Stem Cell-Based Modeling in Pain and ALS

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th>Citation</th>
<th>Wainger, Brian. 2015. Stem Cell-Based Modeling in Pain and ALS. Master’s thesis, Harvard Medical School.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:17613734">http://nrs.harvard.edu/urn-3:HUL.InstRepos:17613734</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
# Table of Contents

- Introduction .................................................. 2
- Reprogramming Fibroblasts Into Nociceptors .............. 5
- Hyperexcitability of ALS Patient-Derived Motor Neurons .. 44
- Conclusions .................................................... 78
- Acknowledgments .............................................. 80
Introduction

Translational research in neurological disorders has largely focused on single or oligo gene rodent models whose value in understanding human disease may be limited. For example, in amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig’s Disease, 90% of cases are sporadic while 10% are familial and result from mutation in one of more than a dozen known ALS-causing genes. Only 2% of ALS cases are due to mutation in superoxide dismutase 1 (SOD1), despite the fact that the overwhelming majority of preclinical research is based on mice overexpressing a human mutant SOD1 protein. However, ALS is a heterologous disease on clinical, genetic, molecular and pathological levels. Thus, the relevance of SOD1 models to the clinical syndrome of ALS is unclear. Relatedly, by overexpressing dominant human mutations in mice, the accuracy of the model phenotype may suffer from artifacts of overexpression and heterologous expression as well as inability to model complex effects of human genetic backgrounds.

Advances in stem cell technology allow disease modeling using human neurons derived from patients. The seminal work of Shinya Yamanaka and colleagues established the generation of induced pluripotent stem cells (iPSCs), cells that are at least extremely similar to embryonic stem cells, can be made from adult patients and do not carry the inherent ethical limitations of human embryonic stem cells. Unlike rodent models, the stem cell modeling approach enables the study of both familial and sporadic disease, thus potentially either providing confirmation that discoveries are indeed relevant to broad populations or instead identifying subgroups of patients likely to benefit from specific treatment targets.

We have explored the use of derived neuronal cell types in modeling pain and motor neuron disease. In the first case, we use the technique of lineage reprogramming, which was established as a tool by which to transdifferentiate human fibroblasts into neurons by Marius Wernig and colleagues. We find that we can derive nociceptor neurons from mouse and human fibroblasts using a group of five
transcription factors. We show how the derived nociceptor neurons may be used to model disease pathophysiology with the future goal of performing phenotype-based drug screens.

In the second case, we take advantage of existing techniques for differentiating motor neurons from ALS patient iPSCs. We use stem cell modeling to explore physiological differences between ALS and healthy control-derived motor neurons, and we show how iPSC-based phenotypes can be used as platforms to identify and evaluate therapeutic candidates.
Modeling pain *in vitro* using nociceptor neurons reprogrammed from fibroblasts

(This work has been published previously: Nature Neuroscience 2015, 18, 17-24)

Brian J. Wainger\(^1,2,3\), Elizabeth D. Buttermore\(^1,3\), Julia T. Oliveira\(^1\), Cassidy Mellin\(^1\), Seungkyu Lee\(^1,3\), Wardiya Afshar Saber\(^1\), Amy Wang\(^1\), Justin K. Ichida\(^4,5\), Isaac M. Chiu\(^1,3\), Lee Barrett\(^1\), Eric A. Huebner\(^1,3\), Canan Bilgin\(^1\), Naomi Tsujimoto\(^4\), Christian Brenneis\(^1\), Kush Kapur\(^1\), Lee L. Rubin\(^4\), Kevin Eggn\(^4,6\), and Clifford J. Woolf\(^1,3\)

\(^1\)FM Kirby Neurobiology Center, Boston Children’s Hospital and Harvard Stem Cell Institute

\(^2\)Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital

\(^3\)Department of Neurobiology, Harvard Medical School

\(^4\)Harvard Stem Cell Institute, Department of Stem Cell and Regenerative Biology, Harvard University

\(^5\)Department of Stem Cell Biology and Regenerative Medicine, Eli and Edythe Broad CIRM Center for Regenerative Medicine and Stem Cell Research, University of Southern California

\(^6\)The Howard Hughes Medical Institute, USA

Clifford J. Woolf

criff.woolf@childrens.harvard.edu

Phone: 617 919-2393

Fax: 617 919-2772

*These authors contributed equally to this work.*
Reprogramming somatic cells from one cell fate to another can generate specific neurons suitable for disease modeling. To maximize the utility of patient-derived neurons, they must model not only disease-relevant cell classes but also the diversity of neuronal subtypes found in vivo and the pathophysiological changes that underlie specific clinical diseases. Here, we identify five transcription factors that reprogram mouse and human fibroblasts into noxious stimulus-detecting (nociceptor) neurons that recapitulate the expression of quintessential nociceptor-specific functional receptors and channels found in adult mouse nociceptor neurons as well as native subtype diversity. Moreover, the derived nociceptor neurons exhibit TrpV1 sensitization to the inflammatory mediator prostaglandin E2 and the chemotherapeutic drug oxaliplatin, modeling the inherent mechanisms underlying inflammatory pain hypersensitivity and painful chemotherapy-induced neuropathy. Using fibroblasts from patients with familial dysautonomia (hereditary sensory and autonomic neuropathy type III), we show that the technique can reveal novel aspects of human disease phenotypes in vitro.

Directed differentiation from pluripotent stem cells and lineage reprogramming of fibroblasts can both be used to derive a wide range of different neuronal subtypes. While the known sequence of morphogen exposure and consequent molecular changes in the development of specific neurons can guide directed differentiation strategies, the selection of transcription factors for lineage reprogramming from fibroblasts remains essentially empirical. No single transcription factor has proved essential for driving cell fates in all neuronal reprogramming studies to date, despite the fact that specific factors such as Ascl1 or Ngn2 seem particularly potent in deriving a range of different neuronal subtypes. Brn2, Ascl1 and Myt1l (abbreviated BAM) generate generic neurons on their own and specific neuronal subtypes when combined with additional factors. Moreover, the developmental stage at which a particular transcription factor acts in vivo may determine whether that factor facilitates or inhibits the patterning of reprogrammed neurons.
Nociceptors are the first-order neurons in the pain sensory transduction pathway and play the critical initial step in the detection of noxious stimuli (nociception) and the development of inflammatory and neuropathic pain. Nociceptor neurons employ a host of highly specific ionotropic receptors and ion channels, including TrpV1, TrpA1, TrpM8 and P2X3 receptors to transduce stimuli, as well as slow, tetrodotoxin (TTX)-resistant sodium channels (NaV1.8 and NaV1.9) that generate their characteristic broad action potentials. Efforts to derive nociceptors using a small molecule-based directed differentiation strategy from human neural crest precursors have produced neurons that recreate some but not all of these characteristic receptors and channels.

Mutations in nociceptor-specific membrane proteins underlie a wide range of pain diseases, including rare but severe channelopathies due to NaV1.7 or TrpA1 mutations, common small fiber neuropathies due to activating mutations in NaV1.7 or NaV1.8, as well as a variety of pain-predisposing polymorphisms; however, the biological effects of these mutations on nociception have not been studied in human sensory neurons. Nociceptors normally activate only following intense, potentially damaging stimuli in order to provide a protective warning of imminent tissue injury. However, they also have the remarkable capacity to become sensitized after exposure to inflammatory mediators or by chemotherapeutic drugs, resulting in a reduced activation threshold so that innocuous stimuli can generate a pain response. Pain hypersensitivity can play a physiologically useful role in minimizing further injury and in promoting healing once damage has occurred; however, such transient sensitization, when it persists, promotes the development of chronic pain.

Nociceptor neuron development occurs through dorsalization within the neural tube, followed by neural crest induction and migration and then nociceptor specification within the still-multipotent neural crest lineage. The generation of nociceptor progenitors expressing the TrkA neurotrophin receptor (Ntrk1) and postnatal nociceptors expressing TrpV1 requires the basic helix-loop-helix transcription factor Ngn1 (Neurogenin1), which is normally present from approximately days E9–
E13 in the embryonic mouse\textsuperscript{21}. Although developing nociceptors express multiple Trk-family receptors, maturing nociceptors express only TrkA. Brn3a (POU4F1) promotes Runx1 expression, which together with Isl1 (Islet 1) and Klf7 maintain TrkA expression in developing nociceptors\textsuperscript{22-25}. A subset of nociceptors that become the peptidergic subclass of nociceptors maintain TrkA expression and express calcitonin gene-related peptide (CGRP, CalcA) and substance P. For non-peptidergic nociceptors, most of which bind isolectin B4, the glial cell derived neurotrophic factor (GDNF) receptor Ret replaces TrkA in a process dependent on Runx1, and loss of Runx1 markedly reduces TrpV1 expression\textsuperscript{23}.

We set out to produce nociceptor neurons through transcription-mediated lineage conversion of fibroblasts. From an initial set of 12 factors, we find that expressing five factors is sufficient to generate functional mouse nociceptor neurons. In a direct comparison between the induced nociceptors and primary adult mouse nociceptors, we show that the induced neurons mimic bona fide nociceptors with regard to the function of the specific individual receptors and channels, such as TrpA1, TrpM8, P2X3 and NaV1.8, as well as with regard to the population diversity. We demonstrate that the induced neurons also model inflammatory peripheral sensitization, a critical process that underlies transient pain hypersensitivity and contributes to the pathological transition to chronic pain, as well as sensitization following exposure to the chemotherapeutic drug oxaliplatin. Finally, we derive human nociceptor neurons from patients with familial dysautonomia (FD) and show that these neurons reveal potentially disease-relevant phenotypes \textit{in vitro}.

\section*{RESULTS}

\textbf{Selection and Optimization of Transcription Factors}

We first developed nociceptor reporter mice by taking advantage of an existing TrpV1 Cre-driver\textsuperscript{26} and floxed \textit{tdTomato} mice to generate TrpV1-Cre\textsuperscript{+/-}:tdTomato\textsuperscript{+/-} reporter mice, from which we obtained mouse embryonic fibroblasts (MEFs). Activation of the \textit{tdTomato} reporter signaled the conversion of the MEFs to TrpV1-expressing cells (\textbf{Supplementary Fig. 1}). We began with nine transcription factors selected to promote lineage conversion to nociceptors in combination with
the three BAM factors (12 total, Table 1). These factors were chosen using a combination of the prior literature, transcription factor expression profiles in FACS-sorted adult mouse nociceptors (NaV1.8-positive) as compared to proprioceptors (parvalbumin-positive) (Chiu et al, submitted), an expression profile similarity to NaV1.8 in the BioGPS database, and postnatal dorsal root ganglion (DRG) expression in the Allen Brain Atlas.

As expected, there was no baseline activation of the tdTomato reporter in MEFs (not shown). Staining of MEFs for neuronal precursor markers using antibodies to Nestin, Sox1, and Ki67, as well as for neuron-specific class III β-tubulin (Tuj1), were all negative (Supplementary Fig. 2). After transducing the fibroblasts with a combination of all 12 individual retroviruses containing the selected transcription factors, we detected a small number of tdTomato-positive cells with a neuronal morphology after two weeks (Supplementary Fig. 3a). In order to identify those transcription factors that were either critical for or inhibitory to lineage reprogramming into TrpV1-expressing cells, we then sequentially eliminated each factor, one at a time. Surprisingly, the omission of some transcription factors strongly supported by the literature for a role in promoting TrpV1 expression, such as Runx1, did not eliminate TrpV1 reporter expression (Supplementary Fig. 3b). In fact, omission of Brn3a led to a marked increase in the number of tdTomato-positive cells (Supplementary Fig. 3c). We identified from this iterative process three factors that were critical to the TrpV1 lineage reprogramming process in that their omission led to a near complete absence of tdTomato and neuronal Class III β-tubulin (Tuj1)-positive cells bearing a neuronal morphology: Ascl1, Myt1l and Klf7 (Supplementary Fig. 3d–f).

When we combined the three BAM factors with Isl2, Ngn1, and Klf7, we again observed only a small number of tdTomato, Tuj1-positive cells (Fig. 1a). Because prior studies and our initial drop out experiments detected specific factors that could inhibit the lineage reprogramming process, we performed single factor dropouts from these six factors and found that omission of Brn2 led to a striking increase in the number of tomato-positive neurons (Fig. 1b), giving a yield of approximately 14% of plated fibroblasts that were both tdTomato- and Tuj1-positive (less than
0.1% were tdTomato-positive but Tuj1-negative. Removal of any other factor from the six sharply reduced the number of tdTomato-positive neurons (Fig. 1c–h). Next, we evaluated Ngn1 alone and in combination with the BAM factors; however, the yield was much lower than with the optimized five factor combination (Supplementary Fig. 4). Indeed, further removal of any of the five factors resulted in a marked decrease in tdTomato, Tuj1-positive cells (Supplementary Fig. 5).

Molecular Characterization of Induced Mouse Nociceptors
To determine if tdTomato-positive reprogrammed neurons phenocopied bona fide nociceptors, we evaluated the expression of protein markers specific for nociceptor neurons. Nearly all tdTomato-positive neurons stained for the pan-neuronal marker Tuj1 and had a neuronal-like morphology with many long branching axons, and most Tuj1-positive neurons were tdTomato-positive (Fig. 2a). Staining with an anti-TrpV1 antibody confirmed the translation of the TrpV1 protein in the vast majority of tdTomato-positive neurons (Fig. 2b). In mouse dorsal root ganglia, most TrpV1-expressing neurons are C-fibers that express the marker peripherin (Prph)29, while only a small percentage of A-δ fibers are also TrpV1-positive26. In the induced neurons, a majority of tdTomato-positive neurons expressed peripherin (66.9 ± 4.1%, n=16 wells from 4 separate transductions) (Fig. 2c) and many CGRP (22.3 ± 6.6%, n=4 wells from 2 separate transductions) (Fig. 2d); however, a smaller number of cells stained for the intermediate filament NF200, a marker, in this context, of myelinated A-δ nociceptors (Fig. 2e). In contrast, the derived nociceptors did not stain for smooth muscle actin (SMA), a marker of muscle, despite reports of TrpV1 expression in muscle30 (Supplementary Fig. 6a,b). Furthermore, neurons derived from the three BAM factors did not express nociceptor markers, consistent with their high specificity (Supplementary Fig. 6c–g).

Because specific antibodies do not exist for many quintessential nociceptor proteins, we utilized quantitative RT-PCR to compare nociceptor marker mRNA levels in tdTomato-positive induced nociceptors and tdTomato-positive adult mouse nociceptors relative to levels in MEFs (Fig. 2f).
For this analysis, we used patch pipettes to pick tdTomato-positive induced and primary mouse neurons, as well as MEFs, and plotted the levels of specific transcripts in induced and primary nociceptors relative to MEFs. The fibroblast marker $S100A4$ was expressed at a similar very low level in both the induced and primary nociceptors, consistent with a non-fibroblast identity of the induced nociceptors. NaV1.7 ($Scn9a$), which is found in nociceptor and autonomic peripheral neurons, was present in both the induced and primary nociceptors, as was TrkA ($NTRK1$), which is turned on in developing nociceptors and persists in the peptidergic subset of mature nociceptors, although the expression of NaV1.7 and TrkA in the induced neurons was several fold less than in the primary DRGs. Together, these immunohistochemistry and PCR data suggest that the induced neurons express a complement of bona fide nociceptor-specific markers.

**Functional Properties of Induced Mouse Nociceptors**

In order to investigate the functional properties of the induced nociceptors, we performed calcium imaging with a battery of agonists and evaluated the number of responders within the tdTomato-positive population with a stable baseline and response to potassium chloride (KCl, which activates voltage-gated calcium channels through depolarization and serves as a measure of neuronal functional integrity) (**Fig. 3a**). We chose concentrations of agonists for TrpM8 (250 µM menthol), TrpA1 (100 µM mustard oil) and TrpV1 (1 µM capsaicin) that only activated their respective cognate receptors$^{31}$. 39% of the cells responded to capsaicin, 9% to mustard oil and 3% to menthol (**Fig. 3a,b**; n=227 tdTomato-positive cells that responded to KCl). We observed occasional cells that responded to both mustard oil and capsaicin, a single cell that responded to menthol and mustard oil but not capsaicin, and one cell that responded to all three agonists. We did not observe any tdTomato-positive cells that responded to menthol alone, but we did identify a small number of tdTomato-negative cells that responded to menthol but not the other Trp agonists (**Supplementary Fig. 7a**). In contrast, 0/50 KCl-responding neurons derived from the BAM factors alone responded to capsaicin (not shown). Using the same experimental procedure, we then asked how the frequencies of the different combinations of receptors within individual neurons compared between induced nociceptors and adult mouse nociceptors. In tdTomato-
positive primary DRG neurons dissected and cultured from adult \textit{TrpV1::tdTomato} mice, we found that 36\% of the neurons responded to capsaicin, 2.5\% to mustard oil and 2.5\% to menthol (\textit{Supplementary Fig. 7b,c}; \(n=249\) tdTomato-positive cells that responded to KCl). Thus, the nociceptor lineage reprogramming not only yielded physiologically functional TrpV1, TrpA1 and TrpM8 proteins in the induced neurons, but the frequencies and combinations of the different receptors in the induced neurons closely mimicked those of adult mouse nociceptors.

While calcium imaging provides detailed information about calcium entry through Trp channels, it does not evaluate whether activation of these channel evokes action potential firing in the neurons. By culturing the induced neurons on extracellular multi-electrode arrays, we found that both capsaicin and mustard oil application evoked robust action potential firing from the induced neurons (\textit{Fig. 3c}; 3/3 arrays for capsaicin and 3/3 arrays for mustard oil).

We next used whole-cell patch clamp recordings to define the electrophysiological properties of the induced nociceptors and found that capsaicin (1 \textmu M) elicited inward currents in 6/11 tdTomato-positive induced neurons, consistent with but somewhat higher than the calcium imaging results (\textit{Fig. 4a}). The P2X3 subtype of ionotropic purinergic receptors is expressed specifically in nociceptor neurons\textsuperscript{32}. Application of the P2X3-specific agonist \(\alpha, \beta\)-methylene-ATP (30 \textmu M) elicited rapidly-adapting inward currents in 8/16 neurons (\textit{Fig. 4b}) that were blocked completely by A-397491, a specific P2X3 antagonist, in 4/4 neurons (not shown)\textsuperscript{33}.

Perhaps the most nociceptor-specific functional marker is the TTX-resistant NaV1.8 sodium channel, which produces a portion of the current in the nociceptor action potential upstroke\textsuperscript{34}. In voltage-clamp, we found that depolarizing voltage steps elicited inward sodium currents both before and after the application of 300 nM TTX (\textit{Fig. 4c}; 14/15 recorded induced nociceptors had TTX-resistant sodium currents greater than 50 pA). In agreement with our expression studies, the slow channel kinetics of the TTX-resistant currents are typical for NaV1.8 as opposed to the fast NaV1.5 cardiac sodium channel, which is present in developing embryonic nociceptors\textsuperscript{35}.
Furthermore, five of the 14 neurons with TTX-resistant sodium currents also exhibited a persistent sodium component, which previous studies have found to be due to NaV1.9\textsuperscript{34,36} (Fig. 4c). The ability to generate action potentials in the presence of TTX is a feature of nociceptors but not of other DRG or central neurons. The induced neurons fired single TTX-resistant action potentials that overshot 0 mV in 7/12 neurons (Fig. 4d). NaV1.8 is responsible for the characteristic broad action potential shape of the nociceptor action potential\textsuperscript{8}, which we found to be a property of the induced neurons (mean action potential width 3.32 ± 0.33 ms; n=13); as expected, adult primary tdTomato-positive nociceptors fired broad action potentials, but not large tdTomato-negative primary non-nociceptor DRG neurons (Fig. 4e). In addition to differences in action potential morphology, the firing pattern of nociceptor neurons to prolonged depolarizing currents is tonic, compared to the phasic firing of most large A-β DRG neurons\textsuperscript{37}. Induced nociceptors fired tonic action potential trains in response to depolarizing current steps in 12/13 cells, consistent with the tonic firing found in tdTomato-positive adult primary mouse nociceptors, and in contrast to the single action potentials elicited in non-nociceptor, large tdTomato-negative adult DRG neurons (Fig. 4f).

While hyperpolarization-activated cyclic nucleotide-sensitive (HCN) currents are not specific for nociceptor neurons, they play an important role within these cells in neuropathic and inflammatory pain\textsuperscript{38}, and thus their presence may be important for disease-modeling. We found that the induced nociceptors produced typical sag depolarizations in response to hyperpolarization (Fig. 4g) in 11/17 tdTomato-positive induced neurons, consistent with ZD7288-sensitive HCN currents recorded in voltage clamp (2/2, not shown).

A critical function of peptidergic neurons, most of which express TrpV1\textsuperscript{7}, is to release neuropeptides such as CGRP and Substance P. To assess the fidelity of the induced nociceptors in this capacity, we measured CGRP levels in supernatant following a depolarizing stimulus and found that induced nociceptors, but not BAM-derived neurons, released CGRP after KCl stimulation (Fig. 4h; n=4; Mann-Whitney U-test p=0.03). The concentrations of CGRP released
by the induced neurons were comparable to those released by primary DRG neurons (Supplementary Fig. 8), thus indicating that the induced neurons have synaptic vesicle release mechanisms in place.

**Induced Nociceptors Model Inflammatory Sensitization**

The transition from high-threshold baseline nociception to low-threshold clinical pain hypersensitivity commonly involves peripheral sensitization of nociceptors. For the induced nociceptors to be valuable in vitro models of in vivo pathophysiology, they must replicate not only the specific functional channels and receptors of the cells but also the process of sensitization that leads to pathological pain. Prostaglandin E2 (PGE2) activates the PKA pathway and sensitizes the TrpV1 receptor, reducing its threshold and decreasing desensitization. In the tdTomato-positive induced neurons, a low concentration (300 nM) of capsaicin rarely yielded a detectable response (mean change in fluorescence absorption ratio of 0.028 ± 3.0*10^-3) (Fig. 5a,b). However, after treatment with PGE2 (1 µM) for two minutes, a second identical capsaicin (300 nM) application yielded a mean response of 0.18 ± 6.0*10^-3 (n=41 cells; paired t-test \( p=1.5\times10^{-4} \)). Plotting the magnitudes of the initial capsaicin and PGE2-sensitized capsaicin responses revealed that although the majority of neurons exhibited small or undetectable initial responses to capsaicin, they produced robust signals after PGE2 sensitization (Fig. 5c).

TrpV1 sensitization also may contribute to painful chemotherapy-induced neuropathy due to oxaliplatin. Using MEA recording, we compared capsaicin responses in induced nociceptors treated with either 50 µM oxaliplatin or vehicle control, and found marked sensitization in the oxaliplatin-treated nociceptors (Fig. 5d,e).

**Induction of Human Nociceptors**

To derive nociceptors from human fibroblasts, we initially included NeuroD1 in the nociceptor induction protocol, as this transcription factor was important in prior human lineage reprogramming studies. However, we found that the reprogramming efficiency, was greater...
without NeuroD1 (five factors) than with NeuroD1 (six factors) (20.7 ± 1.4 cells per field for five factors; 9.7 ± 1.1 cells per field for six factors, n=6 wells/group; t-test p=1.0x10^{-4})

(Supplementary Fig. 9). Furthermore, more neurons exhibited larger sodium currents (67% of patched five factor neurons had peak transient sodium currents greater than 500 pA, versus 29% of six factor neurons) and five factor neurons were healthier (resting Vm -49.3 ± 2.2 mV, n=33 five factor neurons; Vm -37.3 ± 3.2, n=20 six factor neurons; Mann-Whitney U-test p-value = 0.001). Using healthy control (HC) subject fibroblasts, the 5 factors yielded Tuj1-positive neurons at an efficiency of 5% of plated fibroblasts, and 16% of the Tuj1-positive neurons were also peripherin-positive (Fig. 6a,b), efficiencies that were somewhat lower than the mouse induced nociceptors. A small number of the Tuj1-positive neurons were NF200-positive (Fig. 6c). We recorded from the neurons using whole-cell patch clamp. Although we did not have a reporter for a particular neuronal subtype, the induced human neurons fired broad action potentials (mean action potential width 3.88 ± 0.41 ms; n=17; Fig. 6d), consistent with functional nociceptors. In 38 voltage clamp recordings, we applied TTX to neurons with a large total sodium current (greater than 1 nA) and detected TTX-resistant sodium currents in 10/10 neurons (Fig. 6e). As in both our mouse induced nociceptors and primary mouse and human nociceptors\(^{34,36}\), the induced human neurons had different combinations of slow- and persistent TTX-resistant sodium currents, consistent with NaV1.8 and NaV1.9 contributions, respectively (Fig. 6e).

In order to evaluate the potential of the human neurons for disease modeling, we reprogrammed fibroblasts from three HC and three unrelated, age-matched subjects with familial dysautonomia (FD, hereditary sensory and autonomic neuropathy type III, Riley-Day syndrome), due to a homozygous donor splice site mutation that results in deletion of intron 20 from the I-κ-β kinase complex-associated protein (IKBKAP) RNA\(^{40}\). We found that single FD-derived neurons picked using patch pipettes exclusively expressed the abnormally spliced transcript, something not previously identified, while the HC-derived neurons expressed only the normal transcript (Fig. 6f). FD fibroblasts expressed a mixture of abnormally spliced and normal transcripts, consistent with
prior studies\textsuperscript{41,42}, while HC fibroblasts expressed only the normal transcript (Fig. 6f; Supplementary Fig. 10).

Although we detected peripherin-positive, Tuj1-positive neurons from all HC and FD subjects (Fig. 6g,h), the neurons from FD subjects showed a trend toward decrease in number (Fig. 6i; 16.5 ± 1.1 HC neurons/well, n=60 wells; 14.1 ± 1.1 FD neurons/well, n=60 wells; difference between HC neurons/well and FD neurons/well 2.3 ± 1.5, n=60 wells; random intercept mixed-effects model \( p=0.26 \) ) and a robust reduction in neurite outgrowth per cell (Fig. 6j; 725 ± 24 \( \mu \)m per HC neuron, n=60 wells; 433 ± 25 \( \mu \)m per FD neuron, n=60 wells; difference between HC neuron outgrowth per cell/well and FD neuron outgrowth per cell/well 291.3 ± 32.6 \( \mu \)m, n=60 wells; random intercept mixed-effects model \( p=0.012 \) ), as well as number of branches per neuron (Fig. 6k; 7.9 ± 0.3 branches per HC neuron, n=60; 4.7 ± 0.3 branches per FD neuron, n=60 wells; difference between HC branches per neuron/well and FD branches per neuron/well 3.3 ± 0.4, n=60 wells; random intercept mixed-effects model \( p=0.017 \) ) compared to HC-derived neurons.

DISCUSSION

The subjective nature of pain as a human experience confounds its clinical study, raises questions about the relevance of animal models and complicates the development of effective treatments\textsuperscript{43}. Furthermore, limited physiological studies of primary human nociceptors highlight differences between human and rodent nociceptors, including the function of individual channels and receptors as well as their distribution within different nociceptor subtypes\textsuperscript{44}, and thus emphasize the importance of investigating human nociception using human nociceptors. Modeling key mechanistic aspects of human pain processing with derived human cells may enable phenotypic screens for analgesics based on basal and sensitized neurons from chronic pain subjects. Such approaches would improve upon current drug screens that employ heterologously-expressed targets in non-neuronal cells and consequently do not reflect the native
scaffolding and molecular signaling present in human neurons, the pathophysiological changes that drive clinical pain and genetic backgrounds that may increase pain susceptibility.

We find that a small number of transcription factors can quite efficiently convert fibroblasts into neurons that express the key specific functional receptors found in bona fide adult nociceptors. While TrpV1 is expressed in a tiny fraction of central neurons\textsuperscript{30}, NaV1.8 and TrpA1 are not expressed within the central nervous system. The collective expression of subsets of these markers defines specific subpopulations\textsuperscript{7}, and indeed to a first approximation, our neurons recreate the combinatorial patterns that define the diversity of TrpV1-expressing nociceptive neuronal cohorts found in primary mouse nociceptors. Interestingly, we seem not to have derived a single nociceptor type but instead have engineered multiple subtypes of cells with properties and frequencies similar to those found in vivo. Three possible explanations will need to be investigated in future studies: first, there may be an autonomous program driving the native lineage diversity that is replicated by our transcription factors; second, non-cell autonomous communication among neurons could influence their identity; third, different identities could reflect different relative levels of transcription factor expression.

While the transcription factor combinations that facilitate induction of specific fates have typically incorporated factors known to play defined roles in the development of those neurons\textsuperscript{1}, some of the factors we used do not have any well-defined developmental role. We chose Isl2 due to its strong differential expression in FACS-sorted nociceptors compared to proprioceptors and an expression pattern similarity to NaV1.8\textsuperscript{27}, although nothing is known about its role in nociceptor development. Interestingly, the in situ expression of Isl2 appears much more nociceptor-specific than that of Isl1\textsuperscript{27,28}, for which a role in nociceptor development and TrpV1 expression has been documented\textsuperscript{24}, although another report found broader RNA expression of Isl2\textsuperscript{45}. Our results also raise questions as to why specific transcription factors facilitate or inhibit the reprogramming both to neuronal and nociceptor lineages. In contrast with prior studies\textsuperscript{4,5,39}, we found that Brn2 inhibited lineage reprogramming of mouse fibroblasts to neurons, and NeuroD1 decreased the
efficiency and quality of human neurons. With regard to the nociceptor lineage, the roles of Brn3a and Klf7 in maintaining TrkA expression during embryonic development appear similar in the literature. However, we found that Brn3a markedly inhibits lineage reprogramming to nociceptors while Klf7 promotes it. Runx1, which is active and critical in the later embryonic stages of nociceptor development, does not facilitate the reprogramming process. Ngn1 has a well-characterized role in nociceptor development and eventual TrpV1 expression, but is involved much earlier in development (E9–E13) compared to the other studied factors. Thus, while developmental studies may inform the choice of transcription factors for lineage reprogramming, reprogramming studies may themselves provide insight into important developmental pathways and their regulators, recognizing of course that reprogramming may not recapitulate transcription factors expressed in normal development.

The intricacy and specificity of primary nociceptor neuron physiology and the fortunate ability to culture adult primary sensory neurons provide an unusual and well-controlled opportunity to evaluate how closely lineage-reprogrammed neurons resemble the molecular expression, function and maturity of primary adult neurons. We found that the reprogrammed neurons produced functional TrpV1, TrpA1 and TrpM8-expressing neurons in similar relative percentages to those found in primary tdTomato-positive adult mouse nociceptors. In addition, the induced neurons yielded not only functional TTX-resistant action potentials, but also the broad action potential morphology and phasic firing pattern characteristic of nociceptors. Indeed, their function globally was remarkably close to that of adult primary nociceptors by every measure we made, although we cannot exclude other contributions, such as neuronal maturity, to firing pattern and action potential morphology. Future studies will explore the synaptic capacity, neurotrophin dependence, and requirement for sustained viral transgene expression in the derived nociceptors.

Patient-derived neurons would have optimal utility as a drug screening tool if the derived neurons replicate the sequence of pathophysiologica events that result in specific clinical diseases.
Reprogrammed nociceptors may be particularly useful as an in vitro model for pain, because the pain sensitization process mimicked by the induced nociceptors is one of the major factors that drive the transition to pathological pain. The development of a fluorescent nociceptor-specific marker will enable further optimization of human induced nociceptor maturation as well as more extensive molecular and physiological characterization of the human neurons. Whether nociceptors reprogrammed from individuals with chronic pain or peripheral neuropathy will reveal phenotypic differences compared to control subject-derived nociceptors can be evaluated in future research.

Analysis of FD patient tissue samples\textsuperscript{40,41} and FD iPSC-derived neural precursors\textsuperscript{42} have consistently shown the presence of both abnormally and normally spliced transcripts in the same sample. The detection of both splice forms could reflect either the presence of both normal and aberrant splicing in the affected neuronal types or simply the heterogeneity of affected and unaffected tissue types in the samples. Our finding that the normal $IKBKAP$ splice variant is apparently absent in the FD-derived neurons indicates the latter is likely the case and may have implications regarding the extent of splice correction necessary for disease treatment, and may explain the observation in a mouse FD model that small amounts of IKAP are sufficient to revert the phenotype\textsuperscript{46}. The reduction in outgrowth and branching in familial dysautonomia compared to control-derived neurons may reflect the presence of processes similar to those responsible for the progressive decrease in unmyelinated sensory neurons that is observed clinically\textsuperscript{40} and the loss of TrkA-positive neurons in a mouse model\textsuperscript{47}. The ability to obtain FD nociceptors from patient fibroblasts will facilitate future studies to examine the mechanisms of disease and to screen and evaluate potential treatments. Our experiments illustrate how derived neurons with major features of primary nociceptors can be generated and employed as a model for “pain a dish”.
Online Methods

A methods checklist is available with the supplementary materials.

**Fibroblasts.** *TrpV1-Cre<sup>+/−</sup>:tdTomato<sup>+/−</sup>* transgenic mice were generated by crossing *TrpV1-Cre<sup>+/−</sup>* mice with *tdTomato<sup>+/−</sup>* reporter mice on a C57Bl6 background (both from Jackson Laboratories). Mouse embryonic fibroblasts (MEFs) were harvested from *TrpV1::tdTomato* embryos, of either gender, at E12.5, passaged once and frozen at -120˚C. Human fibroblasts (all from Coriell Institute) were obtained from three healthy control subjects (GM00969, 2 year old Caucasian female; GM03348, 10 year old Caucasian male; GM00316, 12 year old Caucasian male) and from three age-matched subjects with familial dysautonomia (GM04663, 2 year old Caucasian female; GM04959, 10 year old Caucasian female; GM04899, 12 year old Caucasian female). The use of human lines was approved under the Boston Children’s Hospital Institutional Review Board.

**Viruses and transductions.** Complimentary DNAs for the 9 candidate factors (obtained from the Dana Farber/ Harvard Cancer Center DNA Resource Core except Ngn1, Tlx3 and Runx1, which were obtained from Q. Ma) were each cloned into the pMXs retroviral expression vector modified to contain a WRE using Gateway technology (Invitrogen). 293T cells were co-transfected with individual viruses and pHDMG and pIKLMV packaging plasmids using Lipofectamine 2000 (Life Technologies). Media was changed to new DMEM (GIBCO), 20% FBS (Invitrogen), 50 U/mL Penicillin/Streptomycin (CellGro) after 16 hours. At that time, fibroblasts were thawed and plated on 24-well plates (25K cells/well), 6-well plates (150K cells/well), 35mm dishes (150K cells/well), or p515A multi-electrode array (MEA) probes (Alpha Med Scientific) (12K cells/MEA) that were previously coated with poly-D-lysine (Sigma), gelatin (Cell Signaling) and laminin (Sigma). Viruses were harvested 24 hours later, concentrated approximately 5 fold using Amicon ultra centrifugal filter units (Millipore) and applied to fibroblasts with 5 µg/ml polybrene (Sigma) (Day 0, transduction). Cortical mouse glia obtained from P0–P2 C57Bl6 mice were added on Day 2 for all
but the calcium imaging experiments. Media was switched on Day 4 to N3 media: DMEM/F-12 (GIBCO), N2 and B27 supplements (Life Technologies), glutamax (Invitrogen), pen/strep, FGF (20 ng/mL, Millipore) with 5% FBS, along with the neurotrophic factors BDNF, CNTF, GDNF (R&D Systems) at 10 ng/ml each. The TGFβ-Inhibitor RepSox (7.5 µM; Millipore), which has been shown to improve survival of different neuronal types over long-term culture (Ichida and Eggan, unpublished), was added for calcium imaging and human transductions. Media was changed every two days, and on Day 10, NGF was also added to the media (50 ng/mL; Invitrogen).

**Immunohistochemistry.** Cells were fixed with 4% paraformaldehyde (PFA), washed three times with 1X PBS, incubated in blocking buffer (1% Blocking Reagent (Roche), 0.5% BSA, 0.1% TritonX-100) for one hour at room temperature and stained with primary antibodies overnight at 4°C in blocking buffer. The next day the cells were washed three times with 1x PBS, stained with secondary antibodies for one hour at room temperature and washed three times with 1x PBS before imaging, which was performed using the microscope setup described below.

Primary antibodies included: mouse anti-β tubulin III (Sigma T8660, 1:1000, validated51), rabbit anti-peripherin (Millipore AB1530, 1:800, validated52), rabbit anti-TrpV1 (Alomone Labs ACC-030, 1:200, validated53), rabbit anti-CGRP (Calbiochem/Millipore PC205L, 1:300, validated54), chicken anti-neurofilament, heavy chain (Millipore AB5539, 1:1000, validated55), mouse anti-Nestin (Abcam ab6142, 1:500, validated56), mouse anti-smooth muscle actin (Sigma A5228, 1:300, validated57), goat anti-Sox1 (Santa Cruz #SC17317, 1:50, validated58), mouse anti-Ki67 (Sigma P6834, 1:500, validated59). Secondary antibodies included: goat anti-chicken AlexaFluor 568 (Life Technologies A11041), goat anti-chicken AlexaFluor 488 (Life Technologies A11039), goat anti-mouse AlexaFluor 488 (Life Technologies A11029), goat anti-mouse AlexaFluor 568 (Life Technologies A11031), goat anti-rabbit AlexaFluor 488 (Life Technologies A11008), goat anti-rabbit AlexaFluor 568 (Life Technologies A11011), donkey anti-goat AlexaFluor 488 (Life Technologies A11055).
Primary DRG culture. DRGs were dissected from adult *TrpV1-Cre::tdTomato* mice (12–13 weeks) into Hank’s balanced salt solution (HBSS) (Life Technologies). DRG were dissociated in 1 mg ml⁻¹ collagenase A plus 2.4 U ml⁻¹ dispase II (enzymes, Roche Applied Sciences) in HEPES-buffered saline (Sigma) for 90 min at 37 °C and then triturated down to single cell level using glass Pasteur pipettes of decreasing size. DRGs were the centrifuged over a 10% BSA gradient and plated on laminin-coated cell culture dishes (Sigma). DRGs were cultured 24 hours in B27-supplemented neurobasal-A medium plus 50 ng/ml NGF (Invitrogen), 2 ng/ml GDNF (Sigma), 10uM arabinocytidine (Sigma) and penicillin/streptomycin (Life Technologies).

qPCR. To compare expression levels of select genes in *TrpV1-tdTomato*-positive induced nociceptors, *TrpV1-tdTomato*-positive primary DRGs and *TrpV1-tdTomato* MEFs, individual tdTomato-positive neurons and fibroblasts were picked using a micropipette. RNA was harvested from sets of 50 cells with the RNeasy Micro Kit (Qiagen) and reverse transcribed with SuperScript VILO cDNA synthesis kit (Life Technologies). Quantitative PCR was completed using mouse-specific TaqMan Gene Expression Assays (Life Technologies) and the TaqMan Gene Expression Master Mix (Life Technologies). A minimum of two technical replicates for each of three biological replicates (independent cell collections) were completed for each gene.

Single cell RT-PCR. Single human induced nociceptors were picked using individual patch pipettes and placed into Single Transcript Amplification (RT-STA) mixture from the CellsDirect One-Step qRT-PCR Kit (Life Technologies) using primers for normally and aberrantly spliced *IKBKAP* and *GAPDH*. RT-STA reaction products were used for PCR using the same *IKBKAP* and *GAPDH* primers and resulting products were run on 1% agarose gels.

Calcium imaging. Cells were loaded with Fura2-AM (10ug/mL, Molecular Probes) by incubating at room temperature for one hour and then de-stained for 15 minutes in saline. For primary DRGs from adult *TrpV1-Cre⁺⁻::tdTomato⁺⁻* mice, cells were imaged after 24 hours in culture using an
identical protocol. Cells were imaged using a Nikon Eclipse Ti microscope with a Xenon lamp, Andor DL-604M camera and standard 340 nM and 380 nM filters controlled by a Ludl Mac6000 shutter using Nikon Elements software. Exposure times were 300-600 ms and images were taken every three seconds. One minute of baseline imaging was recorded prior to the addition of the agonists, during which control vehicle was applied after 30 seconds (not shown). Menthol (250 µM) was applied at one minute, followed by Mustard oil (100 µM) at two minutes and Capsaicin (1 µM) at three minutes and finally KCl (40 mM) at four minutes. For Trp channel experiments, each agonist was applied for 20 seconds and then washed out with external solution. In the PGE2 sensitization experiments, capsaicin (300nM) was applied for 20 seconds after two minutes of recording, followed immediately by PGE2 (1µM) for two minutes, a conditioned capsaicin (300nM) application for 20 seconds and KCl (40 mM) after 4.5 minutes. Analysis of tdTomato positive cells was performed using custom Matlab (Mathworks) software to include cells that responded to KCl (1.5 x baseline), had a stable baseline during control vehicle application and a response to agonist with an amplitude of at least 10% of baseline, with subsequent agonist responses required to be both at least 10% of initial baseline and 10% above a second baseline value obtained during the immediately preceding wash period.

CGRP ELISA. Induced nociceptors, BAM-derived neurons and primary DRGs were exposed to KCl (20 mM, 40 mM, 60 mM, or 80 mM), capsaicin (0.1 µM), or vehicle for 10 minutes at 37°C. The supernatants were collected and analyzed using the Rat CGRP Enzyme Immunoassay Kit (Bertin Pharma/Cayman Chemical, #589001). Plates were read at 405nm for 0.1s on a Wallac Victor² 1420 Multilabel Counter (Perkin Elmer), and data were analyzed using the Wallac 1420 Workstation.

MEA recording. TrpV1-Cre+/−::tdTomato+/− MEFs were plated on poly-D-lysine/laminin coated p515A probes (Alpha Med Scientific) at typical densities of 12,000 cells per probe, transduced with retroviruses and cultured for four weeks. Recordings from 64 extracellular electrodes were made using a Med64 (Alpha Med Scientific) MEA recording amplifier with a head stage that
maintained a temperature of 37°C. Data were sampled at 20 kHz, digitized, and analyzed using
Mobius software (Alpha Med Scientific) with a 2 kHz 9-pole Bessel low pass filter using a sodium-
based extracellular solution: 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM
glucose, 10 mM HEPES 10, pH 7.4. The probes were recorded for one minute before the
application of the agonists to obtain a baseline and two minutes after the application of capsaicin
(1 µM concentration) or mustard oil (100 µM), which were applied at a 10x concentration at the
edge of the well (far from the electrodes). Three replicates for each agonist, capsaicin and
mustard oil, were completed from two separate transductions. For oxaliplatin sensitization
experiments, cells were treated with either 50 µM oxaliplatin or vehicle control for 10 minutes and
then recorded for one minute during treatment with 300 nM capsaicin.

**Patch electrophysiology.** Whole-cell patch recordings were performed on induced tdTomato-
positive nociceptors, derived from *TrpV1-Cre<sup>+/−</sup>:tdTomato<sup>+/−</sup> MEFs, four–five weeks post-
transduction and assessed for responses to capsaicin and α,β-methylene ATP (30 µM, Sigma),
total and TTX (300 nM, Sigma)-resistant sodium currents, HCN depolarizing current sags and
action potentials. Whole-cell current-clamp and voltage-clamp recordings were performed using a
Multiclamp 700B (Molecular Devices) at room temperature (21-23°C). Data were sampled at 20
kHz and digitized with a Digidata 1440A A/D interface and recorded using pCLAMP 10 software
(Molecular Devices). Data were low-pass filtered at 2 kHz. Patch pipettes were pulled from
borosilicate glass capillaries on a Sutter Instruments P-97 puller and had resistances of 2-4 MΩ.
The pipette capacitance was reduced by wrapping the shank with Parafilm and compensated for
using the amplifier circuitry. Series resistance was 5-10 MΩ and compensated by at least 80%.

For voltage-clamp recordings, voltages were elicited by 200-ms depolarizing steps from a holding
potential of -80 mV to test potentials ranging from -100 mV to 30 mV in 10 mV increments.
Responses to capsaicin (1 µM) and α,β-methylene ATP (30 µM) were measured in voltage clamp
at a holding potential of -80 mV. Electrode drift was measured at the end of each recording and
was typically 1–2 mV. The potassium-based intracellular solution contained 150 mM KCl, 2 mM
MgCl$_2$, 10 mM HEPES, 4 mM MgATP, 0.3 mM NaGTP, 10 mM Na$_2$PhosCr, 1mM EGTA, pH 7.4.

For isolation of voltage-gated sodium currents internal KCl was replaced by CsCl to block potassium currents and 100 µM CdCl$_2$ was applied to block calcium currents. 300 nM TTX was used to block TTX-sensitive voltage-gated sodium channels. HCN currents were measured by sequential hyperpolarizing steps in current clamp with an increment of -10 pA steps.

**Quantification of cell number, axon length and axonal branching.** All quantifications of cell count and morphological properties were performed by a researcher blind to genotype/condition. Reprogramming efficiency and percentage of TrpV1::tdTomato-positive, Tuj1-positive, peripherin-positive and CGRP-positive neurons were determined using MetaXpress software. The number of TrpV1::tdTomato-positive (mouse, minimum of 3 wells from each of three separate transdifferentiations) and Tuj1-positive (human, minimum of 12 wells from each of three separate transdifferentiations) neurons was divided by the number of fibroblasts plated to calculate the reprogramming efficiency. Axon length and branching were quantified from the six age-matched human fibroblast lines (three HC and three FD) with images from eight wells (two transductions) and four wells (one transduction) of each line (20 wells total/line).

To quantify the number of TrpV1-tomato-positive neurons resulting from infection of different combinations of transcription factors, tomato-positive neurons were counted by hand in at least four separate wells for each condition from two separate transductions and averaged ([Fig. 1](#), [Supplementary Fig. 4](#)). Due to the variability of retroviral transductions with large numbers of viruses, we did not quantify experiments with transductions of 11–12 viruses at once ([Supplementary Fig. 3](#)). For comparison of 5 Factors ± NeuroD1, images were taken of 6 wells of each condition from one transduction.

**Statistical Analyses.** Figures show mean ± SEM for all analyses; all tests are two-tailed using a significance threshold of 0.05. We used a Mann-Whitney U test to evaluate CGRP release after log transformation to equalize variance between groups ([Fig. 4h](#)). This gave a W statistic of 16 (4
assays/group) and p-value of 0.03. We used a paired t-test to evaluate baseline and PGE2-sensitized capsaicin responses in the induced mouse nociceptors (Fig. 5a). This gave a T statistic of 4.61 with 20 degrees of freedom (21 cells) and a p-value of 9.7*10^{-4}. For comparison of transdifferentiation efficiency using 5 vs 6 factors, we used an unpaired t-test to compare average number of cells/field in 6 wells/group and got a T statistic of 6.1 with 10 degrees of freedom and p-value 1.0*10^{-4} (Supplementary Fig. 9).

For morphological analyses of human HC and FD-derived neurons, we performed analyses of cell number, axonal outgrowth, and number of branches (Fig. 6). Distribution of cell number and number of branches were not normal (Shapiro-Wilk test). We looked at the distribution of all the measures graphically and found that they are approximately normal. Variance of cell number, axon outgrowth, and number of branches were not different between HC and FD-derived neurons (Levene test). Using random intercept model and taking matching of cell lines into account provided FD disease effect estimates (standard errors) compared to HC: -2.3 (1.5) cell numbers, p=0.2632; -291.3 (32.6) µM axon outgrowth/cell, p=0.0123; -3.3 (0.4) branches/cell, p=0.0165. Post-hoc analysis to compare individual lines was performed using Tukey-Kramer for the three pairs of lines. Comparisons of FD line 1 to matched HC line 1 gave estimate of differences as 2.1 (cells), -292.3 (outgrowth), -3.5 (branches) respectively. Comparisons of FD line 2 to matched HC line 2 gave estimate of differences as -7.2 (cells), -274.7 (outgrowth), -3.2 (branches) respectively. Comparisons of FD line 3 to matched HC line 3 gave estimate of differences as -1.9 (cells), -307.1 (outgrowth), -3.1 (branches) respectively. However, the model with interaction parameter for cell line type and disease group only provided generalized inverse estimates of the standard errors. Hence, the confidence intervals and p-values for these individual cell lines are not reported. All statistical analyses were performed in software R ver 3.1.0 and SAS ver 9.1 (Cary, NC). No power analyses were used to pre-determine sample sizes, but our sample sizes are similar to those used by others in the field.
Figure Legends

Figure 1. Combinations of transcription factors result in nociceptor production. (a) Few tdTomato, Tuj1-positive neurons are produced by the combination of six factors (6 TFs): Brn2, Ascl1, Myt1l, Ngn1, Isl2 and Klf7. (b) Removal of Brn2 markedly increases the number of tdTomato, Tuj1-positive neurons. (c-g) Omission of Ascl1 (c), Myt1l (d), Ngn1 (e), Isl2 (f) or Klf7 (g) from the six factors disrupts the generation of nociceptron neurons. Representative images for each transcription factor drop out were taken from n=4 wells from two separate transductions. Scale bars: 100 µm. (h) Quantification of the single factor dropout studies from n=4 wells from each of two separate independent transductions for each category.

Figure 2. Induced nociceptors (iNoc) express characteristic nociceptor genes. (a,b) Tuj1 (a) and TrpV1 (b) expression in fibroblast-derived nociceptor neurons. (c) Most induced nociceptors stain for the C-fiber marker peripherin (Prph). (d) A number of induced nociceptors express the peptidergic-marker CGRP. (e) A small number of induced nociceptors express the intermediate filament marker NF200 found in myelinated fibers. Representative images were selected from immunostaining that was repeated in n=4 wells from two independent transductions. Scale bars: 100 µm. (f) RT-qPCR data showing expression levels of nociceptor-specific genes in 50-picked tdTomato-positive primary adult mouse nociceptors (DRGs, black circles) and 50-picked tdTomato-positive induced nociceptors (red circles), relative to their levels in MEFs, from a minimum of two independent biological replicates (biological replicates represented as independent circles).

Figure 3. Induced neurons respond to different Trp channel agonists. (a) Sample calcium imaging responses to sequential application of menthol (250 µM), mustard oil (100 µM), capsaicin (1 µM), and potassium chloride (40 mM) in a single dish of induced tdTomato-positive derived nociceptors. Traces are representative recordings from n=227 tdTomato-positive / KCl-responding cells cultured in 19 dishes from 3 independent transductions (b) Venn diagram
showing subgroups of tdTomato-positive cells that responded to KCl (40 mM, grey), capsaicin (Cap, 1 µM, red, 39%), mustard oil (MO, 100 µM, lower small circle, green, 9%) and menthol (ME, 250 µM, upper small partial circle, blue, 3%; note that no tdTomato-positive cells responded to menthol alone) (c) Sample electrodes from extracellular multi-electrode array recordings of induced neurons before (left) and after (right) the application of capsaicin (1 µM, upper) and mustard oil (100 µM, lower). Sample recordings for each agonist are indicative of results from three experiments across two independent transductions, in which all replicates showed an increase in firing after agonist application.

**Figure 4.** Whole-cell patch clamp recordings of tdTomato-positive induced nociceptors. (a) Current recording in response to treatment with 1 µM capsaicin (6/11 induced neurons responded). (b) Current recording following the application of 30 µM α, β-methylene-ATP (8/16 induced neurons responded). (c) Inward currents following step depolarization before (left) and after (middle) the application of 300 nM tetrodotoxin (TTX) (14/15 induced neurons had TTX-resistant sodium currents greater than 50 pA). Right panel shows a different neuron without a persistent TTX-resistant sodium current (d) Action potential firing elicited by depolarizing current in the presence of 300 nM TTX (7/12 cells fired single TTX-resistant action potentials with peak greater than 0 mV). (e, f) Examples of individual action potentials (e) and trains (f) elicited from induced nociceptors (iNoc), tdTomato-positive primary adult nociceptors (Primary noc) and tdTomato-negative primary adult non-nociceptors (Primary non-noc) (12/13 induced neurons fired tonically). (g) Examples of sag depolarizations in response to hyperpolarizing current injections in induced nociceptors (11/17 induced neurons produced a sag depolarization). (h) CGRP was released from induced nociceptors (5F), but not BAM-derived neurons, in response to KCl (80 mM), but not vehicle. Mean (SEM) for 5F and BAM following KCl stimulation are 390.4 (52.5) and 10.3 (2.6) pg/ml (n=4; Mann-Whitney U-test p=0.03).

**Figure 5.** Sensitization of induced nociceptors treated with the inflammatory mediator PGE2 and the chemotherapeutic drug oxaliplatin. (a) Sample calcium imaging recordings of induced
nociceptors treated with 300 nM capsaicin before and after treatment with 1 µM PGE2 from recordings of n=41 tdTomato-positive / KCl-responding cells. (b) Plot of individual and mean response amplitudes for initial and PGE2-sensitized capsaicin treatments (paired t-test p=1.5x10^-4). (c) Plot of initial versus PGE2-sensitized capsaicin response amplitudes for individual induced neurons. (d) Sample traces from extracellular multi-electrode array recordings of induced neurons in response to 300 nM capsaicin following 10 minute exposure to vehicle control (n=5 MEAs) or oxaliplatin (50 µM; n=4 MEAs) on induced neurons from two separate transductions. (e) Quantification of spikes per minute from induced nociceptors in response to capsaicin alone (control) and capsaicin following oxaliplatin treatment. Error bars are ± SEM.

**Figure 6.** Human fibroblast-derived neurons for human disease modeling. (a) Low magnification of TuJ1 (left) and peripherin (Prph, right) staining of healthy control (HC)-derived neurons. Scale bars: 500 µm. (b) High magnification of TuJ1 staining of HC-derived neurons. Scale bar: 100 µm. (c) NF200-positive cell derived from HC fibroblasts. (d) Current recording of an action potential train from a HC-derived neuron (17/33 induced neurons with peak Na current > 500 pA fired at least one action potential with peak greater than 0 mV). (e) Total (left) and TTX-resistant (middle) sodium currents from a single HC-derived neuron. Right panel shows persistent TTX-resistant sodium current recordings from a separate HC-derived neuron characteristic of Nav1.9. (f) RT-PCR for *IKBKAP* and *GAPDH* from single human induced neurons (left) and single human fibroblasts (right) show normal (black arrow) and abnormally spliced (red arrowhead) transcripts. Full-length gels are presented in Supplementary Fig. 10. (g) Low magnification of TuJ1 (left) and peripherin (right) staining of neurons derived from a patient with Familial Dysautonomia (FD). Scale bars: 500 µm. (h) High magnification of TuJ1 staining of FD-derived neurons. Scale bar: 100 µm. For all images, representative images were selected from human neurons generated in n=6 wells from three separate transductions. (i) Quantification of TuJ1-positive neurons in HC and FD-derived nociceptors (random intercept mixed-effects model p=0.26). (j) Neurite outgrowth per cell for HC and FD-derived TuJ1-positive nociceptors (random intercept mixed effects model p=0.012). (k) Number of branches per cell for HC and FD-derived TuJ1-positive nociceptors.
(random intercept mixed effects model p=0.017). For i-k, images were analyzed from three pairs of age-matched HC and FD patient lines from each of three separate transductions (n=20 wells/line). Error bars are ± SEM.

**Acknowledgments**

We thank M. Costigan for assistance with RT-PCR and A. Yekkirala and J. Sprague for help with calcium imaging, Q. Ma and E. Turner for constructs, J. Gardner and J. McNeish for helpful advice and support, and K. Wainger for assistance with figure preparation. We also thank the Boston Children’s Hospital IDDRC Molecular Genetics Core Facility for RNA Bioanalyzer analyses and the Harvard Medical School ICCB Screening Facility for assistance with ImageXpress and MetaXpress analyses. This research was supported by NIGMS T32 GM07592 and National Institute of Neurological Disorders and Stroke (1K08-NS082364) (B.J.W.), Conselho Nacional de Desenvolvimento Científico e Tecnológico (J.T.O.), GlaxoSmithKline Regenerative Medicine DPU (C.J.W.), NINDS NS038253 (C.J.W.) and the Dr. Miriam and Sheldon G. Adelson Medical Foundation (C.J.W.).

**Author Contributions**

B.J.W. conceived, designed, and performed lineage reprogramming experiments and physiological experiments, analyzed data and wrote the manuscript. E.D.B. designed, performed and analyzed reprogramming, qPCR, single cell RT-PCR, immunohistochemistry, and CGRP ELISA experiments, and wrote the manuscript. J.T.O. performed and optimized induced nociceptor technique. C.M. performed and analyzed physiological studies and edited the manuscript. S.L. performed CGRP ELISA and single cell RT-PCR assays. W.A.S. performed reprogramming and immunohistochemistry experiments. A.J.W. performed initial cloning and transduction experiments. J.K.I provided essential advice for nociceptor reprogramming strategy.
and edited the manuscript. I.M.C gave critical advice regarding the genetic reporter, choice of transcription factors, performed cell sorting experiments and edited the manuscript. L.B advised and performed molecular biology experiments. E.H. performed image quantification and analysis. C.B. assisted with reprogramming and immunohistochemistry. N.T. assisted with human motor neuron culture and together with C.B. performed culture and characterization using initial approaches. K.K. performed statistical modeling of human nociceptor data. L.L.R advised regarding reprogramming experiments and edited the manuscript. K.E. provided advice and reagents for reprogramming and edited the manuscript. C.J.W designed experiments, interpreted findings and wrote the manuscript.
Table 1. Candidate transcriptions factors for lineage conversion to nociceptor neurons. Lit, literature; Exp, transcriptome of sorted nociceptors compared to proprioceptors; BioGPS.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Family</th>
<th>Role in Reprogramming/Sensory System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascl1</td>
<td>Lit</td>
<td>Basic helix-loop-helix / achaete-scute</td>
<td>Neuronal lineage reprogramming&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Drgx</td>
<td>Lit</td>
<td>Helix-turn-helix / Paired box</td>
<td>Survival of peptidergic and non-peptidergic nociceptors&lt;sup&gt;48&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isl2</td>
<td>Exp, BioGPS</td>
<td>Homeo-domain / LIM region</td>
<td>Unknown</td>
</tr>
<tr>
<td>Klf7</td>
<td>Lit, Exp, BioGPS</td>
<td>Zinc-finger / Krueppel like</td>
<td>TrkA maintenance&lt;sup&gt;49&lt;/sup&gt;</td>
</tr>
<tr>
<td>Myt1L</td>
<td>Lit</td>
<td>Zinc-finger</td>
<td>Neuronal lineage reprogramming&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ngn1</td>
<td>Lit</td>
<td>Basic helix-loop-helix</td>
<td>TrkA and subsequent TrpV1 expression&lt;sup&gt;21&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pknox2</td>
<td>Exp</td>
<td>Homeo-domain / TALE</td>
<td>Unknown</td>
</tr>
<tr>
<td>Pou4f1</td>
<td>Lit</td>
<td>Homeo-domain / POU (Class IV)</td>
<td>Neuronal lineage reprogramming&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Runx1</td>
<td>Lit</td>
<td>ß-scaffold / Runt</td>
<td>Non-peptidergic identify and TrpV1 expression&lt;sup&gt;23&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tlx3</td>
<td>Lit</td>
<td>Helix-turn-helix / homeo-domain</td>
<td>Glutamatergic identity&lt;sup&gt;20&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
REFERENCES


Figure 1 Woolf

- 6 TFs – Brn2
- Ascl1 – Ngn1 – Isl2
- Myt1l – Klf7

Number of tdTomato-positive neurons / well

0 400 800 1200

- 6 TFs – Brn2 – Ascl1 – Ngn1 – Isl2 – Klf7
Figure 2: Woolf
Figure 4 Woollf

a. Capsaicin
b. a,b methylene ATP
c. - TTX
   + TTX
   + TTX
d. + TTX

e. iNoc, Primary noc, Primary non-noc
f. iNoc, Primary noc, Primary non-noc

g. 20 mV
   100 ms
h. CGRP (pg mL⁻¹)
   iNoc, BAM, KCl, Vehicle
Figure 5 Woolf

(a) Graph showing the response to capsaicin before and after PGE2.

(b) Scatter plot comparing the response to capsaicin before and after PGE2.

(c) Scatter plot comparing the initial response to capsaicin with the response after PGE2.

(d) Graph showing spikes per minute in control and Oxaliplatin conditions.

(e) Graph showing spikes per minute in control and Oxaliplatin conditions.
Figure 6 Woollf

- Images of Tuj1 and Prph stained neurons.
- Graphs showing the effect of TTX on cell outgrowth and number of branches.
- Western blot images showing expression levels of IKBKAP and GAPDH in HC and FD lines.

Legend:
- Tuj1: Neurofilament protein
- Prph: Prophagocytosis receptor
- HC Lines: Hippocampal cell lines
- FD Lines: Fibroblast cell lines
- IKBKAP: Inhibitor of nuclear factor kappa-B kinase, alpha
- GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

Statistical analysis:
- p-values for cell outgrowth and branches per cell.
Intrinsic Membrane Hyperexcitability of ALS Patient-Derived Motor Neurons

(This work has been published previously: Cell Reports 2014, 7, 1-11)

Brian J. Wainger¹,²*, Evangelos Kiskinis³*, Cassidy Mellin¹, Ole Wiskow³, Steve S.W. Han³,⁴, Jackson Sandoe³, Numa P. Perez¹, Luis A. Williams³, Seungkyu Lee¹, Gabriella Boulting³, James D. Berry⁴, Robert H. Brown Jr⁵, Merit E. Cudkowicz⁴, Bruce P. Bean⁶, Kevin Eggnan³,⁴,⁷ & Clifford J. Woolf¹,⁶

¹FM Kirby Neurobiology Center, Boston Children’s Hospital and Harvard Stem Cell Institute
²Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital
³Harvard Stem Cell Institute, Department of Stem Cell and Regenerative Biology, Harvard University and the Stanley Center for Psychiatric Research, Broad Institute
⁴Department of Neurology, Massachusetts General Hospital
⁵Department of Neurology, University of Massachusetts Medical Center
⁶Department of Neurobiology, Harvard Medical School
⁷The Howard Hughes Medical Institute, USA

*These authors contributed equally to this work.

Corresponding Authors:
Kevin Eggnan
eggan@mcb.harvard.edu
Phone: 617 496-5611
Fax: 617 384-8234

Clifford J. Woolf
clifford.woolf@childrens.harvard.edu
Phone: 617 919-2393
Fax: 617 919-2772
SUMMARY:
Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease of the motor nervous system. We show using multi-electrode array and patch clamp recordings that hyperexcitability detected by clinical neurophysiological studies of ALS patients is recapitulated in induced pluripotent stem cell-derived motor neurons from ALS patients harboring superoxide dismutase 1 (SOD1), C9orf72 and fused-in-sarcoma mutations. Motor neurons produced from a genetically corrected, but otherwise isogenic, SOD1+/+ stem cell line do not display the hyperexcitability phenotype. SOD1A4V+/+ ALS patient-derived motor neurons have reduced delayed-rectifier potassium current amplitudes relative to control-derived motor neurons, a deficit that may underlie their hyperexcitability. The Kv7 channel activator retigabine both blocks the hyperexcitability and improves motor neuron survival in vitro when tested in SOD1 mutant ALS cases. Therefore, electrophysiological characterization of human stem cell-derived neurons can reveal disease-related mechanisms and identify therapeutic candidates.

HIGHLIGHTS:

• iPSC-derived motor neurons from ALS patients are hyperexcitable compared to controls.
• Correction of the disease-causing mutation corrects the phenotype.
• Retigabine rescues the hyperexcitability phenotype in multiple ALS variants.
• Retigabine improves in vitro survival of SOD1A4V+/+ ALS motor neurons.
INTRODUCTION:

ALS is a devastating, untreatable disease of upper and lower motor neurons (Kiernan et al., 2011). The excitotoxicity neurodegeneration hypothesis posits that excessive glutamatergic synaptic activity in ALS leads to calcium overload and cell death (Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006). However, nerve conduction studies evaluating axonal threshold (strength-duration time constant and recovery cycle times) in ALS patients demonstrate increased axonal membrane excitability, well away from any synapses (Bostock et al., 1995; Kanai et al., 2006; Nakata et al., 2006; Vucic and Kiernan, 2006), and the degree of hyperexcitability correlates with patient survival (Kanai et al., 2012). Increased membrane excitability may be important then as a contributor to disease, and modeling suggests that either increased persistent sodium or reduced delayed-rectifier potassium currents could be responsible for the axonal hyperexcitability (Kanai et al., 2006; Tamura et al., 2006). However, whether excitability results from autonomous changes in motor neurons cannot be determined by this technique (Fritz et al., 2013).

About 10% of ALS cases are familial, and of these, superoxide dismutase 1 (SOD1) mutations account for about 20%. Motor neurons from SOD1<sup>G93A</sup> mice, which overexpress this human mutant SOD1 protein, also show hyperexcitability (Kuo et al., 2004; Pieri et al., 2003; van Zundert et al., 2008), at least in part due to increased persistent sodium currents. Because of the distinct clinical and pathological features of SOD1 ALS compared to other variants (Ince et al., 2011), it is unclear if primary motor neuron hyperexcitability represents a general feature of ALS or a specific characteristic of SOD1-mediated disease. Hyperexcitability in motor neurons of other familial ALS etiologies, such as C9orf72 hexanucleotide repeat expansions and fused-in-sarcoma (FUS) mutations, have not yet been similarly evaluated because of a lack of mouse models.

Induced pluripotent stem cell (iPSC) technology enables neurons of specific disease-relevant subtypes to be derived from disease patients and control subjects, and thereby provides an <em>in vitro</em> platform for discovering human neuron phenotypes that may reflect the individual diseases.
However, the number of subject cell lines employed in studies utilizing this technique has so far been small (Sandoe and Eggan, 2013). Thus, it is currently difficult to know how consistent such findings are across large numbers of patients, and if the results represent disease-specific phenotypes or differences among cell lines. We address these issues here in two ways. First, we use a gene-targeted correction of the disease-causing SOD1 mutation to produce otherwise isogenic stem cells bearing wild-type SOD1 alleles (Kiskinis et al., 2014) and show that the gene correction abrogates the phenotype of ALS motor neuron hyperexcitability. Second, we demonstrate that hyperexcitability is present among motor neurons derived from eight ALS patients of three separate genetic etiologies compared to five control patients, together constituting the largest sample group to date for these types of studies. ALS-derived motor neurons have reduced delayed-rectifier voltage-gated potassium currents compared to controls. Furthermore, retigabine, an activator of Kv7 potassium channels reduces excitability to levels seen in controls. Retigabine increases the in vitro survival of SOD1^{A4V/+} ALS motor neurons, supporting the hypotheses that motor neuron hyperexcitability may contribute to motor neuron degeneration in ALS. The hyperexcitability phenotype and blockade of firing by retigabine are present across a wide range of familial ALS patients harboring additional SOD1 mutations, C9orf72 repeat expansions and FUS mutations. An iPSC-based disease-modeling approach can validate a clinically-relevant phenotype in human motor neurons, reveal mechanisms underlying the phenotype and help evaluate actions of candidate drugs on the disease-specific phenotype.

RESULTS

Hyperexcitability of SOD1^{A4V}-Derived Motor Neurons Using Multi-Electrode Array Recording

We performed an initial set of electrophysiological phenotyping experiments using iPSC-derived motor neurons from two control subjects (11a, 18a) and two unrelated familial ALS patients (39b and RB9d) harboring the same aggressive SOD1^{+/-A4V} mutation. All iPSC lines were generated via 3-factor (OCT4, SOX2, KLF4) retroviral reprogramming, had a normal karyotype, and differentiated into motor neurons after robust neuralization based on dual SMAD inhibition.
(Chambers et al., 2009) and specification through exposure to retinoic acid and induction of sonic hedgehog signaling (Boulting et al., 2011; Kiskinis et al., 2014; Figure 1A).

We recorded spontaneous firing of iPSC-derived motor neurons using extracellular multi-electrode arrays (MEAs), whereby the action potentials of individual neurons are detected by a grid of 64 extracellular electrodes embedded in each culture well. In four separate experiments, control and SOD1 ALS iPSC lines were cultured synchronously, differentiated into motor neurons in parallel, and plated in equal numbers on MEAs, which allowed recording of the spontaneous firing (Hanson and Landmesser, 2004) in hundreds of control and ALS patient-derived neurons per differentiation after culturing for four weeks. We observed significantly more spontaneous action potentials in SOD1+/A4V relative to control cultures (p<0.05, t-test; Figure 1B,C). Action potentials were sorted by spike morphology and timing to derive clusters corresponding to individual neurons (Figure S1) (Cohen and Kohn, 2011), and a significantly higher average mean firing rate was observed in the SOD1+/A4V neurons (p<10^{-15}, t-test; Figure 1D,E).

We performed two experiments to confirm that the hyperexcitability on the MEAs resulted from motor neurons. First, to test if the difference in spontaneous action potential firing resulted from a larger number or more active population of inhibitory neurons in control cultures, we applied GABAergic and glycinergic transmission blockers. The blockers did not increase action potential firing rates (p=0.61, t-test, for bicuculline; p= 0.24, t-test, for strychnine; Figure S2), suggesting that activity of inhibitory neurons was minimal and that the heightened spontaneous firing in the MEA recordings of ALS-derived motor neurons reflected an intrinsic increase in excitability.

Second, we inserted a Hb9::GFP reporter into the AAVS1 locus for iPSCs 18a and 39b (Figure S3A), allowing fluorescence-activated cell sorting (FACS) purification of GFP-positive motor neurons for recording on the arrays (Figure S3B-C). We recorded every four days and observed that 39b Hb9::GFP motor neurons consistently fired more action potentials than 18a Hb9::GFP motor neurons over the entire time course (mixed model ANOVA F-test p=1x10^{-4} for difference
between lines; post-hoc t-tests after Bonferroni correction for multiple comparisons for day 12, p=0.0055; day 16, p= 0.0030; day 20, p=0.0057; day 24, p=0.0029; day 28, p=0.015; Figure 1F). Thus, the hyperexcitability must be due to motor neurons, because only Hb9-positive motor neurons were plated on the arrays.

**Correction of the SOD1<sup>44V</sup> Mutation Eliminates the Hyperexcitability Phenotype**

To test if increased action potential firing was a direct effect of the SOD1<sup>44V</sup> mutation, we took advantage of a gene-targeted derivative of the 39b iPSC line in which the A4V-encoding mutation had been corrected to a wild-type sequence by homologous recombination, 39b-SOD1<sup>+/+</sup> (abbreviated 39b-Cor; Kiskinis et al., 2014). Because substantial motor neuron death begins in ALS motor neurons after 15 days of neuronal maturation in our culture conditions (Kiskinis et al., 2014) and because the hyperexcitability phenotype in the ALS motor neurons was detectable at this early time point (Figure 1F), we compared MEA recordings of 39b and isogenic-derived 39b-Cor motor neurons at 14 days, to avoid the possibility that increased firing reflected either neuronal death or select survival of hyperexcitable neurons (Table S1). While the baseline spike rate was lower at 14 than 28 days, patient-derived 39b neurons had a higher spontaneous firing rate than neurons in which the SOD1 mutation was corrected (p=0.01 for total rate, t-test; Figure 1G; average mean firing rate 1.30 ± 0.10 Hz for n=122 39b-Cor and 1.50 ± 0.08 Hz for n=208 39b; p<0.05, t-test). We conclude that the hyperexcitability phenotype reflects the presence of the disease-initiating mutation and precedes progressive motor neuron death.

**Confirmation of ALS Motor Neuron Hyperexcitability and Mechanistic Exploration Using Whole-Cell Patch Clamp**

To examine the electrophysiological properties of identified individual motor neurons, we transduced developing neurons with an Hb9::RFP lentiviral reporter and recorded only from RFP-positive motor neurons (Marchetto et al., 2008) using whole-cell patch clamp (Figure 2A). Both control- and SOD1 ALS-derived motor neurons were electrically excitable. To quantify the degree of excitability, we assayed the number of action potentials fired in response to a slow ramp
depolarization. The number of action potentials fired by ALS motor neurons was significantly greater than control motor neurons (p<0.05, Mann-Whitney U test; Figure 2B, upper panels, Figure 2C, upper panel). Resting membrane potential, action potential threshold (rheobase) and input resistance did not differ between ALS and control motor neurons (Table S2), indicating that excitability differences were not due to differences in electrophysiological health or baseline capacity for action potential generation.

When we compared motor neurons derived from 39b-Cor and 39b cell lines in three separate additional parallel experiments, we again observed a marked difference in the number of action potentials elicited during ramp depolarization (p<0.05, Mann-Whitney U test; Figure 2B, lower panels, Figure 2C, lower panel), demonstrating that the A4V mutation was essential for the phenotype. There was variability in the number of action potentials in motor neurons from the same line tested across multiple differentiations, but the increased number of action potentials in ALS motor neurons relative to control motor neurons was always preserved. This result underscores the importance of performing repeated parallel differentiations in which equal numbers of control and ALS motor neurons are analyzed from each differentiation.

In addition to quantifying the electrical excitability of individual neurons, patch clamp recording enables quantitative investigation of specific currents that determine excitability. To identify electrophysiological mechanisms responsible for the increased firing of mutant motor neurons, we performed voltage-clamp experiments using Hb9::RFP-positive motor neurons to examine current components. As an index of excitatory and inhibitory voltage-dependent ion channels, we quantified the ratio of outward delayed-rectifier potassium current to inward transient sodium current. In four repeated differentiations of motor neurons from control and SOD1+/A4V iPSC lines, we observed that the ratio of delayed-rectifier potassium to transient sodium current was consistently smaller in SOD1+/A4V motor neurons (p<0.001, t-test; Figure 2D-E). The difference was driven primarily by the reduced delayed-rectifier potassium channel component, as the difference in steady-state potassium current amplitude normalized to individual cell capacitance
between ALS subjects and healthy controls was significant (control 137.0 ± 14.4 pA/pF, n=23, versus ALS 94.4 ± 10.7 pA/pF, n=25; p<0.05, t-test) whereas the peak sodium current normalized to capacitance was not (control 190.3 ± 23.0 pA/pF, n=21, versus ALS 237 ± 21.2 pA/pF, n=23; p= 0.2, t-test). Since voltage-gated potassium channels repolarize the membrane potential back to negative values after an action potential, a decrease in such currents likely contributes to increased action potential firing in ALS motor neurons.

Correction of the disease-causing SOD1^{A4V} mutation also increased the relative steady-state delayed-rectifier potassium current amplitude, showing that this phenotypic difference specifically resulted from the A4V mutation (p<0.005, t-test; Figure 2F). We found a marked reduction in delayed-rectifier current magnitude in 39b compared to 39b-Cor motor neurons (p< 0.05, t-test; Figure 2G) but no difference in sodium current peak amplitudes (p=0.8, t-test; Figure 2H). Thus, correction of the deficit in delayed-rectifier potassium current in 39b-Cor motor neurons may enable repolarization of the membrane potential back to normal hyperpolarized values and reduction of excitability to levels in wild-type motor neurons.

Retigabine Blocks Motor Neuron Hyperexcitability and Increases in vitro Survival of SOD1^{A4V} ALS motor neurons

Motor neurons express many types of voltage-activated potassium channels and pharmacological dissection and quantification into distinct components is challenging. Regardless of which currents produce the hyperexcitability in diseased motor neurons, Kv7 (KCNQ) channels are attractive targets for correcting the hyperexcitability because of their activation at subthreshold voltages and subsequent powerful control of excitability (Brown and Passmore, 2009). Given this and the reduced delayed-rectifier potassium currents in ALS-derived motor neurons, we hypothesized that retigabine, a specific activator of subthreshold Kv7 currents and clinically-approved anticonvulsant (Porter et al., 2007), might block hyperexcitability in the SOD1^{A4V} motor neurons. In whole-cell patch clamp, retigabine significantly increased the minimal current step necessary for action potential generation (rheobase) by 3.6 ± 2.4 pA (p<0.05, Wilcoxon signed
rank test; Figure 3A). Retigabine also stopped spontaneous firing of Hb9::RFP-positive motor neurons and hyperpolarized the resting membrane potential by 6.0 ± 2.2 mV (p=0.001, t-test; Figure 3B). Because these experiments were performed with blockers of glutamatergic, GABAergic, and glycineric receptors, retigabine must have a direct effect on motor neuron excitability. We used MEA recordings to determine a dose-response for inhibition of spontaneous firing by retigabine of SOD1<sup><ins>A4V</ins></sup> ALS-derived neurons. Retigabine suppressed ALS neuron spontaneous firing with an EC<sub>50</sub> of 1.5 µM (Figure 3C), a concentration consistent with its pharmacological activity as an anti-epileptic agent and similar to its EC<sub>50</sub> for Kv7 channels (Wickenden et al., 2000). In line with this finding, analysis of RNA-Seq data from FACS-sorted motor neurons (Kiskinis et al., 2014) confirms expression of Kv7 channels (Table S3).

To evaluate the possibility that hyperexcitability is an upstream modulator of motor neuron degeneration in ALS, we tested if retigabine affects the survival of control and SOD1<sup><ins>A4V</ins></sup> motor neurons over 30 days in culture. As observed by Kiskinis et al. under basal conditions, the loss of SOD1<sup><ins>A4V</ins></sup> motor neurons was greater than SOD1<sup><ins><ins>/+</ins></ins></sup> control motor neurons (Kiskinis et al., 2014). Two weeks of treatment with retigabine (1 µM) increased the number of ALS motor neurons <i>in vitro</i> by 25% (p<10<sup>-4</sup>, t-test; Figure 3D) to levels found in controls.

To investigate how retigabine increases the survival of SOD1<sup><ins>A4V</ins>+/</sup> ALS motor neurons, we determined whether it affects pathways suspected to contribute to motor neuron death in ALS (Robberecht and Philips, 2013). We chose to look at endoplasmic reticulum (ER) stress because of the demonstration that ER stress pathways are activated in SOD1<sup><ins>A4V</ins>+/</sup> ALS compared to SOD1<sup><ins><ins>/+</ins></ins></sup> motor neurons (Kiskinis et al., 2014). After two weeks of treatment with retigabine (1 µM), XBP1 splicing was markedly decreased in retigabine compared to vehicle-treated 39b SOD1<sup><ins>A4V</ins>+/</sup> ALS motor neurons (Figure S4A-B). In addition to reduced XBP1 splicing, we observed a decrease in PUMA and increase in EIF2B3 transcript levels, consistent with down-regulation of ER stress in response to retigabine treatment (Figure S4C).
Motor Neuron Hyperexcitability is Present in Distinct ALS Forms and is Blocked by Kv7 Activators

In order to investigate whether motor neuron hyperexcitability generalized to additional ALS variants, we performed MEA recordings of motor neurons derived from iPSC lines made from two unrelated familial ALS patients with C9orf72 hexanucleotide repeat expansion (19f and RB8b) (Kiskinis et al., 2014), which is responsible for 40-50% of familial ALS and approximately 10% of sporadic cases (Robberecht and Philips, 2013). Motor neurons derived from these patients also showed significant hyperexcitability compared to controls in both total firing rate (p<0.05, t-test; Figure 4A-B) and average mean neuronal firing rate (p<10^-5, t-test; Figure 4C).

We reasoned that comparing neuronal firing properties of a large group of ALS patient and control-derived motor neurons would help evaluate the robustness of the motor neuron hyperexcitability and, together with the A4V gene correction experiments, eliminate artifacts due to cell line variation. SOD1-derived motor neurons (four lines from four unrelated subjects harboring three different mutations), C9orf72-derived motor neurons (two lines from two unrelated subjects), fused-in-sarcoma (FUS)-derived motor neurons (two lines from two unrelated subjects harboring two different mutations) were all hyperexcitable relative to motor neurons derived from six iPSC lines made from five individual healthy controls (ANOVA, p <10^-7; Tukey’s post-hoc tests for control vs SOD1 p<0.01, control vs C9orf72 p<0.01, control vs FUS p<0.05; Figure 5A). Furthermore, spontaneous action potential firing in the ALS variant-derived motor neurons was uniformly blocked by retigabine (Figure 5B). Consistent with an on-target effect of retigabine, we found that a chemically distinct but less potent Kv7 current-enhancer, flupirtine (Brown and Passmore, 2009), also blocked spontaneous motor neuron firing (Figure 5C). These results demonstrate the broad relevance of motor neuron hyperexcitability for familial ALS and its sensitivity to Kv7 agonists across iPSC lines, patients and genotypic etiologies.

DISCUSSION
Neurons derived from patient iPSCs can be used to investigate physiological changes in specific neural subtypes relevant to neurodegeneration and reveal important disease mechanisms and candidate therapeutics. We found consistent hyperexcitability in motor neurons from a broad group of familial ALS patients, whose disease-causing mutations collectively span the majority of familial ALS cases. Differential excitability of particular motor neurons has been proposed to explain the selective vulnerability of specific motor neuron pools in ALS, and our data provide a possible mechanistic basis for this hypothesis (Bae et al., 2009; Saxena and Caroni, 2011).

Motor neuron hyperexcitability may contribute, therefore, to motor neuron death, although connections between hyperexcitability and motor neuron death in other ALS variants will now require investigation. Our results are consistent with multiple studies of excitotoxicity (Cleveland and Rothstein, 2001; Fritz et al., 2013) but, potentially, not with two recent studies. One postulated that increased excitability in the SOD1G93A mouse model may be a compensatory mechanism (Saxena et al., 2013). However, systemically-administered glutamatergic and cholinergic modulators may affect multiple neuronal types, making it difficult to assess how the findings relate to motor neuron excitability, which was not measured. A second study found decreased numbers of elicited spikes in iPSC-derived motor neurons from ALS patients with C9orf72 repeat expansion compared to controls (Sareen et al., 2013). The differences in spike count here may reflect the longer differentiation time (66-79 days), differences in surviving neuronal populations or alterations in resting membrane potential in C9orf72 compared to control neurons. For example, the more depolarized resting potential in C9orf72 expansion-derived neurons could result in greater sodium channel inactivation and reduced capacity for generation of multiple action potentials – indeed depolarization-induced action potential blockade may be a late phase of progressive hyperexcitability. Mouse SOD1G93A motor neurons show hyperexcitability even at an embryonic age (Kuo et al., 2004; Pieri et al., 2003; van Zundert et al., 2008), and neurophysiological studies reveal increased excitability in C9orf72 repeat expansion subjects (Williams et al., 2013) in addition to other familial and sporadic ALS cases (Blair et al., 2010; Mills and Nithi, 1997; Vucic and Kiernan, 2006; Vucic and Kiernan, 2010).
Previously, attention has focused on persistent sodium currents as a mechanism of motor neuron hyperexcitability (Kuo et al., 2005; Vucic and Kiernan, 2010), and riluzole, the only approved drug for ALS, blocks this current (Urbani and Belluzzi, 2000). Our findings suggest an additional important role of voltage-activated potassium channels. Channel compartmentalization may differ between in vivo and cultured-neuron systems, however, a recent immunohistochemical study of human spinal cords found decreased protein levels of a delayed-rectifier potassium channel selectively in the ventral roots of sporadic ALS but not control subjects (Shibuya et al., 2011). Mutations in voltage-gated potassium channels cause other neurodegenerative diseases (Waters et al., 2006), and oxidation of potassium channels (with consequent modulation of voltage-dependence and kinetics of channel gating) has been suggested as a broad mechanism of aging and neurodegeneration (Sesti et al., 2010). DPP6, which is involved in trafficking Kv4 voltage-gated potassium channels in CA1 hippocampal neurons, is linked to ALS (Sun et al., 2011; van Es et al., 2008). Hyper-methylation and down-regulation of potassium channel genes were observed in epigenetic analyses of post-mortem ALS patient spinal cords (Figueroa-Romero et al., 2012).

Both enhanced persistent sodium and reduced potassium currents could converge to produce hyperexcitability, and both may offer complementary pharmacological targets to control it. While our study evaluated hyperexcitability as an innate or autonomous property of motor neurons, differences in excitability may also reflect interactions between motor neurons and glia or Schwann cells (Fritz et al., 2013). Hyperexcitability has also been observed in motor neurons from a mouse model of spinal muscular atrophy (Mentis et al., 2011), and there is evidence for both cell autonomous (Gogliotti et al., 2012) and non-autonomous (Imlach et al., 2012) modulation of excitability. Interestingly, spinal muscular atrophy has also been linked to DPP6, raising the possibility of potassium channel contributions (van Es et al., 2009).
The pathways connecting disease-causing mutations and decreased potassium channel function, and between hyperexcitability and motor neuron death remain to be clarified. Calcium overload through voltage-gated calcium channels (Chan et al., 2007) and activation of ER stress (Saxena and Caroni, 2011) are possibilities. The finding that decreasing motor neuron activity reduces ER stress suggests that hyperexcitability may be upstream of the unfolded protein response, explaining at least partially how hyperexcitability may contribute to motor neuron death in ALS. Because ER stress modifiers increase motor neuron activity, a vicious cycle may result in ALS from reciprocal positive feedback between hyperactivity and ER stress (Kiskinis et al., 2014).

Despite the asymptomatic early decades typical of ALS patients, we observed a disease-specific phenotype in iPSC-derived neurons cultured for only weeks. That this property manifests so quickly in culture may reflect the absence of supporting cells and the inhibitory circuitry normally present in vivo. Identification of a screenable electrophysiological phenotype that contributes to motor neuron death and manifests quickly could facilitate investigation of ALS pathophysiology and identification or validation of therapeutics for individual patients. Furthermore, the phenotype may offer a personalized medicine approach to treatment, using response of stem cell-derived neurons as a guide for individual patient treatment selection; this strategy will require motor neurons derived from large cohorts of patients for validation. Studies of motor neuron excitability can now be expanded to determine whether all forms of ALS converge onto a single mechanistic pathway. More generally, our study illustrates the potential for using iPSCs differentiated into specific disease-relevant cell types to identify disease phenotypes, novel biomarkers and potential treatments.
FIGURE LEGENDS

Figure 1. Multi-Electrode Array (MEA) Recording Reveals Increased Spontaneous Firing in ALS-Derived Neurons Compared to Control-Derived Neurons.

(A) Schematic of differentiation and recording.

(B) Representative recordings from 4 out of 64 MEA electrodes in control (11a, 18a) and ALS (39b, RB9d)-derived neurons cultured for 28 days on the arrays. (C) Total action potential firing rate during one minute of recording from MEAs (11a, n=3; 18a, n=3; control mean 6,510 ± 3,131 spikes/minute; 39b, n=3; RB9d, n=3; ALS mean 20,528 ± 5,069 spikes/minute; p<0.05, t-test).

(D) Mean firing rate histograms of individual neurons from MEAs in B. See also Figure S1.

(E) Average of mean firing rate for patient-derived neurons (11a, n=381; 18a, n=191; control mean 1.17 ± 0.04 Hz; 39b, n= 520; RB9d, n=662; ALS mean 1.76 ± 0.05 Hz; p<10^{-15}, t-test).

(F) Total action potential firing rate during one minute recordings from MEAs of FACS-sorted 18a Hb9::GFP and 39b Hb9::GFP motor neurons recorded every four days (repeated measures ANOVA F-test p=1x10^{-4} for difference between lines; post-hoc t-tests with Bonferroni correction for multiple testing indicated as * for p<0.05 and ** for p<0.01). See also Figures S2-S3.

(G) Total action potential firing rate during one minute of recording from MEAs cultured for 14 days on the arrays (39b-Cor, n=4; mean 775 ± 712 spikes/minute; 39b, n=4; 39b mean 6,278 ± 1,758 spikes/minute; p=0.01, t-test). Error bars throughout figure are SEM.

Figure 2. ALS Patient-Derived Motor Neurons are Hyperexcitable and Have Reduced Delayed-Rectifier Potassium Currents Compared to Control-Derived Motor Neurons.

(A) An iPSC-derived motor neuron identified by Hb9::RFP lentiviral transduction (right) and during patch clamp recording (left) after culture for 28 days. Scale bar 20 µm.

(B) Representative current clamp recordings during ramp depolarization from control and ALS patient-derived motor neurons (upper four panels); sample recordings from separate experiments comparing the isogenic correction of the 39b SOD1^{AV} mutation (39b-Cor) and 39b (lower two panels).
(C) Upper panel: Average number of action potentials elicited by ramp depolarization from control (11a, n=12; 18a, n=11; control mean 2.5 ± 0.4) and ALS (39b, n=13; RB9d, n=12; ALS mean 4.2 ± 0.5) motor neurons obtained from four separate differentiations (p<0.05, Mann-Whitney U test).

Lower panel: Separate experiments showing average number of action potentials during ramp depolarization from 39b-Cor (n=17; mean 4.1 ± 0.5) and 39b (n=19; mean 6.4 ± 0.9) motor neurons from three additional differentiations (p<0.05, Mann-Whitney U test).

(D) Sample voltage clamp recordings from control and ALS-derived Hb9::RFP-positive motor neurons cultured for 28 days.

(E) Average delayed-rectifier (DR) steady-state potassium current amplitude relative to peak sodium current amplitude in control (11a, n=12; 18a, n=11; control mean 0.88 ± 0.087) and ALS (39b, n=13; RB9d, n=12; ALS mean 0.44 ± 0.054) patient-derived motor neurons from four differentiations (p<0.001, t-test).

(F) Experiments from three separate differentiations showing average delayed-rectifier steady-state potassium current amplitude relative to peak sodium current amplitude in 39b-Cor (n=18; mean 0.54 ± 0.061) and 39b (n=19; mean 0.32 ± 0.036; p<0.005, t-test).

(G) Direct measurement of delayed-rectifier voltage-gated potassium current isolated by holding at -30 mV, stepping to a test-potential of +40 mV for 2 s and normalizing steady state current amplitude to cell capacitance in 39b-Cor (n=19; mean 42.6 ± 4.3 pA/pF) and 39b (n=18; mean 30.3 ± 3.1 pA/pF; p<0.05, t-test) derived motor neurons using cells from two additional separate differentiations.

(H) Peak sodium current amplitude normalized to cell capacitance in 39b-Cor (n=16; mean 400.4 ± 44.7 pA/pF) and 39b (n=15; mean 387.1 ± 50.5 pA/pF; p=0.8, t-test) derived motor neurons.

Error bars throughout figure are SEM.

Figure 3. Retigabine Reduces Motor Neuron Excitability and Increases Survival.

(A) Rheobase measurements in a 39b Hb9::RFP-positive ALS-derived motor neuron in whole-cell patch clamp before (left) and after (right) the application of 10 µM retigabine (baseline rheobase 4.8 ± 1.5 pA vs post-retigabine rheobase 8.4 ± 2.2 pA; n=11; p<0.05, Wilcoxon signed rank test).
(B) Representative current clamp recording showing effect of 10 µM retigabine on membrane voltage and spontaneous firing (baseline Vm -60.4 ± 2.9 mV vs post-retigabine Vm -66.3 ± 3.6 mV, n=11; p=0.001, t-test). In (A-B), CNQX (15 µM), D-AP5 (20 µM), bicuculline (25 µM), and strychnine (2.5 µM) were added to the external solution.

(C) Dose response curve for retigabine on suppression of spontaneous action potentials in MEA recording and Hill plot fit of mean data from 39b (n=4) and RB9d (n=4) with EC50 1.5 ± 0.8 µM.

(D) Effect of vehicle (open circles) and 1µM retigabine (filled circles) treatment from days 14-28 of culture on the survival of Islet-positive, TuJ1-positive motor neurons measured at day 30 (total control n=11; total ALS n=9; F-test for effect of retigabine on all cells p=3.8x10^-4; effect of retigabine in ALS motor neurons, red, 25.3% (SD 5.6; t-test p=6.4x10^-5); effect of retigabine in control motor neurons, black, 6.1% (SD 5.1, p=0.23). Cell counts are from individual wells for four separate differentiations. See also Figure S4. Error bars throughout figure are SEM.

Figure 4. Hyperexcitability of C9orf72 Repeat Expansion-Derived Motor Neurons

(A) Representative recordings from four/64 MEA electrodes recorded from control (11a, 18a) and C9orf72 expansion ALS-derived neurons (19f, RB8b) cultured for 14 days.

(B) Total action potential firing rate during one minute of recording from MEAs (11a, n=1; 18a, n=3; control mean 4,752 ± 2,786 spikes/minute; 19f, n=3; RB8b, n=3; ALS mean 20,022 ± 3,775 spikes/minute; p<0.05, t-test).

(C) Average of mean firing rate for control and C9orf72-derived neurons (11a, n=82; 18a, n=203; control mean 1.11 ± 0.06 Hz; 19f, n=407; RB9d, n=929; ALS mean 1.50 ± 0.04; p<10^-5, t-test). Error bars throughout figure are SEM.

Figure 5. Motor Neuron Hyperexcitability and Block by Retigabine are Broad Properties of ALS Variants.

(A) Multi-electrode array recordings of motor neurons derived from control (11a, n=8; 15b, n=2; 17a, n=5; 18a, n=7; 18b, n=10; 20b, n=6), SOD1 (25b, D90A, n=3; 27d, G85S, n=7; 39b, A4V, n=3; RB9d, A4V, n=7), C9orf72 expansion (19f, n=2; RB8B, n=13) and FUS (MGH5b, frameshift
mutation at residue 511, n=10; RB21, H517Q, n=4) subjects cultured for 14 days. ANOVA, p < 10^{-7}; Tukey’s post-hoc tests for control vs SOD1 p<0.01, control vs C9orf72 p<0.01, control vs FUS p<0.05. For subject 18, motor neurons from two different iPSC lines were recorded. Error bars are 95% CI. See also Figure S5.

(B) Dose response curve for retigabine on suppression of spontaneous action potentials in MEA recording and Hill plot fit of mean data from SOD1 (n=10; EC50 1.9 ± 0.5 µM), C9orf72 (n=9; EC50 2.6 ± 0.8 µM) and FUS (n=4; EC50 1.9 ± 1.1 µM).

(C) Dose response curve for flupirtine on suppression of spontaneous action potentials in MEA recording and Hill plot fit of mean data from SOD1 (n=5; EC50 9.8 µM), C9orf72 (n=4; EC50 19.4 µM) and FUS (n=2; EC50 15 µM). Error bars for B and C are SEM.
EXPERIMENTAL PROCEDURES

iPSC Lines, Culture and Motor Neuron Differentiation

iPSC generation from patient fibroblasts obtained under IRB approval, characterization and motor neuron differentiation were performed as described in Kiskinis et al. (Kiskinis et al., 2014) and Figure S5, with iPSC line scoring as done previously (Bock et al., 2011). For transfection of 18a and 39b lines with a Hb9::GFP reporter (Figure S3), a 1kb Hb9 promoter fragment (gift from Hynek Wichterle) controlling the expression of myristoylated GFP was inserted into a donor plasmid specific for the AAVS1 locus (Sigma). Subsequently, 2.5 million iPS cells were accutased and electroporated using the Neon transfection system (100μl tip; 1600V Voltage, 20ms Width, 1 Pulse; Life Technologies) with 2 μg of AAVS1 ZFN plasmid and 6 μg of modified AAVS1 donor plasmid. After nucleofection cells were plated on matrigel with mTeSR1 in the presence of ROCK inhibitor. After 48hrs, puromycin selection was applied and surviving clonal colonies were individually passaged and gDNA was extracted. PCR was used to confirm proper targeting of the cassette. Primer sequences are available upon request. Faithful expression of the reporter was verified using expression of the motor neuron marker Isl1 (Figure S3C).

iPSCs were maintained on culture dishes as described previously (Boulting et al., 2011) with modifications (Kiskinis et al., 2014) in a 24-day protocol based on initial neuralization with SB431542 (10 μM, Sigma Aldrich) and Dorsomorphin (1μM, Stemgent), and motor neuron patterning with RA (Sigma) and a small smoothened Agonist 1.3 (Calbiochem). For Figure 1F, FACS-purified neurons were grown on a confluent monolayer of primary cortical mouse glia prepared from P0-P2 mouse pups (as described in Boulting et al., 2011), which may increase firing rates compared to experiments without glia (Boehler et al., 2007).

MEA Recording

After 24 days of differentiation, equal numbers of control and ALS neurons were plated on poly-D-lysine/laminin coated p515A probes (Alpha Med Scientific) or M768-GLx 12-well plates (Axion BioSystems) at typical densities of 40,000-80,000/ probe or well. All probes were visualized
immediately before each recording session to confirm a full monolayer of cells. Initial experiments (11a, 18a, 39b, and RB9d comparison) were performed as close as possible to the time of patch recordings (4 weeks). However, because we wished to evaluate firing at a time point prior to significant motor neuron death (Kiskinis et al., 2014), we performed subsequent experiments (39b-Cor and 39b comparison and all later experiments) at day 14 after dissociation (Table S1).

Recordings from 64 extracellular electrodes were made using a Med64 (Alpha Med Scientific) or Maestro (Axion BioSystems) MEA recording amplifier with a head stage that maintained a temperature of 37°C. For Med64 recordings, data were sampled at 20 kHz, digitized, and analyzed by spike clustering and spike extraction algorithms using Mobius software (Alpha Med Scientific) with a 2 kHz 9-pole Bessel low pass filter, 10 µV action threshold detection limit, and 30% cluster similarity radius. These standard settings were maintained for all analyses. We confirmed that we obtained similar results across a wide range of action potential threshold and cluster similarity radius settings. Correlation analysis to detect and correct for clusters detected by multiple electrodes was performed using custom Matlab software. Total action potential firing rates and mean neuronal firing frequencies were then determined and plotted. In order to record in larger replicates, we used the Axion Maestro MEA device, in a 12-well format with 64 extracellular electrodes in each well. For Maestro recordings, data were sampled at 15 kHz, digitized, and analyzed using Axion Integrated Studio software (Axion BioSystems) with a 200 Hz high pass and 2500 kHz low pass filter and an adaptive spike detection threshold set at 5.5 times the standard deviation for each electrode with 1 second binning. These standard settings were maintained for all Axion MEA recording and analysis.

For retigabine dose response curves, action potential numbers during one minute of recording in each concentration of retigabine were normalized to the initial action potential number during one minute of recording in standard extracellular saline solution. The EC50 value was determined by fitting the mean normalized data values to the Hill equation, $y = 1/((EC50/x)^{nH} + 1)$ where $nH$ is the Hill coefficient.
Patch Electrophysiology

Whole-cell patch recordings were performed on iPS-derived motor neurons identified by transduction with an Hb9::RFP lentivirus. Lentiviral transduction was typically performed 7-10 days before recording. Two large comparisons were performed, one consisting of 11a, 18a, 39b, and RB9d, and the second consisting of 39b-Cor and 39b. Each comparison was made from pooled data from multiple separate experiments, each consisting of synchronous and parallel iPSC culture and differentiation, embryoid body dissociation, plating and maturation of control- and ALS-derived neurons. Equal numbers of control and ALS motor neurons were recorded from each experiment. Comparison of the original four lines (11a, 18a, 39b, and RB9d) was made using four separate parallel differentiation experiments, while comparison of the isogenic correction comparison (39b-Cor and 39b) was performed using three separate parallel differentiation experiments.

For each experiment, neurons from control and ALS lines were dissociated after 24 days of differentiation and plated onto poly-d-lysine/laminin coated glass coverslips (20,000-40,000/coverslip) and allowed to mature for four weeks from start of differentiation. We chose four weeks as the best timepoint because this yielded the most homogeneous population of mature-appearing Hb9::RFP-positive motor neurons (at the requisite low cell density for patch clamp) with the most mature electrophysiological properties. Whole-cell current-clamp and voltage-clamp recordings were performed using a Multiclamp 700B (Molecular Devices) at room temperature (21-23°C). Data were sampled at 20 kHz and digitized with a Digidata 1440A A/D interface and recorded using pCLAMP 10 software (Molecular Devices). Data were low-pass filtered at 2 kHz. Patch pipettes were pulled from borosilicate glass capillaries on a Sutter Instruments P-97 puller and had resistances of 2-4 MΩ. The pipette capacitance was reduced by wrapping the shank with Parafilm and compensated for using the amplifier circuitry. Series resistance was typically 5-10 MΩ, always less than 15 MΩ, and compensated by at least 80%. Neurons were excluded from analysis if holding current at -80 mV exceeded 100 pA, input resistance was less than 250 or
greater than 2000 MΩ, or spikes elicited from -65 mV had peaks below 0 mV. Resting membrane potential was determined by averaging for 20s of recording, and afterwards a small holding current (typically with amplitude less than 5 pA) was used to clamp the resting membrane potential as close as possible to -65 mV. Rheobase was measured by applying 1 s steps in increments of 2.5 pA until an action potential was generated. Current ramps were elicited from an initial hyperpolarizing current of 10 pA for 1 s followed by a 210 pA/s depolarizing ramp of duration 1 s. Spikes on the ramps were counted if the peak voltage exceeded -10 mV. Action potential properties (Table S2) were determined using custom-written analysis software in Igor Pro (Wavemetrics) with DataAccess (Bruxton) for importing the files. For voltage-clamp recordings, voltages were elicited by 100-ms depolarizing steps from a holding potential of -80 mV to test potentials ranging from -80 mV to 50 mV in 10 mV increments. For the latter gene correction experiments the step length was increased to 200 ms to assay delayed-rectifier currents after more complete decay of transient potassium currents. For retigabine patch applications, resting membrane potential was recorded immediately before and 10 seconds after the application of 10 µM retigabine (in each case, membrane potential was an average of values sampled for 20 seconds). For all patch experiments, series resistance was monitored by brief -5 pA hyperpolarizing steps during current clamp recordings and by 5 mV hyperpolarizing steps during voltage clamp recordings. Electrode drift was measured at the end of each recording and was typically 1-2 mV. The extracellular solution was sodium-based and contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES 10, pH 7.4. The intracellular solution was potassium-based and contained 150 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 4 mM MgATP, 0.3 mM NaGTP, 10 mM Na₂PhosCr, 1mM EGTA, pH 7.4. For isolation of delayed-rectifier potassium channels (Figure 2G), 300 nM TTX and 100 µM CdCl₂ were used to block voltage-gated sodium channels and voltage- and calcium-activated potassium channels, respectively. For isolation of voltage-gated sodium currents (Figure 2H), internal KCl was replaced by CsCl to block potassium currents and 100 µM CdCl₂ was used to block calcium currents.
Cell Survival and ER Stress Assays
Dissociated neurons (20k) were plated on poly-D-lysine/laminin coated 8-well chamber slides (BD Biosciences) containing a confluent monolayer of primary cortical mouse glia (Boulting et al., 2011). For survival analysis, slides were fixed at 3 and 30 days, and cultures were stained for counting. The number of ISL-positive, TUJ1-positive motor neurons (counter blinded to cell line identity) was normalized to the number on day 3 for each line. Retigabine (1 µM) or vehicle control was added from day 15 onwards. 11 total experiments (control motor neurons) and nine total experiments (ALS motor neurons) were performed from the same four separate differentiations. ER stress experiments were performed as described in Kiskinis et al. In brief, in two separate independent biological replicates, 300 ng of RNA was used to generate cDNA, of which 2 µL and AmpliTaq Gold Polymerase (Applied Biosystems) were used for PCR amplification. The amounts of spliced and unspliced bands were quantified using Image J. Quantitative RT-PCR was performed in triplicate using the iSCRIPT kit (Biorad) for cDNA synthesis and SYBR green (Bio-Rad) labeling followed by amplification using the iCycler system (Bio-Rad).

Drugs
Drugs included retigabine (Santa Cruz Biotechnology), bicuculline, strychnine, D-AP5, CNQX, flupirtine, TTX (all from Tocris Bioscience). CsCl and CdCl\textsubscript{2} were from Sigma.

Statistical Analysis
p<0.05 was considered statistically significant. Comparisons were made between control and ALS populations using t-tests (two-tailed, unpaired)/ANOVA for continuous data and rank tests for non-parametric data (discrete measurements of number of spikes on ramps and rheobase). For analysis of MEA firing over time (Figure 1F), we used a mixed repeated measures ANOVA model with fixed effects of cell line and time and random effects of individual replicate. For effect of retigabine on specific cells (Figure 3A-B), paired tests were used. For effect of retigabine on survival (Figure 3D), we fitted a linear regression model with effects of retigabine treatment, cell
line and their interaction. F-tests for difference between lines gave p=0.0011, for effect of retigabine p=3.8x10^{-4} and for effect modification (p=0.015). For ALS subjects, the effect of retigabine was an increase in cell count of 25.3% (SD 5.6, p=6.4x10^{-5}). The effect estimate for 39b was 16.9% (SD 12.9, p=0.03) and for RB9d 39.9% (SD 11.3, p=0.001). For control subjects, the effect of retigabine was an increase in cell count of 6.1% (SD 5.1, p=0.23). For multiple genotype comparisons (Figure 5A), one-way ANOVA after log transformation to normalize variance and post-hoc Tukey tests were used to analyze multiple ALS variants. Error bars are SEM unless indicated.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes three figures and three tables.

ACKNOWLEDGEMENTS

We thank W. David and S. Cash for comments, suggestions, and review of the manuscript, K. Kapur for assistance with statistical analysis and K. Wainger for assistance with figures. This work was supported by NIH 5 T32 GM007592-33, Harvard NeuroDiscovery, ALS Association and American Brain Foundation Clinical Research Fellowship (B.J.W); Charles King Trust Postdoctoral Fellowship (E.K.); American Brain Foundation/ALS Association and KL2 MeRIT fellowship/Harvard Catalyst (S.S.W.H); ALS Therapy Alliance, P2ALS, Angel Fund, Pierre L. de Bourgknecht ALS Research Foundation, Al-Athel ALS Research Foundation, ALS Family Charitable Foundation and NIH/NINDS (1R01NS050557 and NINDS ARRA Award RC2-NS070-342) (R.H.B.); P2ALS, Project ALS, Target ALS, NINDS GO grant (5RC2NS069395-02), NINDS R24 (1U24NS078736-01) and HHMI (K.E.); NIH (5 R01 NS038253-10; 2 R01 NS038153-15), Target ALS and New York Stem Cell Foundation (C.J.W.). None of the authors of this manuscript have a financial interest related to this work.
REFERENCES


Targeting RNA foci in iPSC-derived motor neurons from ALS patients with a C9ORF72 repeat expansion. Sci Transl Med 5, 208ra149.


Figure 2

- **A**: Images of neurons with tracings.
- **B**: Graph showing number of spikes over time with error bars.
- **C**: Ratio of K(DR) to Na current density.
- **D**: Graph for Na current density.
- **E**: Graph for K(DR) current density.
Figure 3
Figure 4
Figure 5

A

Percentage of Spikes Blocked

Retigabine (µM)

C9orf72

FUS

SOD1

Percent of Spikes Blocked

Control

B

C

Total Spikes / Minute (x10^3)

Retigabine (µM)

Percent of Spikes Blocked

Flupirtine (µM)

Control

SOD1

C9orf72

FUS

10

1

0.1

Flupirtine (µM)

Percent of Spikes Blocked

D90A

G85S

A4V

A4V

M511FS

H517Q
Conclusions and Future Goals

The presented work shows how stem cell techniques can be used to model pain and motor neuron disease. The first project illustrates the development and validation of a model using lineage reprogramming of fibroblasts. The second project demonstrates how an existing protocol for deriving neurons of a disease-relevant type can be used to identify a disease phenotype and use the phenotype to evaluate candidate therapeutics.

The integration of iPSC-based modeling into clinical research and practice, which we hope to develop in the future, could have a transformative effect on the study and practice of medicine. First, future selection of subjects for clinical trials could be based on prospective evaluation of in vitro phenotypes using iPSC-derived neurons. One potential reason for clinical trial failure is subject heterogeneity and the inability to recruit individual subjects most likely to respond to a specific treatment. Analysis of subject-derived neurons may enable improved identification of subgroups most likely to respond. Thus, clinical studies may prospectively include subjects based on their in vitro motor neuron response to a drug. Such optimized selection of study subjects using in vitro iPSC-derived neuron phenotypes as a biomarker for drug response would help identify effective treatments that otherwise might be lost in the noise of non-responders.

Second, the use of iPSC-derived disease-relevant subtypes may serve as a new type of diagnostic to help decide whether a subject is likely to benefit from a specific treatment versus another. Just as specific genetic analyses may identify disease subgroups likely to benefit from a specific treatment, analyses of iPSC-derived motor neurons may reveal information that can guide treatment response. Furthermore, iPSC-derived motor neurons may potentially also be used to predict prognosis.

Third, instead of performing drug discovery on cell lines heterologously expressing a target, we will employ screens based on the phenotypic properties of disease iPSC-derived neurons. The generation of a large collection of subject iPSC lines and derived neurons together with clinical phenotyping in the same subjects will be an invaluable resource for drug discovery using human neurons. In
particular, the validation of potential hit compounds across the large range of genetic and clinical subject variants will enable us to focus on hit molecules that are either broadly effective across a large number of disease subjects or extremely potent in a smaller group of cases.
Acknowledgements

Clifford Woolf and Lab Members:
Elizabeth Buttermore, Julia Oliveira, Cassidy Mellin, Seungkyu Lee, Isaac Chiu, Amy Wang, Lee Barrett

Kevin Eggan and Lab Members:
Evangelos Kiskinis, Justin Ichida, Jackson Sandoe, Steve Han, Ester Son, Gabriella Boulting, Luis Williams

Jeanine Wiener-Kronish, Jim Rathmell, Jianren Mao

Merit Cudkowicz, James Berry, Eric Macklin, Bob Brown

Thesis Committee:
Darin Dougherty
Florian Eichler
Clifford Woolf

Nick Andrews

MPCTI Program:
Anthony Hollenberg, Paul Conlin, Jonathan Williams, Lauren Dewey Platt

Keren, Ellie & Asher Wainger