Differentiation and Production of Action Potentials by Embryonic Rat Retina Stem Cells In Vitro

Li-Feng Chen,1,2 Zheng Qin Yin,1,2 Shan Chen,3 and Zhong-Shan Chen1

PURPOSE. Transplantation of retinal stem cells (RSCs) is a potential therapy for retinal degeneration. However, success critically depends on whether RSCs can differentiate into fully functional daughter cells.

METHODS. Whole-cell patch clamp techniques were used to characterize the postinduction developmental profile of membrane potentials and ionic currents in RSCs cultured from embryonic day 17 Long Evans rats. These results were compared to recordings obtained from cultured cells obtained from postnatal day 1 rat pups.

RESULTS. Outward rectifying potassium currents ($I_{\text{K}}$) were observed in approximately 15% of cells at day 3 and in all cells by day 7. Voltage-dependent sodium currents took longer to emerge (day 7), and TTX-sensitive channels were not fully mature until day 15. Broad excitatory potentials, characteristic of immature action potentials, could be reliably stimulated by day 10, and spontaneous action potentials were recorded at day 25, which were indistinguishable, in terms of width and amplitude, from the comparison group of developing retinal neurons.

CONCLUSIONS. The maturation of presumptive retinal ganglion cell electrophysiological properties appears to take at least 15 days under these culture conditions. Knowledge of the timing of voltage-dependent ion channel development could improve the success rate in future transplantation protocols. (Invest Ophthalmol Vis Sci. 2008;49:5144-5150) DOI:10.1167/ iovs.08-1907

Repair or substitution of defective cells by activated retinal stem cells (RSCs) has become one of the most promising treatments for degenerative abnormalities such as retinitis pigmentosa.1,2 However, the success of stem cell transplantation critically depends on whether the stem cells can differentiate into functional daughter cells. The neural stem cells that originate from the embryonic and adult central nervous system can be made to differentiate into neurons and neural glial cells and then labeled with specific antigens.3 However, a criterion for neuronal differentiation based on the presence of neuronal antigens cannot satisfactorily predict neuronal function.4 Thus, knowledge of functional criteria based on the presence of characteristic active membrane properties and functional synapses could play an important role5 in determining the efficacy of a therapeutic application.

The developmental physiology of RSCs is poorly represented in the literature. Neural stem cells/progenitors that give rise to neurons and glia have been identified in different regions of the brain, including the retina.6 Previous investigations have demonstrated that isolated RSCs/progenitors can exhibit an electrophysiological phenotype similar to that of glial Müller cells or a neuronal phenotype.7 However, close comparison between induced RSC differentiation and cultured retinal cells derived directly from postnatal or adult animals has not been available.

Das et al.8 reviewed the development of cell membrane properties of retinal stem/progenitors, including the differential expression of genes and physiological properties and the development of voltage-dependent channels. However, data were presented for only the first few days after the induction of differentiation, and, though the development of potassium and sodium channels in these first few postinduction days was charted, it was intuited from the presence of outward and inward currents, respectively, rather than from determining the effects of known channel blockers.

The purpose of the present study was to investigate the development of electrophysiological properties of rat RSCs derived from embryonic day 17 Long Evans rats with the use of whole-cell patch-clamp recording after the induction of differentiation in vitro (DIV). These results were compared to recordings from cultured retinal neurons. The data demonstrate the value of whole-cell patch clamp recording in providing information on the developmental state of ion channels and neuronal function not available through anatomic techniques.

MATERIALS AND METHODS

All procedures were approved and monitored by the Third Military Medical University Animal Care and Use Committee and have been conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Stem Cell Culture

Embryos from timed-pregnancy Long Evans rats (Taconic, Hudson, NY; http://www.taconic.com; n = 20) were harvested on embryonic day (E) 17 and were placed in D-Hanks balanced salt solution (DBS; Hyclone, UT). Eyes were carefully enucleated and placed in DBSS in a separate culture dish. Care was taken to enucleate eyes with a minimum of extraneous tissue. The optic nerve and remaining mesenchymal tissue were carefully removed before the retina was isolated. This approach prevented the possible contamination of the retina with brain tissue. The retina was carefully teased away from the retinal pigment epithelium (RPE), and the central portion of the retina around and including the optic nerve head was removed and discarded. The isolated retina was incubated in 2 mL of 0.2% trypsin (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C in 5% CO2 in an incubator (NuAire, Plymouth, MN) and was dissociated to a single-cell suspension.

From the 1Southwest Eye Hospital/Southwest Hospital, Third Military Medical University, Chongqing, People’s Republic of China; and the 2Department of Ophthalmology, Second Affiliated Hospital Jiangxi Medical College, Nanchang, People’s Republic of China.

2Contributed equally to the work and therefore should be considered equivalent authors.

Supported by National Basic Research Program of China Grants 2007CB512203 and 2009CB724302 and by Nature Science Foundation of China Grant 30772371.

Submitted for publication February 20, 2008; revised May 18 and June 12, 2008; accepted August 27, 2008.

Disclosure: L.-F. Chen, None; Z.Q. Yin, None; S. Chen, None; Z.-S. Chen, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Zheng Qin Yin, Southwest Eye Hospital/ Southwest Hospital, Third Military Medical University, Chongqing 400038, China; qinzinyin@yahoo.com.cn.
Isolated cells were cultured as a cell suspension at $1 \times 10^6$ cells/cm$^2$ in serum-free medium containing DMEM/F-12, B27 supplement (1:50; Invitrogen, Carlsbad, CA), 20 ng/mL human recombinant bFGF (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin in uncoated flasks at 37°C in 5% CO$_2$ in an incubator. After 6 to 8 days in culture, primary neurospheres were collected, and passaging was performed using 0.025% trypsin combined with gentle mechanical trituration. The resultant single cells were plated at the same density and were identically cultured. After three passages, 10% fetal bovine serum was added to the medium to induce stem cell differentiation. Cell morphology was observed at different time points (days 0–15) after induction and differentiation. In a separate series of experiments, retinal neurons from postnatal day 1 Long Evans rats were cultured for a corresponding period and under similar conditions on coverslips at a density of 1 cell/sphere or from isolated differentiated cells with comparison group neurons (Figs. 2A, 2D from DIV7 cells). To confirm the presence of presumptive dendritic spines, rats were stained with lucifer yellow. Dendritic fields were larger and thicker and longer as culture duration increased. RSCs and comparison group in culture. Cells were photographed after recordings and injection with lucifer yellow. Dendritic fields became larger and thicker and longer with longer differentiation times (or time in culture). (A–D) Differentiated cells at DIV0, DIV3, DIV7, DIV15, respectively, from RSCs. (E–G) Comparison group neurons at days 3, 7, 15 in culture, respectively. Scale bar, 50 μm.

Differentiation of Retinal Stem Cells

Retinal spheres from the third passage were harvested after 7 days in culture and were plated on poly-D-lysine–coated glass coverslips in 24-well culture dishes with retinal culture medium supplemented with 10% fetal bovine serum (FBS) but in the absence of EGF and bFGF. Three separate cultures were started, each containing three 24-well plates. As many cells as possible were sampled on the coverslips at the specified duration of differentiation (RSCs) or length of time in culture (comparison group).

Criteria for Neuronal Choice

Neuronal-like cells were chosen for patch clamp recordings based on the criterion that their cellular processes should be five times longer than the cell body.9

Electrophysiological Recordings

Our procedures for making whole-cell patch clamp recordings have been described in detail.10,11 Third-generation RSCs were divided into six groups according to the length of time in culture after induction and differentiation (days 0, 3, 7, 10, 15, and 25); cells were cultured on poly-D-lysine–coated coverslips in media containing 10% FBS. Cells were superfused at 1 mL/min with 119 mM NaCl, 2.5 mM KCl, 2.5 mM CaCl$_2$, 1.3 mM MgCl$_2$, 26.3 mM NaHCO$_3$, 1 mM Na$_2$HPO$_4$, 20 mM glucose, 2 mM sodium pyruvate, and 4 mM sodium lactate (pH 7.2–7.6). Superfusate was continuously bubbled with 95% O$_2$/5% CO$_2$.

Whole-cell patch clamp recordings were obtained from cells in retinal spheres (1 cell/sphere) or from isolated differentiated cells with an amplifier (Axopatch 200B; Axon Instruments, Foster, CA) in current and voltage clamp modes.15 Patch electrodes (World Precision Instruments, Sarasota, FL) were pulled with a micropipette puller (P-97; Sutter, Novato, CA) and had tip resistances of 6 to 8 MΩ. Pipettes were filled with 120 mM KCl, 20 mM HEPES, 10 mM EGTA, 2 mM MgATP, and 0.2 mM NaGTP, and the pH was adjusted to 7.2 with KOH. Series resistance was less than 20 MΩ and was monitored continuously and adjusted with compensation. Data were filtered at 5 kHz and sampled at 10 kHz; acquisition and analysis were performed by pCLAMP software (Axon Instruments).

Statistical Analysis

All data were assessed with software (SPSS ver. 10.0; SPSS Inc., Chicago, IL) using one-way ANOVA, post hoc two tailed t-tests for comparing means or χ² analysis.

RESULTS

Morphology

Morphologic changes were observed after 3 days in culture in the induced RSCs and the cultured postnatal day 1 Long Evans rat retinas. This consisted of the presence of tiny protuberant growths and neurites (Fig. 1; lucifer yellow was injected during whole-cell patch recordings and was used for visualization of cell processes). Different dendritic morphologies were observed even at the same stage of differentiation or on the same day in culture; however, there was a clear tendency for dendritic fields to become larger and dendrites to become thicker and longer as culture duration increased. RSCs and comparison group neurons (Figs. 1D, 1G) were qualitatively similar with respect to the processes of differentiation and development (i.e., both showed a similar degree of dendritic field complexity and the emergence of presumptive dendritic spines).

Development of Potassium Currents

Thirty-seven of the 68 RSC cells recorded (54%; DIV0–15) demonstrated the presence of outward-rectifying currents ($I_{K_+}$; Figs. 2A, 2D from DIV7 cells). To confirm the presence of $I_{K_+}$, tetraethylammonium ions (TEA; 15 mM) was added to the bath solution, revealing a slowly activating $I_{K_+}$ at volt-
ages more positive than −40 mV (Figs. 2A–C; n = 10 from DIV7), consistent with the criterion of a slowly activating, outward-rectifying IK, 11 IK, was partially blocked by Cd2+ (100 μM, Figs. 2D–F; n = 14 from DIV7), suggesting that a calcium-activated IK, makes a small contribution to the current measured at voltages more positive than −20 mV (Fig. 2F).

Percentages of cells with IK, peak currents blocked by TEA or Cd2+ for RSCs and comparison group cells are shown in Figure 3A. We compared the effectiveness of TEA and Cd2+ to block potassium channels in both groups of cultured neurons. Overall, Cd2+ produced weaker blocking with no difference between RSCs and comparison groups. TEA caused nearly 100% block in RSC DIV7 and DIV15 cells and in all comparison group cells. IK, was not detected in floating undifferentiated RSCs (n = 14) on DIV0. However, after 3 days of RSC differentiation, IK, was observed as a small, persistent outward rectification and was present in approximately 15% (3 of 20) of cells. From DIV7 onward, all patched cells showed outward-rectifying IK, (34/34). In contrast, all retinal neurons in the comparison group had IK, currents from day 3 onward (n = 27; Fig. 3B).

**Development of Sodium Currents**

Voltage-dependent sodium currents, I Na+, could be recorded by DIV7 (Figs. 4A, 4D) in the RSCs. The voltage was maintained at −70 mV, and a stimulation pulse (60 ms) was stepped from −70 mV to 20 mV in 10 mV/step. The levels at which Na+ channels were blocked by 1 μM TTX, a specific Na+ channel blocker, are shown in Figure 5. The concentration of TTX was chosen on the basis that this level routinely blocked 100% of I Na+ in retinal slice preparations in our laboratory. In the present study, cells with ≥60% blocking were defined as TTX-blocked cells, and those with ≤40% blocking were defined as TTX-resistant cells or partially blocked cells (the ratio of I Na+ peak value TTX vs. normal was used as the measure of blocking). In the DIV7 RSC group, 12 of 18 (67%) neuronal-like cells showed instantaneous inward currents, whereas the remaining six cells demonstrated only IK, currents. Four of the 12 cells expressing sodium channels were sensitive to TTX, and eight of 12 cells were resistant to TTX, as defined (i.e., ≤40% blockade by TTX; Figs. 4B, 4C). In the DIV15 RSC group, 10 of 16 (63%) neuronal-like cells showed instantaneous inward currents; 70% of those were sensitive to TTX.

In contrast, Na+ channels were present in the comparison group on day 3 (57%), and higher proportions of cells showed the presence of channels by days 7 (80%) and 15 (75%) compared with the RSCs (67% and 63%, respectively; Fig. 5B). The observed relative maturity of Na+ currents at DIV15 indicated that this was unlikely to change with additional time in culture; therefore, Na+ channel recordings were not performed at DIV25.

The percentages of cells expressing Na+ channels under undifferentiated conditions (DIV0) and differentiated conditions (DIV3–15) were compared. No significant differences were observed between RSC DIV7 and DIV15, RSC DIV7 and comparison day 7, or RSC DIV15 and comparison day 15 (χ2 test; respectively, χ2 = 0.971, P = 0.324; χ2 = 0.56, P = 0.454; χ2 = 1.797, P = 0.18). To determine whether RSCs are slower to develop TTX-sensitive Na+ channels than the comparison group cells of the same age, we compared the percentage of cells with Na+ currents that were blocked by TTX with a two-tailed t-test and found no significant differences between RSCs and comparison cells at DIV7 or DIV15.
Development of Action Potentials

Action potentials (APs) could not be elicited from RSCs on DIV0 ($n = 15$), DIV3 ($n = 13$), or DIV7 ($n = 10$). Note that cells do not express antigens specific to RGCs until DIV7 (data not shown), at which time voltage-dependent currents could be recorded (see also Kohyama$^{14}$). In all cases after DIV7, cells with a morphology resembling that of retinal ganglion cells were selected (e.g., Fig. 1C; see also Yamasaki and Ramoa$^{15}$); thus, sampling was biased against nonspiking cell types such as presumptive bipolar cells. At DIV10, only broad potentials of relatively small amplitude were recorded, characteristic of immature action potentials.$^{16}$ Data regarding AP amplitudes, width, and resting membrane potentials are shown in Table 1. These potentials, evoked by 1000-ms, 80-pA intracellular current pulses, were seen in 7 of 15 cells, whereas the remaining cells showed no response. For DIV15, 6 of 14 cells gave relatively mature APs (DIV10), but potentials were markedly less mature than those seen at DIV25 and in comparison group neurons, which had clear APs (6 of 20 and 7 of 20, respectively). RSCs at DIV25 and comparison group neurons showed a similar firing pattern when stimulated (Fig. 6).

Quantification was not performed on the percentage of cells with APs because of uncertainties about damage to the cell during recording (i.e., the absence of an AP might or might not have indicated damage to the cell). Hence, we placed emphasis on the apparent maturity of the AP when it was present rather the proportion of cells with an AP. Statistical analysis on the amplitudes of APs in developing RSCs (when they could be reliably evoked, such as DIV10, DIV15, DIV25) and comparison group cells was carried out using ANOVA. A significant effect of developmental age was found ($F_{(3,19)} = 39.4; P < 0.0001$) with post hoc significant differences ($P < 0.0001$) between the early developing RSCs and later times and the comparison group. Baseline was taken as the membrane potential at the end of the current step; however, amplitude (AMP) measurements were determined by measuring the height from the beginning of the depolarization to its peak. Amplitudes for the RSCs at DIV25 compared with comparison cells were not significantly different. The effect of development was also indicated by a correlation analysis of the three longer development times (10, 15, 25 days), in which AP amplitude was strongly correlated with the age of development ($R^2 = 0.818; F_{(1,14)} = 62.8; P < 0.0001$).

Spontaneous APs occurred in differentiated RSCs by DIV25. These were similar to those recorded from comparison group neurons after 7 days in culture (Fig. 7), and analysis of AP width and amplitude showed no significant differences (Fig. 7C). AP width was defined as the width from the beginning of the depolarization to its return to baseline (amplitude, $P = 0.163, n = 12$; AP width, $P = 0.349, n = 12$).

DISCUSSION

Previous studies on RSCs, as on other stem cells, have relied almost exclusively on morphologic and immunohistochemical criteria for the identification of neurons and their state of development.$^{6}$ As recently shown, however, morphologic and immunohistochemical data cannot define whether these cells are normally functioning neurons.$^{3,6}$ We have used patch clamp techniques to confirm that RSCs express electrophysiological characteristics typical of differentiated retinal neurons and have determined their time course of development in culture.

Das et al.$^{8}$ and we$^{17}$ have systematically labeled cells with neuronal antigens or, as in the present study, used lucifer yellow injections to visualize dendritic cell morphology. However, these methods gave no indication of the actual functional status of the labeled cells. Although one may argue that these cells are in the process of developing into mature neurons, many cultured or transplanted cells do not complete their development, and neuronal precursors and cell lines differ widely with respect to their capacity for maturation.$^{18,19}$ Because of this a more stringent standard for neuronal identification has been proposed based on the identification of functional neuronal characteristics, such as electrical excitability (e.g., APs) and the ability to communicate information with other neurons by way of synapses. Thus, it is important to use techniques that establish the stage of functional maturity during differentiation and that can be applied when evaluating the clinical usefulness of successfully differentiated cultures as potential transplant material.

We evoked apparently mature APs when RSC differentiation had proceeded beyond DIV15. In our cultures, APs were reli-
ably evoked by day 10, though later than reported by Das et al., who studied RSCs for 6 days after induction compared with 25 days in this study, thus demonstrating that cultured RSCs harvested from embryonic rats can produce APs in vitro. However, spontaneous APs were not apparent until DIV25, at which stage they were indistinguishable from cultured retinal neurons.

The present results show that the neuronlike cells induced to differentiate from RSCs developed active electrophysiological features, including inward sodium currents and outward potassium currents. APs could be induced and could develop spontaneously. These latter cells exhibiting spontaneous APs are most likely retinal ganglion cells. During the process of stem cell differentiation, voltage-dependent ion channels gradually developed in a time-dependent manner and ultimately supported mature AP firing similar to that observed in the cultured postnatal cells. Distinct voltage-dependent currents emerged during different stages of stem cell development. These findings are similar to results recorded from cultured bone marrow stem cells, for which the gradual maturation of physiological parameters and antigen expression were concomitant. Full maturation of K⁺ and Ca⁺ channels seemed to occur after 14 to 28 days in culture and is roughly in agreement with the present findings that the appearance of Na⁺ channels (DIV7) and their maturation at approximately DIV15 does not occur until after antigen expression (unpublished data, 2006). Our data also showed a significant correlation between channel and AP maturity and length of time in culture. Physiological recording from mouse thalamic cells in slice preparations have also noted the significant correlation between AP size and postnatal age. This time-dependent feature may be a useful criterion for classifying the functional stage of maturity for cultured cells, particularly as considerable morphologic variation was encountered in the differentiated cells.

Interestingly, during development we found a mixed population of TTX-sensitive and TTX-insensitive cells. A similar finding was reported by Wu et al., whereby Na⁺ channels were expressed in different types of dorsal root ganglion (DRG) neurons. That study provides complementary evidence that there were distinct differences in the expression levels of TTX-S and TTX-R Na⁺ channels between IB4-negative and IB4-positive small-diameter DRG neurons. This difference in the density of TTX-R Na⁺ channels is responsible for the distinct membrane properties of these two types of nociceptive neurons, suggesting that all RSCs develop Na⁺ channels at the same differentiation time; however, the density and maturity of the channels differ between cell types, leading to the different electrophysiological properties of the channels. Previous work has shown that there are several types or classes of rat RGCs and that these cell classes differ in their maturation during early postnatal life. The latter could not be resolved with the whole-cell patch clamp method. Similar findings were reported by Sun et al.

**FIGURE 4.** (A, D) Voltage-dependent sodium currents (INa) could be recorded on DIV7 after the induction of RSCs. Inward INa was present in differentiated RSCs from DIV7 onward. Sodium currents could be recorded in a voltage clamp configuration (holding potential, −70 mV, voltage step from −80 to +100 mV, 20 mV/step). (B) TTX (1 μM) blocked Na⁺ currents. (C) I/V curves of Na⁺ currents without (○) and with 1 μM TTX (●). Currents were effectively blocked by TTX. (E, F) An example of a cell that was only partially blocked by 1 μM TTX (●).
concerning differentiating neural stemlike cells derived from the nonhematopoietic blood fraction of the human umbilical cord. Further work is necessary to unravel the developmental differences between these sodium channels and their effects on the development of cell morphology and function, including their effect on the evoked action potential arising from the two neural-like cells types containing different amounts of TTX-sensitive and -insensitive Na\textsuperscript{+} channels.

One question that remains is why APs cannot be evoked despite the expression of Na\textsuperscript{+} currents at DIV7. We suspect that it is related to properties of immature Na\textsuperscript{+} channels at some differentiation stages and that only a sufficient density of mature Na\textsuperscript{+} channels at the axon hillock can support evoked APs. During the development of rat retinal ganglion cells, the size and complexity of the dendritic trees were found to increase rapidly during an initial stage of development lasting from late fetal life until approximately postnatal day 12,\textsuperscript{15,21} which is consistent with the morphologic and physiological maturation at DIV15. Given the more mature starting point of RGCs from the postnatal day 1 Long Evans rat comparison group, the earlier appearance of K\textsuperscript{+}, Na\textsuperscript{+} channels, and APs in these neurons is consistent with the normal development of RGCs. Before eye opening, waves of correlated AP firing spread across the RGC layer in response to Ca\textsuperscript{+} flux before the functional maturation of the photoreceptors; these waves of spontaneous APs appear to be crucial for the early topographic organization of connections within the thalamus and cortex.

Successful stem cell transplantation for degenerative retinal disorders depends on a deep understanding of the characteristics of the stem cell.\textsuperscript{24–26} Oriented induction in vitro may be necessary before cell transplantation; access to the electrophysiological function of induced RSCs should assess assessment of the suitability of the RSCs. Our results suggest that functional maturation (i.e., APs) is not achieved until at least 15 days in culture and that RSCs, when differentiated to 25 days, can also develop spontaneous APs, similar to that seen in the comparison group (Figs. 6, 7C). The suitability of these cells for transplantation and their viability for integration within a host retina will require further detailed study.

**TABLE 1.** Characteristics of Action Potentials and Resting Membrane Potentials

<table>
<thead>
<tr>
<th>DIV (d)</th>
<th>Width (ms)</th>
<th>AMP (mV)</th>
<th>RMP (mV)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>45.24 ± 7.95</td>
<td>16.86 ± 4.05</td>
<td>−39.28 ± 8.7</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>29.17 ± 6.18</td>
<td>45.92 ± 9.84*</td>
<td>−56.38 ± 2.77*</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>19.63 ± 3.48†</td>
<td>55.92 ± 6.14*</td>
<td>−54.83 ± 3.81*</td>
<td>6</td>
</tr>
<tr>
<td>Comp</td>
<td>16.01 ± 2.66†</td>
<td>56.64 ± 11.46</td>
<td>−56.73 ± 3.61*</td>
<td>7</td>
</tr>
</tbody>
</table>

Comp, comparison group.

* P < 0.001 vs. DIV10.
† P < 0.05 vs. DIV15.
Their technical assistance.

The authors thank Sheila and David Crewther and Thomas FitzGibbon for their valuable comments on the research and earlier versions of the manuscript, and Jian Liu, Yu-Xiao Zeng, and Chuang-Huang Weng for their technical assistance.

Acknowledgments

The authors thank Sheila and David Crewther and Thomas FitzGibbon for their valuable comments on the research and earlier versions of the manuscript, and Jian Liu, Yu-Xiao Zeng, and Chuang-Huang Weng for their technical assistance.

References