Neuroscience 149 (2007) 38–52

DIFFERENTIATING EMBRYONIC STEM–DERIVED NEURAL STEM CELLS SHOW A MATURATION-DEPENDENT PATTERN OF VOLTAGE-GATED SODIUM CURRENT EXPRESSION AND GRADED ACTION POTENTIALS

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Abstract—A population of mouse embryonic stem (ES)–derived neural stem cells (named NS cells) that exhibits traits reminiscent of radial glia-like cell population and that can be homogeneously expanded in monolayer while remaining stable and highly neurogenic over multiple passages has been recently discovered. This novel population has provided a unique *in vitro* system in which to investigate physiological events occurring as stem cells lose multipotency and terminally differentiate. Here we analysed the timing, quality and quantity of the appearance of the excitability properties of differentiating NS cells which have been long-term expanded *in vitro*. To this end, we studied the biophysical properties of voltage-dependent Na^+ currents as an electrophysiological readout for neuronal maturation stages of differentiating NS cells toward the generation of fully functional neurons, since the expression of neuronal voltage-gated Na^+ channels is an essential hallmark of neuronal differentiation and crucial for signal transmission in the nervous system. Using the whole cell and single-channel cell-attached variations of the patch-clamp technique we found that the Na^+ currents in NS cells showed substantial electrophysiological changes during *in vitro* neuronal differentiation, consisting mainly in an increase of Na^+ current density and in a shift of the steady-state activation and inactivation curves toward more negative and more positive potentials respectively. The changes in the Na^+ channel system were closely related with the ability of differentiating NS cells to generate action potentials, and could therefore be exploited as an appropriate electrophysiological marker of ES-derived NS cells undergoing functional neuronal maturation.

Key words: neural stem cells, sodium currents, neuronal differentiation, whole-cell patch-clamp, action potentials, TTX.

Recent advances in stem cell technology have led to the purification and *in vitro* propagation of stem cells from fetal and adult tissues of various species, including humans (Weissman et al., 2001). In relation to the CNS, many strategies have been exploited to try to achieve a renewable and stable source of neural stem (NS) cells that can be propagated while remaining highly neurogenic over passages.

Classically, NS cells have been grown as neurosphere cultures, heterogeneous aggregates comprising cells population at different stages of maturation, including a minor fraction of NS cells. The neurosphere is considered to act as a niche in which a limited number of stem cells can undergo self-renewal, and whose progeny can express neuronal antigens *in vivo* and *in vitro* (Reynolds and Weiss, 1992; Gritti et al., 1999). However, although neurospheres have been valuable in many experimental paradigms (including transplantation studies), their cellular complexity together with the reduced stability of the system over time (Rossi and Cattaneo, 2002; Pluchino et al., 2005; Morshead et al., 2002) make them an inappropriate system for systematically defining the gradual physiological changes characterising the transition of a stem cell to a fully mature neuron.

In the light of these limitations, a number of research groups have adopted approaches aimed at generating sources of neural stem cells with optimal properties. Recently, some of the current authors have contributed to the development of a novel and efficient strategy for isolating and propagating a NS population in pure cultures. Such cells were first described by Conti et al. (2005), and were demonstrated to undergo neuronal differentiation after long-term *in vitro* expansion. In particular, the NS-derived neurons express neuronal markers such as β3-tubulin, MAP2 and synaptophysin, thus indicating the prospect of their functional maturation, a finding that was further substantiated by the recording of action potentials in long-term differentiated neurons (Conti et al., 2005).

NS cells can be derived from embryonic stem (ES) cells as well as from fetal and adult brain (Conti et al., 2005; Pollard et al., 2006). By virtue of their homogeneity and stable neurogenic potential, they represent an ideal system for comprehensively investigating the electrophysiological properties of stem cell derivatives while allowing an analysis of the appearance of specific and specialised ion currents over time. Our analysis may ultimately provide an electrophysiological signature and end-point assays capable of defining the propensity of a given NS-derivative to give rise to the desired neuron. This kind of information is often missing in the literature, mainly because the

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Abbreviations: AHP, afterhyperpolarisation potential; EBSS, Earle's balanced salt solution; EGTA, ethylene glycol-bis(2-aminoethylether)-tetraacetic acid; ES, embryonic stem; Hepes, 2-hydroxyethyl-piperazine butane sulfonic acid; NS, neural stem; P, postnatal day; PBS, phosphate-buffered saline; t_p, time to peak; TTX, tetrodotoxin; τ_m, time constant of activation.

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doi:10.1016/j.neuroscience.2007.07.021

neurosphere-based NS cell cultures were not amenable to such detailed electrophysiological characterisation because of the intrinsic heterogeneity of the neurosphere system (Morshead et al., 2002; Toma et al., 2001).

Here we investigated the timing, quality and quantity of the appearance of the excitability properties of differentiating NS cells previously generated from ES cells and long-term expanded *in vitro*. To this purpose, we analysed the biophysical properties of voltage-dependent Na^+ currents as an electrophysiological tool for testing neuronal maturation of differentiating NS cells toward the generation of fully functional neurons, since the expression of neuronal voltage-gated Na^+ channels is a fundamental stage for the generation of tetrodotoxin (TTX) -sensitive action potentials (Kandel, 2000) and for the establishment of key properties for the transfer of information in the nervous system.

We believe that our findings may provide evidence of a strict correlation between the ability of NS cells to generate graded action potentials and the expression of voltage-gated Na^+ channels during *in vitro* differentiation, which indicates that, when exposed to appropriate neuronal differentiating conditions, NS cells are fully capable of becoming electrophysiologically active and acquiring properties typical of mature neuronal cells.

EXPERIMENTAL PROCEDURES

ES-derived NS cell culture and differentiation

The ES-derived NS cells (LC1 cell line) were established from 46C ES cell line and normally grown as previously described (Conti et al., 2005). Briefly, established NS cells were routinely grown in Euromed-N medium (Euroclone, Celbio, Pero, Italy) supplemented with N2 mix (1%, GIBCO, Invitrogen, San Giuliano Milanese, Italy) and epidermal growth factor (EGF) and fibroblast growth factor (FGF-2) (10 ng/mL each; Peprotech, Tebu-Bio, D.B.A.Italia s.r.l., Segrate, Italy). They were passaged using Accutase solution (Sigma, Milano, Italy) with the cells being divided into ratios of between 1:3 and 1:5 every 2–3 days. In this study we employed LC1 cells that have been expanded *in vitro* for at least 20 passages. Neuronal differentiation procedure was carried out as elsewhere extensively described (Conti et al., 2005).

Primary cultures of hippocampal neurons

Hippocampal neurons were obtained from 18- to 19-day-old rat fetuses and maintained in culture for 4–8 days. The pregnant rats were anaesthetised with ether and killed by decapitation. All experiments conformed to Italian and international guidelines on the ethical use of animals. The number of animals used and their suffering was minimised. The fetuses were removed from the uterus and decapitated. The brains were removed and transferred to a Petri dish containing Earle's balanced salt solution (EBSS, Gibco). Cells were mainly isolated from the CA1–CA3 regions of the hippocampus by trituration in EBSS without enzymatic treatment, obtaining a yield of about 3×10^5 cells per hippocampus. The neurons were plated at a density of 1.5×10^5 cells mL^{-1} in poly-D-lysine-coated culture plates, containing α-DMEM (Sigma) with 25 mM Hepes and enriched with 2 mM glutamine, 10% glucose and 10% horse serum; they were grown in a 5% CO_2 humidified atmosphere at 37 °C. The predominant neuronal cell type was represented by pyramidal neurons, well distinguishable from other cell types present in the cultures.

Immunocytochemical analysis of endogenous sodium channel

ES-derived NS cells were seeded onto polyornithine-coated glass coverslips and grown in differentiative conditions for a number of days (Conti et al., 2005). After this period, the cultures were rinsed twice with phosphate buffered saline (PBS) and fixed in paraformaldehyde 4%. The cells were then permeabilised with 0.5% Triton X-100 for 15 min. After a brief rinse with PBS, the cells were incubated for 60 min at room temperature in blocking buffer (1% bovine serum albumin and 5% non-fat milk in PBS) and then incubated overnight at 4 °C with affinity-purified anti-sodium channel (Chemicon, Italy) rabbit polyclonal antibody (1:200 in blocking buffer). Secondary goat anti-rabbit IgG antibody conjugated with Alexa 546 (Molecular Probes, Invitrogen, Italy) was added for 2 h in blocking buffer. The coverslips were then rinsed and incubated with a Hoechst Solution (Molecular Probes) and subsequently mounted in antifading solution (Dako, Italy). The immunostained cells were examined by means of a Zeiss microscope.

Solutions for electrophysiological recording

For whole-cell patch recordings seals between the electrodes and cells were established in a bath solution consisting of (in mM) 155 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 3 KCl, and 10 Hepes/NaOH (pH 7.4). To avoid contamination of voltage responses with low threshold Ca^{2+} spikes, the bath solution contained also the Ca^{2+} channel blocker $NiCl_2$ (1 mM) when required. After the whole-cell configuration had been established, the pipette filling solution for current-clamp and total ionic current recordings in voltage-clamp contained (in mM) 128 KCl, 10 NaCl, 11 EGTA, and 10 Hepes/KOH (pH 7.4). The patch pipette for the study of voltage-gated Na^+ currents under voltage clamp conditions was filled with (in mM) 120 CsCl, 10 NaCl, 20 TEA-Cl, 10 EGTA, 2 $MgCl_2$, 4 Mg-ATP, and 10 Hepes/CsOH (pH 7.4). In order to dissect the Na^+ currents, the extracellular solution contained (in mM) 135 NaCl, 1 $CaCl_2$, 2 $MgSO_4$, 10 glucose, 5 tetraethylammonium-Cl, and 10 Hepes/NaOH (pH 7.4). A multi-barrel delivery system positioned close to the cell allowed the rapid exchange of the external solutions.

For cell-attached patch recordings the pipette solution consisted of (in mM): 145 Na-aspartate, 5 4-aminopyridine, 1 $MgC1_2$, 35 TEA-Cl, 1 $BaCl_2$ and 10 Hepes/NaOH (pH 7.4). Membrane potential was zeroed with a solution containing (in mM): 135 potassium gluconate, 1 $MgCl_2$, 0.2 $CdCl_2$, 5 NaCl, 10 Hepes/KOH, 5 EGTA and 300 nM TTX (pH 7.4).

Patch-clamp recordings and data analysis

The patch pipettes were made of borosilicate glass tubing (Hilgenberg GmbH, Malsfeld, Germany) and fire-polished to a final resistance, when filled with internal solutions, of 0.5–2 MΩ for whole-cell recording and 4–5 MΩ for single channel recording. Electrodes for single channel recording were also Sylgard (Dow-Corning) coated. All of the experiments were performed at room temperature (22–24 °C). Both whole-cell and single channel currents were recorded using an Axopatch 200A amplifier (Axon Instruments Inc., Burlingame, CA, USA), digitised at sampling intervals of 10–50 μs using a DigiData 1200 AD/DA converter (Axon Instruments Inc.). Stimulation, acquisition, and data analysis were carried out using PCLAMP (Axon Instruments Inc.) and ORIGIN software (Microcal Software Inc., Northampton, MA, USA). Fast capacitive transients were reduced on line by means of analog circuitry. Residual capacitive and leak currents were removed by P/4 subtraction for whole-cell recordings, whereas for single channel recordings they were removed by subtracting from each active sweep an idealised current obtained by fitting the averaged silent traces (nulls) with a multi-exponential function. The currents were filtered at 5 kHz. During whole-cell current recording series resistance compensation for the pipette resis-