showed that botulinum toxin also blocked contractions (Fig. 5f and Supplementary Video 4).

Although sites with large and frequent contractions could be found in cultures from both ALS and control lines, we subjectively noted fewer contractions in organoid cultures derived from ALS iPSC lines. This observation led us to hypothesize that the organoids captured an impairment of NMJ function in ALS lines, as loss of NMJs is one of the earliest hallmarks of ALS\(^1\). To evaluate the suggested reduction of contractions in ALS organoids, we performed an unbiased contractility assay in 7–8-
week-old organoid cultures. While analysis sites for the pharmacology assay were specifically selected for high contraction rates, sites for the contractility assay were selected based only on the presence of muscle fibers detectable in brightfield and blind to line identity of the two control and three ALS iPSC lines using six individual sites per organoid culture from three independent differentiations of each line. We quantified the rate of skeletal muscle contraction across the control and ALS iPSC lines (Fig. 5f) by recording brightfield videos of muscle, identifying fluctuations in pixel intensity using automated frame subtraction, and then manually verifying whether fluctuations corresponded to muscle contraction all blind to line identity. We detected contractions in 12 out of the 15 organoid cultures analyzed (five out of six of the control and seven out of nine of the ALS organoid cultures). The percentage of contractile sites per organoid culture, as well as the overall contraction rate, were similar across groups (n = 5 control and 7 ALS organoid cultures obtained from three independent biological differentiations, Percentage of contractile sites: ANOVA type 3, F = 1.458, P = 0.255; contraction rate: ANOVA type 3, F = 0.343, P = 0.571). We observed large contractions (encompassing over half a field of view) in seven out of 15 organoid cultures (three out of six of the control and four out of nine of the ALS organoid cultures). Distinguishing between small and large contractions revealed that, despite the similar percentages of sites with large contractions (n = 3 control and 4 ALS organoid cultures obtained from three independent biological differentiations, ANOVA type 3, F = 1.753, P = 0.243), there was a reduction of 72.8% in the frequency of large contractions in ALS compared to control cultures (n = 3 control and 4 ALS organoid cultures obtained from three independent biological differentiations, ANOVA type 3, F = 8.289, P = 0.035).

Gene editing of iPSC lines reduces within- and among-line variability of sensorimotor organoids. While the five iPSC lines consistently generated organoid cultures containing ALS-relevant cell types, namely, motor neurons, muscle, astrocytes, and microglia, we were concerned that the large within- and among-line variation observed in cell counts could decrease sensitivity or confound the interpretation of disease modeling studies. To reduce the influence of individual genetic backgrounds on the observed variability of sensorimotor organoids, we designed a strategy of editing three familial ALS mutations into a single control line, yielding three isogenic pairs of ALS and matched control lines. We used CRISPR/Cas9 to introduce the point mutations *TARDBP*G298S, *SOD1*G85R and *PFN1*G118V into the FA000011 control line. Individual clones with a single heterozygous mutation and no modification of the opposite allele were selected and expanded to generate the following iPSC lines: TDP-43+/G298S, SOD1+/G85R, PFN1+/G118V, whereas clones with no detectable editing were selected as paired controls (TDP-43+/+, SOD1+/+, PFN1+/+) (Fig. 6a). Whole exome sequencing of the isogenic ALS lines and paired controls detected variants, but none indicating similar rates of neurite growth between each pair of isogenic lines (Fig. 8b and Supplementary Fig. 1). This lower variation was also evident in the qualitatively similar architecture of the developing organoid cultures across the isogenic lines (Supplementary Fig. 10 compared to Supplementary Fig. 1).

Mutations in familial ALS genes give rise to distinct NMJ phenotypes. Having found deficits in muscle contractions as an initial ALS disease modeling phenotype, we focused our studies with the isogenic pairs on understanding potential structural contributions to explain the physiological impairment. We labeled neurons in isogenic organoid cultures by transduction with an hSYN:EGFP AAV9 vector (Fig. 8a) at week 2 and tracked neuronal growth longitudinally from weeks 3–9 of culture. There was a significant growth of neurite area over time (n = 6 organoid cultures obtained from three independent biological differentiations; linear model F = 8.743, P = 1.3 × 10⁻⁹) with no significant interaction between time and line (F = 0.562, P = 0.985), indicating similar rates of neurite growth between each pair of isogenic lines (Fig. 8b and Supplementary Fig. 11).

Because the ALS lines showed neither a deficit in neurite outgrowth nor a difference in the percentage of skeletal muscle area (Fig. 6f) when compared to their isogenic controls, we examined the NMJs by transducing separate cultures with hSYN: EGFP at 13 weeks and labeling with α-bungarotoxin 2 weeks later. The number of labeled cells was similar among lines (n = 6 organoid cultures obtained from three independent biological differentiations; Kruskal–Wallis, P = 0.522; Supplementary Fig. 12a). Innervated NMJs, as defined by the number of EGFP+ and α-bungarotoxin+ puncta divided by the total α-bungarotoxin+ puncta, were reduced in the SOD1 and PFN1 mutant lines but not the TDP-43 mutant line (n = 6 organoid cultures obtained from three independent biological
Fig. 6 Gene-edited iPSC lines are paired with their isogenic controls and generate sensorimotor organoids with similar compositions. a Strategy for CRISPR/Cas9-mediated insertion of point mutations in either TARDBP (TDP-43), SOD1, or PFN1 into the FA0000011 control line. b Multiple correspondence analysis of TDP-43+/+ (dark green), TDP-43+/G298S (light green), SOD1+/+ (dark blue), SOD1+/G85R (light blue), PFN1+/+ (red), PFN1+/G118V (pink), and the original healthy control line FA0000011 (orange). c Distribution of individual spheres according to their expression of myogenic (TBXT) or neurogenic (SOX2) transcription factors from three independent biological differentiation replicates (colors). d Spinal motor neurons expressing HB9, ChAT, and β-III Tubulin, in organoid cultures of the SOD1+/G85R iPSC line at 4 weeks (confirmed in three independent biological differentiation replicates). Scale bar, 40 μm. e–g. Quantification of GFAP+ cells (e), SAA+ area (f), and IBA1+ cells (g) in organoid cultures at 6 weeks, normalized to paired isogenic controls, per differentiation. Bars indicate mean and SEM. (n = 6–9 organoid cultures obtained from 2–3 independent biological differentiation replicates, in distinct colors, of each of six lines; Kruskal-Wallis, P = 0.934 (e), P = 0.432 (f), P = 0.983 (g)).

discussions; one-way ANOVA: F = 5.938, P = 6.3 × 10^{-4}; two-tailed t-tests; TDP-43 pair: P = 0.560; SOD1 pair: P = 0.010; PFN1 pair: P = 0.047; Fig. 8c). The size of the innervated NMJs, but not that of the uninnervated α-bungarotoxin clusters (Supplementary Fig. 12b), was reduced in the TDP-43 mutant line compared to its control, whereas the SOD1 and PFN1 pairs did not show effects of innervation state on NMJ area (n = 6 organoid cultures obtained from three independent biological differentiations; one-way ANOVA: F = 3.001, P = 0.026; two-tailed t-tests; TDP-43 pair: P = 0.009; SOD1 pair: P = 0.203; PFN1: P = 0.659; Fig. 8d, e). Thus, these results show a structural deficit in ALS NMJs, albeit without the temporal and cellular resolution of neuronal outgrowth and NMJ formation necessary to distinguish developmental versus degenerative contributions.

**Discussion**

Although both iPSC-derived neuronal cultures and organoids yield synchronized networks of neurons\(^{[32,60]}\), the ability to capture and interrogate specific synapses may prove critical to successful translational applications. Using a fusion of free-floating sphere culture followed by plating and growth under adherent conditions, we established sensorimotor organoid cultures and derived the NMJ together with other neuronal and non-neuronal cells that play cell-autonomous and non-cell-autonomous roles in sensorimotor diseases. In modeling ALS, for which NMJ loss is an early and critical component, we found evidence of compromised NMJs for disease-causing mutations in genes that span the broad categories of familial ALS-causing gene functions, namely, proteostasis, RNA binding proteins, and axonal transport\(^{[61]}\).
The reproducibility of neural organoids presents a challenge of uncertain magnitude. Although recent studies are encouraging at least for more selectively patterned organoids, large variation in the types and numbers of cells generated—both among-line but particularly poor within-line reproducibility—has been a cause for loss of confidence in organoid models, a hindrance in the transition from bench to clinic, and a major focus of research effort. To demonstrate the robustness of the sensorimotor organoid model, we characterized cultures generated using a total of 11 stem cell lines, including healthy controls and individuals with ALS, as well as gene-edited lines harboring ALS mutations and isogenic controls. We consistently observed a broad range of cells from neuronal and mesodermal lineages as well as motor neuron-dependent muscle contractions using the

![Graphs showing among-line and within-line variance in sphere potency, area, IBA1+ cells, GFAP+ cells, SAA+ area, and TDP-43+/+ and SOD1+/+ cells for non-isogenic and isogenic conditions.](image)
Fig. 7 Isogenic iPSC lines reduce among- and within-line variability of organoid cultures. Analysis of among-line (a)-(e) and within-line (f)-(j) variance for individual sphere potency (a), (f) and area (b), (g), as well as GFAP+ cells (c), (h), IBA1+ cells (d), (i), and SAA + area (e), (j) within mature organoid cultures from two to three independent biological differentiation replicates for each line. a–e left, Mean and SEM of fold change from batch average, along with points for individual spheres (a), (b) or cultures (c), (e). Right, Mean and SEM among non-isogenic and isogenic lines with points for each unique iPSC line. Significance assessed by F-test for Equality of Variances, with group variances (ω²) indicated at bottom, for n = 5 non-isogenic, 6 isogenic lines: (a) F = 2.0 × 10⁵, P = 6.8 × 10⁻¹⁰; (b) F = 2.0 × 10⁵, P = 6.8 × 10⁻¹⁰; (c) F = 4.37, P = 0.137; (d) F = 106.33, P = 1.0 × 10⁻⁸; (e) F = 18.90, P = 0.006. f–j, left, Mean and SEM of fold change from batch average within-line, along with points for individual spheres (f), (g) or cultures (h)–(j). Right, Mean and SEM of within-line variances for non-isogenic and isogenic lines with points for each unique iPSC line. Significance assessed by one-way ANOVA for n = 5 non-isogenic, 6 isogenic lines: (f) (non-isogenic vs isogenic) 79.9 ± 24.0 vs 7.0 ± 2.1, F = 11.21, P = 0.009; (g) 0.328 ± 0.095 vs 0.015 ± 0.003, F = 13.2, P = 0.005. Colors indicate individual iPSC lines.

Fig. 8 Isogenic ALS mutations show distinct NMJ phenotypes. a Live neuronal labeling by expression of hSYN::EGFP delivered by an AAV9 vector. Scale bar, 100 µm. b Quantification of neurite area as fold change of initial, from 3 to 9 weeks in culture. The line indicates mean and shaded area indicates SEM. (Effect of time on neurite area, n = 6 organoid cultures obtained from three independent biological differentiation replicates of each of six lines, linear model, F = 8.743, P = 1.3 × 10⁻¹⁰, individual comparisons: TDP-43 pair: F = 37.040, P = 2.0 × 10⁻⁸; SOD1 pair: F = 16.3725, P = 1.0 × 10⁻⁴; PFN1 pair: F = 18.639, P = 3.6 × 10⁻⁶; interaction between time and iPSC line on neurite area, n = 6 organoid cultures obtained from 3 independent biological differentiations of each of six lines, linear model, F = 0.562, P = 0.985). c Normalized percentage of NMJs innervated at three months in culture (normalized to the paired isogenic controls). Bars indicate mean and SEM (n = 6 organoid cultures obtained from three independent biological differentiation replicates, indicated by colors, of each of six lines, Univariate ANOVA: F = 5.938, P = 6.3 × 10⁻⁴; separate two-tailed t-test; TDP-43 pair: P = 0.560; SOD1 pair: P = 0.010; PFN1 pair: P = 0.047). d Normalized area of the innervated NMJs at three months in culture (normalized to the paired isogenic controls). Bars indicate mean and SEM (n = 6 organoid cultures obtained from three independent biological differentiation replicates, indicated by colors, of each of six lines, Univariate ANOVA: F = 3.001, P = 0.026; separate two-tailed t-test; TDP-43 pair: P = 0.009; SOD1 pair: P = 0.203; PFN1: P = 0.659). e Example of the reduction in NMJ area observed in the TDP-43+/G298S iPSC line compared to the isogenic control. Scale bar, 100 µm.

five non-isogenic lines studied, although with considerable variability. The variation of both sphere makeup and derived cell types was dramatically reduced in the six isogenic lines compared to either the single parental line or the other lines we previously examined. Both the among-line and, strikingly, the within-line variances were reduced (Fig. 7), leading us to hypothesize that the proximity to clonal selection within only a small number of passages may be responsible. Intuitively, accumulating variation
during iPSC replication and passaging might well increase the variation in differentiated organoid types. An alternative hypothesis, that the reduced variation in the isogenic lines reflects an unidentified consistent effect of gene editing at the three independent sites, seems less likely. The possibility that limited passaging after clonal selection can reduce variance will have to be tested formally in dedicated experiments and other organoid models, both neural and non-neuronal, with large numbers of independent differentiations to increase precision in variance estimates. If validated, the ability to reduce within-line variance by five to tenfold may prove broadly useful in organoid modeling, albeit requiring additional confirmation that genetic alterations due to clonal selection have been minimal.

Whether there may be implications for more routine differentiation of specific cellular types from iPSCs also remains to be seen, but less patterned differentiation strategies may be more vulnerable to greater iPSC variation.

Human in vitro disease modeling of the NMJ may help construct mechanistic hypotheses for early pathogenesis in neuromuscular diseases as well as facilitate the identification and validation of targets and personalized preclinical therapeutic candidates. For ALS in particular, a growing body of evidence supports the importance of axonal biology in the disease and the NMJ has provided the earliest and among the most important pathological and functional disease readouts for both mechanistic understanding and therapeutic development. A recent study generated NMJs by co-culturing iPSC-derived motor neurons with myoblasts made from a single control iPSC line using a custom microfluidic device. Optogenetic stimulation of motor neurons harboring ALS-causing mutations in TDP-43 yielded impaired entrainment of the control-derived muscle compared to stimulation of control motor neurons. In our data, we observed a reduction in the rate of large spontaneous contraction in all three ALS lines, a component of which may reflect additional non-cell-autonomous effects of either the disease muscle itself or other cell types, as discussed below.

Comparison of pairs of isogenic lines bearing familial ALS mutations showed defects at the level of the NMJ and no impairment in neuronal outgrowth, supporting the primacy of the NMJ across ALS variants. One caveat was that the hSYN:: EGFP virus did not report specifically motor neuron outgrowth, although the high percentage of motor neurons in the culture should nonetheless have preserved sensitivity to large effects in motor neurons. While increased numbers of lines will be needed for confirmation, the distinct phenotypes observed—namely a reduced percentage of innervated NMJs in the SOD1G85R and PEN1G118V mutations and a decreased area of innervated NMJs in the TARDBP6298S mutation—is similar to prior reports demonstrating that different ALS genetic variants can affect the NMJ in distinct ways.

Future studies will be necessary to identify the mechanistic connections between these mutations and the NMJ physiological and structural phenotypes.

The strategy we employed to generate neuromesodermal precursors and subsequent organoids was similar to that recently described by Martins et al. using control human embryonic stem cell lines and an iPSC line. While that technique yielded single, three-dimensional free-floating organoids, our cultured organoid strategy produced cultures that enabled live-cell imaging and facilitated quantification of cell types in all lines, measurement of within- and among-line variation, and consequently disease modeling based on comparing NMJs derived from control and disease iPSC lines.

Limitations of our study included the ambiguity of developmental versus degenerative contributions to the observed phenotypes. In the future, longitudinal experiments may help address this issue. For example, such time course experiments might show a period of NMJ similarity between control and ALS organoid cultures followed by phenotype onset, and therefore support a degenerative etiology; however, we would be cautious in concluding this without a better understanding of the timing and duration of neurogenesis within organoid cultures along lines. Separately, longitudinal changes in organoids may not reflect in vivo development, in part due to the cellular stresses exerted by organoid culture. Thus, longitudinal studies showing an apparent developmental phenotype could simply reflect differential sensitivity to stressors during what would otherwise be a common developmental program. These caveats aside, studies dedicated to the temporal evolution of the ALS NMJ phenotype combined with concomitant monitoring of key progenitors may provide insight into the mechanisms affecting NMJ physiology, number, and size.

While the structural complexity of the model may help better approximate features of the human disease, elucidating how different mechanisms act and interact—in disease-driving or compensatory ways—may prove challenging. For example, our observed reduction in muscle contraction within ALS organoid cultures may be due to decreased motor neuron hyperexcitability. However, progressive hyperexcitability may lead to depolarization block and reduced motor neuron firing, consistent with other studies showing a reduction in motor neuron firing, and thus explain the reduction in muscle contractions observed in this report. In this interpretation, hyperexcitability and apparent hypoxic similarity due to depolarization block are on a continuum as opposed to opposite extremes. Alternatively, hyperexcitability could itself contribute to NMJ degeneration and weakened physiological function through a range of potential different implicated mechanisms, including ER stress, dipeptide formation, and TDP-43 pathology.

Diseases of the sensorimotor system bear large non-cell-autonomous effects from many cell types. For spinal muscular atrophy and ALS, astrocytes, microglia, and muscle may all contribute to motor neuron injury, in chronic pain, the effects of microglia show unexpected sex-dependent differences. Given the extensive number of causal and modifier genes for ALS, Charcot-Marie Tooth neuropathies, and hereditary sensory and autonomic neuropathies (HSANs), one might anticipate that non-cell-autonomous contributions vary among disease and even disease subgroups. While co-culture experiments have demonstrated some non-cell-autonomous toxicities, specification of non-cell-autonomous phenotypes and pathology can require elaborate interactions among multiple diverse cell types, for example, microglia, astrocytes, and neurons. Compared with efforts to culture several cell types together or use remaining multipotent cells to perform sequential differentiations, the organoid approach may actually improve variance by avoiding separate batch effects that arise from the generation of each cell type independently. We did not address the integration of cell types beyond motor neurons and muscle at the NMJ, such as to what extent the astrocytes or microglia exerted non-cell-autonomous effects or whether the vascular cells contributed broad vascularization and even capacity for blood-brain barrier modeling as has been seen in co-culture studies. Our study provided only limited molecular and transcriptional resolution of cell subtypes present at later points in the culture. Given the observed NMJ phenotypes, we now anticipate that additional single-cell sequencing as well as tissue-specific cell sorting or Ribotag labeling of individual cell classes will help evaluate the presence and maturation of distinct cell types, as well as identify within-batch cell type-specific transcriptomic ALS features and thus help improve understanding of non-cell-autonomous effects for different disease-causing mutations and genetic backgrounds.

In addition to motor neurons, the model generated dorsal spinal cord derivatives. We found nociceptor-like cells arranged...
in ganglia, and a large percentage of these cells yielded robust TTX-resistant sodium currents and capsaicin-induced calcium influx, substantial improvements in both percentage of cells responding and amplitudes over prior iPSC, lineage reprogramming, and organoid studies. Although we have not yet applied the model to diseases with preponderant sensory phenomenotypes, it may prove useful for aggressive inherited conditions such as HSANs or acquired ones such as painful diabetic neuropathy.

The generation of distinct neuronal subtypes, such as nociceptors and motor neurons, provides an opportunity to test hypotheses of both disease heterogeneity and specificity. Our results suggest that different ALS variants may affect the NMJ in different ways, and larger numbers of distinct gene variant lines will be necessary to confirm this result. One would expect that motor neuron diseases would generally spare sensory neurons while sensory disorders, like HSANs, would not affect motor neurons. These hypotheses can now be investigated in a within-batch experimental setup. We propose that the sensorimotor organoid model will yield value in modeling a broad range of sensory and neuromuscular diseases.

**Methods**

**Ethics statement.** The study was approved by the Institutional Review Board at Massachusetts General Hospital. Consents allowing for broad research use had previously been obtained from research subjects (prior to Genomic Data Sharing description initiated in 2015). iPSC lines were obtained from the Target ALS Repository and Harvard University. The embryonic stem cell line NEU3 HB9; GFP (kindly shared by Kevin Eggan) was also approved by the same Institutional Review Board, used in pilot experiments, but not in experiments within the manuscript.

**Human iPS cells.** Human iPSC cells included the previously available non-isogenic control lines and. The culture for 10 min at 37 °C (day 0). Dissociated single cells were resuspended in organoid differentiation media consisting of STEMdiff APEL2 medium (Stem Cell Technologies, 05275) and 5.32% Protein Free Hybridoma Medium II (Thermo Fisher Scientific, 12040077), supplemented with the GSK-3 Inhibitor IX (0.5 μM) (Santa Cruz Biotechnologies, sc-595234), FGFR2 (10 ng/ml) (Thermo Fisher Scientific, 13256029), Forskolin (20 μM) (Santa Cruz Biotechnologies, sc-3562) and Y27632 (10 μM) (Abcam, ab120129) and counted using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific, AMQAF1000). Cells were then seeded as a 3D non-adherent culture at a density of 300,000 cells/mL for each well of an AggreWell 800 plate (Stem Cell Technologies, 34815), which had been first treated with Anti-Adherence Rinsing Solution (Stem Cell Technologies, 07010).

One day after seeding (day 1), the media was supplemented with 30% by volume base media and 30 ng/ml FGFR2, 60 μM Forskolin, and 1.5 μM GSK-3 Inhibitor IX. From day 2 to day 6, daily media changes were performed. Cultures were centrifuged at 200 rcf for 5 min, and the media was aspirated and replaced with base media and FGFR2 (10 ng/ml) Forskolin (20 μM), and GSK-3 Inhibitor IX (0.5 μM). On day 7, the spheres were counted with a Cytation 5 Cell Imaging Reader (Biotek, Gen5 (v3.00, Biotek)), resuspended and centrifuged at 200 rcf for 5 min, and plated at a density of 600 spheres per 35 mm dish on plasticware coated with Matrigel (Corning, 354277). From day nine onwards, the media was replaced daily with DMEM with High Glucose (Thermo Fisher Scientific, 11965092) supplemented with 2% Horse Serum (Thermo Fisher Scientific, 1605010) and 1% Pen/Strep (Life Technologies, 15070-063) for up to 3 months.

**Immunocytochemistry.** Organoid cultures were fixed for 20 min at room temperature with 4% (w/v) formaldehyde (Thermo Fisher Scientific, 28908) and rinsed gently three times with PBS (Thermo Fisher Scientific, 10010049). Cultures were then permeabilized with 2% Triton X-100 (Millipore Sigma, 9400) in PBS for 20 min, followed by incubation with primary antibodies (Supplementary Table 3) in the same 2% Triton X-100 solution overnight at 37 °C. Primary antibodies were removed the following day, and cultures were washed three times with PBS, followed by overnight incubation at 37 °C with Alexa Fluor secondary antibodies (ThermoFisher Scientific) at 1:500 dilution. Organoids were then washed three more times with PBS and mounted with Prolong Diamond (Thermo Fisher Scientific, P36962) per the manufacturer’s instructions. Cultures were imaged the following day with either a Cytation 5 Cell Imaging Reader (Biotek) or an ImageXpress Micro Confocal (Molecular Devices). For analysis of NMJs, α-bungarotoxin directly conjugated with Alexa Fluor 647 (ThermoFisher Scientific, B3450) was reconstituted per manufacturer’s instructions, diluted at 1 μg/ml in PBS, and applied to fixed organoids for 2 h at room temperature. Immunostaining during the suspension period of organoid development (day 2) was performed by first plating the spheres and following the protocol described above. Quantification of individual cell populations was done through analysis of the whole well in MetaXpress (Version 6.5.4.332, Molecular Devices).

Immunostaining for FACS sorted cells was performed similarly, but secondary antibodies were incubated for 2 h at room temperature prior to mounting with Prolong Diamond (Thermo Fisher Scientific, P36962) and imaging in Cytation 5 Cell Imaging Reader (Biotek).

**FACS sorting.** Dissociation of the organoid culture was done at week 4 by incubating the culture in 0.25% Trypsin-EDTA (Thermo Fisher Scientific, 25200056) for 20 min at 37 °C. The resulting cell suspension was dissociated mechanically by pipetting 10 times, followed by centrifugation for 200 rcf for 5 min. Briefly, the cell pellet was resuspended into 200 μl of Fc-block antibody solution (1:100 of Human Fc Receptor Binding Inhibitor, Purified, Thermo Fisher Scientific, 14-9161-73; in PBS with 2% FBS, GE Healthcare, SH30910.03H) for 30 min at 4 °C while protected from light. Directly conjugated antibodies (NCAM or IG control) were then diluted in PBS with 2% FBS. The cell suspension was split in a 9:1 ratio, and 1% was reserved as IG control. The antibody solutions were either added to 180 μl of each sphere and the percentage of each sphere covered by the SOX2 and TBXT mask areas for all spheres, and mask the nuclear, SOX2, and TBXT channels. The nuclear area of each sphere and the percentage of each sphere covered by the SOX2 and TBXT mask areas were then calculated. The potency of individual cells was calculated as the difference between the percentages of SOX2 and TBXT mask areas for all spheres without the cell yielding a ratio (cold-only sphere) to 100 (SOX2-only sphere), with spheres expressing equal amounts of SOX2 and TBXT (neuromesodermal) having a score of 0.

**Analysis of sphere phenotype.** The whole well acquisition was performed for spheres stained for TBXT, SOX2, and DAPI and at day 2 using an ImageXpress Micro Confocal (Molecular Devices). MATLAB (version 2018b; MathWorks, Natick, MA) was used to stitch the individual acquired images, identify individual spheres, and mask the nuclear, SOX2, and TBXT channels. The nuclear area of each sphere and the percentage of each sphere covered by the SOX2 and TBXT masks were then calculated. The potency of individual cells was calculated as the difference between the percentages of SOX2 and TBXT mask areas for all spheres without the cell yielding a ratio (cold-only sphere) to 100 (SOX2-only sphere), with spheres expressing equal amounts of SOX2 and TBXT (neuromesodermal) having a score of 0.

**Gene editing of isogenic iPSC pairs.** Gene editing of isogenic iPSC pairs was performed in collaboration with the Harvard Stem Cell Institute iPS Core Facility. Briefly, the FA000001 control iPSC line was characterized for morphology and pluripotency at passage 13 (Supplementary Data 1) and transfected with pCas9GFP. GFP (kindly shared by Kevin Eggan) was also approved by the same Institutional Review Board, used in pilot experiments, but not in experiments within the manuscript. Organoids were cotransfected with pSPgRNA (pSPgRNA was a gift from Charles Gersbach, Addgene c, P36962) per the manufacturer’s instructions. Cultures were imaged the following day with either a Cytation 5 Cell Imaging Reader (Biotek) or an ImageXpress Micro Confocal (Molecular Devices). For analysis of NMJs, α-bungarotoxin directly conjugated with Alexa Fluor 647 (ThermoFisher Scientific, B3450) was reconstituted per manufacturer’s instructions, diluted at 1 μg/ml in PBS, and applied to fixed organoids for 2 h at room temperature. Immunostaining during the suspension period of organoid development (day 2) was performed by first plating the spheres and following the protocol described above. Quantification of individual cell populations was done through analysis of the whole well in MetaXpress (Version 6.5.4.332, Molecular Devices).

Immunostaining for FACS sorted cells was performed similarly, but secondary antibodies were incubated for 2 h at room temperature prior to mounting with Prolong Diamond (Thermo Fisher Scientific, P36962) and imaging in Cytation 5 Cell Imaging Reader (Biotek).
**Single-cell RNA-seq** Single-cell RNA-seq was performed by microfluidic inDrop encapsulation, barcoding, and library preparation by the Single Cell Core at Harvard Chan Biomatics Core at the Harvard T. H. Chan School of Public Health, Boston, MA. RAW text was processed using the bcbio-nextgen single-cell RNA-seq pipeline (https://bcbio-nextgen.readthedocs.io/en/latest/content/pipelines.html#single-cell-novel-seq). The pipeline uses tools from the umis repository (https://github.com/vals/umis/) to generate a cell by gene count matrix. FASTQ files were formatted to parse out non-biological segments of the reads (i.e., cellular barcode, sample barcodes, and UMIs). Excess cellular barcodes were removed to reduce artifacts. The reads were aligned to the GRCm38 Ensemble Release 90 transcriptome with RapMap[105]. Duplicate UMIs were collapsed, and the number of reads per transcript was counted for each cellular barcode. Samples were assessed for quality and filtered using the distributions of reads per cell, UMIs per cell, genes per cell, mitochondrial ratios per cell, UMIs vs. genes detected, UMIs vs. read counts, and novelty scores. Cells clustering was performed using the Seurat R package (v 2.3.4)[106] in R (v. 3.5.0). Cells with less than 200 unique genes were removed from the analysis, and genes expressed in less than five cells were filtered out. Raw expression values were logged normalized and each gene was scaled and centered after regression of contributions from batch, cell cycle phase, the total number of reads, and the number of mitochondrial genes. We then performed principle components analysis of genes with highly variable expression across all cells (n = 1,882) to identify sets of genes that captured transcriptional variation. Using the top 15 principle components, clusters were created with a resolution setting of 1.6, and clusters were visualized using tSNE dimensionality reduction. Marker genes for each cluster were identified as genes expressed in at least 50% of cells in a cluster and enriched relative to all non-cluster cells based on the negative binomial model.

**SPRING analysis** was performed using the webtool (SPRING viewer) R package (v. 1.6)[107] the number of PCA dimensions chosen was 50, with gene filtering settings of a minimum count of 5, a variability percentile threshold of 80%, and 5 nearest neighbors. No minimum was applied for cell filtering and a minimum of 3 cells was used for gene filtering.

**qPCR** Global gene expression analysis of 10-week-old organoid cultures was performed by qPCR. RNA was extracted with Trizol and 2 µg of RNA were converted with iScript High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814). qPCR was performed with IQ™ SYBR Green Supermix (Biorad, 1708882) in a Biorad CFX96 with the following program: 95 °C (20 s), 37 °C for 90 min and rinsed three times with PBS. Cells were then transferred to a sodium-based extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 D-Glucose, 10 Hepes, pH 7.4. The organoid cultures were then imaged using a Nikon Ti-Eclipse microscope and an Andor Zyla sCMOS camera with a SYBR Green fluorescence intensity. For the comparison of ALS and control lines, custom MATLAB (version 2018b; MathWorks, Natick, MA) scripts were written to set the threshold between frames using frame subtraction. The absolute value of pixel intensity variation was then averaged for the frame. Large changes in the average pixel intensity per frame marked putative sites of muscle contraction. Each potential contraction was then manually verified by a blinded observer to exclude air bubbles, noise, and artifacts. The validated contractions were then readily classified as small (encompassing less than half of the field of view) or large (encompassing more than half of the field of view).

**Whole-cell electrophysiology** Cells were washed twice with the same solution described for calcium imaging prior to recording to remove media and debris. Cells were visualized using an inverted Nikon Ti-Eclipse microscope and recordings were made using an EPC-10 amplifier (HEKA) controlled by PatchMaster software (PatchMaster v2x90.2, HEKA). During recording, cultures were continually perfused with ACSF containing (in mM): 127 NaCl, 3 KCl, 2 CaCl2, 1 MgCl2, 1.3 NaH2PO4, 10 Glucose, 25 NaHCO3, and gassed with 5% CO2 in oxygen. Intracellular pipette solution contained (in mM): 140 KMeSO4, 10 NaCl, 1 CaCl2, 1 EGTA, 3 MgATP, 0.4 NaGTP, 10 Hepes, TTX (300 µM) was applied for at least 10 min prior to recording to allow complete penetration into the tissue. Voltage-activated currents were elicited by 100 ms voltage steps to 0 mV from a holding potential of −80 mV.

**Neurite outgrowth analysis** Organoids were transduced with an hSYN: EGF-AAV9 viral vector (a gift from Bryan Roth (Addgene viral prep # 50465-AAV9; http://www.addgene.org/50465/)) at week 2 of culture. A selection of 20 independent sites arranged in a fixed grid, constituting 7.8% of the total area of individual organoid cultures, was imaged longitudinally and bi-weekly.
from week 3 to week 9 in culture. Images were acquired with an Image X-Press Micro Confocal (Molecular Devices), and each individual site was acquired as a z-stack of 12 individual planes separated by 1 µm. Neurite outgrowth quantification was performed in Fiji (Image 1.5.2p9)33. Maximum intensity projections of each individual stack were loaded into ImageJ. First, neurites were identified by applying a 2 sigma Gaussian blur, then applying a rolling ball filter with a 5 pixel radius to remove large objects. Long, non-circular neurites were then identified using Minimum Error automatic thresholding and selecting objects greater than 100 pixels² and circularity values less than 0.5. Second, cell bodies were identified by applying a 2 sigma Gaussian blur, identifying objects using Otsu automatic thresholding, and selecting objects greater than 100 pixels² and with circularity values greater than 0.5. Additional non-neurite noise resulting from autofluorescence of remaining large spheres was identified by applying a 10 sigma Gaussian blur and Triangle automatic threshold. Neurite signal overlapping with either cell bodies or sphere autofluorescence was subtracted to yield the full mask of neurites, which was refined further to large neurite tracks by selecting objects greater than 1000 pixels² and with circularity less than 0.2. For each neurite mask, the total area of the mask was measured. The neurite area for each image was loaded into R (v3.5.0) and the total neurite area within each well was quantified by summing the area of each neurite mask. The neurite area was normalized to the initial time point for each well.

**Analysis of percentage of innervation and area of NMJs**. Organooid cultures were transduced with an hSyn:EGFP AAV9 viral vector (a gift from Bryan Roth (Addgene viral prep # 50465-AAV9; http://n2t.net/addgene:50465; RRID: Addgene_50465)) at week 13 of culture. At 15 weeks of culture, organooid cultures were fixed and subjected to 4% (w/v) formaldehyde (Thermo Fisher Scientific, 28098) and rinsed gently three times with PBS. Organooid cultures were then labeled with a 1 µg/ml solution of α-bungarotoxin directly conjugated with an Alexafluor™ 647 dye (Thermo Fisher Scientific, B35450) in PBS, for 2 h at room temperature. After incubation, the excess dye was rinsed with PBS, and the organoid cultures were imaged. A random selection of 40 independent sites arranged in a fixed grid and constituting 15.6% of the total area of individual organoid culture wells were imaged for each organoid culture. Images were acquired with an Image X-Press Micro Confocal (Molecular Devices), and each individual site was acquired as a z-stack of five individual images separated by 1 µm. Quantification of EGFP expressing cells was performed manually, blinded to the iPS-line and genotype. Detection of individual clusters of α-bungarotoxin, quantification of the intensity of α-bungarotoxin and EGFP, and quantification of the area of the α-bungarotoxin clusters were done with MetaXpress (Version 6.5.4.532, Molecular Devices). Analysis results were exported to R (v3.5.0). Clusters consistent with dimensions (10–50 µm) within the range of that of a human NMJ107, intensity of α-bungarotoxin labeling above the intensity threshold of 1000 were quantified for the total amount of α-bungarotoxin, with EGFP labeling above the intensity threshold of 5000 were considered innervated. The percentage of innervated NMJs was calculated as the ratio between innervated and total α-bungarotoxin clusters per organoid culture. The areas of NMJs and innervated α-bungarotoxin clusters were calculated as the average area of either innervated or uninnervated α-bungarotoxin clusters per organoid culture. Displayed data were normalized to the mean of matching isogenic controls.**

**Statistics and reproducibility**. All experiments and analyses were performed blind to cell line/drug identity. Sample sizes were chosen based on typical numbers in the field, with at least two independent differentiations for each experiment, with the exception of FACs sorting of neuronal cells. For all data, the Shapiro–Wilk test was used to test for normal distribution of the data. For normally distributed data, significance was assessed by one-way ANOVA and post hoc two-tailed t-tests for pairwise comparisons. For non-normal data, the non-parametric Kruskal–Wallis test was used, with post-hoc Mann–Whitney tests.

To compare amplitudes of TTX-R sodium and potassium currents from individual cells grouped by the presence or absence of TTX-R currents, we performed Mann–Whitney tests and reported median and IQR. In Fig. 3h, group sizes were 4 and 10; U = 0; P = 0.002 for sodium currents, and group sizes were 4 and 10; U = 19; P = 0.945 for potassium currents. For Fig. 3l, ANOVA (type 3 sum of squares in R) was used to compare contractile sites and contraction rates between control and ALS organoid cultures. For all statistics, significance was determined by 2-sided p-values under 0.05.

For the analysis of variance in non-isogenic and isogenic iPSC lines, measurements of individual sphere potency and area, as well as GFP+ astrocytes, IBA+ primitive microglia, and SAA+ skeletal muscle area from mature organoid cultures were collected from 5 to 6 wells across three differentiation batches. For quantification of among-line variance, values for each well were normalized as fold change from the batch average across all lines. The mean value for each line was calculated and the variance of values among the 5 non-isogenic lines and 6 isogenic lines were compared using an F-test for Equality of Variances. For quantification of within-line variance, for each well values were normalized as fold change from the batch average for each line independently. The variance of values, that is the sum of squared deviations from the normalized average, were calculated for each line and the difference between average within-line variance among isogenic and non-isogenic lines was assessed using a one-way ANOVA.

**All confocal images shown are representative of data from at least three independent differentiation of each iPS line.**

**Reporting summary**. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**. Data supporting the findings of this study are provided within the paper and its supplementary information. A source data file is provided with this paper. The single-cell RNA-seq and whole-exome sequencing data generated in this study have been deposited in the db-GAP database under accession code phs024400.v1.p1. 570 These data are available under restricted access to comply with the conditions of the informed consent form agreed with the donors involved in the study. Access can be obtained by applying for controlled access through the db-GAP website. CRISPR-Cas9 edited iPSC lines carrying the ALS-associated mutations TDP+–G289S, PFN1+–G148V, SOD1+–G148R, paired isotype controls, and all additional information is available upon reasonable request to the authors. Source data are provided with this paper.

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Acknowledgements

This work was supported by NIH grants F32-NS114319 (A.H.), DP2-NS106664 and K08-NS082364, the New York Stem Cell Foundation, and Target ALS (B.J.W.). B.J.W. is a New York Stem Cell Foundation—Robertson Investigator. Electron microscopy was performed in the Microscopy Core of the Center for Systems Biology/Program in Membrane Biology, which is partially supported by an Inflammatory Bowel Disease Grant DK043351 and a Boston Area Diabetes and Endocrinology Research Center (BADERC) Award DK057521. Single-cell RNA-seq encapsulation and cDNA library preparation was performed at the Single Cell Core at Harvard Medical School, Boston, MA, cDNA libraries were sequenced at the Center for Cancer Computational Biology at the Dana-Farber Cancer Institute, and quality control and data analysis was done in collaboration with the Harvard Chan Bioinformatics Core at the Harvard T. H. Chan School of Public Health, Boston MA. Generation of isogenic ALS lines was performed at Harvard Stem Cell Institute iPSC Core Facility. We thank members of the Lager-Tourenne and Albers labs as well as J. Sanes, P. Arlotta, and K. Hochedlinger for helpful discussion, C. Marques for assistance with figures, and A. Cohen for constructs.

Author contributions

J.D.P., D.M.D., A.-C.D., A.H., Y.S., and B.J.W. designed the experiments and wrote the paper. All authors reviewed and edited the manuscript. J.D.P., A.-C.D., D.M.D., Y.S., J.H., K.D., and V.C. performed experiments. D.M.D. analyzed and interpreted single-cell RNA-seq data together with the Harvard Chan Bioinformatics Core, E.B., D.M.D., and A. H. quantified muscle contractions. J.D.P., D.M.D., and A.H. quantified micrographs and performed statistical analyses.

Competing interests

B.J.W. serves as a SAB member for Quralis and has consulted for Apic Bio and Q-State Biosciences. The remaining authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-24776-4.

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Peer review information Nature Communications thanks Ilary Allodi and the other anonymous reviewer(s) for their contribution to the peer review of this work.

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