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Maturation of Spinal Motor Neurons Derived from Human Embryonic Stem Cells

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ABSTRACT

Our understanding of motor neuron biology in humans is derived mainly from investigation of human postmortem tissue and more indirectly from live animal models such as rodents. Thus generation of motor neurons from human embryonic stem cells and human induced pluripotent stem cells is an important new approach to model motor neuron function. To be useful models of human motor neuron function, cells generated in vitro should develop mature properties that are the hallmarks of motor neurons in vivo such as elaborated neuronal processes and mature electrophysiological characteristics. Here we have investigated changes in morphological and electrophysiological properties associated with maturation of neurons differentiated from human embryonic stem cells expressing GFP driven by a motor neuron specific reporter (Hb9::GFP) in culture. We observed maturation in cellular morphology seen as more complex neurite outgrowth and increased soma area over time. Electrophysiological changes included decreasing input resistance and increasing action potential firing frequency over 13 days in vitro. Furthermore, these human embryonic stem cell derived motor neurons acquired two physiological characteristics that are thought to underpin motor neuron integrated function in motor circuits; spike frequency adaptation and rebound action potential firing. These findings show that human embryonic stem cell derived motor neurons develop functional characteristics typical of spinal motor neurons in vivo and suggest that they are a relevant and useful platform for studying motor neuron development and function and for modeling motor neuron diseases.

ATTACHMENTS

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MATERIALS TEXT

ES Medium comprised of Dulbecco's Modified Eagle Medium:

A	B
Nutrient mixture F-12 (DMEM:F12, Invitrogen) with 20% Knockout Serum Replacer (Invitrogen)	
Betamercaptoethanol (BME; Sigma)	110 uM
L-Glutamine	
Non Essential Amino Acids (NEAA; Invitrogen)	
Basic fibroblast growth factor (bFGF; Invitrogen)	20 ng/ml

ROCK inhibitor (Ri) Y-27632 **Stemcell**

Technologies Catalog #72308

Recombinant mouse Noggin (R&D)
DMEM F:12
N2 supplement (Invitrogen)
Heparin (Sigma)
Wnt3a-L-cell conditioned medium (ATCC)

all-trans retinoic acid (RA, [M]**100 Nanomolar (nM)** , Sigma)

Ascorbic acid ([M]**0.4 mg/ml** , Sigma)

db-cAMP ([M]**1 Milimolar (mM)** , Sigma)

Recombinant mouse Sonic hedgehog (SHH) protein ([M]**100 ng/ml** SHH-C25II, R&D)

Recombinant human brain-derived neurotrophic factor (BDNF; 10 ng/ml, R&D)

Neurobasal Medium:

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A	B
N2 and B27 (Invitrogen)	
L-Glutamine	
NEAA	
Ascorbic acid	
db-cAMP	
Neural Differentiation Medium (NDM)	
Recombinant human BDNF	10 ng/ml
Glial cell-derived neurotrophic factor (GDNF)	
Insulin-like growth faction 1 (IGF-1)	
Ciliary neurotropic factor (R&D)	
SHH	200 ng/ml
RA	100 nM

EmbryoMax 26freezing medium (Millipore)
Poly-ornithine/laminin coated glass coverslips

[M]**1 mg/ml** mouse laminin (Invitrogen)

BME ([M]**25 Milimolar (mM)** , Sigma)

Glutamate ([M]**25 Milimolar (mM)** , Sigma)

Forskolin ([M]**20 Milimolar (mM)** , Sigma)

IBMX ([M]**100 Milimolar (mM)** , Fisher) at 250 K cells per 35 mm coverslip or 46 K cells per 15 mm coverslip

4% paraformaldehyde (PFA)
Phosphate buffered saline (PBS)
Triton X-100 (Wash buffer)

[M]**50 Milimolar (mM)** glycine

10% normal donkey serum
Blocking buffer (chicken anti-GFP 1:1000,

GFP Polyclonal Antibody **Invitrogen - Thermo Fisher Catalog #A10262** ; mouse antiHB9,1:50, MNR2/815C10-s, Developmental Studies Hybridoma Bank)
DyLight coupled donkey anti primary-species IgG antibodies (Jackson Immunoresearch, 1:1000)
DAPI (Invitrogen)

ESC Culture 20m

1 Grow a human embryonic stem cell line with a motor neuron reporter (BAC-Hb9::GFP) under standard pluripotency maintenance conditions: on irradiated CF-1 mouse embryonic fibroblast feeder cells (0.015 M cells/cm2, GlobalStem) seeded on gelatinized (Millipore) tissue culture plastic.

2 Feed cells daily with ES Medium comprised of Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (DMEM:F12, 20m Invitrogen) with 20% Knockout Serum Replacer (Invitrogen), [M]**110 Micromolar (µM)** betamercaptoethanol (BME; Sigma), L-Glutamine and Non Essential Amino Acids (NEAA; Invitrogen), and [M]**20 ng/ml** basic fibroblast growth factor (bFGF; Invitrogen) (ESC medium), and passage weekly using [M]**50 µg/ml** dispase for **00:20:00** (Invitrogen) followed by manual trituration.

3 Karyotype parallel passages of ESCs at subsequent passages

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Motor Neuron Differentiation 1h

4 1h

After normal passage, incubate washed ESC colonies for **01:00:00** in ESC medium with **10 Micromolar (µM)** Rho associated kinase (ROCK) inhibitor (Y-27632, Ascent Scientific).

5 Trypsinize to single cells and seed in suspension at **0.4 M** cells/ml in ES medium with **10 Micromolar (µM)** ROCK inhibitor, and **300 ng/ml** recombinant mouse Noggin (R&D).

6 6d

Add fresh ROCK inhibitor, FGF, and Noggin daily for the first **144:00:00** .

7 Pellet embryoid bodies (EBs) at 100 G on day 4 and resuspend in DMEM F:12 plus N2 supplement (Invitrogen), NEAA, L-Glutamine, 2 mg/ml Heparin (Sigma), bFGF, Noggin, and ROCK inhibitor.

8

Pellet EBs and feed with fresh medium every other day until day 31. Add ROCK inhibitor last at day 5.

9 Discontinue Noggin and bFGF at day 10.

10

Add 1:10 dilution of Wnt3a-L-cell conditioned medium (ATCC), all-trans retinoic acid (RA, **100 Nanomolar (nM)** , Sigma), ascorbic acid (**0.4 µg/ml** , Sigma), db-cAMP (**1 Micromolar (µM)** , Sigma), and recombinant mouse Sonic hedgehog (SHH) protein (**100 ng/ml** SHH-C25II, R&D) from day 10 onward.

11 Discontinue Wnt3a-conditioned medium on day 18.

12

Increase SHH to **200 ng/ml** and add recombinant human brain-derived neurotrophic factor (BDNF; **10 ng/ml** , R&D).

13 At day 25, switch base medium to Neurobasal with N2 and B27 (Invitrogen), LGlutamine, NEAA, ascorbic acid, db-cAMP, (Neural Differentiation Medium (NDM)), with **10 ng/ml** each recombinant human BDNF, glial cell-derived

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neurotrophic factor (GDNF), insulin-like growth faction 1 (IGF-1), and ciliary neurotropic factor (R&D), [M]**200 ng/ml** SHH, and [M]**100 Nanomolar (nM)** RA.

14 Dissociate EBs were using trypsin on day 31 and cryopreserve using EmbryoMax 2x freezing medium (Millipore) for future use.

15 Thaw separate vials for electrophysiology and morphology time series, seed on poly-ornithine/laminin coated glass coverslips, in complete day 25 NDM including all supplements.

16 

However, reduce RA to [M]**10 Nanomolar (nM)** and reduce SHH to [M]**20 ng/ml** and add the following supplements: [M]**1 µg/ml** mouse laminin (Invitrogen), BME ([M]**25 Micromolar (µM)** , Sigma), glutamate ([M]**25 Micromolar (µM)** , Sigma), forskolin ([M]**20 Micromolar (µM)** , Sigma), and IBMX ([M]**100 Micromolar (µM)** , Fisher) at 250 K cells per **35 mm** coverslip or 46 K cells per **15 mm** coverslip.

17 Change half the medium every 4 days.

Immunocytochemistry 50m

18 Fix cultures for **00:30:00** in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at **4 °C** 30m

19  20m

Wash 3 times for **00:05:00** in PBS, quench and permeabilize in wash PBS plus 0.1% Triton X-100 (Wash buffer) plus [M]**50 Milimolar (mM)** glycine for **00:15:00** .

20 Block samples with Wash buffer plus 10% normal donkey serum for **01:00:00** . 1h

21 

Incubate with primary antibody in blocking buffer (chicken anti-GFP 1:1000, Invitrogen A10262; mouse antiHB9,1:50, MNR2/815C10-s, Developmental Studies Hybridoma Bank) overnight.

22 

Wash cells, incubate with DyLight coupled donkey anti primary-species IgG antibodies (Jackson Immunoresearch, 1:1000).

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23

Finally, wash cells and counterstain with DAPI (Invitrogen).

Imaging and Image Analysis

24

Mount coverslips in Fluoromount G and image on an inverted Zeiss AxioObserver Z1 using a 206 Plan-APOCHROMAT 0.8 NA objective and a 14-bit, gray scale Photometrics HQ2 CCD camera.

25 Export images as 16 bit gray scale images for analysis.

26 Score cells as HB9+ or GFP+ based on having >10 K mean gray levels of $HB9^-$ or >40 K mean gray levels of GFP-channel fluorescence intensity over local background, respectively, using the Metamorph Multiwavelength Cell Scoring module.

27 Trace neurites using the Neurite Outgrowth module (Metamorph) based on GFP fluorescence intensity of >3,000 g.l. over local background.

28 Manually adjuste a 20x field of view to capture the maximum neurite outgrowth for each cell.

This field size was sufficient to capture all neurites for almost all cells. At day 36 for example, the day at which the peak of total median outgrowth per cell occurs, less than 9% of GFP+ cells had neurites extending beyond the field of view.

29 Group morphological measurements by days in culture and use SigmaPlot11 for statistical analysis.

Electrophysiology

30 Make whole-cell patch recordings from Hb9::GFP+ motor neurons.

31 Transfer coverslips to the stage of a TE2000-E microscope (Nikon) and continuously perfuse at a low flow rate of **1 ml/min** with bath recording solution containing (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES, 2 $MgCl_2$ and 5.5 glucose, pH adjusted to pH **7.3** with NaOH, osmolality **325 mOsmol/kg**

32 Pull patch pipettes with a resistance of 3-5 MΩ from borosilicate glass capillaries (0.86 mm ID, 1.5 mm OD) using a P-97 pipette puller (Sutter Instrument Co).

Intracellular solution had the following composition (in mM): 120 potassium methanesulfonate, 10 NaCl, 10 EGTA, 1 $CaCl_2$, 10 HEPES, 0.5 NaGTP, 5 MgATP, 0.1% biocytin, pH adjusted to 7.2 with KOH,

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osmolality 280 mOsmol kg^{-1}.

33 Fill some of the electrophysiologically recorded cells with biocytin during recording, then fix, stain and image as above. Correct junction potential before recording.

34 Acquire data using an Axopatch 200 B amplifier and pClamp 10 software (Molecular Devices, Sunnyvale, CA, USA).

35 Filter data at 2 kHz and digitized at 20 kHz.

36 Evoke action potentials by injecting depolarizing currents of 1 s duration and analyze using AxoGraph X software (AxoGraph Scientific, Sydney, Australia).

37 Measure action potential characteristics in hESMNs from resting membrane potential.

The criterion for identification of a first action potential is when a voltage response to depolarizing current injection has obvious threshold, visible as a rapidly rising membrane potential that is positive to 0 mV. Rheobase is defined as the minimum current step amplitude required to evoke an action potential. Voltage threshold for action potentials is measured as in [44].

38 Measure action potential half-width at rheobase.

39 In a given cell, calculate ISIs from recordings in which the maximum number of action potentials are evoked.

40 Calculate spike frequency adaptation (SFA) ratio as: ISI_{last}/ISI_{1st}.

41 Perform the calculation only on data from neurons in which more than 5 action potentials could be evoked by a 1 s depolarizing current step.

42 Analyze Sag as the response following a hyperpolarizing current step that fit the following criteria:

42.1 The ratio between the size of voltage change at the steady state and the peak during a 1 sec current injection is greater than 1.1.

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42.2 The voltage change peaked during the first half of the current injection period.

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