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ION CHANNELS AND IONOTROPIC RECEPTORS IN HUMAN EMBRYONIC STEM CELL DERIVED NEURAL PROGENITORS

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Abstract—Human neural progenitor cells differentiated from human embryonic stem cells offer a potential cell source for studying neurodegenerative diseases and for drug screening assays. Previously, we demonstrated that human neural progenitors could be maintained in a proliferative state with the addition of leukemia inhibitory factor and basic fibroblast growth factor. Here we demonstrate that 96 h after removal of basic fibroblast growth factor the neural progenitor cell culture was significantly altered and cell replication halted. Fourteen days after the removal of basic fibroblast growth factor, most cells expressed microtubule-associated protein 2 and TUJ1, markers characterizing a post-mitotic neuronal phenotype as well as neural developmental markers Cdh2 and Gbx2. Real-time PCR was performed to determine the ionotropic receptor subunit expression profile. Differentiated neural progenitors express subunits of glutamatergic, GABAergic, nicotinic, purinergic and transient receptor potential receptors. In addition, sodium and calcium channel subunits were also expressed. Functionally, virtually all the hNP cells tested under whole-cell voltage clamp exhibited delayed rectifier potassium channel currents and some differentiated cells exhibited tetrodotoxin-sensitive, voltage-dependent sodium channel current. Action potentials could also be elicited by currents injection under whole-cell current clamp in a minority of cells. These results indicate that removing basic fibroblast growth factor from the neural progenitor cell cultures leads to a post-mitotic state, and has the capability to produce excitable cells that can generate action potentials, a landmark characteristic of a neuronal phenotype. This is the first report of an efficient and simple means of generating human neuronal cells for ionotropic receptor assays and ultimately for electrically active human neural cell assays for drug discovery. Published by Elsevier Ltd on behalf of IBRO.

Key words: human embryonic stem cells, human neural progenitors, glutamate receptors, sodium channels.

Human embryonic stem cells (hESCs) can differentiate into human neural progenitor (hNP) cells in the absence of bone morphogenic protein signaling and in the presence of basic fibroblast growth factor (bFGF). Once derived, bFGF is required for hNP cell replication over extended culture (Carpenter et al., 2003; Shin et al., 2006; Elkabetz et al., 2008). These hNP cells present an ideal neural cell source for high throughput screening of a large variety of pharmacological compounds (Kiryushko et al., 2004) and the differentiated progeny of these cells were recently used to screen for compounds that act as a potentiator of AMPA activity (McNeish et al., 2010). Achieving efficient and cost effective human *in vitro* neuronal differentiation through the minimal use of growth factors and other alterations in the differentiation procedures could facilitate neural drug discovery and developmental toxicology studies using hESC neural derivatives and enabling higher throughput in these assays.

Of particular interest to the field of drug development are the ion channels, which are required for normal functional neural activity. *In vivo*, mature functional neurons develop from progenitors by migrating into place, developing neurotransmitter specificity, developing electrical excitability and forming axons, dendrites and functional synaptic connections. Ion channels also have been shown to play an essential role in many aspects of neural development (Yen et al., 1993).

Developmental characterization of many ion channel subunits has been done in rodents; however, characterization in human tissue is lacking (Jeng et al., 1991; Numakawa et al., 2002). Fully functional neurons have been derived from mouse embryonic stem cells (mESCs) [reviewed by (Wu et al., 2007)], but other studies have reported that cells differentiated from mESCs had the morphology, synaptic contacts and other biochemical markers characteristic of differentiated neurons, but lacked the voltage-dependent sodium channels required for functional synaptic transmission (Benn et al., 2001; De Filippis et al., 2007). hNP cell differentiation *in vitro* may provide a good experimental system for elucidating the factors necessary for regulation of ion channels throughout neural development.

Ionotropic glutamate receptors are critical for neural migration (Behar et al., 1999), synaptogensis (Gallo et al.,

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Abbreviations: bFGF, basic fibroblast growth factor; CFSE, carboxyfluorescein succinimidyl ester; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle medium; hERG, human *Ether-à-go-go* related gene potassium channel; hESCs, human embryonic stem cells; hNPs, human neural progenitors; LIF, leukemia inhibitory factor; MAP2, microtubule-associated protein 2; MEF, mouse embryonic fibroblast; NMDA, N-methyl-D-aspartic acid; PBS+/+, phosphate buffered saline with calcium and magnesium; RFU, relative fluorescence unit; SAS, statistical analysis software; TRP, transient receptor potential; TTX, tetrodotoxin.

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1995) and neural survival (Platel et al., 2008). Glutamate receptors are also important in mediating excitotoxicity and apoptosis (Buckingham et al., 2008; Milanese et al., 2010). Glutamate receptor mediated excitotoxicity occurs in a wide range of maladies including but not limited to stroke, traumatic brain injury and seizure, and could be associated with neurological diseases such as Huntington's disease, amyotrophic lateral sclerosis or Parkinson's disease (Erceg et al., 2008; Ladewig et al., 2008; Blandini, 2010; Tallaksen-Greene et al., 2010).

Previously, we derived a karotypically normal, stable, adherent monolayer of hNP cells from WA09 hESCs (Shin et al., 2006). These cells have been characterized for their ability to maintain multipotency and for their expression of neural stem cell marker NESTIN and lack of expression of stem cell marker POU5F1 (Dhara et al., 2008). The hNP cells have also been previously differentiated into all three subtypes of the neural lineage: neurons, oligodentrocytes and astrocytes (Shin et al., 2006). Upon further differentiation in basal conditions (without bFGF) but with leukemia inhibitory factor (LIF), greater than 90% of the cells were TUJ1 and microtubule-associated protein 2 (MAP2) positive (Shin et al., 2006; Harrill et al., 2010). To define the potential of hNP cells and derivatives in functional neural drug discovery, glutamate receptor activity as well as function of other ion channel subtypes requires electrophysiological studies.

Here, we demonstrate that post-mitotic differentiated hNP cells express developmental regionalization genes, as well as markers of functional neural receptors. These differentiated hNP cells can evoke action potentials that can be blocked with tetrodotoxin (TTX) as well as increase the intracellular calcium response when exposed to AMPA receptor potentiator, cyclothiazide. GABAergic and glutamatergic ionotropic receptor expression was found to be up regulated as early as after 2 weeks of hNP cell differentiation. These results suggest that these differentiated hNP cells are capable of differentiation into a functional neuronal phenotype.

EXPERIMENTAL PROCEDURES

hESC cultures

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. hESC line WA09 (H9) was obtained from WiCell Research Institute in Madison, WI, USA. These hESCs are an NIH approved line derived from embryos donated from *in vitro* fertilization clinics by James Thomson (Thomson et al., 1998). hESCs were cultured on mouse embryonic fibroblast (MEF; Harlan, Indianapolis, IN, USA) feeders inactivated by mitomycin C in medium consisting of Dulbecco's modified Eagle medium (DMEM)/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% knockout serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 50 μg/ml penicillin (Invitrogen), 50 μ/ml streptomycin (Invitrogen), 0.1 mM β-mercaptoethanol and 4 ng/ml bFGF (R&D, Minneapolis, MN, USA). They were maintained in 5% CO_2 and at 37°C. Cells were passaged every 3 days by mechanical dissociation and re-plated on fresh feeder layer cultures to prevent undirected differentiation with daily medium changes as previously described (Kalia et al., 2004).

hNP cell cultures

hNP cells were derived from hESC line WA09 by our laboratory as previously described (Shin et al., 2006). Briefly, after 1 week of culture on MEF feeder layers, WA09 hESCs were cultured with derivation medium containing DMEM/F12 medium supplemented with 2 mM L-glutamine, 2 U/ml penicillin, 2 μg/ml streptomycin, N2 (Invitrogen), and 4 ng/ml bFGF (R&D) for 7 days. Rosettes were selected utilizing hook passaging from culture dishes and re-plated on polyornithine (20 μg/ml) and laminin (5 μg/ml) coated dishes. These rosettes were propagated for 3 days on polyornithine and laminin coated dishes in growth medium consisting of Neurobasal medium (Invitrogen) supplemented with 2 mM L-glutamine, 2 U/ml penicillin, 2 μg/ml streptomycin, B27 (Invitrogen), 20 ng/ml bFGF, and 10 ng/ml LIF (Millipore, Billerica, MA, USA). Medium was changed every other day and cells were passaged every fourth day or as needed. Cells used for this experiment were passages 22–39. To initiate hNP cell differentiation 24 h after the last passage, growth medium was switched to growth medium lacking bFGF (differentiation media). Differentiation medium was changed every 3 days. Cells were collected at 14 days, 35 days and 125 days for analysis.

Cell proliferation analysis by carboxyfluorescein succinimidyl ester (CFSE)

Cell proliferation was analyzed using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) following manufacturer's instructions. Briefly, cells were incubated for 10 min in 10 μM of CFSE solution at 37°C. CFSE staining was quenched with ice-cold media. Cells were washed, re-suspended in fresh hNP cell media, re-plated at a density of 1×10^6 cells per 35 mm dish and incubated for 0, 8, 16, 24 and 48 h. At each time point, cells were harvested, washed twice with PBS+/+ (phosphate buffered saline containing calcium and magnesium; ThermoScientific, Whatham, MA, USA) and analyzed on Dako CyAn flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with a 488 nm laser. Non-stained cells were used as a control. CFSE data were analyzed using the FlowJo software (TreeStar, Ashland, OR, USA) proliferation model. The half-life of mean fluorescence was determined by fitting the mean fluorescence with the following equation:

$$I = A\left(2^{-\frac{t}{\tau}}\right) + I_0 \qquad \text{(equation1)}$$

where I is the mean fluorescence intensity, I_0 is the fluorescence intensity at steady state, τ is the characteristic time of half intensity, A is the amplitude and t is time.

Immunocytochemistry

Cells were fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS+/+ for 20 min and processed for immunocytochemistry. Cells were washed three times with PBS+/+ followed by three washes for 5 min each with permeabilization buffer consisting of 25 μl (.05%) Tween 20 (EMD Chemicals, Gibbstown, NJ, USA) in a 50 ml high salt buffer solution consisting of 1 M Tris base and 0.25 M NaCl. Cells were then blocked in 6% goat serum (Jackson Immuno Research Laboratories, West Grove, PA, USA) for 45 min. The following primary antibodies were used: mouse anti NESTIN (1:200, Neuromics, Edina, MN, USA), mouse anti TUJ1 (1:200, Neuromics) and mouse anti MAP2 (1:500, Millipore). AlexaFluor 488 and 594 goat anti-mouse (1:1000, Invitrogen) were used as secondary antibodies to visualize primary antibody staining. Cell nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Fluorescence was visualized using spinning disk confocal microscope (Olympus, Center Valley, PA, USA).