cAMP promotes differentiation of rodent neuronal progenitor cells

Guilherme Lepski,1,2 Cinthia Elim Jannes,1 Guido Nikkhah1
1Department of Functional and Stereotactic Neurosurgery, Albert Ludwigs University, Freiburg im Breisgau; 2Department of Neurosurgery, Eberhard Karls University, Tübingen, Germany

Abstract

Numerous studies have described neuronal differentiation of neuronal progenitor cells derived from fetal tissue in vitro, but the biochemical mechanisms underlying this process remain largely unknown. In the present study, the role of cAMP in promoting functional maturation of neuronal progenitor cells (NPCs) from the subventricular zone (SVZ) of rodent fetal brain was investigated. NPCs were extracted from telencephalic vesicles of E14 rat embryos and then expanded in medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). A mature neuronal fate was induced by: i) withdrawal of growth factors (basal condition); ii) addition of brain-derived neurotrophic factor (BDNF); or iii) addition of isobutylmethylxantine (IBMX). Whole cell patch clamping assessed electrophysiological properties. Immunocytochemistry for MAP2 confirmed neuronal differentiation. Quantification of neuronal cells, and determination of their electrophysiological properties, was performed in the cited experimental groups. IBMX significantly enhanced the yield of MAP2-positive neurons in the culture system (3.7-fold increase). Application of a one-week differentiation protocol under IBMX induced functional maturity. MAP2-positive cells presented large Na- and K-voltage-dependent currents, fired bursts of action potentials, and exhibited spontaneous synaptic activity. Further, IBMX proved more effective than BDNF for promoting neuronal maturation, leading to higher evoked peak currents and current densities, as well as greater yields of cells firing action potentials and presenting active synaptic contacts. Our data indicates the importance of cAMP-dependent mechanisms for maturation of neuronal progenitor cells in vitro. This knowledge can enable future manipulation of neurogenesis in vitro in order to promote the intrinsic regenerative capacity of the central nervous system.

Introduction

Various different stem cell types have been considered as potential matrices to generate new neurons for central nervous system (CNS) restoration. The most important of these types include neural stem cells (NSCs), extracted from adult and fetal neuronal tissue,1,2 embryonic stem cells (ESC), from the internal layer of blastocysts,3,4 mesenchymal stem cells (MSCs), from adult bone marrow,5,6 and even somatic cells, genetically induced to an immature state, the so-called induced pluripotent stem cells (iPSC).7 We have previously demonstrated that the ability to generate functional neurons varies significantly depending on the stem cell type used.3 Among the different stem cell types, NSCs from fetal tissue have been the most intensively investigated category to date. Studies have shown that neural progenitor cells (NPCs) and NSCs from fetal brains are able to survive in the host tissue, differentiate into mature neurons, and form active synaptic contacts.8-11 Additionally, NSCs are also able to release neurotransmitters and restore functional deficits in Parkinson’s disease,12-14 Huntington’s disease,15 spinal cord injuries,16 stroke,17,18 and motor cortical lesions.19 Although much knowledge has been gained in recent years regarding stem cell biology and plasticity, few studies have demonstrated complete electrophysiological maturation of these cells in vitro.19,20 while the mechanisms underlying this process remain largely unknown.

The key roles of cAMP and the PKA-pathway in neuronal differentiation have been described in previous studies.12-25 However, some controversy remains over these functions, with some authors suggesting that ATP acts preferentially on cell proliferation, serving as a negative regulator of terminal neuronal differentiation.14,24-27 cAMP on the other hand, has been shown to promote neurite elongation in NT2 embryonal carcinoma stem cells,28 human neuroblastoma cell lines29 and pheochromocytoma PC12 cells,30 during neuronal differentiation. This effect is thought to occur through phosphorylation of cAMP response-element binding protein (CREB) at the Ser133 position. CREB phosphorylation induces differentiation of hippocampal progenitor cells in vitro,30 and is also believed to regulate specific phases of adult neurogenesis in the subventricular zone/olfactory bulb system in vivo, since co-expression of pCREB and bromodeoxyuridine has been demonstrated in neuroblasts within the subventricular zone.31

In order to clarify the role of cAMP in neuronal differentiation of progenitor cells derived from the rodent subventricular zone, the immunocytochemical and electrophysiological properties, after induction of a neuronal phenotype, were explored under three experimental conditions: i) basal medium, after withdrawal of growth factors; ii) brain-derived neurotrophic factor (BDNF), and iii) isobutylmethylxantine (IBMX). The yield of MAP2-positive neurons was found to increase 1.6 fold under BDNF, and 3.7 fold under IBMX. Electrophysiological recordings with patch clamp revealed significantly higher amplitudes of peak Na- and K-currents in cells differentiated under IBMX, as well as a higher number of cells firing action potentials and exhibiting spontaneous synaptic activity. These observations emphasize the importance of cAMP and the PKA-pathway in promoting functional maturation of neural progenitor cells.

Materials and Methods

Isolation and differentiation of neuronal progenitor cells from E14 rat embryos

All experiments were approved by the Research Ethics Committee of the Albert-
Ludwig University, Freiburg. Neural progenitor cells were originally isolated from E14 Sprague-Dawley rat fetuses, as previously described. Pregnant females were anesthetized with an intraperitoneal injection of ketamine 10% (Essex Pharma, Munich, Germany), their abdominal cavities opened, and uteruses removed. Fetuses were then extracted and dissected under a Leica M55 stereomicroscope (Leica Microsystems, Heidelberg, Germany) with external light source (Leica KL1500 LCD, Leica Microsystems). The telencephalic vesicle was then dissected, immersed in Hank’s balanced salt solution without MgCl₂ or CaCl₂ (HBSS) (Gibco, Invitrogen, Carlsbad, CA, USA), freed from meninges and surrounding vessels, then digested with trypsin 0.05% (Worthington Biochemical Corp., Lakewood, NJ, USA) and DNase 0.05% (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37°C for 10 minutes. In addition, the tissue was mechanically triturated with a plastic 1mL pipette to obtain a single-cell suspension. Total cell number and viability were assessed with the trypan-blue dye exclusion method.

Neuronal differentiation in vitro

For neuronal differentiation, a previously tested protocol for human fetal neuronal progenitors was used. Briefly, after at least 5 passages, cell aggregates were enzymatically dissociated with Accutase™ (PAA Laboratories, Cülbe, Germany) at 37°C for 12 minutes. They were then mechanically triturated with a fire-polished pipette and plated at high density onto poly-L-ornithin-coated (Sigma) coverslips in differentiation medium. The medium consisted of Neurobasal (NB) (Gibco), L-glutamine (1%, Invitrogen), N2 (1%, Gibco), and P5 (5% Gibco), 200 µg/mL basic fibroblast growth factor (bFGF, Sigma), 20ng/mL epidermal growth factor (EGF, PeproTech EC Ltd, London, UK), and 5 µg/mL heparin (Sigma). The flasks were incubated at 37°C, 5% CO₂, 21% O₂, and 95% humidity. The medium was changed every other day. Cells were fixed and immunostained immediately after electrophysiological recording, which was performed on the seventh differentiation day. Cultures were fixed on the seventh differentiation day with 4% fresh PFA for 1h, immediately after electrophysiological recording, and were processed for immunocytochemistry.

Electrophysiology

Standard whole-cell patch recording methods were used to examine the physiological properties of cultured fetal-derived neuronal cells during in vitro differentiation. The recording conditions are described elsewhere. The intracellular saline solution consisted of 140 mM KCl, 10 mM EGTA, 2 mM MgCl₂, 2 mM Na₂ATP, and 10 mM Heps. The standard extracellular recording saline comprised 125 mM NaCl, 25 mM NaHCO₃, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 25mM Glucose, 2mM CaCl₂, and 1mM MgCl₂ (equilibrated with 95% O₂, 5% CO₂). All recordings were made at room temperature (21-25°C). Recordings were performed under visual control using an inverted microscope and phase contrast optics (Zeiss model IM35, Jena, Germany). Neuronal cells were identified based on their neuritic ramifications, which were usually visible near the cell bodies, especially in large cells. The neuronal phenotype was subsequently confirmed by immunocytochemistry. Voltage-clamp and current-clamp recordings were obtained using an Axopatch 200a patch-clamp amplifier (Axon Instruments Inc., Foster City, CA, USA). Signals were filtered at 5-10 KHz and digitized at 10-20 KHz using a 1041plus interface (Cambridge Electronic Design Ltd., Cambridge, UK) connected to a personal computer, which was used for stimulus generation and data acquisition. Data acquisition and analysis were achieved using custom software (FPulse, U. Frib, Institute of Physiology, University of Freiburg, Germany), running under Igor Pro 5.02 (WaveMetrics Inc., Lake Oswego, OR, USA). For calculation of cell capacitance, 10-50 ms pulses were applied to the cells after whole cell configuration. The decay of the averaged voltage-clamp traces was fitted with a monoexponential curve using a nonlinear least-squares fit algorithm. Cell capacitance was then calculated using the equation \( R_s = \frac{V}{dV/dt} \), where \( R_s \) refers to series resistance. Sodium and potassium currents were evoked by applying 15 voltage pulses from a holding potential of -80 mV, starting at -70mV with 10mV increments up to +70mV. Sodium peak current was determined from 5 data points at the maximum inward current value. Similarly, potassium peak current was calculated from 100 data points.

![Figure 1. Multi-lineage differentiation of neuronal progenitor cells derived from rodent subventricular zone.](image)

A) Mature oligodendrocytes are stained in red with RIP, mature neurons in green with MAPs, and scale bar shown is 50 µm. B) MAP-2-positive neurons are stained in red, whereas astrocytes are stained in green with the antibody anti-GFAP. The Scale bar is 80µm. C) Young neurons are shown stained in green with anti-β III-tubulin. The Scale bar is 50 µm. D) The percentages of neural progenitors (nestin-positive), young neurons (β III-tubulin-positive), and mature neurons (MAP2-positive), were determined under the three experimental conditions, namely, in the control situation without growth factors, in BDNF and finally, in IBMX. Note the larger proportion of mature neuronal cells under IBMX, with no significant difference in the number of immature cells. *P<0.05; **P<0.01; ***P<0.001. E) Fate distribution in cells cultured under IBMX for 7 days. It is important to note that neuronal precursors also stain positively for GFAP.
points at the maximal outward current value. The leakage and capacitive currents were subtracted on-line using a P/-4 protocol as described previously. Current densities were calculated by dividing peak current by cell capacitance. The activation curves were obtained from the respective peak currents assuming ohmic behavior. The activation of Na⁺- and K⁺-currents (I-V relationship) was fitted with a Boltzmann equation, multiplied by a driving force considering the reversal potential for K⁺ and for Na⁺, calculated as -95mV and 80mV, respectively, according to the Nernst equation with the solutions used.

In current clamp mode, action potentials were elicited by clamping the cells at -80mV and applying 10 voltage pulses, starting from -20 and increasing to +70pA at 10pA increments. During the recording session, the cells were filled with biocytin (Molecular Probes, 1mg/mL). All patched cells were fixed and double-stained for biocytin and MAP2, for phenotypic characterization.

Fixation and immunostaining
Immunostaining of cell cultures was carried out as described elsewhere. Briefly, cultures were fixed in fresh 4% PFA (pH 7.2) for one hour, incubated in a blocking solution containing 4% goat serum (Sigma) and 0.1% Triton X-100 (Sigma), then incubated with anti-MAP2ab (1:200, mouse monoclonal, Millipore/Chemicon Corp., Billerica, MA, USA) and antisynaptophysin (1:1000, mouse monoclonal, Millipore/Chemicon) overnight at 4ºC. After washing, cells were incubated in secondary antibodies and 4.6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:10,000, Sigma). Biocytin staining after electrophysiological recording was revealed by fluorescein-avidin D (1:500, Vector Labs, Burlingame, CA, USA). The secondary antibodies used were Alexa Fluor 488 and 594-goat anti-mouse (1:150, Molecular Probes). Fixed tissue, without primary and secondary antibodies, served as negative controls.

Confocal microscopy and cell quantification
Images were obtained with a Leica TCS SP2 confocal system (405 nm diode, Ar/AKr, HeNe 543/594 lasers) using the 20x/0.7 or 63x/1.2 objectives, and digitized at 2048x2048 pixels. For quantification, ten consecutive images across the horizontal diameter of each coverslip were systematically scanned from left to the right for each culture condition. Cells positive for a specific marker were counted relative to the total number of DAPI-stained nuclei using ViewFinder 2.1 software (ERDAS Imagine, Leica Geosystems Geospatial Imaging, Norcross, GA, USA). Experiments were performed in triplicate, and counting was performed in a double-blind manner, using cultures from which some cells had been previously patched.

Statistical analysis
The Mann-Whitney U-non-parametric Test was used to compare means between the groups, since a normal distribution could not be assumed. All data are reported as mean±s.e.m., with significance level (P) indicated as follows: *P<0.05; **P<0.01; ***P<0.001.

The SPSS 13.0 Software package (IBM Corporation, Somer, NY, USA) was used for statistical computations. Graphs were constructed with Igor 5.02 (WaveMetrics).

Figure 2. cAMP enhances the yield of MAP2-positive neurons in culture. A-D) Confocal pictures of differentiated cells derived from E14.5 telencephalic tissue, cultured under various conditions after 7 days in vitro. Cell nuclei are stained with DAPI in blue; green represents MAP2-Alexa Fluor 488. Scale bars A-D are 150 um. E) Quantification of MAP2 positivity, expressed in percentage of positive cells in relation to total cells. Note that NT3 and BDNF, when added to basal medium (BM), promote an increase in the yield of neuronal cells of the same magnitude. IBMX has a more marked effect however, causing a 5-fold increase in number of neuronal cells. Interestingly, the addition of serum to the culture system reduces the percentage of neuronal cells, possibly by inducing the growth of immature cell types. *P<0.05; **P<0.01; ***P<0.001.
Results

cAMP enhances yield of MAP2-positive neurons in culture

The expression of MAP2 in differentiated cells was first investigated under the three experimental conditions for 7 days in vitro. After withdrawal of growth factors, 6.38±.18% of the cells expressed MAP2 in basal medium. This number increased to 10.26±1.26 (P<0.05) under BDNF, and similarly to 10.85±1.28 in NT3 (P<0.05). A more pronounced increase in the neuronal cell percentage was observed under IBMX (23.58±4.07, P<0.001). Additionally, IBMX was able to induce differentiation of both oligodendrocytes (RIP-positive) and astrocytes (GFAP-positive) (Figure 1A-C,E). Notably, the proportion of immature neural progenitors (nestin-positive) and young neuronal cells (β III-tubulin) did not differ significantly across the experimental groups (Figure 1D).

Interestingly, when fetal bovine serum was added to the differentiation medium, the effect of BDNF and IBMX was masked. Specifically, the yield of MAP2-cells was 4.28±1.30 in basal medium, 2.09±0.56 in BDNF, 3.48±1.50 in NT3 and finally, 4.87±1.28 in IBMX (Figure 2). These data indicate the importance of BDNF and NT3 for neuronal differentiation, and point to a more significant contribution of IBMX.

cAMP promotes functional maturation of neural precursor cells within seven days in vitro

Patch clamp recordings of electrophysiological events revealed that cAMP was able to induce full maturity in a 7-day time window. On the 7th differentiation day, large K-currents (6.4±1.5 nA) and Na-currents (2.5±0.6 nA) were recordable. Accordingly, K- and Na-current densities, which correspond to the density of ionic channels on cell surfaces, were 861±193 pA/pF and 383±109 pA/pF, respectively. At this stage, 50% of the cells fired action potentials, and 16.7% displayed spontaneous synaptic activity. Cells with action potentials fired at 21.6±8.3 Hz, and the peak amplitude was measured as 49±14 mV. Figure 3 illustrates a high proportion of MAP2-positive cells (panel A), as well as the co-staining of MAP2 and synaptophysin in the dendrites of neuronal cells (panel B). During recording sessions, cells were filled with biocytin and then stained for MAP2, thus confirming neuronal fate. Figure 3C illustrates two recorded cells with long and ramified neuritic extensions. Inward and outward currents are represented in Figure 3D, and voltage-dependent activation is depicted in Figure 3E. Bursts of action potentials and spontaneous synaptic activity were also observed (Figure 3F and 3G, respectively).

Figure 3. cAMP promotes functional maturation of neural precursor cells within seven days in vitro. A-C) Confocal pictures of differentiated cells, cultured under IBMX in basal medium for 7 days. A-B) Green represents mature neuronal cells stained with MAP2, revealed with Alexa-Fluor488, whereas red represents pre-synaptic vesicles stained with synaptophysin-Alexa594. Note some pre-synaptic vesicles along MAP2-positive dendrites of mature cells; some vesicles can be seen in non-stained dendrites, probably from immature cells. C) Patched cells were filled with biocytin (green, FITC) during electrophysiological recording, allowing subsequent confirmation of neuronal fate, since positivity for MAP2 (red) was demonstrated. Scale bars are as follows: A:50 um, B:25 um, C:70 um. D) Currents evoked in voltage clamp mode, after application of 15 voltage pulses from a holding potential of -80 mV, at 10 mV increments; capacitive and leakage currents were subtracted using a p/-4 protocol (see text). E) Voltage-dependent activation of sodium (left) and potassium (right) currents, i.e. I-V plots. Curve fitting with a theoretical model predicted by a non-raised Boltzmann function multiplied by a driving force, considering the Nernst potential for each ion with the solutions used (for more details see text and references). F) Bursts of action potentials, evoked after application of 20 pA for 100 ms. G) Spontaneous synaptic currents recorded in the same cell illustrated in D-F.
Isobutylmethylxantine is more effective than brain-derived neurotrophic factor for inducing functional maturity

Analysis of the electrophysiological properties of cells differentiated in basal medium, BDNF, and IBMX, revealed strong evidence of increased maturity in the IBMX-group (Figure 4). First, the membrane time constant was significantly lower (28.1±7.1 for IBMX, 26.9±6.8 for BDNF, and 40.2±15.3 for basal medium, P IBMX x BM <0.01). Second, current peaks were considerably higher for sodium, measuring 2.5±0.6 nA in the IBMX-group (P<0.001), 511±138 pA in the BDNF-group (P<0.01), and 633±50 pA in the basal medium group. Similarly, amplitudes of K-peak currents were considerably higher in the IBMX-group, although this difference did not reach statistical significance (Figure 4A). Current densities are reliable indicators of ionic channel density on cell surfaces. Both Na and K- current densities were significantly higher in the IBMX-group, as shown in Figure 4B and 4D (P<0.001 for Na and K). Moreover, a higher yield of neuronal cells in the IBMX group fired action potentials (IBMX 50%, BDNF 21.7%, BM 13.5%) and exhibited spontaneous synaptic activity (16.7%, 4.3% and 0, respectively).

Discussion

Clonal-expanded neuronal precursor cells from the subventricular zone represent a viable option for neural restoration and offer several advantages, namely: i) clonal expansion allows the obtention of a large number of cells from a single fetal brain, thereby resolving the dilemma over limited availability of fetal tissue for transplantation purposes; ii) clonal expansion does not prevent acquisition of a mature neuronal fate, despite reduced neurogenic potential due to aging; iii) clonal-expanded neuronal precursors from the SVZ are able to develop into electrophysiologically active neurons both in vitro and in vivo, after transplantation. However, the assumption that other stem cell types have similar neurogenic potential as NPCs from the SVZ requires further experimental confirmation. Comparisons of adult mesenchymal and neuronal stem cells have clearly demonstrated the limited neurogenic potential of the former type. Another relevant issue is biological safety, especially concerning possible clinical applications. In this regard, a physiological rule can be applied: the more immature the stem cell, the higher the chances of malignant degeneration. This is because the proliferative potential is high and difficult to control. In fact, ESCs have been shown to induce the formation of malignant teratomas in up to 25% of implanted animals.

Various studies have demonstrated the importance of the cAMP and PKA-pathway in neuronal differentiation. P1 receptors are activated by adenosine and act through G protein. P2 receptors on the other hand, are activated by ATP and can be metabotropic, G-protein-coupled (P2Y), or ionotropic, causing transient influx of Ca2+ (P2X). In cells, both second messengers Ca2+ and cAMP can induce phosphorylation of CREB at Ser133 through protein kinases. CREB is a known transcription factor expressed during neurogenesis in the rostral migratory stream. In vitro, CREB was shown to induce differentiation of hippocampal...
References


