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Modeling Motor Neuron Resilience in ALS Using Stem Cells

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SUMMARY

Oculomotor neurons, which regulate eye movement, are resilient to degeneration in the lethal motor neuron disease amyotrophic lateral sclerosis (ALS). It would be highly advantageous if motor neuron resilience could be modeled in vitro. Toward this goal, we generated a high proportion of oculomotor neurons from mouse embryonic stem cells through temporal overexpression of PHOX2A in neuronal progenitors. We demonstrate, using electrophysiology, immunocytochemistry, and RNA sequencing, that in vitro-generated neurons are bona fide oculomotor neurons based on their cellular properties and similarity to their in vivo counterpart in rodent and man. We also show that in vitro-generated oculomotor neurons display a robust activation of survival-promoting Akt signaling and are more resilient to the ALS-like toxicity of kainic acid than spinal motor neurons. Thus, we can generate bona fide oculomotor neurons in vitro that display a resilience similar to that seen in vivo.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is characterized by a loss of motor neurons in cortex, brain stem, and spinal cord with subsequent spasticity, muscle atrophy, and paralysis. Motor neurons innervating the extraocular muscles, including the oculomotor, trochlear, and abducens nuclei, are however relatively resistant to degeneration in ALS (Comley et al., 2016; Gizzi et al., 1992; Nijssen et al., 2017; Nimchinsky et al., 2000). This resilience has been attributed to cell-intrinsic properties (Allodi et al., 2016; Brockington et al., 2013; Comley et al., 2015; Hedlund et al., 2010; Kaplan et al., 2014). It would be highly advantageous if differential motor neuron vulnerability could be modeled in vitro. This could aid in the identification of gene targets responsible for oculomotor neuron resilience. However, it is not self-evident that in vitro-generated oculomotor neurons would be more resistant to degeneration in culture than spinal motor neurons, as multiple cell types contribute to motor neuron degeneration in ALS, including, for example, astrocytes (Yamanaka et al., 2008), microglia (Boillée et al., 2006), and oligodendrocytes (Kang et al., 2013), and may play a role in selective vulnerability.

During development, distinct neuronal populations are defined through diffusible morphogens with restricted temporal and spatial patterns and subsequent activation of distinct intrinsic transcription factor programs. These programs can be mimicked to specify different CNS regions and neuronal populations from stem cells in vitro. When mouse embryonic stem cells (mESCs) are exposed to SHH (sonic hedgehog) signaling and retinoic acid, which ventralize and caudalize the CNS during development, spinal motor neurons constitute 15%–30% of the cells in culture, and interneurons the large remainder (Allodi and Hedlund, 2014; Wichterle et al., 2002). Oculomotor neurons constitute only about 1% of the cells in the midbrain; thus, patterning mESC cultures with fibroblast growth factor 8 (FGF8) and SHH to direct cultures toward a midbrain/hindbrain fate (Allodi and Hedlund, 2014; Hedlund et al., 2008) results in cultures with too few oculomotor neurons to allow for any quantitative analysis. However, when combining morphogens with overexpression of the intrinsic determinant Phox2a in neural precursors, a large proportion of ISLET1/2+ brain stem motor neurons can be generated (Mong et al., 2014). Furthermore, a combination of the transcription factors Ngn2, Isl1, and Phox2a can specify mESCs into cranial motor neurons (Mazzoni et al., 2013). However, the exact identity of motor neurons generated this way has not been previously demonstrated, and it is currently unclear whether these are indeed oculomotor neurons or rather a mix of brain stem motor neurons, including, for example, trigeminal and facial motor neurons.

Toward the goal of developing stem cell-based in vitro models of differential vulnerability we generated cultures with a high proportion of oculomotor neurons through temporal overexpression of PHOX2A in mESC-derived neuronal progenitors and compared these with mESC-derived spinal motor neurons. Careful characterization of these distinct motor neuron populations using immunocytochemistry, electrophysiological recordings, and RNA sequencing demonstrated that mESC-derived oculomotor neurons were highly similar to their in vivo counterparts in both rodent and man. Finally, we exposed these to ALS-like conditions using kainic acid and demonstrated that oculomotor neurons were more resilient than spinal motor neurons.
motor neurons. Thus, we can generate bona fide oculomotor neurons in vitro that display a resilience to ALS similar to that seen in vivo.

RESULTS

Bona Fide Oculomotor Neurons Can Be Generated from mESCs

To characterize the specific identity of brain stem motor neurons generated in culture and compare their properties with those of spinal motor neurons, we generated neurons from mESCs. Spinal motor neurons were derived by adding 100 nM retinoic acid and the smoothed agonist SAG to the culture for 5 days. Brain stem motor neurons were specified by overexpressing Phox2a under the nestin enhancer and patterning the culture with FGF8 and SAG for 5 days (Figure 1A). Immunocytochemical analysis demonstrated that spinal motor neurons expressed the transcription factors ISLET1/2 and HB9 (MNX1) (64.1% ± 5.2% ISLET1/2+HB9+ cells, mean ± SEM, n = 4) (Figures 1B, 1D, and 1E). PHOX2A-overexpressing motor neurons appeared to be of an oculomotor identity as these cells expressed ISLET1/2 but lacked HB9 (6.3% ± 1.3% ISLET1/2+HB9+ cells, mean ± SEM, n = 4), which is present in all cranial motor neuron nuclei except the oculomotor nucleus (Figures 1C, 1D, 1F, and 1G) (Guidato et al., 2003; Lance-Jones et al., 2012). Henceforth we therefore refer to the brain stem motor neurons as oculomotor neurons. In oculomotor neuron cultures 47.5% ± 5.9% (mean ± SEM, n = 4) of βIII-TUBULIN+ cells were ISLET1/2+ and 62% ± 5.2% (mean ± SEM, n = 4) of the ISLET1/2+ cells were PHOX2A+, which demonstrates that 25% of all neurons generated were oculomotor neurons (Figure 1G).

Oculomotor neurons (derived from Islet-GFP mESCs) and spinal motor neurons (derived from Hb9-GFP mESCs) were analyzed for their electrophysiological properties at day-9 and day-11 time points (Figure S1). Cells were able to fire overshooting action potentials in response to depolarizing current pulses. At day 9 few spikes were evoked. Neuronal maturation from days 9 to 11 was seen as a change in the electrical properties whereby day-11 oculomotor neurons could fire trains of action potentials (Figures S1A and S1B) and the width of action potentials was decreased when compared to day 9 (Figure S1C). The increase in the size of neurons was reflected by the increased cell capacity (Figure S1D), decreased input resistance (Figure S1E), and increased rheobase (Figure S1F) from days 9 to 11. Similar changes were seen in spinal motor neurons, which fired a higher number of action potentials (Figures S1G and S1H) and showed a reduced width of action potentials (Figure S1I) at day 11 compared to day 9. Moreover, the changes in cell capacity (Figure S1J), input resistance (Figure S1K), and rheobase (Figure S1L) showed signs of neuronal maturation over time. To further investigate neuronal maturation over time, we performed choline

Figure 1. PHOX2A Overexpression Drives the Generation of Bona Fide Oculomotor Neurons from Stem Cells

(A) Time line depicting in vitro protocols for the generation of resistant oculomotor neuron (OMN) and vulnerable spinal cord motor neuron (SC MN). The mESC column reports the four cell lines used in this study. Patterning was performed for 5 days following EB formation. mRNA-seq coupled with FACS was performed on EB dissociation day (9 of the protocol). The survival assay was performed 5 days after EB dissociation (day 14 to 21).

(B and C) SC MNs generated from E14.1 ESCs express HB9, ISLET1, and NF200 (B). OMNs generated from E14.1 ESCs overexpressing the transcription factor Phox2a under the nestin enhancer co-express ISLET1 and NF200 in the absence of HB9 (C). Scale bar, 60 μm.

(D) Percentage of HB9+/ISLET1+ cells in SC MN (64.1% ± 5.2%, mean ± SEM, n = 4) and OMN (6.3% ± 1.3%, mean ± SEM, n = 4) cultures, respectively (at least 120 Islet1+ cells counted per condition, bar and whiskers represent means ± SEM).

(E) Microphotographs showing ISLET1+/TUJ1+ cells in OMN cultures.

(F) OMNs also express the specific marker Phox2a as indicated by asterisks. Scale bar, 100 μm (applies also to E).

(G) Quantification of ISLET1 over TUJ1 cells demonstrates that half the neuronal population appears to be ISLET1+ (47.5% ± 5.9%, mean ± SEM, n = 4; total number of TUJ1+ cells counted: 1,325). Quantification of PHOX2A over ISLET1+ cells (experiments performed in quadruplicates with two technical replicates each, total number of ISlet1+ cells counted: 746) indicates that 62% ± 5.2% (mean ± SEM, n = 4) of the ISLET1+ population is also PHOX2A+. All quantifications were performed 5 days after EB dissociation, and experiments were conducted in quadruplicates including two technical replicates per experiment (bar and whiskers represent means ± SEM).

(H) PCA of OMN and SC MN samples based on all genes expressed confirmed cell differential identities. mRNA-seq analysis of OMNs and SC MNs isolated by FACS was performed after 5 days of patterning (day 9 of the protocol).

(I) A total of 1,017 DEGs was found between the two different cell types (adjusted p < 0.05, n = 6). Heatmap shows the top 500 most significant DEGs by adjusted p value.

(J–L) Heatmap of expected progenitors and motor neuron transcripts but also OMN and SC MN specific transcripts obtained in the two generated motor neuron populations (J and L). Using the top 100 DEGs obtained from our RNA-seq analysis (K), we could separate datasets originating from in vivo microarray studies on early postnatal (J) and adult (L) rodent OMNs and SC MNs.

(M and N) Venn diagrams showing gene sets enriched either in brain stem cultures or OMN-specific as revealed by PAGODA analysis (M), and gene sets preferentially found in spinal cord cultures or MN specific (N).
acetyltransferase (ChAT) staining on oculomotor and spinal motor neuron cultures, confirming expression of the transferase from day 14 in vitro in both ISLET1+/HB9− and ISLET1+/HB9+ cells (Figures S1M and S1N).

To further confirm the identity of the cells generated, we sorted the motor neurons based on GFP expression using fluorescence-activated cell sorting (FACS). For this purpose we used a Hb9-GFP mESC line to select for spinal motor neurons and an Islet-GFP:NeuPhox2a mESCs line for the purification of oculomotor neurons (Figures 1A and S2A–S2D). Poly(A)-based RNA sequencing of the sorted samples in an unbiased way. Here we set out to identify gene clusters regulated in vivo neuron populations closely resemble their counterparts.

Sorted oculomotor neurons clustered closely together in the principal component analysis (PCA) and were separated from spinal motor neurons along the fourth principal component (Figure 1H). Hierarchical clustering of the 1,017 genes that were differentially expressed between oculomotor neurons and spinal motor neurons at an adjusted p value of <0.05 demonstrated that each group is defined by a specific set of markers, with a majority of genes being more highly expressed in spinal motor neurons (Figure 1I and Table S1). Analysis of Hox mRNAs clearly demonstrated the more caudal nature of spinal motor neurons compared with oculomotor neurons (Figure S1F). The oculomotor neurons and spinal motor neurons clustered separately based on known marker gene expression, e.g., Phox2a, Phox2b, Thx20, and Rgs4 for oculomotor neurons and Olig1/2, Hb9, and Lhx1/3 for spinal motor neurons. Oculomotor neuron cultures were also devoid of noradrenergic neuron markers Slc6a2 (NET) and Th (Figure 1J).

Using the top 100 differentially expressed genes (DEGs) between our in vitro-generated oculomotor neurons and spinal motor neurons, we could separate a published microarray dataset (Mazzoni et al., 2013) of in vitro-generated brain stem and spinal motor neurons (Figure S1G). Importantly, we could also use the 100 top DEGs between our oculomotor and spinal motor neurons to separate microarray data originating from mouse oculomotor and spinal motor neurons isolated from postnatal day 7 (P7) mice (Kaplan et al., 2014) (Figure 1K) and from adult rats (Hedlund et al., 2010) (Figure 1L). Thus, our in vitro-generated motor neuron populations closely resemble their in vivo counterparts.

To further delineate the nature of our in vitro culture systems, we set out to identify gene clusters regulated in groups of samples in an unbiased way. Here we analyzed all GFP-positive (Hb9+ for spinal and Islet1+ for oculomotor) and -negative fractions from both spinal- and midbrain-specified cultures using PAGODA (Fan et al., 2016). This analysis revealed gene sets enriched in the spinal and oculomotor cultures (GFP+ and GFP− samples), as well as in the motor neuron groups specifically (GFP+)(Figures 1M and 1N; Table S1). Of the oculomotor-enriched genes found with DESeq2, a subset was enriched in all midbrain-specified cultures (GFP+ and GFP− cells), such as Phox2a and Engrailed1 (En1). Fgf10, known to be expressed in the developing midbrain, including the oculomotor nucleus (Hattori et al., 1997), and Fgf15, with known enrichment in midbrain and rhombomere 1 (Partanen, 2007), were also enriched in our midbrain-specified cultures (Figure 1M). The genes that were highly enriched specifically in oculomotor neurons (GFP+) included Eya1, Eya2, Tbx20, and Phox2b (Figure 1M). Vice versa, of the genes found to be differentially expressed in spinal motor neurons versus oculomotor neurons with DESeq2, a subset was enriched in all samples of the spinal-specified cultures versus the midbrain-specified cultures. This included Hox genes that regulate positional identity, e.g., Hoxc4, Hoxa4, Hoxa5, Hoxb5, Hoxb6, Hoxb8, and Hoxd8, as well as spinal V1 interneuron markers Sp8 and Prdm8. Another subset was found to be highly specific for only the spinal motor neurons, including Mnx1 (Hb9) and Lhx1 (Figure 1N).

Different axon guidance-related genes are expressed by oculomotor and spinal cord motor neurons during development (Björke et al., 2016; Gutekunst and Gross, 2014; Stark et al., 2015; Wang et al., 2011). To further characterize the in vitro-generated oculomotor neurons, we analyzed their mRNA expression of morphological markers that are important for axon guidance. We found that oculomotor neurons were enriched in Plxn4, Sema6d, Cdh6, and Cdh12, while spinal motor neurons were enriched for Epha3, Ephx4, Sema4a, and Sema5b (Figure S2G). We subsequently analyzed the expression level of these axon guidance RNAs in microarray data from mouse oculomotor and spinal motor neurons isolated from P7 mice (Kaplan et al., 2014) and found that Sema5d was preferentially maintained in oculomotor postnatally (Figure S2H).

Thus, our in vitro-generated oculomotor neurons have characteristics distinct from spinal motor neurons regarding genes that govern axon guidance, and some of these characteristic are maintained in vivo.

In conclusion, we have generated a high proportion of bona fide oculomotor neurons in vitro that can be utilized to understand their normal function as well as resilience to degeneration in motor neuron diseases.

**Stem Cell-Derived Oculomotor Neurons Are Relatively Resistant to Excitotoxicity**

To investigate whether in vitro-generated oculomotor neurons are relatively resistant to ALS-like toxicity similarly
Figure 2. In Vitro-Generated Oculomotor Neurons Are Relatively Resistant to ALS-like Toxicity

(A) OMNs and SC MNs express similar mRNA levels of glutamate ionotropic receptor AMPA, NMDA, and kainate type subunits. The heatmap shows log2 RPKM values of these subunits, and no separate clustering of the two cell types is observed.

(B and C) Immunohistochemistry performed on generated OMNs (B) and SC MNs (C) at D1 in vitro in control conditions; similar levels of glutamate ionotropic receptor kainate type subunit 5 (GRIK5) are found in both cell types. Scale bar in (C), 60 μm (applies also to B).

(D and E) Microphotographs presenting SC MN (D) and OMN (E) response to kainic acid-induced toxicity (20 μM) for a week. Scale bar in (D), 100 μm (applies also to E).

(F) Curves represent percentages of MN survival over time in OMN and SC MN cultures. OMNs were visualized as NF200+ISLET1+HB9− cells, while SC MNs as NF200+ISLET1+HB9+ cells. OMNs show increased survival to kainic acid toxicity at D7 (bar and whiskers represent means ± SEM, two-way ANOVA and Tukey’s multiple comparison test, F(9, 56) = 2.333, *p = 0.0261, SC MNs n = 4, OMNs n = 5) when compared with SC MNs (experiments were performed at least in quadruplicate, with technical replicates and with at least 130 motor neurons counted per condition in each experiment).
to their in vivo counterparts, we used kainic acid, which is a neuroexcitatory amino acid that acts by activating glutamate receptors. Glutamate excitotoxicity is thought to be a downstream event in motor neuron degeneration in ALS and is thus considered appropriate to model this disease. First, we evaluated the mRNA levels of glutamate ionotropic receptors in our cultures to ensure that generated neurons could respond to kainic acid. mRNA-sequencing data of sorted motor neurons demonstrated that oculomotor neurons and spinal motor neurons expressed similar levels of AMPA, NMDA, and kainate receptor subunits (Figure 2A). Grik5 (glutamate receptor, ionotrophic kainate 5) was the subunit expressed at highest level (Figure 2A) and was also detectable at the protein level in both oculomotor (Figure 2B) and spinal (Figure 2C) motor neurons. Thus, both motor neuron types should respond to kainic acid. Exposure of the cultures to kainic acid for 1 week demonstrated that oculomotor neurons were more resilient to the elicited long-term excitotoxicity than spinal motor neurons (ANOVA, *p < 0.05; Figures 2D–2F).

Stem cell-derived cultures are not easily temporally restricted in a precise way. Cultures that contain postmitotic neurons may also harbor neuronal progenitors that later on are specified into neurons. It can thus be challenging to distinguish survival from renewal in a stem cell-based in vitro system. We therefore analyzed the turnover rate in our cultures by pulsing these with bromodeoxyuridine (BrdU). Analysis of the number of BrdU^+ ISLET1/2+ neurons demonstrated that a significantly higher number of motor neurons were generated over time in the spinal motor neuron cultures compared with the oculomotor neuron cultures (Figure S2I). Consequently, the resilience of oculomotor neurons should be considered even more pronounced as these cultures had a comparatively low turnover rate. Furthermore, we investigated the presence of astrocytes in the cultures and found that only a minority of cells expressed glial fibrillary acidic protein (Figures S2J and S2K). As the processes of motor neurons show the first signs of pathology in ALS, we investigated length of neuronal processes in both oculomotor and spinal motor neuron cultures exposed to kainic acid for 7 days. The result clearly demonstrated that oculomotor neurons were unaffected by kainic acid while spinal motor neurons displayed a shortening of neurites (Figure 2G). To further evaluate oculomotor neurites, we used Sholl analysis to interrogate arborization complexity. The number and branching of oculomotor neurites were not negatively affected under kainic acid treatment (Figures 2H–2K). As axonal fragmentation and degeneration is seen early on in motor neurons in ALS, our data clearly demonstrate that oculomotor neurons cope well under conditions of excitotoxicity and maintain normal morphology.

In conclusion, in vitro-generated oculomotor neurons are highly resilient to ALS-like excitotoxicity. Thus, the pattern of selective motor neuron vulnerability seen in vivo can be replicated in vitro.

Oculomotor Neurons Have High Levels of Calcium-Buffering Proteins and Akt Signaling, which Could Contribute to Resilience

To explain the resilience of in vitro-generated oculomotor neurons to excitotoxicity, we conducted a directed comparative analysis of the RNA-sequencing data from purified oculomotor neurons versus spinal motor neurons. It has been suggested that preferential expression of calcium binding proteins in oculomotor neurons plays a role in their resilience (Comley et al., 2015; Van Den Bosch et al., 2002). We focused on transcripts with implications in calcium handling. Our analysis demonstrated that Cald1, Esyt1, Camk2a, and Hpca11 were preferentially expressed in oculomotor neurons (Figure S3A). ESY1 protein levels were also preferential to oculomotor neurons and unaffected by kainic acid treatment (Figures S3B–S3D). This could render oculomotor neurons with an increased capacity to buffer Ca^{2+} intracellularly.

We have previously shown that AKT signaling is important in motor neuron resilience toward ALS after insulin-like growth factor-2 treatment (Allodi et al., 2016). We therefore analyzed our RNA-sequencing data from sorted oculomotor and spinal motor neurons for Akt signaling effectors, which could aid in explaining the relative resilience of oculomotor neurons in vitro. This analysis demonstrated
an elevated expression of Akt1 and Akt3 in oculomotor neurons compared with spinal motor neurons (adjusted p < 0.05, Figure 3A). Consistent with our RNA-sequencing findings, immunocytochemistry against pAKT and quantification of fluorescent intensities in ISLET1/2+ oculomotor and spinal motor neurons derived from mESCs demonstrated that oculomotor neurons had higher levels of pAKT protein than spinal motor neurons (Figures 3B–3D). These levels were maintained after excitotoxicity elicited by kainic acid (two-way ANOVA, ***p < 0.0001; Figure 3D).

To further validate this pathway in oculomotor neuron resistance, we analyzed the protein levels of activated β-CATENIN (non-phosphorylated), a downstream effector of AKT signaling, using immunocytochemistry (Figures 3E and 3F). The semi-quantitative analysis demonstrated that oculomotor neurons had elevated levels of β-CATENIN compared to spinal cord motor neurons (two-way ANOVA, ***p < 0.0001; Figure 3G). β-CATENIN expression was maintained at relatively high levels in oculomotor neurons compared to spinal motor neurons also during kainate toxicity, with expression preferentially localized to processes (Figures 3F and 3G).

Our data thus indicate that AKT signaling, which is a known survival pathway, could contribute to oculomotor neuron resistance in mouse in vivo as well as in a dish.

**AKT Signaling Is Elevated in Human Postmortem Oculomotor Neurons**

To evaluate whether our in vitro data recapitulate motor neuron resistance in human, we used the spatial transcriptomics method LCM-seq (laser-capture microdissection coupled with RNA sequencing) (Nichterwitz et al., 2016, 2018) to analyze individually isolated oculomotor neurons (Figures S4A–S4D) and spinal motor neurons (cervical and lumbar spinal cord) (Figures S4E–S4H) from human postmortem tissues. In addition, we isolated motor neurons from the Onuf's nucleus in the sacral spinal cord (Figures S4I–S4L and Table S2), as these are highly resilient to degeneration in ALS and maintained until end stage of disease (Mannen et al., 1977), similar to oculomotor neurons. Analysis of marker gene expression showed that all motor neuron groups expressed high levels of neurofilaments and VACHT (SLC18A3) (Figure S4M). Motor neurons also expressed ISL1/2 and ChAT, while being almost devoid of contaminating glial markers, e.g., SLC1A3, AQP4, CCL3,
**DISCUSSION**

In ALS, motor neuron subpopulations show differential vulnerability to degeneration. In particular, cranial motor neurons of the oculomotor, trochlear, and abducens nuclei (cranial nerves 3, 4, and 6), which innervate the extraocular muscles around the eyes, are highly resistant throughout disease progression. In our study we demonstrate that a high proportion of bona fide oculomotor neurons can be generated from mESCs through temporal overexpression of PHOX2A, and that these are relatively resilient to ALS-like toxicity.

Oculomotor neurons are generated in the ventral midbrain through the specification by SHH (secreted from the floor plate and the notochord), FGF8, and WNTs (both secreted by and around the isthmic organizer). These morphogens in turn regulate transcription factors that influence differentiation of ventral midbrain neurons, including oculomotor neurons, dopamine neurons, red nucleus neurons, and GABA-positive interneurons (reviewed in Nijssen et al., 2017). Oculomotor neurons are specified by the transcription factors PHOX2A, PHOX2B, and LMX1B (Deng et al., 2011; Pattyn et al., 1997). PHOX2A is required to drive oculomotor neuron fate as demonstrated by the lack of both oculomotor and trochlear nuclei in Phox2a knockout mice (Pattyn et al., 1997). PHOX2A is also sufficient to generate a complete oculomotor complex as shown by studies in chick (Hasan et al., 2010; Pattyn et al., 1997). PHOX2B, on the other hand, is sufficient to induce ectopic generation of oculomotor neurons in the spinal cord, but is not required to induce oculomotor neuron specification in the midbrain (Dubreuil et al., 2000; Pattyn et al., 1997). *In vitro* studies have shown that (1) mESCs can be directly programmed into cranial neurons using the proneuronal gene Ngn2, in combination with Islet1 and Phox2a (Mazzoni et al., 2013), and (2) overexpression of either PHOX2A or PHOX2B alone in mESC-derived neural progenitors exposed to SHH and FGF8 can promote a midbrain/hindbrain motor neuron fate (Mong et al., 2014). However, while it was demonstrated that cranial motor neurons were generated in *vitro* it was never previously determined whether oculomotor neurons were specifically produced.

Here, we show that PHOX2A overexpression in neural progenitors in combination with patterning using FGF8 and SHH resulted in the generation of 50% ISLET+ neurons of which more than half were PHOX2A+. The transcriptome and proteome of oculomotor neurons is distinct from other motor neurons (reviewed in Nijssen et al., 2017). Notably, oculomotor neurons can be distinguished by their lack of the transcription factor HB9, which defines other somatic motor neurons (Guidato et al.,...
As the vast majority of ISLET+ cells in our midbrain cultures lacked HB9, we conclude that the PHOX2A+ cells generated were indeed oculomotor neurons and that Phox2a overexpression is sufficient to induce an oculomotor fate from stem cells in vitro. This conclusion was further supported by our...
RNA-sequencing experiments on purified oculomotor and spinal motor neuron cultures and our bioinformatics cross-comparison with other in vitro and in vivo datasets from rodent and man (Brockington et al., 2013; Hedlund et al., 2010; Kaplan et al., 2014), which confirmed that the oculomotor neurons we generated were molecularly similar to their in vivo counterpart. Furthermore, our analysis of axon guidance molecules demonstrated differential expression of distinct morphological markers in oculomotor and spinal motor neurons. Finally, the electrical properties of the mESC-derived motor neurons matured over time in culture, and cells also expressed the cholinergic marker ChAT. Altogether this demonstrates that we can generate bona fide oculomotor neurons.

Furthermore, we found that mRNAs of Fg10 and Fg15 were enriched in our oculomotor cultures. These two morphogens are normally expressed in the developing midbrain, and Fg10 is even present within oculomotor neurons themselves (Hattori et al., 1997; Partanen, 2007). It is therefore highly conceivable that these factors are involved in further specifying the cultures. We therefore believe that these factors can be used to further improve the in vitro differentiation protocol, but this remains to be further investigated.

Multiple studies have demonstrated that differential motor neuron vulnerability appears to be regulated by cell-intrinsic differences in gene expression (Allodi et al., 2016; Brockington et al., 2013; Hedlund et al., 2010; Kaplan et al., 2014). We therefore hypothesized that in vitro-generated oculomotor neurons would be more resilient to ALS-like toxicity than spinal motor neurons. Indeed, our experiments on neuronal vulnerability clearly demonstrated that oculomotor neurons were more resistant to the ALS-like toxicity elicited by kainic acid than spinal motor neurons. Furthermore, the fact that oculomotor neuron processes remained intact indicates that these cells were indeed highly resilient, as axonal degeneration is an early sign of pathology in ALS.

It has been shown that oculomotor neurons have a higher calcium-buffering capacity than other motor neuron groups (Vanselow and Keller, 2000), which could render the cells more resilient to excitotoxic insults. Our finding that several transcripts with implications for calcium handling were preferentially expressed in stem cell-derived oculomotor neurons as well as oculomotor neurons in man were thus very compelling and consistent with their resilience. ESYT1, for example, is an ER integral membrane protein that presents a cytosolic synaptotagmin-like domain that can bind organelle membranes and a C2 domain able to bind the plasma membrane. These interactions are involved in exchange of lipids and vesicles but also, in the ER, control of Ca\textsuperscript{2+} homeostasis. Thus, ER-plasma membrane junctions can control Ca\textsuperscript{2+} release from ER in response to extracellular Ca\textsuperscript{2+} levels (Giordano et al., 2013), an event that could have a beneficial effect during excitotoxicity.

Furthermore, we have previously shown that AKT signaling plays an important role in induced resistance in vulnerable motor neurons (Allodi et al., 2016). We now demonstrate that Akt signaling is more highly activated in resistant oculomotor neurons in vitro as well as in vivo in human oculomotor neurons. This observation indicates that oculomotor neurons preferentially activate a cell-intrinsic survival program. Notably, cross-comparison of human oculomotor neurons with resilient human Onuf’s nucleus motor neurons showed that elevated AKT signaling was unique to oculomotor neurons. When comparing human oculomotor and Onuf’s motor neurons with vulnerable spinal motor neurons it became evident that the majority of DEGs were unique to each resilient nucleus. However, there were commonly regulated genes across these two resilient nuclei, including MIF. MIF was recently shown to inhibit the toxicity of misfolded SOD1 (Shvil et al., 2018), indicating that these two neuron groups may be better at handling toxic aggregation-prone proteins.

In conclusion, we have demonstrated that bona fide oculomotor neurons can be generated from stem cells in vitro. We also show that these neurons are relatively resilient to ALS-like toxicity. This finding enables modeling of neuronal resistance and susceptibility in ALS in vivo, which could further elucidate mechanisms that could be highly advantageous to activate in vivo to rescue vulnerable motor neurons from degeneration.

**EXPERIMENTAL PROCEDURES**

**Ethics Statement**

All work was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and with national legislation and institutional guidelines. Animal procedures were approved by the Swedish animal ethics review board (Stockholm Norra Djurförsöksnämnden). Ethical approval for the use of the human post mortem samples was obtained from the regional ethical review board in Stockholm, Sweden (Regionala Etikprövningsnämnden, Stockholm, EPN). Human CNS samples were obtained from the Netherlands Brain Bank (www.brainbank.nl), the National Disease Research Interchange (www.ndiresource.org), and the NIH Neurobiobank with the written informed consent from the donors or next of kin.

**Generation of mESC Lines and Specification into Oculomotor and Spinal Motor Neurons**

Three different mESC lines were used in this study: E14.1 (ATCC), Hbb-GFP (gift from Dr. S. Thams, Karolinska Institutet), and Islet1-GFP (gift from Prof. S. Pfaff, UCSD). To increase oculomotor neuron differentiation efficiency, we generated E14.1/NesEPhox2a and Islet1-GFP/NesEPhox2a mESC lines by Lipofectamine (Invitrogen) transfection. NesEPhox2a construct was a gift from Prof. J. Ericson.
et al., 2008). A concentration of 20 μM was found sufficient to induce consistent motor neuron death also when motor neurons were cultured on astrocytes. Kainate toxicity was induced by exposing embryo bodies (EBs) to the smoothened agonist SAG (500 nM, Calbiochem #364590-63-6) and retinoic acid (100 nM, Calbiochem #302-79-4) for 5 days, while oculomotor neurons were patterned by SAG (250 nM) and FGf8 (100 ng/mL, R&D Systems #423-F8) for 5 days. Medium containing morphogens was replaced daily. EB dissociation and motor neuron culture maintenance were carried out as previously described (Wichterle et al., 2002). Oculomotor neurons and spinal motor neurons were kept in Neurobasal medium + Glutamax ( Gibco) with a cocktail of trophic factors (5 ng/mL each of glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, neurotrophin-3, and ciliary neurotrophic factor). All cultures, including survival experiments, were treated with the mitotic inhibitor 5-fluoro-2’-deoxyuridine (final concentration 2 μM) to reduce cell proliferation.

**Fluorescent-Activated Cell Sorting and RNA Sequencing of Stem Cell-Derived Neurons**

Islet1-GFP/NesEPHox2a and Hb9-GFP ESCs were differentiated into oculomotor and spinal motor neurons, respectively. GFP+ motor neurons were sorted, and positive and negative fractions were collected for mRNA sequencing or plated for subsequent immunocytochemistry analysis. FACS was performed using a 100-μm nozzle, a sheath pressure of 15–25 pounds per square inch and an acquisition rate of 1,000–2,000 events per second (Hedlund et al., 2008). 5 × 10³ cells were collected in 5% Triton X-100 (Sigma-Aldrich) in water and then prepared for RNA-seq as previously described (Nichterwitz et al., 2016). Samples were sequenced on Illumina HiSeq2000 and HiSeq2500 platforms with read lengths of 43 and 51 bp, respectively.

**Laser-Capture Microdissection and RNA Sequencing of Human Motor Neurons**

LCM-seq of motor neurons from human postmortem tissues was performed as previously described (Nichterwitz et al., 2016, 2018).

**RNA-Seq Data Processing**

Reads from cell-culture samples were mapped to the mouse mm10 reference genome with HISAT2 (Kim et al., 2015) using the publicly available infrastructure (available at usegalaxy.org) on the main Galaxy server (Afgan et al., 2016). Human LCM samples were mapped to hg38/GRCh38 (Genencode version V29) using STAR version 2.5.3 (Dobin et al., 2013). Transcript-level counts and RPKM values were obtained using rpkmforgenesis.py (Ramskold et al., 2009). After mapping, the basic exclusion criteria were <500,000 mapped reads and, for LCM samples, lack of both motor neuron marker transcripts ISL1 and ChAT.

**ALS-like Toxicity and Motor Neuron Survival**

Oculomotor and spinal motor neurons were cultured for 5 days and then exposed to kainic acid. Three concentrations of kainic acid were tested: 20, 50, and 100 μM. A concentration of 20 μM was found sufficient to induce consistent motor neuron death also when motor neurons were cultured on astrocytes. Kainate toxicity was induced for 7 days. Quantifications were performed blind to the motor neuron population and toxicity condition. Pictures were taken with a Zeiss LSM700 confocal microscope (40× magnification and 1.5 digital zoom) using the ZEN 2010S SP1 software and then further analyzed by ImageJ software. At least four experiments per condition were quantified in technical duplicates. Twelve random areas were quantified per each coverslip (at least 130 cells were quantified for each condition). To evaluate proliferation and generation of motor neurons, we exposed cultures to pulses of BrdU (Sigma #B5002) for 24 h. BrdU was dissolved in dimethyl sulfoxide (DMSO; Sigma #276855) and added to the cultures at a final concentration of 10 μM. After 24 h of exposure to BrdU, cells were washed twice with fresh culture medium. Proliferation was assessed at day 0 (D0), day 2 (D2), and day 3 (D3) of the survival protocol and all samples were fixed with 4% paraformaldehyde at day 7 (D7). Quantifications were performed in triplicates with technical duplicates.

**Morphological Analysis**

Arborization and Sholl analysis were conducted utilizing Fiji software. To compare arborization area of oculomotor neurons and spinal motor neurons, we transformed the microphotographs to a grayscale 8-bit image, and assessed quantification after applying a threshold for signal intensity/background correction. Threshold values were kept constant among images, and microphotographs with higher background noise were excluded. Between 10 and 12 pictures per condition per experiment were quantified. Experiments were performed in quadruplicates. The complexity of oculomotor neuron arborization after kainic acid toxicity was obtained by Sholl analysis (O’Neill et al., 2015) by calculating the average of neurites intersecting defined concentric cycles spaced 25 μm from each other starting from the center of the cell body as a function of distance from soma. Individual oculomotor neurons were analyzed after culturing them at lower density (spinal motor neuron analysis could not be performed due to their poor survival at lower densities). Several microphotographs were taken at 40× magnification with a Zeiss LSM700 confocal microscope, mounted with Adobe Photoshop software, and analyzed with Fiji software. The intersection averages were analyzed by multiple t test.

**Statistical Analysis**

All statistical analyses were performed with GraphPad software unless otherwise specified. Two-way ANOVA was used when taking into account multiple variables and making multiple comparisons, followed by appropriate post hoc analysis. Unpaired Student’s t test was used to compare two groups. Further details on statistical tests used can be found in figure legends as well as information on the total number of motor neurons counted per experiment. All experiments were performed at least in triplicate with technical duplicates. All results are expressed as mean ± SEM unless otherwise specified.

**ACCESSION NUMBERS**

The original RNA-sequencing data from this study are available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GEO: GSE118620 (in vitro-generated neuron data) and GEO: GSE93939 (human LCM-seq data).
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2019.04.009.

AUTHOR CONTRIBUTIONS


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