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Two outward potassium current types are expressed during the neural differentiation of neural stem cells

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Research Highlights

(1) In this study, we applied the modified mechanical dissociation method, rather than enzymatic digestion as used in previous studies, to obtain neural stem cells. This method caused minimal damage to the cells and maintained maximal cell viability.
(2) The proliferating neural stem cells selected were capable of differentiating into neural cells, and the differentiation process was accompanied by the expression of potassium currents. After 2 weeks of differentiation and development, the potassium current density increased sharply.
(3) Neonatal rat hippocampal neural stem cells can be induced to differentiate into functional neurons *in vitro*, and the differentiated neurons express two types of outward K^+ currents similar to mature neurons *in vivo.*

Abstract

The electrophysiological properties of potassium ion channels are regarded as a basic index for determining the functional differentiation of neural stem cells. In this study, neural stem cells from the hippocampus of newborn rats were induced to differentiate with neurotrophic growth factor, and the electrophysiological properties of the voltage-gated potassium ion channels were observed. Immunofluorescence staining showed that the rapidly proliferating neural stem cells formed spheres *in vitro* that expressed high levels of nestin. The differentiated neurons were shown to express neuron-specific enolase. Flow cytometric analysis revealed that the neural stem cells were actively dividing and the percentage of cells in the S + G_2/M phase was high. However, the ratio of cells in the S + G_2/M phase decreased obviously as differentiation proceeded. Whole-cell patch-clamp recordings revealed apparent changes in potassium ion currents as the neurons differentiated. The potassium ion currents consisted of one transient outward potassium ion current and one delayed rectifier potassium ion current, which were blocked by 4-aminopyridine and tetraethylammonium, respectively. The experimental findings indicate that neural stem cells from newborn rat campus could be cultured and induced to differentiate into functional neurons under defined conditions *in vitro*. The differentiated neurons expressed two types of outward potassium ion currents similar to those of mature neurons *in vivo*.

Key Words

neural regeneration; neural stem cells; hippocampus; proliferation; differentiation; neurons; patch-clamp; electrophysiological properties; transient outward potassium ion current; delayed rectifier potassium ion current; inactivation; nestin; neuron-specific enolase; grants-supported paper; neuroregeneration

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INTRODUCTION

Since 1992, neural stem cells have been successfully isolated from different regions of the central nervous system, including the hippocampus, subventricular zone and striatum[1-3], and it is thought that they have potential for cell therapy because of their self-renewal and multipotency properties[4-5]. Neural stem cells from the hippocampus appear to survive better than other stem cells following neural transplantation[6-7]. Hippocampal neural stem cells also display long-term survival and up-regulated neurogenesis following integration into host tissue in a rat model of brain injury. Functional neuronal differentiation may aid in the restoration of lost function, perhaps by contributing to trophic support or integrating into functional synaptic networks with contiguous host cells. This raises the possibility that neural stem cells from the hippocampus have great potentials for cell transplantation. To achieve this goal, neural stem cells from the hippocampus should be characterized in detail to determine their capacity for neuronal differentiation.

Although morphological changes and the expression of neural markers in cultured neural stem cells from the hippocampus have been well characterized *in vitro* [8-10], it remains unclear whether these differentiated neurons display functional activities similar to those of neurons *in vivo*. Therefore, it is very important to determine the electrophysiological properties of neural stem cells after neuronal differentiation using the patch-clamp technique[11], which can provide direct evidence of neuronal functionality. The patch-clamp technique can be used to record ionic currents with a glass microelectrode (pipette) in contact with the cell membrane. Under the control of a micromanipulator, the pipette, which is filled with an internal solution, is gently pressed against the membrane to form a seal. Gentle suction leads to the formation of very tight seal, giving electrical resistance in excess of 1 GΩ (called the gigaseal). Thus, the gigaseal ensures that the net current flowing through the pipette is identical to the current flowing through the membrane delimited by the pipette mouth. The gigaseal makes high-resolution current measurements possible while a controlled voltage is applied across the membrane. Thus, the flow of ions through a single channel can be directly observed. Therefore, as an electrophysiological detection method, the patch-clamp technique allows the study of various ion channels in cells[12-15].

Ion channels in cell membranes play major roles in electrical signal transmission in the nervous system. In particular, the potassium ion (K^+) channel maintains a variety of neuronal activities, including membrane potential setting, repolarization/hyperpolarization and extracellular K^+ buffering[16]. Moreover, the expression of K^+ channels in differentiated neurons *in vitro* is particularly important because it directly reflects the response to changes in the microenvironment. For example, in Drosophila central nervous system precursor cells, the K^+ currents are autonomous when cell-cell contacts are made[17]. Moreover, K^+ currents are expressed during the differentiation of mouse neural progenitor cells[18-19]. These studies have demonstrated that the maturation and differentiation of progenitor cells are accompanied by the expression of ion channels, and the activation of ion channels might conversely modulate cell development. It has been shown that K^+ channels are expressed in neurons differentiated from rat embryonic forebrain and neostriatum progenitor cells under conditions that promote differentiation[20-22]. Therefore, the K^+ channel properties provide a basic electrophysiological marker for the functional differentiation of neural stem cells[23]. At present, however, insufficient studies have been performed on the electrophysiological properties of K^+ channels in neural stem cells dissociated from the rat hippocampus, in contrast to the substantial K^+ currents observed in differentiating cells. The electrophysiological properties of differentiated neurons are crucial to their clinical use, because they indicate whether these cells can function as mature neurons. The aim of the present study was to investigate the prolife-

ration and differentiation of neural stem cells from the rat hippocampus *in vitro*, and to characterize K^+ currents recorded during the course of neural differentiation.

RESULTS

Morphological analysis of proliferating and differentiating neural stem cells

The neural stem cells were cultured as shown in Figure 1. Primary progenitor cells from the hippocampus of newborn (24 hours) rats were isolated under sterile conditions and cultured in serum-free medium, and single cells divided and formed small neurospheres after 3 days of *in vitro* culture (Figure 2A). These small neurospheres expanded continuously to form large neurospheres consisting of a few hundred cells. When the enlarging neurospheres reached a critical size, the neurospheres were mechanically dissociated into single cells or small spheres using a micropipette before the cells in the center became necrotic. After a few days, more spheres developed rapidly by multiple divisions of a single cell picked from a preceding neurosphere. Thus, neurospheres were continuously cultured for several passages to form further spheres that could be purified[24] (Figure 2B).

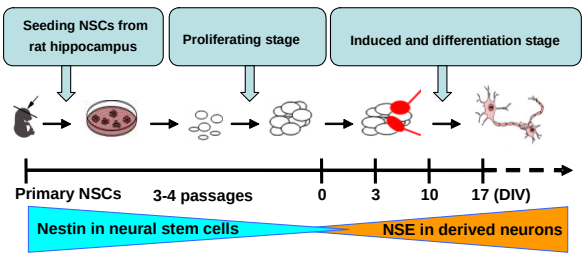

Figure 1 Schematic representation of the proliferation and differentiation of neural stem cells (NSCs) from the hippocampus of newborn rats *in vitro*.

The figure outlines the behavior of NSCs in the course of proliferation and differentiation. Primary progenitor cells were isolated from the hippocampus of newborn rats and underwent self-renewal. These cells generated neurospheres. The neurospheres continuously proliferated for several passages to further form NSCs. The NSCs were induced to differentiate by the removal of growth factors from the medium. Proliferating cells expressed the neural stem cell marker, nestin, and the differentiating cells expressed the neuronal marker, neuron-specific enolase. NSE: Neuron-specific enolase; DIV: days after differentiation *in vitro*.

Neurospheres at passage 3 or 4 were mechanically dissociated and the resulting single cells or small neurospheres were cultured in neuronal induction medium supplemented with 10% fetal calf serum in the absence of growth factors. Cells cultured under these conditions assumed a range of differentiated morphologies (Figure 3A–E).

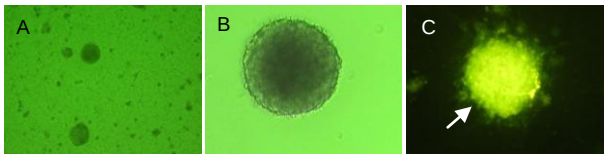

Figure 2 Morphological analysis of proliferating neural stem cells.

(A) Freshly isolated neural progenitor cells aggregated to form free floating small neurospheres after 3 days of culture (inverted microscope, × 200).

(B) A healthy third passage sphere with smooth, round borders undergoing rapid growth (inverted microscope, × 200).

(C) The cells within the proliferative neurosphere were strongly positive for nestin by immunofluorescence microscopy (× 200). The arrow indicates a nestin-positive neurosphere.

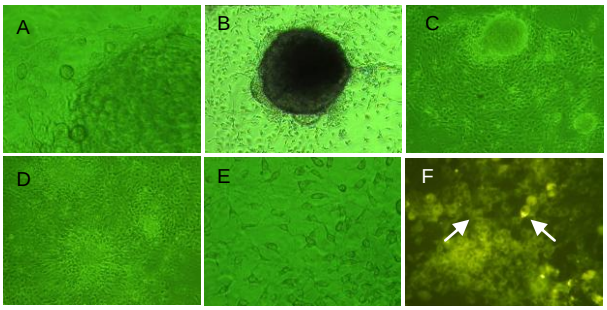

Figure 3 Morphological analysis of differentiating neural stem cells.

(A) Migration of cells from the edge of an adhered neurosphere after 1 day of induction (inverted microscope, × 400).

(B) In the neurosphere center, the cells remained tightly packed after induction for 3 days (inverted microscope, × 100).

(C) Radiating processes developed from the neurospheres and extended to contact adjacent neurospheres after induction for 8 days (inverted microscope, × 100).

(D) The cells spread out from the neurospheres into a monolayer and the center of spheres became high refractive after induction for 12 days (inverted microscope, × 100).

(E) The differentiated cells exhibited extensive branching and interacted to form a network after induction for 19 days (inverted microscope, × 400).

(F) Morphologically mature neurons were positive for neuron-specific enolase (immunofluorescence, × 200). Arrows indicate neuron-specific enolase-positive cells, which showed green fluorescein-5-isothiocyanate (FITC) fluorescence. Green fluorescence was observed in the cytoplasm but not the nuclei.

The floating neurospheres began to adhere after 1–3 days of culture in neuronal induction medium (Figure 3A), and a few cells migrated gradually from the neurospheres (Figure 3B). Radiating processes developed from the edge of the neurospheres and the cells migrated and made contact with adjacent neurospheres over the following days (Figure 3C). The centers of the neurospheres became highly refractive and large numbers of cells spread out from them (Figure 3D). As differentiation proceeded, the differentiated cells showed a typical neuronal morphology characterized by extensive branching and started to form networks with the surrounding cells (Figure 3E).

Identification of differentiated cell types by immunofluorescence microscopy

Specific markers of proliferating and differentiated cells were examined by immunofluorescence microscopy. The proliferating neurospheres expressed high levels of nestin, a specific marker of neural stem cells (Figure 2C), while the differentiated cells expressed neuron-specific enolase, a common marker of neurons (Figure 2F). The immunofluorescence results showed that the self-renewing cells isolated from the hippocampus were neural stem cells, which could be induced to differentiate into neurons.

Analysis of neural stem cell proliferation by flow cytometry

To gain further insight into the cell cycle kinetics accompanying neural stem cell development, we examined the cell cycle at several developmental stages (at passage 3 and on days 3, 10, and 17 after differentiation *in vitro*) by flow cytometry (Figure 4A). In each case, 10 000 cells were analyzed and the cell numbers at different cell cycle stages were expressed as a percentage of the total cell count. The percentage of cells in the S + G_2/M phase is an index of proliferative activity[25-26]. Data were obtained using a FACScan flow cytometer. The percentages of cells in the different phases of the cell cycle (G_0/G_1, S + G_2/M) were analyzed using SigmaPlot software. As shown in Figure 4, the percentage of neural stem cells at passage 3 in the S + G_2/M phase was 36.94 ± 2.07%. Compared with cells before induction, the ratios of cells in the S + G_2/M phase decreased sharply after the induction of differentiation ($P < 0.05$), accounting for 26.39 ± 1.09%, 24.54 ± 1.12% and 23.13 ± 2.31% on days 3, 10 and 17 after differentiation *in vitro*, respectively. This decrease in cell number in the S + G_2/M phase was accompanied by an increase in the cell number in the G_0/G_1 phases. The proliferative activity of neural stem cells was high before induction[27-28]. However, the induction of differentiation caused cell cycle arrest in the G_0/G_1 phases and inhibited DNA replication in the cells. The proliferative activity of the cells gradually decreased with increasing differentiation time, which further demonstrated their differentiation.

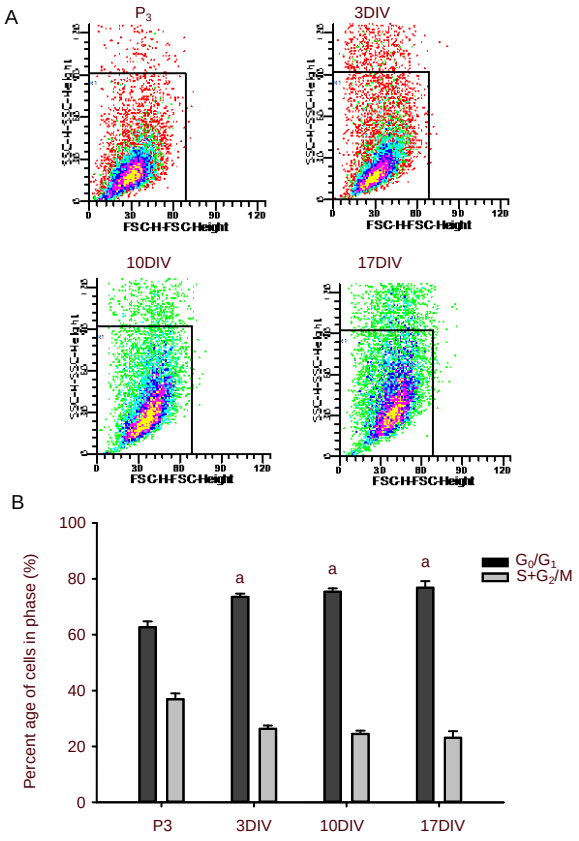

Figure 4 Cell cycle distribution of neural stem cells and differentiated neurons determined by flow cytometry.

(A) Propidium iodide-area plot showing the percentages of cells in the G_0/G_1, S, G_2/M phases of the cell cycle. (B) The graph shows the percentages of cells in the G_0/G_1 and S + G_2/M phases at different developmental stages. The tested cells were fixed with cold ethanol and stained with propidium iodide. The number of cells in each phase was calculated as a percentage, as follows: cell number in each cell cycle phase/total cell count × 100%. Data are expressed as mean ± SEM. All experiments were repeated three times. $^aP < 0.05$, *vs*. S + G_2/M. DIV: Days after differentiation *in vitro*. One-way analysis of variance was used to analyze statistical differences between the control and induced groups, followed by the independent-sample *t*-test.

Measurement of K^+ currents in neural stem cells and differentiated neurons using the patch-clamp technique

The electrophysiological properties of sorted cells at different developmental stages were investigated using the patch-clamp technique in the whole-cell configuration. Under the voltage-clamp pattern, neural stem cells were

electrically unexcitable because depolarizing current pulses failed to elicit K^+ currents. However, the outward K^+ current was recorded in differentiated neurons. A typical current trace is presented in Figure 5A. The outward K^+ current was rapidly activated and steadily increased with depolarization. It had two components: a transient outward component (A-type potassium current; Figure 5B), which peaked and disappeared following bath application of 4-aminopyridine[29-30], and a slowly inactivating component (delayed rectifier potassium current; Figure 5C), which remained after 4-aminopyridine application and was almost completely blocked by tetraethylammonium[29-30]. Therefore, the total outward K^+ currents consisted of transient outward K^+ currents and delayed rectifier K^+ currents, which are sensitive to 4-aminopyridine and tetraethylammonium, respectively. Overall, these results suggest that functional voltage-gated K^+ channels were expressed in the differentiated neurons. The amplitude of the steady current was plotted as a function of the current-voltage relationship (I–V curves; Figure 5D).

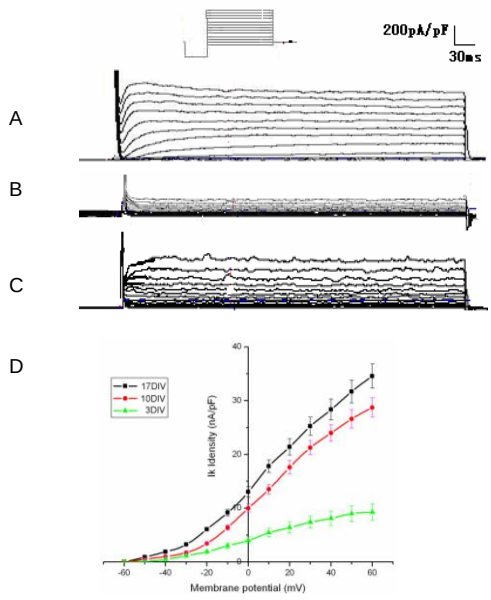

Figure 5 Potassium ion (K^+) currents recorded in differentiated neurons.

(A) Representative recording of outward K^+ currents from differentiated neuron membranes. The currents were recorded with depolarization steps ranging from –70 mV to +50 mV in 10 mV steps applied from a holding potential of –80 mV.

(B) An initial transient current component blocked by 1 mmol/L 4-aminopyridine.

(C) A delayed rectifier current component blocked by 5 mmol/L tetraethylammonium.

(D) The current-voltage curves (I–V curves).

DIV: Days after differentiation *in vitro*.

In the early stage of differentiation (3 days after differentiation *in vitro*), the cells with a less typical neuron-like appearance had a K^+ current amplitude of 8.99 ± 1.46 pA/pF at +50 mV, while the neuron-like cells with a more mature appearance (10 and 17 days after differentiation *in vitro*) had larger K^+ current amplitudes of 26.60 ± 1.69 pA/pF and 31.70 ± 2.14 pA/pF at +50 mV. Our data suggested that the increasing amplitude of the K^+ currents was more obvious when differentiated neurons became more mature in the later stage of the differentiation. The differences were considered significant ($P < 0.05$).

DISCUSSION

The discovery of endogenous neural stem cells in the fetal and adult brain may enable novel therapeutic strategies for neurodegenerative diseases by the development of techniques for isolation, propagation, expansion and differentiation of stem cells[31-33]. The potential use of neural stem cells in the treatment of certain neurological disorders has been investigated in experimental models[34-36]. In the present study, we investigated the morphology and electrophysiological properties of proliferating and differentiating neural stem cells *in vitro* in a broader attempt to provide a theoretic and experimental foundation for the clinical application of neural stem cells.

Our experimental method of harvesting neural stem cells included some slight variations compared with previous approaches[39-40], which used enzymatic or chemical means to dissociate neural stem cells. We used gentle mechanical trituration with fine cell filtration to obtain single cell suspensions from tissue samples. The great advantage of this method is that the harvested cells undergo a minimal degree of manipulation, which is critical for cell viability. Neural stem cells were rapidly expanded after the primary passage and sufficiently purified in proliferative conditions after 3–4 passages. The morphological and immunofluorescence results showed that the self-renewing cells were neural stem cells that could be induced to differentiate into neurons. In the present study, the morphological properties of the cultured neural stem cells were consistent with previous observations reported by Kondon and Dawson[39-40], which showed several distinct phenotypes at different developmental stages. Meanwhile, flow cytometric analysis showed that the induction of differentiation in neural stem cells was associated with a decrease in proliferation, consistent with inhibition of DNA synthesis. Accordingly, the cells were arrested in the G_0/G_1 phase as they differentiated. Therefore, the percentage of cells in the S + G_2/M phase

decreased, suggesting that these cells had lost their ability to proliferate and were indeed induced to differentiate. Overall, our experimental results distinctly showed that neural stem cells could be induced into neurons in our defined culture system without harsh chemicals, cell co-cultures, or genetic manipulations[41].

The functional activity of differentiated neurons is critical when assessing their potential for clinical applications. In mature neurons, K^+ currents regulate neuronal excitability[42]. Thus, the electrophysiological properties of the cells were recorded under voltage-clamp mode in our experiments. The results showed that there were no detectable K^+ currents in the self-renewing neural stem cells. This indicated that the proliferating neural stem cells did not express K^+ channels, at least not functional K^+ channels. In contrast to the neural stem cells, the differentiated cells became electrically excitable after exposure to neuronal differentiation conditions for a few days, when outward K^+ currents started to appear in differentiated cells. The neuronal differentiation process was accompanied by the expression of K^+ currents. The outward K^+ currents consisted of two components: a transient outward K^+ current that was sensitive to 4-aminopyridine, and tetraethylammonium-sensitive delayed rectifier K^+ currents[29-30]. These results indicated that the differentiated neurons expressed potassium channels after neuronal differentiation.

In fact, the amplitude of K^+ currents on the differentiated neurons increased gradually as differentiation proceeded. During the early stage of differentiation *in vitro*, the K^+ currents started to appear at a low density of 8.99 ± 1.46 pA/pF at +50 mV on day 3 of differentiation. Undoubtedly, those early differentiated cells were still functionally immature. Although the K^+ current properties during the early differentiation stage (1–5 days) were unlikely to be comparable with the K^+ current properties of mature neurons, the early stage is still a critical period for regulating neuronal differentiation. The effect of K^+ currents might provide a driving force that triggers rapid cell differentiation after the differentiated cells pass through the transient state. Spontaneous K^+ channel activity was observed in differentiated neurons, particularly in the second week of differentiation. The significantly higher amplitude of K^+ currents exhibited functional neuronal characteristics of 26.60 ± 1.69 pA/pF at +50 mV on day 10 of differentiation. Subsequently, the current density steadily increased on the differentiated neurons as differentiation progressed. The progressive increase in the amplitude of K^+ currents on day 17 of differentiation was similar to that in mature neurons. An increase in outward K^+ currents during neuronal development was observed in several other cell types, such as rat retinal ganglion cells or rat cerebellar granule cells[43-44]. Similar findings were obtained in mouse neural progenitor cells[45-46]. Ryder found that multipotent mouse neural stem cell lines also presented a delayed rectifying K^+ current in the process of neuronal differentiation[45]. Curlew also demonstrated that spontaneous activity indeed occurred in mouse cortical neural progenitor cells during the late embryonic developmental period. This evidence supports our experimental findings.

We presumed that the increasing amplitude of K^+ currents throughout the stages of neural stem cell differentiation might involve several factors. First, long-term treatment with neuronal induction medium regulated the number of K^+ channels and the increased number of ion channels in the cell membrane contributed to cell growth, which could account for the increased amplitude of K^+ currents as differentiation progressed. The differentiation of neural stem cells might be a gradual process regulated by genetic factors or microenvironmental cues. The maturation of K^+ channels in differentiated neurons took 2–3 weeks in differentiation medium, following which the currents are more similar to those observed in mature neurons[47]. Second, the transition between the inactivation and activation of ion channel expression was critical, and was influenced by different configurations of ion channel expression or in some cells by the transient occurrence of synaptic interactions. The activation of K^+ channels might further regulate the maturation of differentiated neurons. Cell maturation was accompanied by stereotypic changes in patterns of K^+ channel expression, and K^+ channel modulation influenced the differentiation of cells. The studies reported by Kraus and Moody[48-49] also showed that the K^+ currents modulated the differentiation of mouse neural progenitor cells. Of course, the exact mechanisms need to be fully investigated.

However, our results were not completely consistent with previous observations by Hogg and Chipperfield *et al* [50-51]. In Hogg's studies, adult hippocampal neural stem cells from Dark rats expressed inwardly rectifying K^+ currents. Undeniably, different types of potassium channels expressed in neuron-like cells have been reported by different laboratories. We consider that the differences in K^+ channel expression and the properties of the membrane currents are related to differences in the types of differentiated neurons, the types of K^+ channels, and the culture conditions. Because the neural stem cells used were derived from different species, they might generate neurons with different functional roles. In

our study, neural stem cells from newborn (24 hours) rats were different from adult neural stem cells derived from Dark rats in Hogg's study. Furthermore, it is conceivable that the changes in the functional properties of K^+ currents in the differentiated neurons might be related to the different expression and assembly of K^+ channel subunits. In rat brain, voltage-gated K^+ channels consist of a large homotetrameric transmembrane complex[52]. Specific expression patterns of the genes in brain have been demonstrated during development[53]. The genes encoding K^+ channel subunits are differentially regulated during development[54]. Moreover, the difference in culture conditions and differentiation state of neural stem cells might help to explain why our results differed from those of other groups. Of course, the electrophysiological properties of neural stem cells from the hippocampus need to be further investigated.

At present, the possibility of propagating and differentiating neural stem cells has led to new developments in reconstructive transplantation for clinical applications, even though neural stem cells are still under investigation for their capacity to differentiate into specific types of neurons[55-57]. However, there are many problems to address regarding the use of neural stem cells[58]. The number of neural stem cells and neurons is inadequate for therapeutic transplants, and the survival of engrafted neurons remains poor. Therefore, it is important to develop *in vitro* cell culture models and to harvest sufficient neural stem cells and functional neurons to overcome some of these obstacles. The electrophysiological characteristics of differentiated neurons *in vitro* might help to design studies and uncover the mechanisms of nervous system development.

In summary, our results strongly suggest that neural stem cells from the hippocampus are capable of differentiating into neurons with typical neuronal morphology and basic electrophysiological properties of neurons in our culture system. The process of cell differentiation and maturation was accompanied by the increased expression of K^+ currents. The differentiated neurons expressed two types of outward K^+ currents (sensitive to 4-aminopyridine or tetraethylammonium), which were essential for the neuronal function of these cells.

MATERIALS AND METHODS

Design

A contrast observation experiment involving *in vitro* cell culture.

Time and setting

Experiments were performed in the Department of Physiology and Neurobiology, Xinxiang Medical University, China, from May 2008 to November 2011.

Materials

Sixty newborn (24 hours) Sprague-Dawley rats were used for the neural stem cell extraction and cultivation experiments. All rats were provided by the Experiment Animal Center of Xinxiang Medical University, China (certificate No. SYXK 2006-0012). All experimental protocols were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[59].

Methods

Isolation, proliferation and purification of neural stem cells

Neural stem cells were isolated from the hippocampus of newborn rats[60-61]. The harvested hippocampal tissue was washed in ice-cold Hank's balanced salt solution and mechanically dissociated with a scalpel and triturated with a small tip. The tissue mixture was passed through a 200-μm strainer to release single cells from surrounding tissue and centrifuged at 1 500 r/min for 5 minutes. The cells were washed three times with Hank's balanced salt solution and resuspended as single cells in serum-free neurosphere medium, comprising Dulbecco's modified Eagle's medium/Nutrient Mixture F12, 2% B27 (Gibco, New York, NY, USA), 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor (Sigma, St. Louis, MO, USA) and 100 U/mL penicillin-streptomycin.

Primary dissociated cells were incubated in a 5% CO_2 humidified atmosphere at 37°C and cultured in 50:50 fresh and conditioned neurosphere medium, which was changed twice a week. Within 3–7 days, the cells grew as free floating small neurospheres, which were formed from clusters of proliferating cells. Typically, large neurospheres were harvested after 20–25 days from the primary passage and dissociated gently into single cells and seeded in new flasks. Generally, the neurospheres rapidly expanded for successive passages every 7 days after the primary passage and these cells continued proliferating until the start of the differentiation experiments.

Differentiation of neural stem cells

After the neural stem cells had expanded sufficiently and were purified by 3–4 passages in proliferative conditions, these neurospheres were dissociated mechanically into single cells or small neurospheres for further culture in

neuronal induction medium. To differentiate neural stem cells, 10% fetal calf serum (Hyclone, Logan, UT, USA) was added to Dulbecco's modified Eagle's medium/Nutrient Mixture F12 and basic fibroblast growth factor and epidermal growth factor (Sigma) were withdrawn from the medium. The medium was replaced every 3 days.

Detection of differentiated cell types by immunofluorescence microscopy

Immunofluorescence microscopy was performed to identify the differentiated cells. The proliferating neurospheres were collected, seeded onto coverslips and allowed to air-dry for 1 hour. The differentiated cells were also seeded onto coverslips and cultured for 3–15 days in neural induction medium. The coverslips were coated with poly-L-lysine[62]. All of the cells on coverslips were analyzed by immunofluorescence microscopy[63]. Briefly, the cells were washed with PBS and fixed with 4% paraformaldehyde. After blocking with 5% bovine serum albumin, the cells were incubated overnight at 4°C with rabbit anti-nestin antibody (1:200; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China) and rabbit anti-neuron-specific enolase antibody (1:300; Beijing Biosynthesis Biotechnology). Between each step, the cells were rinsed with PBS. Finally, the FITC-conjugated goat anti-rabbit IgG (1:100; Beijing Biosynthesis Biotechnology) was added and incubated for 2 hours, following which the cells were washed three times in PBS. The stained cells were analyzed under an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Analysis of cell proliferation by flow cytometry

The proliferative activity of the cells was assessed with a FACScan (Beckman Coulter, Epics)[64]. The cell cycle was examined at several stages by propidium iodide staining. Cells for testing were harvested by centrifugation at 1 500 r/min for 5 minutes, washed thrice with PBS and fixed with 70% cold ethanol at 4°C. Subsequently, the fixed cells were washed twice with PBS and resuspended in a staining solution containing propidium iodide (20 μg/mL) and RNaseA (100 μg/mL). The cell suspensions were incubated at 37°C for 30 minutes in the dark. Data were obtained and analyzed using FACScan and SigmaPlot software to discriminate G_0/G_1, S and G_2/M phase cells. For all samples, 10 000 cells were analyzed. The percentage of cells in S + G_2/M phase was regarded as a measure of proliferative activity[25-26].

Analysis of K^+ currents using the patch-clamp technique

Whole-cell perforated patch recordings were used to detect K^+ currents in sorted cells, which were cultured in medium for proliferating neural stem cells (cells from neurosphere at passage 3) or differentiated cells (on days 3, 10 and 17 after differentiation *in vitro*). The recording micropipettes (resistance 2–5 MΩ) were filled with internal solution (140 mmol/L KCl, 4.0 mmol/L MgATP, 2.0 mmol/L $MgCl_2$, 5.0 mmol/L EGTA, 10.0 mmol/L HEPES; pH 7.4). The cells were maintained in an extracellular solution (145 mmol/L NaCl, 5.4 mmol/L KCl, 2.0 mmol/L $CaCl_2$, 2.0 mmol/L $MgCl_2$, 10 mmol/L glucose, 10.0 mmol/L HEPES; pH 7.4). Application of TTX or $CdCl_2$ was used to block Na^+ currents and Ca^{2+} currents[65-66], respectively. Then, 1 mmol/L 4-aminopyridine was added to the external test solution to inhibit transient outward K^+ currents and 5 mmol/L tetraethylammonium was applied to inhibit slowly inactivating delayed rectifier K^+ currents.

The currents were measured with a voltage-clamp amplifier Axopatch 200B and digitized using a Digidata-1200 AD converter (AXON, New York, NJ, USA). The voltage-clamp protocol consisted of a depolarizing pulse from –70 mV to +50 mV with a 10 mV increment for 60 ms from –80 mV holding potential. All experiments were performed at room temperature.

Statistical analysis

Data are expressed as mean ± SEM. Differences in current changes were tested with one-way analysis of variance followed by independent sample *t*-test (SPSS, Chicago, IL, USA), and considered significant when $P < 0.05$.

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