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Differentiation of rhesus embryonic stem cells to neural progenitors and neurons

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Abstract

Embryonic stem (ES) cells are pluripotent cells capable of differentiating into cell lineages derived from all primary germ layers including neural cells. In this study we describe an efficient method for differentiating rhesus monkey ES cells to neural lineages and the subsequent isolation of an enriched population of Nestin and Musashi positive neural progenitor (NP) cells. Upon differentiation, these cells exhibit electrophysiological characteristics resembling cultured primary neurons. Embryoid bodies (EBs) were formed in ES growth medium supplemented with 50% MEDII. After 7 days in suspension culture, EBs were transferred to adherent culture and either differentiated in serum containing medium or expanded in serum free medium. Immunocytochemistry on differentiating cells derived from EBs revealed large networks of MAP-2 and NF200 positive neurons. DAPI staining showed that the center of the MEDII-treated EBs was filled with rosettes. NPs isolated from adherent EB cultures expanded in serum free medium were passaged and maintained in an undifferentiated state by culture in serum free N2 with 50% MEDII and bFGF. Differentiating neurons derived from NPs fired action potentials in response to depolarizing current injection and expressed functional ionotropic receptors for the neurotransmitters glutamate and γ-aminobutyric acid (GABA). NPs derived in this way could serve as models for cellular replacement therapy in primate models of neurodegenerative disease, a source of neural cells for toxicity and drug testing, and as a model of the developing primate nervous system.

Keywords: Embryonic stem cell; Human; Primate; Neural progenitor; Electrophysiology; Neuron; Neural stem cell

The isolation of ES cells from monkey and human embryos has generated great interest in using these cells as the basis of cell replacement therapies for degenerative diseases, as well as models of human development. ES cells were first isolated from mouse embryos over 20 years ago [1,2]. More recently, monkey ES cells were isolated first from the rhesus monkey followed by the isolation of marmoset and cynomolgous ES cells [3–6]. Shortly after the isolation of monkey ES cells the same methods were used to isolate human ES cells from in vitro fertilized human embryos [7,8].

Currently, immense interest is focused on finding ways to differentiate ES cells into specific cell types that would be candidates for cell replacement therapies for degenerative diseases. However, the very quality that makes ES cells so appealing, the ability to generate virtually every cell type found in the body, is the Achilles heel of stem cell research. Methods need to be defined that will allow for

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the production of specific differentiated cell types for cell replacement therapy to become a reality.

Non-human primate and human ES cells have similar markers of pluripotency including the expression of Oct-4, SSEA-3, SSEA-4, TRA-1–60, TRA-1–81, and alkaline phosphatase activity. Therefore, we feel that information gained from the study of non-human primate ES cells will be applicable to human ES cells. Rhesus animal models for Parkinson's disease, diabetes, and spinal cord injury have been demonstrated [9–12], and rhesus ES cells can be used to generate cells for allografts into these models thus avoiding potential xeno-transplant rejection as a confounding factor in these studies. Transplants of cells derived from non-human primate ES cells into these models will be used to perfect strategies that can then be applied to human cell therapies. However, to date, there are no reports of efficient neural differentiation of non-human primate ES cells.

Research done with MEDII, the conditioned medium from the human hepatocellular carcinoma cell line HepG2, indicated that the stepwise differentiation of mouse ES cells could be done in vitro [13]. It was found that by applying this conditioned medium to adherent cultures of mouse ES cells a uniform conversion of ES cells to a second cell type occurred. Gene expression data confirmed that expression profiles of the two cell types were different with the up-regulation of FGF5 and down-regulation of REX-1, a profile consistent with primitive ectoderm found in the mouse blastocyst [14–17]. In subsequent experiments it was found that the timing and method of exposure to MEDII changed the differentiation outcome. By forming EBs in the presence of 50% MEDII, neurectoderm was formed at the expense of mesodermal derivatives such as beating heart muscle. Further, the stepwise differentiation of this neurectoderm was achieved [18]. Here we report the efficient neural differentiation of rhesus ES cells using the conditioned medium MEDII. Explants of primate ES derived EBs contained large areas of neuralized cells that were MAP-2 and NF200 positive. By sub-culturing the neuralized areas a highly enriched population of neural progenitors has been isolated and characterized. These cells are Nestin, MAP-2, Hu, and Musashi positive. Upon differentiation these cells have a morphology consistent with embryonic neurons including a phase bright cell body with processes. These cells have several electrophysiological properties of neurons including the firing of action potentials and responses to excitatory and inhibitory neurotransmitters.

Materials and methods

ES cell culture. Primate ES cells were maintained in DMEM/F12 with 15% FBS, 5% Knockout Serum Replacer (KSR, Invitrogen), 2 mM L-Glutamine, 0.1 mM MEM non-essential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, 1000 U/ml recombinant human leukemia inhibitory factor (hLIF), 0.1 mM βME, and 4 ng/ml bFGF. Cells were grown on mitomycin C inactivated feeder layers and manually passaged with a pulled Pasteur pipette every 4 days.

Neural induction of primate ES cells. Primate ES cells were dissociated from adherent culture by incubation with 1 mg/ml collagenase IV (Gibco). When colonies of ES cells could be seen lifting off of the feeder layer they were dislodged with a cell scraper and the suspension of ES cells was spun down and re-suspended in primate ES growth medium (lacking bFGF and hLIF) either with or without 50% MEDII medium [18]. EBs were grown for 7 days in suspension.

EB differentiation. For differentiation, EBs were plated on poly-ornithine and laminin coated 35 mm dishes in DMEM with 10% FCS, L-Glut, and Pen–Strep for 7 days. Previous experiments demonstrated that after 7 days of adherent culture significant differentiation occurred and large areas of neurons could be visualized with phase contrast microscopy. Samples were fixed to avoid loss of neurons in culture.

Sub-culture of neural progenitors. For isolation of NPs, EBs were plated on poly-ornithine and laminin coated 35 mm dishes in DMEM/F12 with 50% MEDII, N2, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-Glutamine, 10 ng/ml bFGF, and 1000 U/ml hLIF. After proliferation of rosette areas, usually after 7–10 days, these areas were sub-cultured mechanically with a fire drawn Pasteur pipette. Cultures were maintained in DMEM/F12 with 50% MEDII, N2, Pen–Strep, L-Glut, 10 ng/ml bFGF, and 1000 U/ml hLIF. Further sub-culturing was carried out to enrich the population for NPs (Mitalipova et al., 2003; manuscript in preparation).

Immunocytochemistry. Cells were fixed in 4% para-formaldehyde and 4% sucrose for 15 min at room temperature. After three 1 min washes cells were permeabilized by incubation in 50 mM Tris, pH 7.6, 250 mM NaCl, 3% normal goat serum, 0.3% Triton X-100, and 1% polyvinyl pyrolidine for 30 min. Cells were incubated with primary antibody in permeabilization solution for 1 h. The following primary antibodies were used: rabbit polyclonal Nestin (1:50 dilution, Chemicon), mouse monoclonal MAP-2 (1:1000 dilution, Sigma clone HM-2), mouse monoclonal Hu (1:50 dilution, Molecular Probes), and rabbit polyclonal Musashi (1:500 dilution, Chemicon), and mouse monoclonal NF200 (1:400 dilution, Sternberger Monoclonals SMI-32). Cells were then incubated with Alexa 488 and 595 conjugated secondary antibodies (1:1000 dilutions, Molecular Probes) for 1 h in permeabilization solution. Nuclei were stained with DAPI (1 μg/ml, Roche).

Differentiation of NPs. For early differentiation, NPs were incubated in DMEM/F12 (Gibco) with 1× B27 (Gibco), 50 U/ml penicillin, and 50 μg/ml streptomycin. After 3 days 5-Fluorouracil and Uracil (Sigma) were added at 10 μM each to inhibit overgrowth by mitotic cells. Cells were allowed to differentiate for an additional 5 days before electrophysiology was performed. For more advanced differentiation cells were incubated in Neurobasal (Gibco), 1× B27, 5% FBS, 10 ng/ml hLIF, and 10 ng/ml BDNF (R&D Systems). After 24 h 5-Fluorouracil and Uracil were added at 10 μM each. Cells were differentiated for a total of 4 weeks and then electrophysiology was performed.

Electrophysiology. Whole-cell recordings were made at room temperature on the stage of an inverted phase-contrast microscope using standard procedures. Briefly, patch electrodes were filled with a solution containing (in mM): 140 K-gluconate, 5 KCl, 0.2 EGTA, 10 Hepes, 3 MgATP, and 0.3 Na_2GTP (pH 7.2, ~295 mOsm kg^{-1} H_2O). The external solution contained (in mM): 150 NaCl, 2.5 KCl, 10 Hepes, 10 glucose, 1.5 $CaCl_2$, and 2.5 $MgCl_2$ (pH 7.2, ~310 mOsm kg^{-1} H_2O). Currents were digitized and recorded with a multifunction I/O board and WinWCP software (provided by Dr. J. Dempster, Strathclyde University, Glasgow). Drugs were applied during recordings via a fused silica tube (i.d. 200 μm) connected to multiple reservoirs, the outlet of which was positioned immediately in front of the cell under study.

Results and discussion

To test the ability of MEDII conditioned medium to promote neural differentiation of rhesus ES cells we formed embryoid bodies (EBs) from the cells with and without 50% MEDII in the medium. EBs formed in the absence of MEDII exhibit a small proportion of rosette structures indicative of neural progenitor cells (NP cells)

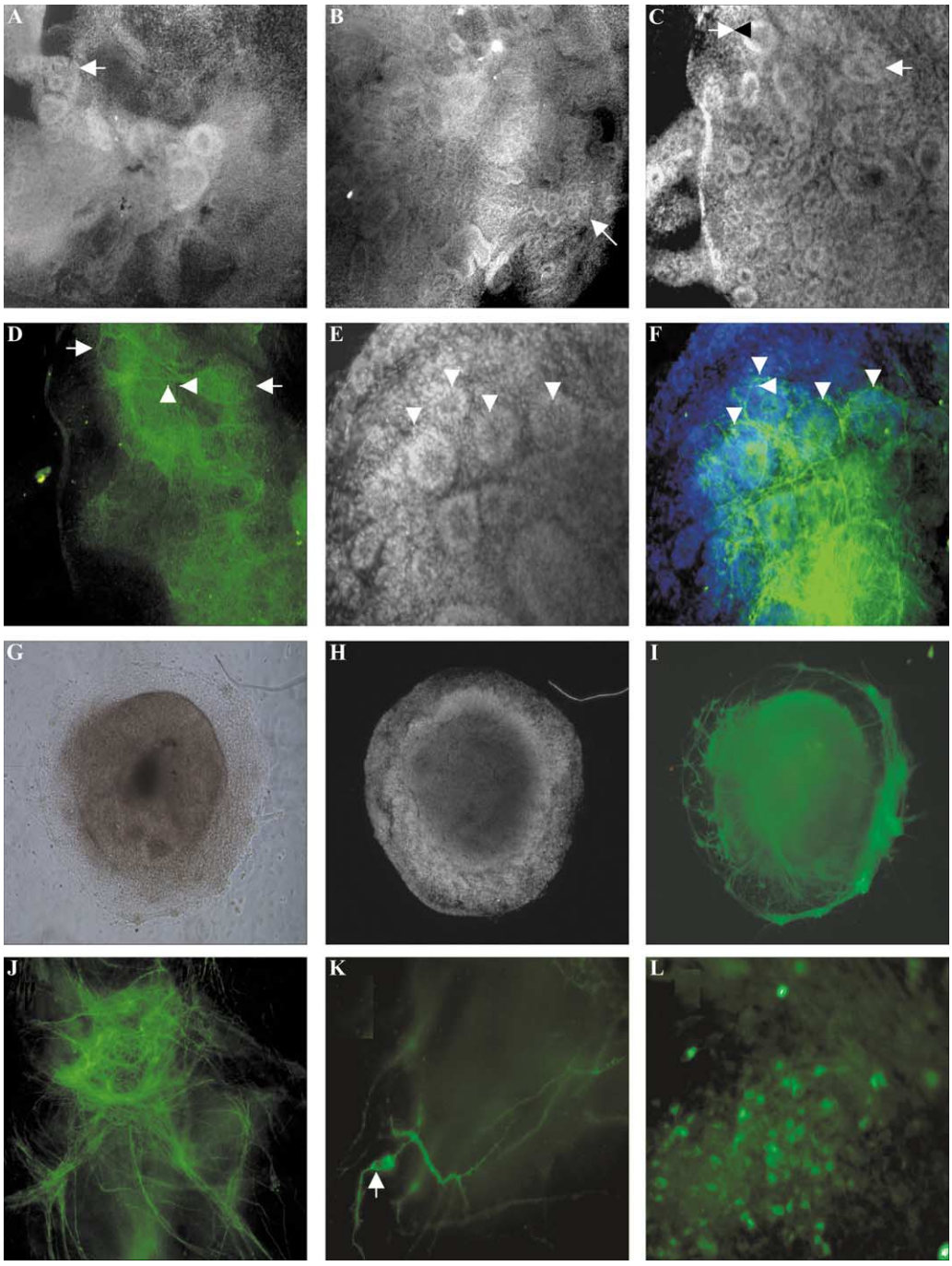

Fig. 1. MEDII conditioned medium promotes the neural differentiation of non-human primate ES cells. (A, B) Neural progenitor rosettes in untreated (A) and MEDII treated (B) EBs. Rosettes (arrows pointing to representative rosettes in A and B) were far more common in cells from MEDII-treated EBs. (C–F) MAP-2 positive neurons were in close association with rosette structures seen in the interior of MEDII-treated EBs. Neurites from two rosettes (arrows in C and D) extend processes toward each other (arrowhead in D). Neurons formed rings around rosettes (arrowheads in E and F) while a radial pattern of staining could be visualized in the center of the encircled rosettes. (G–I) Some MEDII-treated EBs approached approximately 90% neutralization as characterized by MAP-2 staining. (G) Phase contrast, (H) DAPI, and (I) MAP-2 staining of a MEDII-treated EB. (J) Higher magnification view of a network of MAP-2 stained neurons. (K) High power magnification of a MAP-2 positive neuron showing characteristic peri-nuclear staining (arrow) and localization to neurites. (L) NF 200 positive neurons in MEDII-treated EB explants. Magnification: A and B, 40×; C, D, G, H, and I, 100×; E, F, and J, 200×; K, 600×; and L, 400×.

previously described in differentiating human ES cell cultures [19–21]. However, EBs formed with MEDII exhibited what appeared to be many more rosettes than control EBs (Figs. 1A and B). In some EBs treated with MEDII, approximately 90% of the cells in the EB expressed the post-mitotic pan-neuronal marker MAP-2 (Figs. 1G–I). Large networks of MAP-2 positive neurons were easily visualized in MEDII-treated EBs (Figs. 1D, F, and J). High power magnification of MAP-2 staining showed characteristic peri-nuclear staining and small spines on MAP-2 stained neurites (Fig. 1K). Differentiated neurons were also positive for non-phosphorylated neurofilament 200 (NF200) (Fig. 1L). While some neural differentiation occurs in non-MEDII treated EBs, the level of neural differentiation is not high enough to yield enough neurons for subsequent studies. The inclusion of MEDII in the differentiation process increases the amount of neural differentiation of rhesus ES cells. This suggests that MEDII will promote neural cell fates in differentiating non-human primate and human ES cell lines.

We also noticed that areas of the EBs with intense MAP-2 staining were almost always in close association with the rosettes of NP cells (Figs. 1C–F). Close examination shows that MAP-2 staining emanates in a spoke-like pattern from the center of these rosettes. More mature neurons encircle the rosette areas and extend neurites toward other MAP-2 positive neurons that arise from other neighboring rosettes. Neurons with a more immature appearance seem to originate from the center of these rosettes. Therefore, we sought to isolate these rosette areas and determine if they express NP markers [19–21]. By using a manual passaging technique we were able to isolate presumptive NP cells and continue their

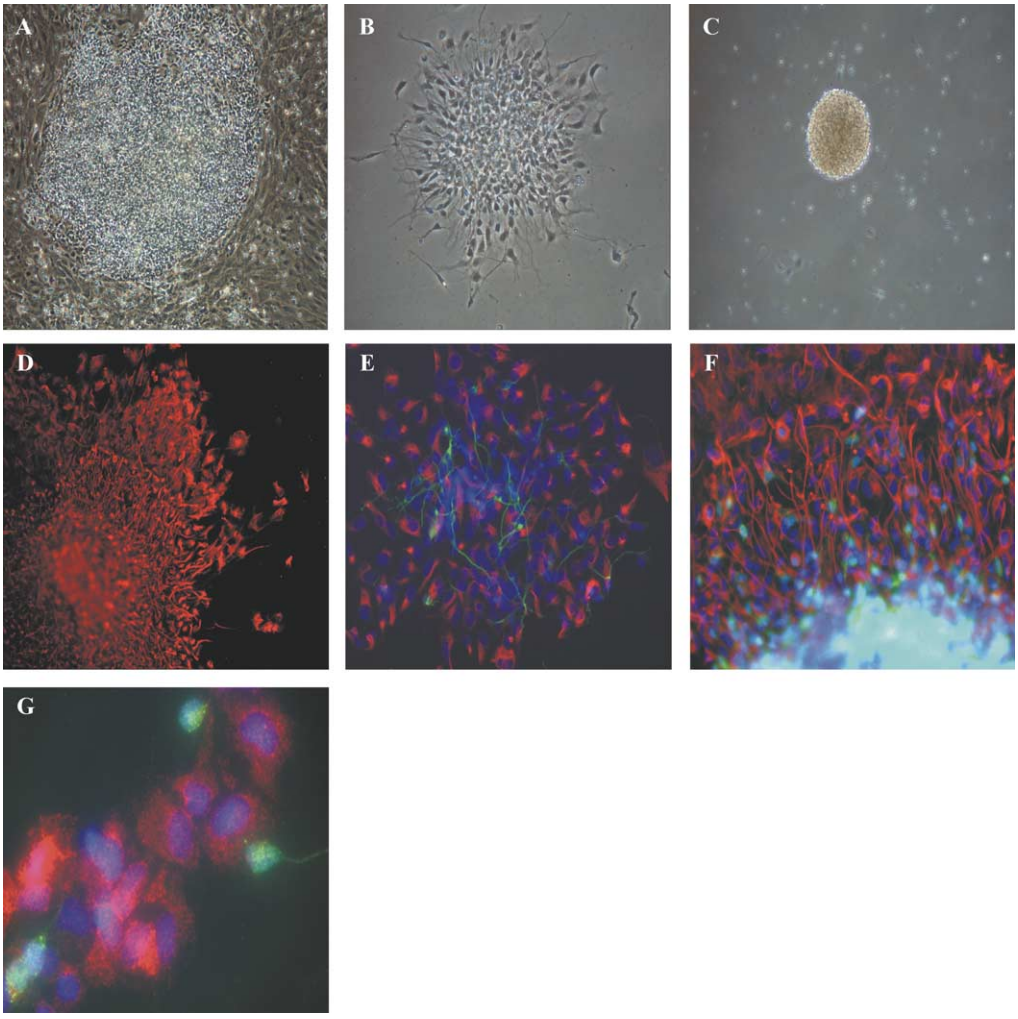

Fig. 2. ES derived NP cells enriched from MEDII-treated EBs. (A, B) Phase contrast images of NPs after first round of isolation (A) and smaller colony (B) after further sub-culturing by mechanical passaging. (C) Representative sphere that arose spontaneously after passaging. (D) Nestin staining of a colony of putative NP cells and associated neurons. (E) MAP-2 (green), Nestin (red), and DAPI (blue) staining of a colony of NP cells. (F) Hu (green), Nestin (red), and DAPI (blue) staining of a colony of NP cells. (G) High magnification view of NP cells and neurons double stained for Musashi (red), DAPI (blue), and Hu (green). Magnifications: (A–D), 100×; (E) and (F), 200×; and (G), 600×.

culture independently. By selecting areas that grew in a rosette shape cultures were obtained that can be seen in Fig. 2; A. By again selecting cells with an NP morphology [19–23], we could expand these cultures and begin to eliminate cells that were not thought to be NPs (Fig. 2B). Occasionally small symmetrical spheres arose after passaging that remained in suspension (Fig. 2C). These resembled neurospheres [24–36]. The ability to form neurospheres from ES-derived neural progenitors could be used advantageously to generate more advanced differentiation derivatives in future studies.

To confirm that the passaged rosette cells were indeed neural progenitors we characterized these cultures by immunocytochemistry for the NP markers Nestin and Musashi [37–42]. Clumps of NPs were positive for Nestin (Figs. 2D–F) as well as Musashi (Fig. 2G). Double staining with the pan-neuronal markers MAP-2 (Figs. 2E and F) and Hu (Fig. 2G) demonstrated the presence of developing neurons within the colonies of NP cells. Therefore we feel that the cells isolated from MEDII-treated EB explants are indeed NP cells.

NP cells isolated from rhesus ES cell EB explants spontaneously formed neurons with withdrawal of mitogen. To determine the functional capacity of these cells, electrophysiology was performed on neurons derived from the NP cells. Whole-cell intracellular recordings were made from phase-bright cells with at least two visible processes (Fig. 3A). In current-clamp mode cells differentiated for 4 weeks maintained a negative resting membrane potential that varied between −23 and −59 mV (mean -39 ± 3 mV, $n = 10$). When cells were held near −60 mV with steady hyperpolarizing current injection, square depolarizing current commands reliably evoked overshooting action potentials. Cells differentiated for a shorter period of time (3 days) also generated action potentials, but were unable to generate repetitive action potential trains, suggesting these cells expressed a relatively low density of voltage-gated sodium channels (data not shown). In contrast, cells differentiated for 4 weeks could fire action potentials repetitively, consistent with a more mature neuronal phenotype (Fig. 3B). In the same cells voltage-clamp experiments were performed to determine if stem cell-derived neurons expressed functional ligand-gated ion channels (Fig. 3C). Rapid bath application of glutamate (1 mM; with 100 μM cyclothiazide to block rapid desensitization) evoked robust inward currents (985 ± 416 pA at −60 mV, $n = 5$) that reversed polarity at a holding potential near 0 mV. Similarly, rapid bath application of γ-aminobutyric acid (GABA; 100 μM) evoked robust inward currents (654 ± 416 pA at −60 mV, $n = 5$; Fig. 3C) that reversed polarity near −50 mV and rapidly desensitized. These results suggest that stem cell-derived neurons express functional ionotropic glutamate (AMPA/NMDA) and GABA ($GABA_A$) receptor/ion channels.

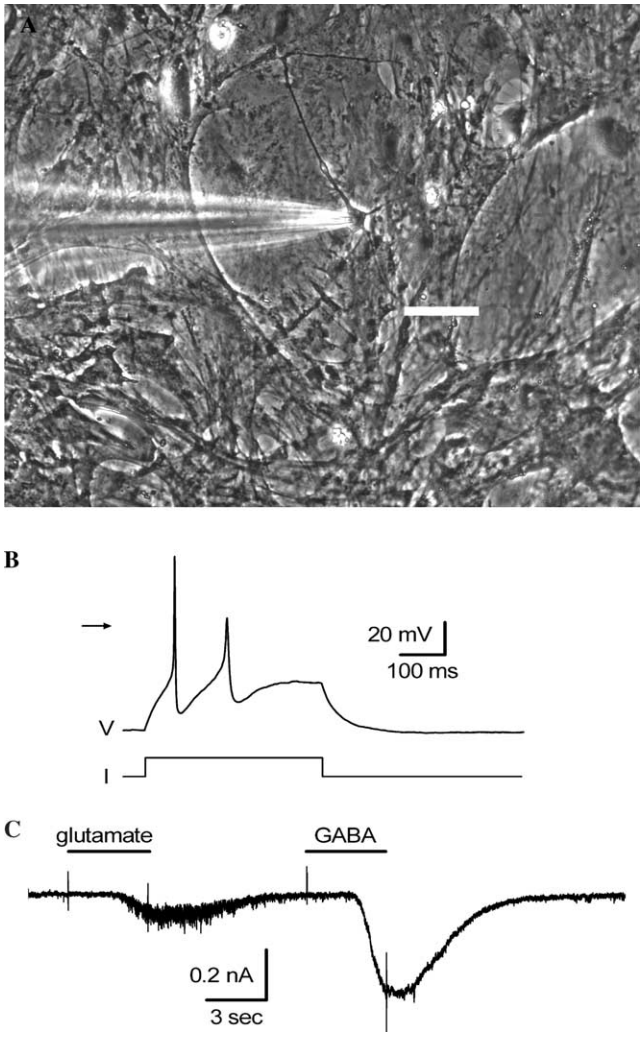

Fig. 3. Electrophysiological properties of stem cell-derived neurons. (A) Phase contrast image of a stem-cell derived neuron 4 weeks after differentiation attached to a whole-cell recording electrode. (B) Current-clamp recording of the voltage response (V) to a square injected current command (I). This cell responded with two overshooting action potentials; 0 mV is indicated by the arrow. (C) Voltage-clamp recording of responses to rapid application (indicated by horizontal bars) of glutamate (100 μM) or γ-aminobutryic acid (GABA; 100 μM). Scale bar in (A) is 50 μm.

In the present study we outline a strategy for the differentiation of rhesus ES cells to neural lineages. Our approach differs from the two previously published reports on differentiation of non-human primate ES cells to neural lineages. The first report described spontaneous differentiation in teratomas [43] and the second used co-culture of the ES cells with the stromal cell line PA6 to induce neural differentiation [44]. In contrast, we have shown that rhesus ES cells can efficiently produce terminally differentiated neurons in a simple cell differentiation culture system without stromal cell co-culture.

To induce neural differentiation of primate ES cells we employed a novel strategy involving the use of the conditioned medium MEDII, which has been shown to drive

efficient neural differentiation of mouse ES cells [18]. In that study the formation of neurectoderm was largely homogeneous with 95.7% of the cells scoring positive for NCAM while only 42.13% of cells expressing NCAM in non-MEDII treated differentiations. Further, development of neurectoderm in vitro followed a time line consistent with developmental progression in vivo. Many efforts to produce dopaminergic neurons in vitro involve using cocktails of growth factors to obtain the desired phenotype. While some of the strategies developed have been successful, cells may not pass through normal developmental stages that occur in vivo. We feel that taking ES cells through a sequential, stepwise differentiation process may produce cells more similar to the naturally occurring cell type. The data presented here suggest that we have achieved the first step of sequential neural differentiation of rhesus ES cells.

In summary, we have used a previously undescribed strategy for the in vitro neural differentiation of non-human primate ES cells. MEDII medium was used to generate relatively homogeneous neural differentiation as well as to facilitate the isolation of neural progenitor cells that differentiate to neurons with functional electrophysiological properties. We feel the electrophysiological data presented are important proof of principle that functional neurons can be derived from rhesus ES cells. The use of primate ES cell derived products in primate models of disease will undoubtedly prove the best model for cell replacement therapy in humans, and we feel the differentiation strategy employed here will be useful in generating a variety of cell types for transplantation into these models. Further experiments will be carried out to determine whether or not the neural differentiation obtained from primate ES cells in this study closely parallels the results obtained with mouse ES cells.

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Note added in proof. H.C. Kuo et al., Differentiation of monkey embryonic stem cells into neural lineages, Biol. Reprod. 68 (2003) 1727–1735, provides additional information on deriving neural like cells from monkey ES cells.

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