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Functional maturation of isolated neural progenitor cells from the adult rat hippocampus

Ron C. Hogg, Hiram Chipperfield, Kathryn A. Whyte, Mark R. Stafford, Mitchell A. Hansen, Simon M. Cool, Victor Nurcombe and David J. Adams

School of Biomedical Sciences, University of Queensland, Brisbane, Queensland 4072, Australia

Abstract

Although neural progenitor cells (NPCs) may provide a source of new neurons to alleviate neural trauma, little is known about their electrical properties as they differentiate. We have previously shown that single NPCs from the adult rat hippocampus can be cloned in the presence of heparan sulphate chains purified from the hippocampus, and that these cells can be pushed into a proliferative phenotype with the mitogen FGF2 [Chipperfield, H., Bedi, K.S., Cool, S.M. & Nurcombe, V. (2002) *Int. J. Dev. Biol.*, **46**, 661–670]. In this study, the active and passive electrical properties of both undifferentiated and differentiated adult hippocampal NPCs, from 0 to 12 days *in vitro* as single-cell preparations, were investigated. Sparsely plated, undifferentiated NPCs had a resting membrane potential of ≈ −90 mV and were electrically inexcitable. In > 70%, ATP and benzoylbenzoyl-ATP evoked an inward current and membrane depolarization, whereas acetylcholine, noradrenaline, glutamate and GABA had no detectable effect. In Fura-2-loaded undifferentiated NPCs, ATP and benzoylbenzoyl-ATP evoked a transient increase in the intracellular free Ca^{2+} concentration, which was dependent on extracellular Ca^{2+} and was inhibited reversibly by pyridoxalphosphate-6-azophenyl-2'-4'-disulphonic acid (PPADS), a P2 receptor antagonist. After differentiation, NPC-derived neurons became electrically excitable, expressing voltage-dependent TTX-sensitive Na^{+} channels, low- and high-voltage-activated Ca^{2+} channels and delayed-rectifier K^{+} channels. Differentiated cells also possessed functional glutamate, GABA, glycine and purinergic (P2X) receptors. Appearance of voltage-dependent and ligand-gated ion channels appears to be an important early step in the differentiation of NPCs.

Keywords: heparan sulphates, intracellular calcium, ion channels, neural progenitors, phenotypic maturation, purinergic receptor

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Introduction

Recent *in vitro* studies have indicated that multipotent, self-renewing progenitors of neurons and glia can be isolated from several adult brain regions (Reynolds *et al*., 1992; Richards *et al*., 1992; Rietze *et al*., 2001; Gage, 2002; Song *et al*., 2002). Moreover, the cells appear to be remarkably plastic: adult hippocampal stem cells can give rise to regionally specific cell types not only in the hippocampus but also in the olfactory bulb, cerebellum and retina (Gage *et al*., 1995; Suhonen *et al*., 1996; Takahashi *et al*., 1998). Thus, neural stem cells from both embryo and adult seem to be 're-programmable', an idea drawn from haematopoeisis, that there is a generic, 'naïve' brain stem cell capable of being manipulated (Anderson, 2001). Several identified growth factors, including epidermal growth factor, fibroblast growth factor (FGF2) and leukaemia inhibitory factor, have been found to be necessary to trigger their

proliferation (Carpenter *et al*., 1997; Carpenter *et al*., 1999; Palmer *et al*., 1999), although FGF2 appears to be crucial (Nurcombe *et al*., 1993; Palmer *et al*., 1999; Ornitz, 2000) for cells that are committed to a neural fate (Qian *et al*., 1997; Qian *et al*., 2000).

We have previously shown that adult hippocampal neural progenitor cells (NPCs) can be cloned from single cells in the absence of laminin, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3) and without the complication of glial cell underlayers, provided they are cultured in the presence of FGF2 (Chipperfield *et al*., 2002). Furthermore, when grown in the presence of FGF2 and purified progenitor cell-derived heparan sulphate (HS), a time-dependent increase in the number of MAP2, β-tubulin III+ve neurons, glial fibrillary acid protein (GFAP)+ve astrocytes and O4+ve oligodendrocytes were observed when compared to clones grown in the absence of HS (Chipperfield *et al*., 2002). Notably, morphologically distinct neurons were readily detected at the earliest periods, with astrocytes and oligodendrocytes only becoming prominent after 10 days. The morphology and growth characteristics of these adult cells were similar to those that have been described previously (Palmer *et al*., 1999). The cells grew from clonal density in FGF-dependent colonies that, on reaching a high density, became detached from the substrate to form 'neurospheres' (Svendsen *et al*., 1998); they were positive for β-tubulin III, MAP-2 expression and synaptophysin.

Recent functional analyses of NPCs from the adult rodent CNS (Song *et al*., 2002) and from human embryonic and fetal brain (Hurelbrink *et al*., 2002; Wu *et al*., 2002) have suggested that isolated NPCs differentiate into mature neurons; moreover, neurons from such multipotent neural precursors appear to form functional synapses (Toda *et al*., 2000; Gage, 2002). Nguyen *et al*. (2002) have recently shown that α-subunit proteins of the glycine ionotropic receptors are expressed by cells within cultured neurospheres derived from postnatal rat striatum. Whole-cell patch-clamp experiments further demonstrated that neural progenitor cells express functional glycine receptors.

The present electrophysiological study shows that freshly isolated NPCs from the adult rat hippocampus undergo maturation of membrane excitability within 7–10 days after the induction of differentiation, and that this maturational process lasts until 18 days *in vitro* (DIV). The appearance of Na^+ and Ca^{2+} channel currents underlying action potential firing indicates that the expression of voltage-gated ion channels develops upon differentiation with time in culture. In addition, the expression of functional purinergic (P2X) receptors in the undifferentiated NPCs may provide a Ca^{2+} influx pathway for the initiation of neuronal differentiation. A preliminary report of some of these results has been presented in abstract form (Hogg *et al*., 2002).

Materials and Methods

NPCs plated on coated coverslips

NPCs were isolated from the hippocampus of adult Dark Agouti rats as described previously (Chipperfield *et al*., 2002). Rats were killed by lethal injection of ketamine and xylazine in accordance with guidelines of the University of Queensland Animal Experimentation Ethics Committee. The cells grew in colonies that, on reaching a high density, became detached from the substrate to form 'neurospheres' (Svendsen *et al*., 1998). The cells were maintained in Neurobasal A (Gibco BRL, Life Technologies, Melbourne, Australia) supplemented with 2% B-27 supplement (Gibco), 0.5 mm glutamine and 10 ng/mL of FGF-2 (Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia). For experiments with undifferentiated precursors, cells were plated onto poly d-lysine-coated 12-mm-diameter round coverslips at a density of 2000 cells/coverslip in maintenance medium and used within 3 days. As described previously (Chipperfield *et al*., 2002), the NPCs were induced to differentiate in the initial experiments by adding a combination of laminin (10 μg/mL), BDNF (10 ng/mL), NGF (10 ng/mL), NT-3 (10 ng/mL) and 1 μg/mL of neural precursor-derived heparan sulphate to the maintenance medium. Subsequently it was found that supplementing the maintenance medium with 10% foetal calf serum (FCS) could induce the progenitor cells to differentiate into cells with a neuronal phenotype. The same batch of FCS was used for all experiments. Differentiated cells were obtained by seeding precursors onto poly d-lysine-coated 12-mm round coverslips at an initial density of 1000 cells/coverslip and culturing for 7–12 days in differentiation media with media changes every 72 h.

Immunofluorescence staining

All cells that were used for immunofluorescence staining were grown on poly d-lysine-coated 12-mm round coverslips. Undifferentiated cells were obtained by seeding progenitors at a density of 2000 cells/coverslip and culturing for 1–2 days before fixation. Differentiated cells were seeded at the same density but were cultured for 7–

12 days in differentiation medium containing 10% FCS. Immunofluorescence staining was carried out at room temperature as described previously (Chipperfield *et al*., 2002). Briefly, cells were fixed with 4% paraformaldehyde in PBS for 15 min, rinsed three times with phosphate-buffered saline (PBS) then incubated for 1 h in blocking buffer (PBS with 5% FCS and 0.5% Triton X-100). Primary antibodies were diluted in blocking buffer at the appropriate concentration and incubated on the coverslip for 2 h followed by three washes in blocking buffer. Secondary antibodies conjugated to fluorescein isothiocyanate, Texas Red or Cy-5 and diluted 1 : 100 in blocking buffer were incubated with the cells for 1 h followed by three washes in PBS. The cells were then mounted in antifade mounting media and visualized on a Bio-Rad M600 confocal microscope. All primary antibodies were diluted to their optimum concentration: mouse antinestin, 1 : 200; mouse anti-MAP2, 1 : 100; mouse anti-growth associated molecule-43 kDa/neuromodulin (GAP-43), 1 : 100; rabbit anti-GFAP 1 : 100 (all Sigma-Aldrich).

The immunohistochemical protocol was based on previous specific P2X purinoceptor immunostaining procedures (Hansen *et al*., 1998; Hansen *et al*., 1999). Neuroprogenitor cell cultures were fixed in 4% paraformaldehyde in borate–acetate buffer (pH 9.5) for 1 h. Preparations were placed in 0.1% dimethyl sulphoxide (DMSO) in phosphate-buffered fetal bovine serum [100 mL PBS, 2 mL foetal bovine serum (FBS), 0.1 mL Triton X-100 and 1 g bovine serum albumin] for 30 min. The preparation was then washed three times in PBS (10 min each) and immersed in 20% FBS in PBS for 1 h to block nonspecific binding sites. This was followed by incubation with the relevant 1 : 100 anti-P2X antibody for 24 h at 25 °C. Slides were rinsed in PBS followed by the addition of the relevant secondary fluorescent antibodies for 90 min at 25 °C. The slides were washed three times in PBS (each for 10 min), coverslipped and sealed. Sections were viewed on a Bio-Rad M600 confocal microscope system, with standard settings. The P2X antibodies used were a gift from Dr Julian Barden (University of Sydney) and have been characterized previously (Hansen *et al*., 1998; Hansen *et al*., 1999).

Western blotting

Cells were grown in poly d-lysine-coated 6-well plates in either maintenance or differentiation medium until semiconfluent (5–7 days). They were then lysed at 4 °C in lysis buffer (PBS, 1% TritonX-100, 10 mm EDTA and 1 mm sodium orthovanadate with protease inhibitors). After centrifugation, a Bradford protein determination was performed and 20 μg/lane was electrophoresed in an 8% SDS-PAGE gel. The protein was then semidry transblotted onto 20-μm nitrocellulose. Membranes were blocked for 1 h in casein blocking buffer, incubated with primary antibody diluted to the appropriate concentration in blocking buffer for 1 h, washed three times and incubated with secondary antibody conjugated to alkaline phosphatase diluted in blocking buffer 1 : 1000 for 1 h. After two washes in blocking buffer, the membranes were equilibrated in NBT buffer and a nitro-blue tetrazolium chloride−5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT–BCIP) colour reaction developed. The primary antibodies used for subsequent quantification were mouse antinestin, mouse anti-GFAP, mouse anti-GAP-43 and mouse antiactin (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia) at dilutions of 1 : 1000.

Whole-cell patch-clamp recording

Undifferentiated and differentiated NPCs were examined using the whole-cell recording configuration of the patch-clamp technique (Hamill *et al*., 1981). At least 200 cells from glia-free cultures (> 150 cells from 0 DIV and > 60 cells from 7–12 DIV) were tested. Neuron-like cells with a large cell body and neurite-like structures were chosen from differentiated cell cultures for patch-clamp examination. Membrane current and voltage were recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments Inc., Union City, CA, USA) from undifferentiated NPCs using the conventional 'dialysed' whole cell recording configuration whereas differentiated NPCs were examined using either the conventional 'dialysed' or perforated-patch whole-cell recording configurations. Patch electrodes were pulled from borosilicate glass capillaries with resistances of < 1.5 MΩ when filled with an intracellular solution. For perforated-patch whole-cell recordings, the patch electrode was backfilled with an intracellular solution containing 240 μg/mL amphotericin B. Access resistances using the perforated-patch configuration were routinely < 4 MΩ following series resistance compensation which was typically 60–80%. Current signals from the amplifier were filtered at 10 kHz through a four-pole low-pass Bessel filter, sampled with a Digidata A/D interface, leak-subtracted on-line using a –P/4 protocol and stored on a hard disk drive of a Pentium III PC computer. Voltage and current protocols were applied using pClamp programs and offline data analyses were performed using Axograph (Axon Instruments) and SigmaPlot (SPSS Inc., Chicago, IL, USA) software. Cells were maintained in physiological saline solution of the following composition (in mm): NaCl, 140; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 1; Hepes–NaOH, 10; and glucose, 10, pH 7.2. A KCl-based internal solution containing (in mm) KCl, 140; EGTA, 5; and Hepes–KOH, 10, pH 7.2, was used for recordings of membrane potentials and currents under dialysed

whole-cell recording conditions. The pipette 'intracellular' solution for perforated-patch whole-cell recordings contained (in mm): K_2SO_4, 75; KCl, 55; $MgSO_4$, 5; and Hepes, 10; pH adjusted to 7.2 with *N*-methyl-d-glucamine. All experiments were performed at 22–23 °C. Changes in the extracellular K^+ concentration were made by isosmotic substitution of NaCl for KCl. Bath application of tetrodotoxin (TTX) and Cd^{2+} were used to block depolarization-activated TTX-sensitive Na^+ currents and Ca^{2+} currents, respectively. Outward K^+ currents were inhibited by replacing K^+ with Cs^+ in the internal solution and Na^+ with tetraethylammonium ions (TEA) in the external solution. Barium (10 μm) was added to the external solution to inhibit inwardly rectifying K^+ currents and Cs^+ (2 mm) to inhibit hyperpolarization-activated cation currents (I_h). Voltage-dependent Ca^{2+} channel currents were recorded using Ba^{2+} (5 mm) as a charge carrier and intracellular Cs^+ and extracellular TEA to suppress outward K^+ currents.

Fura-2 fluorescence ratio imaging

Undifferentiated NPCs plated on poly d-lysine-coated 12-mm glass coverslips at a density of ≈ 2000 cells/well were rinsed once in fresh media then loaded with Neurobasal A containing 5 μm of the fluorescent Ca^{2+} indicator dye Fura-2 acetoxymethylester (Fura-2 AM), and 0.02% Pluronic-127 (Molecular Probes Inc., Eugene, OR, USA). Cells were loaded at room temperature (22–24 °C) for 45 min and then rinsed with physiological salt solution (PSS; in mm) NaCl, 140; KCl, 3; $CaCl_2$, 2.5; $MgCl_2$, 1.2; glucose, 7.7; and Hepes-NaOH, 10, pH 7.2. Cells were allowed to recover in PSS for 20–30 min before ratio imaging to allow for complete desterification of the Fura-2 AM ester. Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed using a Ratiovision™ dual excitation digital imaging workstation (Atto Bioscience, Rockville, MD, USA) attached to a Zeiss upright Axioskop 2 FS microscope (Carl Zeiss, Germany). Light from a 100 W mercury arc lamp (Carl Zeiss, Germany) was passed through a filter switching unit (Atto Bioscience, Rockville, MD, USA) containing 340 nm and 380 nm excitation filters (Omega Optical, Brattleboro, VT, USA). Neutral density filters were placed in front of the 380 and 340 nm filter (OD1.3 and 0.1, respectively) to balance the excitation light intensity and reduce photobleaching. Cells were excited alternately at 340 nm and 380 nm and viewed with a water-immersion ×40 objective lens (Achroplan, 0.75w Ph2, Carl Zeiss Germany). Emitted light from the loaded cells was passed through a 510-nm emission filter (Omega Optical, Brattleboro, VT, USA) and captured using an intensified CCD video camera (Atto). Coverslips were placed in a small volume bath (≈ 250 μL) and immobilized with a small amount of silicone grease. The imaging chamber was superfused at room temperature (22–24 °C) with extracellular solutions of either PSS or Ca^{2+}-free PSS (containing 0.5 mm EGTA) at a flow rate of ≈ 4.5 mL/min. Solutions were rapidly switched using electronically controlled valves connected to a small multiinput solution manifold (Warner Instruments Inc., Hamden, CT, USA). Individual cells were measured at a sampling frequency of 0.3 or 1.0 Hz and images stored on a Pentium III PC. The camera, filter changer and sampling frequencies were controlled via the Ratiovision™ software (Atto Bioscience, version 6.1). Ratio measurements were converted into approximate Ca^{2+} concentrations using R_{min} and R_{max} values and the two-point calibration method described by (Grynkiewicz *et al*., 1985). At the completion of an experiment cells were perfused for 10 min with a Ca^{2+}-free solution (R_{min} solution) containing (in mm) KCl, 150; EGTA, 5; and Hepes-NaOH, 10, pH 7.2. Maximal $[Ca^{2+}]_i$ levels were obtained by bath addition of a high-calcium solution (R_{max}) containing (in mm) KCl, 150; Hepes, 10; $CaCl_2$ 20; and ionomycin (a calcium ionophore), 5 μm. Maximum and minimum $[Ca^{2+}]$ values and a dissociation constant (K_D) of 135 nm were used to estimate $[Ca^{2+}]_i$ (Grynkiewicz *et al*., 1985).

Drugs

All chemical reagents used were of analytical grade. Receptor agonists were applied from an extracellular pipette (3–5 μm diameter) either in response to pressure application (Picospritzer II, General Valve Corp., Fairfield, NJ, USA) or using a rapid piezo application system to minimize rapid desensitization to agonists and receptor antagonists were bath-applied. The following agonists were tested: noradrenaline, acetylcholine (ACh), γ-aminobutyric acid (GABA), glutamate, glycine and adenosine 5'-triphosphate (ATP), adenosine 5'-O-(thio-triphosphate) (ATP-γ-S), α,β-methylene-ATP, benzoylbenzoyl-ATP (BzATP) and uridine 5'-triphosphate. The receptor antagonists pyridoxalphosphate-6-azophenyl-2'-4'-disulphonic acid (PPADS; P2 purinergic receptors), (–)bicuculline methiodide (GABA receptors), dizocilpine maleate (MK-801; glutamate receptors) and strychnine hydrochloride (glycine receptors) obtained from Sigma-Aldrich Pty. Ltd. (Castle Hill, NSW, Australia) were examined on agonist-evoked responses.

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Results

In the present study, we operationally define progenitor cells as 'undifferentiated' when they are nestin-positive cells that are clonal, self-renewing and capable of generating both neurons and glia *in vitro*, and as 'differentiated' when they become nestin-negative, morphologically defined and electrically excitable. Cultures of self-renewing, multipotential adult hippocampal progenitor cells could be induced to differentiate by adding a combination of factors or simply 10% FCS to the FGF-2-containing maintenance medium. The morphology of the differentiated progenitors was markedly different from their undifferentiated counterparts (Fig. 1). These differentiated cells maintained a relatively simple morphology on laminin-coated tissue culture plastic, with an average neurite length of 280 ± 46 μm (n = 66, calculated by image processing as in Chipperfield *et al*., 2002) and an average of 9.9 ± 1.1 neurite branches per cell (n = 101), values similar to those reported by Song *et al*. (2002).

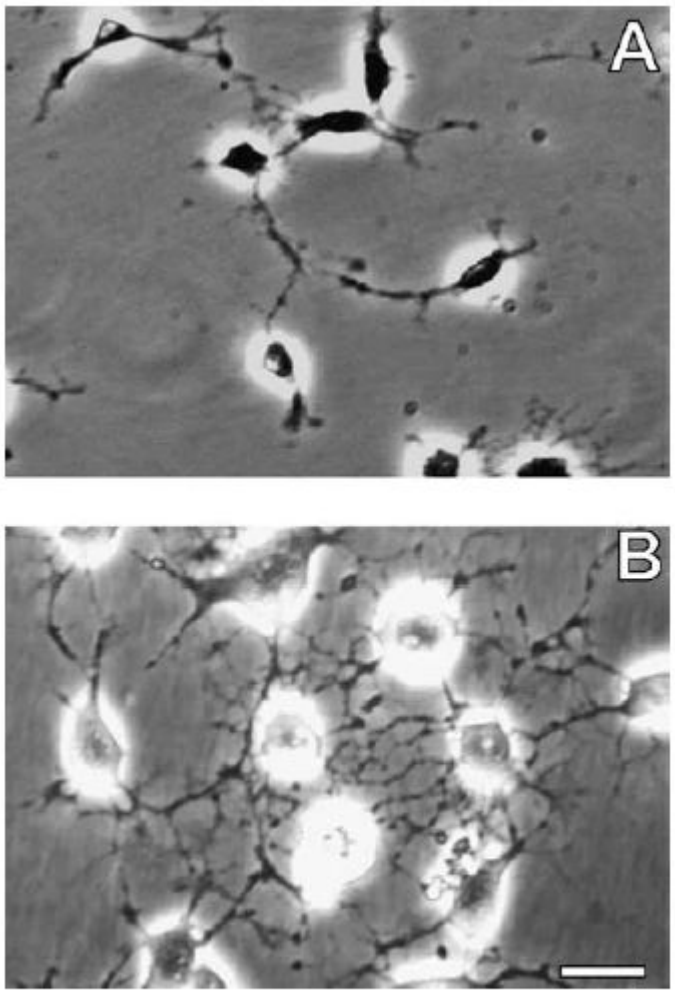

FIG. 1. Phase contrast micrographs of undifferentiated and differentiated adult neural stem cells maintained *in vitro*. (A) Undifferentiated stem cells maintained in FGF medium exhibit short processes and divide every 18 h, whereas (B) the differentiated stem cells, however, exposed to FCS, exhibit extensive neurite branching with an absence of mitotic activity. Scale bar, 20 μm.

The expression of markers of neuronal differentiation was examined using a combination of immunofluorescence staining and Western blotting in both undifferentiated and differentiated cells. Of the undifferentiated progenitors, 5% (235/4750) expressed nestin and 0.5% (20/3920) expressed the glial marker GFAP. These proportions are low because of the relatively short time in culture; if left for a further 48 h, the proportion of

precursors staining for nestin rose by > 70% (not shown), levels commensurate with those reported previously (e.g. Mistry *et al*., 2002). After 7 days in differentiation medium, the number of cells expressing GFAP increased to 21% (33/155), and cells with a neuronal phenotype and expressing the markers MAP2 (103/939, 11%) and GAP-43 (44/336, 13%) were present (Fig. 2). These immunofluorescence results were confirmed using Western blotting where the undifferentiated NPCs expressed relatively high levels of nestin compared to the differentiated cells, while the differentiated cells expressed relatively higher levels of the markers GAP-43 and MAP-2 (Fig. 2). This correlated with our previous demonstration of the markers MAP2, β-tubulin III and synaptophysin in phenotypically mature NPCs (Chipperfield *et al*., 2002).

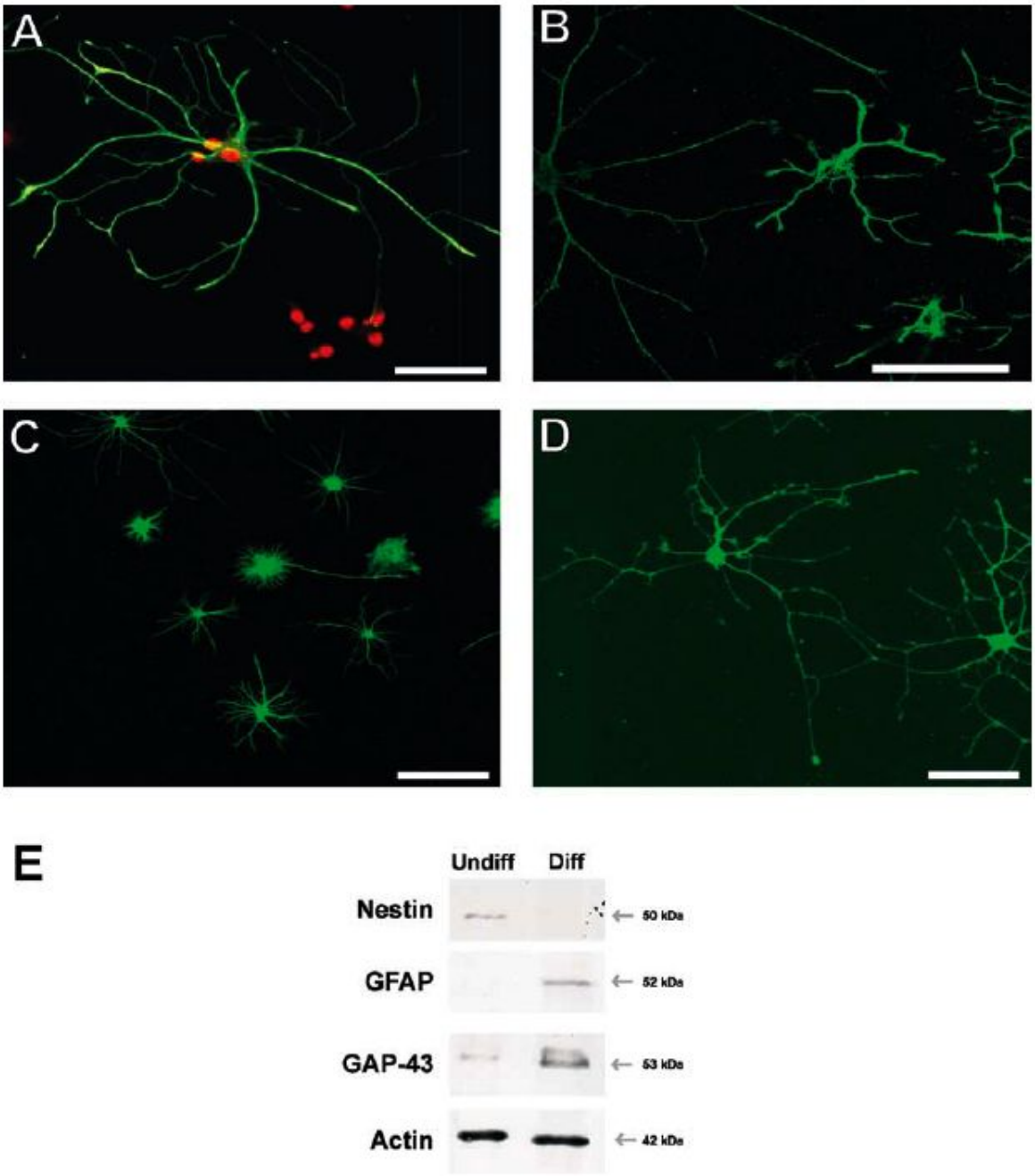

FIG. 2. Markers of undifferentiated and differentiated neural progenitors. (A and B) A proportion of differentiating progenitors transiently expressed nestin (nestin in green, propidium iodide counterstain in red), and showed a range of different morphologies. The other nuclei visible correspond to the nestin-negative population of progenitor cells. (C) The differentiated cells expressed MAP2 and (D) GAP-43. (E) Western blots were performed on equal amounts of protein from undifferentiated (UD) and differentiated (D) cells with actin as a control. The differentiated cells exhibited a marked reduction in the expression of nestin while the expression of GFAP and GAP-43 coincidentally increased. Scale bar, 100 μm (A), 50 μm (B–D).

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Electrical properties of undifferentiated neural progenitor cells

The passive and active electrical properties of isolated, cloned NPCs from the adult hippocampus were investigated using the conventional dialysed whole-cell patch-clamp recording technique. Undifferentiated NPCs had a resting membrane potential (E_m) of -87.7 ± 0.5 mV ($n = 46$) and were electrically inexcitable, as depolarizing current pulses failed to elicit action potentials (Fig. 3A). The increase in membrane resistance during depolarizing current injection is most probably due to inactivation of a resting K^+ conductance. The resting membrane potential changed by 57 mV for a 10-fold change in extracellular K^+ concentration, a value similar to that predicted by the Nernst equation for a K^+-selective electrode (Fig. 3B). Inhibition of the Na^+–K^+ pump by addition of 10–100 μm ouabain to the external solution depolarized the isolated NPC by 21.1 ± 1.9 mV ($n = 8$), indicating that active transport of Na^+ and K^+ contributes significantly to the resting membrane potential. Under voltage-clamp conditions, voltage ramps from −120 to +10 mV reversed the membrane current at −91 mV; there was marked inward rectification at negative membrane potentials (Fig. 3C). The inwardly rectifying current was inhibited reversibly by external Ba^{2+}. Inward K^+ currents evoked by hyperpolarizing voltage steps from a holding potential of 0 mV were blocked in a time- and voltage-dependent manner by 0.1 mm Ba^{2+} applied externally (Fig. 3D) but were insensitive to 4-aminopyridine (4-AP) and TEA ions ($n \geq 5$). The onset of block was more rapid when the current was passing in the inward direction, as would be expected for the steric block of a K^+ channel by Ba^{2+} (Standen & Stanfield, 1978).

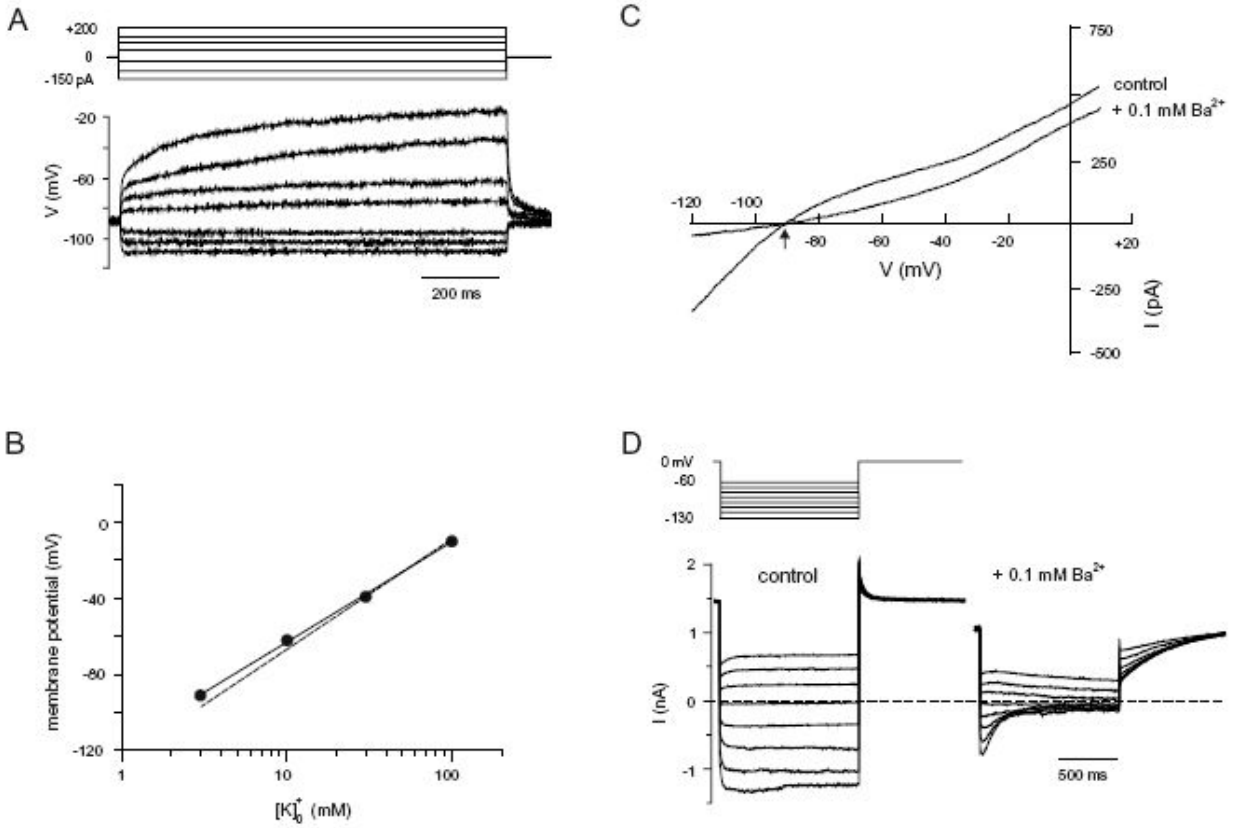

FIG. 3. Undifferentiated neuronal stem cells were nonexcitable and expressed an inward rectifier K^+ current. (A) Under current clamp, an undifferentiated NPC did not elicit an action potential in response to current steps from +200 to −150 pA. Resting membrane potential, −89 mV. Membrane resistance increased slowly during current injection. (B) Membrane potential plotted as a function of the external K^+ concentration. The solid line represents a slope of 57 mV/10-fold change in external K^+ concentration, similar to that predicted by the Nernst equation for a K^+-selective electrode (broken line). (C) Under voltage clamp, voltage ramps from −120 to +10 mV reversed at −91 mV and the marked inward rectification observed at negative membrane potentials was inhibited by 0.1 mM Ba^{2+}. (D) NPCs held at 0 mV and stepped to membrane potentials between −60 and −130 mV exhibited an inwardly rectifying current that reversed close to −90 mV, and was inhibited by 0.1 mM Ba^{2+}.

Electrical properties of differentiated neural progenitor cells

The electrical properties of differentiated NPCs that were exposed to combinations of extracellular factors for 7–10 days (Chipperfield *et al*., 2002) were investigated using the conventional 'dialysed' and perforated-patch whole-cell recording techniques. No systematic difference was noted between these recording configurations. Differentiated NPCs were electrically excitable and exhibited phasic action potential firing in response to depolarizing current pulses (Fig. 4A). The inward rectification observed at hyperpolarized membrane potentials in undifferentiated NPCs was absent. A time-dependent voltage sag was observed in response to hyperpolarizing current pulses; this was inhibited by bath application of Cs^+ (2 mm), which is characteristic of the H-current (Robinson & Siegelbaum, 2003). The E_m of differentiated NPCs was −58.8 ± 1.6 mV (n = 26) and the relationship between E_m and external K^+ concentration was fitted by the Goldman–Hodgkin–Katz voltage equation with $P_{Na}/P_K = 0.08$ (Fig. 4B). The current–voltage (I–V) relationship of differentiated NPCs obtained in response to slow voltage ramps showed marked outward rectification and reversed at ≈ −60 mV (Fig. 4C).

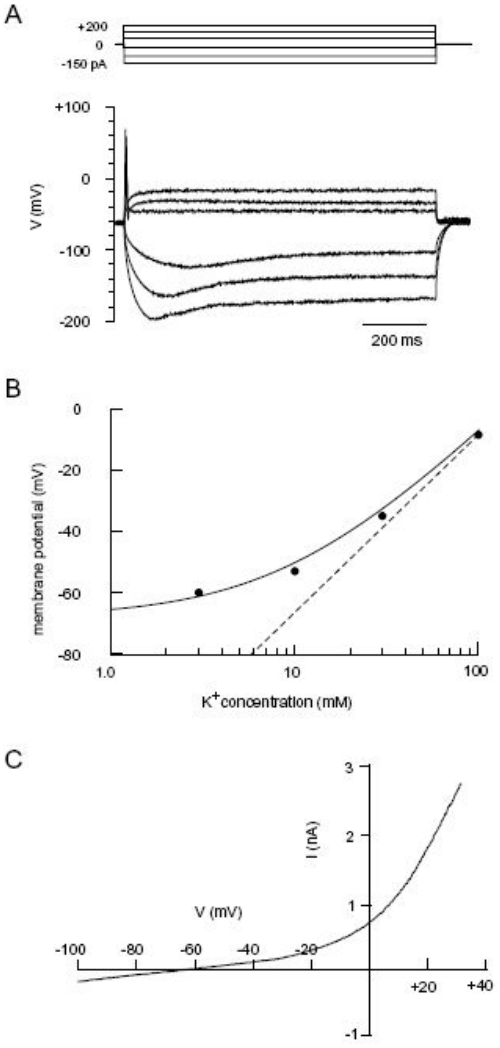

FIG. 4. Differentiated neuronal stem cells were excitable and exhibited marked outward rectification. (A) Under current-clamp conditions, an NPC exhibited phasic action potential firing in response to depolarizing current pulses and a time-dependent voltage sag to hyperpolarizing pulses. Resting membrane potential, −62 mV. (B) Membrane potential plotted as a function of the external K^+ concentration. The solid curve represents the best fit of the data by the Goldman–Hodgkin–Katz equation with $P_{Na}/P_K = 0.8$. The broken line represents that predicted by the Nernst equation for a K^+-selective electrode. (C) Under voltage clamp, differentiated NPCs showed marked outward rectification at positive membrane potentials in response to a depolarizing voltage ramp.

Voltage-clamped cells held at −100 mV exhibited a transient inward current followed by a sustained outward current in response to depolarizing voltage steps (Fig. 5A). The transient inward current was reversibly abolished in the presence of 300 nm TTX, indicating the presence of functional TTX-sensitive voltage-dependent Na^+ channels in differentiated NPCs. The outward current was reduced to ≈ 20% of control in the presence of 5 mm TEA externally and to ≈ 80% of control upon bath application of 0.5 mm 4-AP indicating the presence of delayed-rectifier K^+ channels. The *I–V* relationships for the inward current in the absence and presence of TTX and outward current in the absence and presence of TEA are shown in Fig. 5B. Voltage-dependent Ca^{2+} channel currents were recorded in all differentiated NPCs examined upon step depolarization from −60 to 0 mV in the presence of extracellular TTX and TEA and intracellular Cs^+. Figure 5C shows a representative inward Ba^{2+} current evoked upon depolarization from −60 to 0 mV and inhibition in the presence of external Cd^{2+} (20 μm). The peak Ba^{2+} current density–voltage relationship obtained in differentiated NPCs ($n = 6$) is shown in Fig. 5D and the shape of the *I–V* relationship suggests that both low- and high-voltage-activated Ca^{2+} channel currents are present, consistent with those observed in rat hippocampal pyramidal neurons (Thompson & Wong, 1991).

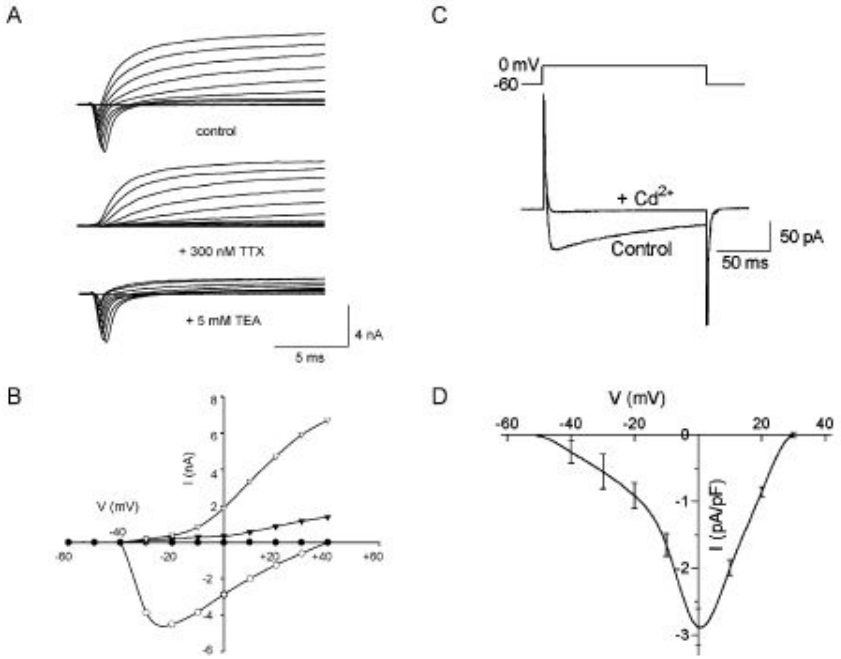

FIG. 5. Differentiated NPCs exhibited depolarization-activated Na^+, Ca^{2+} and K^+ currents. (A) Voltage clamped cells held at −100 mV exhibited a transient inward current followed by a sustained outward current in response to depolarizing voltage steps (+10 mV increments). The inward current alone was abolished in the presence of 300 nM TTX whereas the outward current was reduced by 5 mM TEA leaving the inward current unaffected. (B) *I–V* relationship obtained for the inward (○) and outward (▽) currents, and in the presence of 300 nM TTX (●) and 5 mM TEA (), respectively. (C) Superimposed traces of Ba^{2+} currents evoked by voltage steps to 0 mV from a holding potential of −60 mV in the absence (control) and presence of 20 μM Cd^{2+}. (D) Peak Ba^{2+} current density (pA/pF) plotted as a function of test potential (mV) for six cells. Holding potential, −60 mV.

Expression of purinergic receptors on neural progenitor cells

The functional expression of receptors in undifferentiated NPCs was examined in response to brief focal application of various putative neurotransmitters at concentrations ≥ 100 μm. The only agonist-induced responses observed in undifferentiated NPCs were evoked by ATP. The agonists acetylcholine (ACh), α,β-methylene ATP, noradrenaline, GABA, glutamate and glycine failed to elicit a detectable membrane response in any of the cells tested (n ≥ 10). In more than two-thirds (32/45) of undifferentiated NPCs, ATP and the selective $P2X_7$ receptor agonist benzoylbenzoyl-ATP (BzATP) evoked a depolarizing response (34 ± 2 mV) at the resting membrane potential (Fig. 6A). Under voltage-clamp conditions, ATP and BzATP evoked an inward current at negative membrane potentials that was inhibited by bath application of the nonselective P2 receptor antagonist PPADS (Fig. 6A). ATP and BzATP also evoked an excitatory response which was inhibited by PPADS (10 μm) in > 80% of differentiated NPCs, suggesting that the activation of P2X purinoceptors may play a significant role during differentiation of adult

NPCs. Focal application of glutamate to voltage-clamped differentiated NPCs evoked an inward current and excitatory response, whereas in perforated-patch whole-cell recordings both GABA and glycine evoked an outward current at a holding potential of 0 mV (Table 1). Glutamate-, GABA- and glycine-evoked responses in differentiated NPCs were inhibited by MK-801 (10 μm), bicuculline (10 μm) and strychnine (1 μm), respectively (data not shown). NPC-derived hippocampal neurons expressed GABA, glutamate and glycine receptors after they had been exposed to a combination of FGF2, NT3, BDNF, stem cell-derived HS and laminin over a 10-day period (Table 1).

The expression of P2X receptors was confirmed by confocal microscopy, which demonstrated anti-$P2X_7$ antibody labelling in both undifferentiated and differentiated NPCs (Fig. 6B). The majority of cells demonstrated strong labelling that could be seen extending into the outreaching processes in differentiated NPCs. The absence of anti-$P2X_2$ antibody labelling in undifferentiated NPCs was also demonstrated (Fig. 6B). Confocal microscopy also demonstrated substantial anti-$P2X_3$ antibody labelling but no anti-$P2X_6$ antibody labelling in undifferentiated NPCs. $P2X_7$ and $P2X_3$ were the most strongly and widely expressed purinoceptor subtypes found in undifferentiated NPCs. The expression of anti-$P2X_1$ and anti-$P2X_3$ antibody labelling together with $P2X_7$ in differentiated NPCs is consistent with that recently reported in cultured cerebellar granule neurons from neonatal rat hippocampus (Amadio *et al*., 2002).

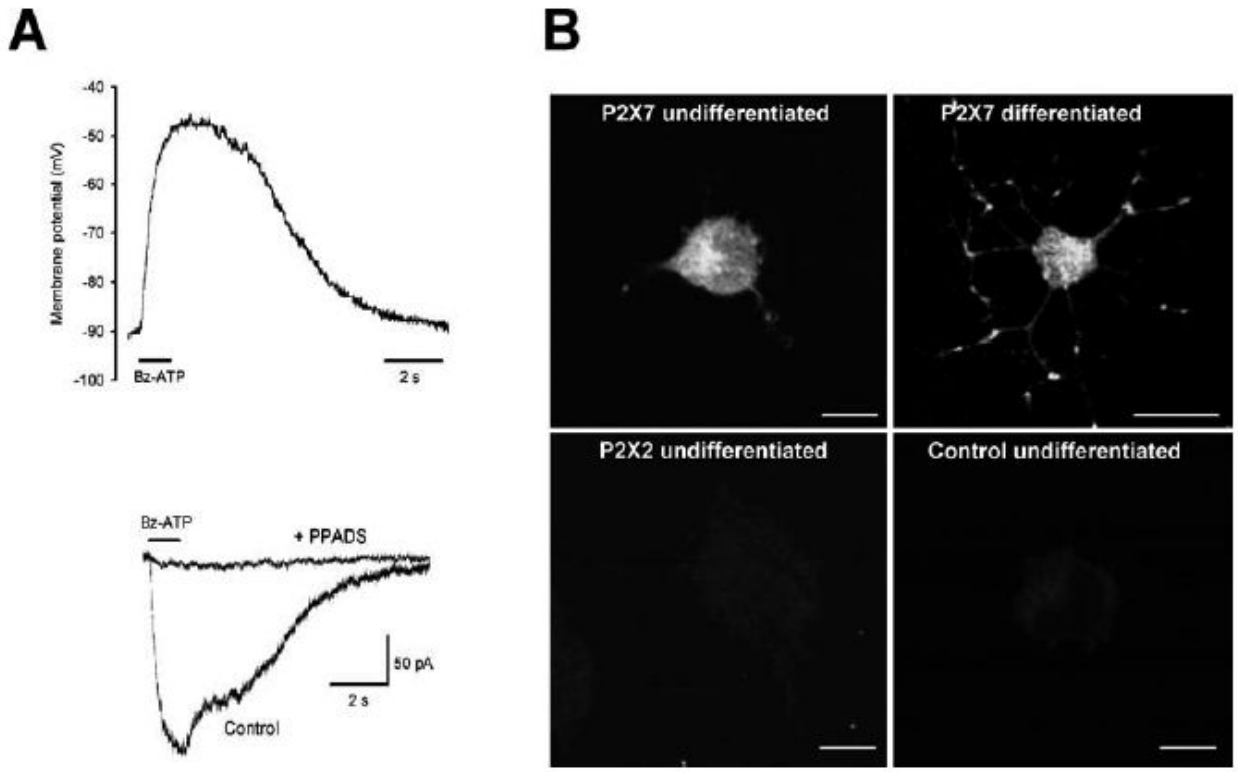

FIG. 6. P2X purinoceptors in undifferentiated and differentiated adult NPCs. (A) Upper, focal application (1 s duration) of 100 μM BzATP to an undifferentiated NPC evoked a depolarizing response. Resting membrane potential, −91 mV. Lower, under voltage-clamp conditions, focal application of 100 μM BzATP evoked an inward current in undifferentiated NPCs that was inhibited by bath application of 10 μM PPADS. (B) Upper, confocal micrographs demonstrating anti-$P2X_7$ antibody labelling in undifferentiated and differentiated NPCs. Anti-$P2X_7$ antibody labelled the processes of the differentiated NPCs. Lower, confocal micrograph demonstrating the absence of $P2X_2$ labelling in undifferentiated NPCs and micrograph of control undifferentiated NPCs not incubated in primary $P2X_7$ antibody. Scale bars, 5 μm (B).

TABLE 1. Functional properties of undifferentiated and differentiated NPCs

Parameter	Undifferentiated	*n*	Differentiated	*n*
E_m (mV)	−87.7 ± 0.5	46	−58.8 ± 1.6	26
C_m (pF)	10.9 ± 0.4	34	22.0 ± 1.1	39
R_i (MΩ)	121.3 ± 5.5	14	770.2 ± 25.9	13
Excitable (action potential)	No	–	Yes	–
Agonist responses†				
ATP	+	9/16	+	10/15
BzATP	+	23/29	+	8/8
α,β-methylene ATP	–	0/15	–	0/5
ACh	–	0/10	–	1/5
Noradrenaline	–	0/11	–	0/6
Glutamate	–	0/12	+	10/10
GABA	–	0/10	+	16/16
Glycine	–	0/14	+	9/17

†All agonists were focally applied at a concentration of ≥ 100 μM.

Purinoceptor agonists evoked transient increases in [Ca2+]i in undifferentiated NPCs

Calcium microfluorimetry was used to monitor $[Ca^{2+}]_i$ in undifferentiated NPCs in response to bath application of purinergic agonists. Cells exhibited a mean resting $[Ca^{2+}]_i$ level of 54.1 ± 10.1 nm (n = 377) and brief exposure (5–15 s) to 100 μm ATP, ATP-γ-S or BzATP evoked a transient increase in $[Ca^{2+}]_i$ in NPCs. Figure 7A shows an averaged response of 22 cells to 100 μm ATP-γ-S and 100 μm BzATP in the presence and absence of extracellular Ca^{2+}. Removal of extracellular Ca^{2+} by bath perfusion with Ca^{2+}-free PSS containing 0.5 mm EGTA abolished the $[Ca^{2+}]_i$ response to ATP-γ-S and BzATP in 88% of cells tested (n = 246). Brief exposure of undifferentiated NPCs to either the P2Y receptor agonist uridine 5'-triphosphate (300 μm) or the cholinergic agonist ACh (300 μm) did not change resting $[Ca^{2+}]_i$ (data not shown). Taken together, these data suggest that the transient increases in $[Ca^{2+}]_i$ evoked by ATP and BzATP are dependent on extracellular Ca^{2+} and are unlikely to be due to P2Y-mediated Ca^{2+} release from internal stores. Bath application of the purinergic P2 receptor antagonist PPADS (10 μm) prior to agonist application inhibited $[Ca^{2+}]_i$ transients evoked by BzATP and ATP by 70 ± 3 (n = 60) and 90 ± 0.5% (n = 42), respectively (Fig. 7B and C). Recovery of the ATP-induced $[Ca^{2+}]_i$ transients occurred following 12–15 min washout of PPADS (n = 20) as shown in Fig. 7B.

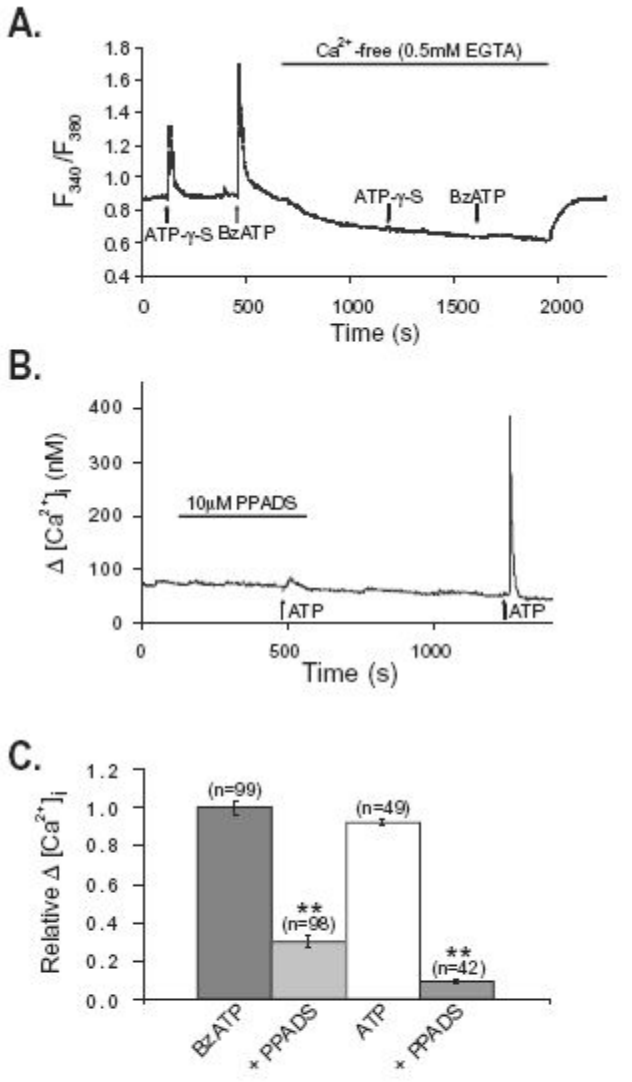

FIG. 7. ATP and BzATP evoked a transient increase in intracellular $[Ca^{2+}]$ in undifferentiated NPCs. (A) Representative trace of ATP-γ-S (100 μM)- and BzATP (100 μM)-evoked $[Ca^{2+}]_i$ transients in undifferentiated NPCs in the presence and absence of external Ca^{2+} (averaged trace, $n = 22$). Ca^{2+}-free bath solution containing 0.5 mM EGTA was applied as indicated by the horizontal bar. Arrows indicate application of agonists for 5–15 s (B) ATP-induced $[Ca^{2+}]_i$ transients inhibited reversibly by 10 μM PPADS bath-applied as indicated (averaged trace, $n = 20$). (C) Bar graph of the relative changes in $[Ca^{2+}]_i$ in response to BzATP and ATP in the absence and presence of 10 μM PPADS. Data are presented as mean ± SEM.

Discussion

In the present study, the passive and active electrical properties of isolated NPCs from the adult rat hippocampus were examined, and the expression of membrane ion channels and receptors in both undifferentiated and differentiated cells characterized. The starting population of adult NPCs employed here are multipotent, retaining a capacity to be clonogenic, to divide, and to differentiate into neurons, astrocytes and oligodendroglia. The major finding is that adult hippocampal NPCs express functional purinergic receptors before any other cell surface receptors as they begin to differentiate under the influence of extracellular factors.

Maturation of neural stem cells

We found that the undifferentiated NPCs had a E_m of ≈ −90 mV that was critically dependent on the extracellular K^+ concentration and the Na+–K^+ ATPase pump. They were electrically inexcitable as depolarizing current pulses failed to elicit action potentials; under voltage clamp conditions they exhibited an inwardly rectifying K^+ current which was inhibited by external Ba^{2+} but was insensitive to 4-AP and TEA. The absence of voltage-gated Na^+ and Ca^{2+} channels combined with the presence of the inwardly rectifying K^+ channel is consistent with previous reports on cultured human NPCs (Piper *et al*., 2000; Cho *et al*., 2002) and, indeed, may be a functional characteristic of undifferentiated NPCs. In ≈ 70% of undifferentiated NPCs, brief focal application of ATP and BzATP evoked an inward current and depolarization at negative potentials whereas ACh, noradrenaline, glutamate, glycine and GABA failed to elicit a membrane response. Immunocytochemistry confirmed the presence of $P2X_7$ and $P2X_3$ purinergic receptors on the plasma membranes of these immature adult cells. The resting membrane potential of ≈ −90 mV would provide a substantial electrochemical gradient (> 200 mV) for Ca^{2+} influx through activated Ca^{2+}-permeable P2X receptor channels in undifferentiated NPCs.

In contrast, after exposure to a combination of specific extracellular neurotrophic, mitogenic, adhesive and carbohydrate factors, differentiated NPCs were electrically excitable and exhibited phasic action potential firing in response to depolarizing current pulses. The E_m of differentiated NPCs was ≈ −60 mV and the resting permeability $P_{Na}/P_K = 0.08$. The *I–V* relationship of differentiated NPCs obtained in response to slow voltage ramps showed marked outward rectification and reversed at ≈ −60 mV. In response to depolarizing voltage steps, a TTX-sensitive transient inward and an outward current inhibited by external TEA indicated the presence of functional voltage-dependent Na^+ and delayed rectifier K^+ channels, respectively, in differentiated NPCs. In the presence of extracellular TTX and TEA and intracellular Cs^+, relatively small-amplitude voltage-dependent Ca^{2+} channel currents, inhibitable with external Cd^{2+}, were recorded in differentiated NPCs.

Unlike the immature cells, differentiated NPCs responded to focal application of glutamate, GABA, glycine and ATP, indicating the presence of functional glutamate, GABA, glycine and purinergic (P2X) receptors. The membrane response evoked by BzATP in undifferentiated cells suggests that the activation of $P2X_7$ purinoceptors may play a significant role during the differentiation of adult NPCs. ATP-gated P2X purinoceptors are a family of cation-permeable channels with a significant permeability to Ca^{2+} (Koshimizu *et al*., 2000). Fura-2 fluorescence ratio imaging of undifferentiated NPCs demonstrates that the P2X receptor agonists ATP and BzATP evoke a transient increase in $[Ca^{2+}]_i$ which is dependent on extracellular Ca^{2+} and is inhibited reversibly by the purinoceptor antagonist PPADS. $P2X_7$ receptor channels, in particular, are capable of conducting significant amounts of Ca^{2+} and allow small peptides to pass through the open pore (Virginio *et al*., 1999). Calcium signalling by $P2X_7$ receptor channels thus provides an effective mechanism for generating increases in intracellular $[Ca^{2+}]$ independent of voltage-dependent Ca^{2+} channels.

Overall, the voltage-gated currents and neurotransmitter responses of both the undifferentiated and differentiated NPCs were very consistent, as described in Table 1. There were no obvious ‘gradations’ in cellular responses, suggesting that the behaviour of the clonally derived cells stayed uniform rather than presenting as a heterogeneous population of differentiated neurons.

Comparison of functional properties

The resting membrane and action potentials of mouse multipotent stem cells have been reported to be similar to those described here, although they displayed a larger after-hyperpolarization with shorter duration of spikes (Song *et al*., 2002). Other neural precursor cells from brain and spinal cord have been reported to have resting membrane and action potentials, although the spinal cord cells showed relatively lower resting membrane potentials with smaller, TTX-sensitive, sustained action potential firing (Piper *et al*., 2000; Nguyen *et al*., 2002). In a preliminary study, a neural stem cell line created from human embryonic telencephalon has recently been shown to express inward and outward K^+ currents, with no evidence for voltage-dependent Na^+ currents (Cho *et al*., 2002). Upon the induction of differentiation after NeuroD transfection, subsequent neuronal excitability was shown to be due to the expression of TTX-sensitive Na^+ currents. The variances between the above studies are probably due to the differences in species, region of CNS, methods of isolation, and age, and the time cells spent in culture. Undifferentiated NPCs share similar properties to astrocytes including the passive membrane currents (Filippov *et al*., 2003) and Ca^{2+} signalling mediated by activation of purinergic receptors (Fumagalli *et al*., 2003).

The recent study by Nguyen *et al*. (2002) used a combination of RT-PCR and immunocytochemistry to show that α_1-, α_2- and β-subunit mRNAs and α-subunit proteins of the glycine ionotropic receptor are expressed by

the majority of postnatal rat striatum-derived, nestin-positive cells within cultured neurospheres. Whole-cell patch-clamp experiments demonstrated that in ≈ 50% of these cells glycine evokes currents that can be reversibly blocked by strychnine and picrotoxin, demonstrating the presence of functional glycine receptors. Formation of functional synapses in the study by Toda *et al*. (2000) occurred in 1.2% of the tested adult rat hippocampal stem cells during the period 28–35 DIV, suggesting that synaptic maturation can be delayed to match the maturation of membrane excitability. While it remains intrinsically difficult to compare these results with our own, where we studied isolated NPCs cultured on substrates, there are a number of intriguing similarities with this study; most notably, maturing NPCs are capable of recruiting and activating classical neurotransmitter systems.

Mistry *et al*. (2002) have previously demonstrated that the continued presence of FGF2 greatly increases the number of both hippocampal progenitors and neurons in culture. Such progenitor-derived neurons expressed functional GABA and glutamate synapses *in vitro* consistent with those expressed in the mature hippocampus. When maintained on microelectrode plates, these progenitors formed elaborate neural networks that exhibited spontaneously generated action potentials after 21 days. This activity was observed only when cultures were exposed to FGF2 and either N-CAM or BDNF. They concluded that mitogenic growth factors were synergizing with N-CAM or neurotrophins to generate spontaneously active neural networks.

Using a transgenic mouse, Belachew *et al*. (2003) have also recently demonstrated that adult hippocampal progenitor cells expressing the NG2 proteoglycan also display a multipotent phenotype *in vitro* and generate electrically excitable neurons, as well as astrocytes and oligodendrocytes. They proceeded to show that the *in vivo* hippocampus contains a sizeable fraction of proliferative postnatal NG2 progenitors whose progeny appear to differentiate into GABAergic neurons capable of propagating action potentials and displaying functional synaptic input.

Extracellular factors and functional maturation

Wu *et al*. (2002) have recently reported that a ‘priming’ cocktail of FGF2, heparin and laminin could be used to selectively harvest large cholinergic neurons from fetal human neural stem cells *in vitro*. Whole-cell patch clamping of these cells established that the priming procedure could eventually induce action potentials that were inhibited by TTX. Primary cultures of embryonic rat hippocampal progenitor cells, maintained in FGF2, were also associated with low levels of Na^+-, Ca^{2+}-, *N*-methyl-d-aspartate (NMDA)- and kainite-mediated currents (Sah *et al*., 1997). The expression of functional channels and receptors in these progenitor cells was up-regulated by BDNF and NT-3. These results resemble those reported here, and emphasize the importance of extracellular influences for the expression of stable neuronal phenotypes from adult stem cells. Such trophic actions are also reflected by neurotrophin-induced changes in intrinsic neuronal excitability. In many kinds of cultured neuron, prolonged exposure to neurotransmitters can elevate and differentially regulate the expression of various voltage-gated ion channels, and acute effects of neurotrophins on excitability have also been noted (Poo, 2001).

The results presented here clearly raise a number of further questions. How important is the presence of functional purinergic receptors for the cascade of differentiation in these cells? Does blocking these cell-surface receptors inhibit the maturation of the progenitors? How do subsequent extracellular influences, such as the neurotrophins, influence the adoption of a particular neurotransmitter phenotype after exposure to heparan sulphates? Can these influences be manipulated in the *in vivo* situation? Answers to these questions will require more rigorous dissections of the responses of adult neural stem cells to subtler manipulations of their environment.

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Abbreviations

4-AP, 4-aminopyridine; ACh, acetylcholine; ATP, adenosine 5'-triphosphate; BzATP, benzoylbenzoyl-ATP; BDNF, brain-derived neurotrophic factor; DIV, days *in vitro*; FCS, foetal calf serum; FGF, fibroblast growth factor; GABA, γ-aminobutyric acid; GAP-43, growth-associated molecule-43 kDa/neuromodulin; GFAP, glial fibrillary acid protein; HS, heparan sulphate; NGF, nerve growth factor; NMDA, *N*-methyl-d-aspartate; NPCs, neural progenitor cells; NT-3, neurotrophin 3; PBS, phosphate-buffered saline; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid; PSS, physiological salt solution; TEA, tetraethylammonium; TTX, tetrodotoxin.

References

Amadio, S., D'Ambrosi, N., Cavaliere, F., Murra, B., Sancesario, G., Bernardi, G., Burnstock, G. & Volonte, C. (2002) P2 receptor modulation and cytotoxic function in cultured CNS neurons. Neuropharmacology, 42, 489– 501.

Anderson, D.J. (2001) Stem cells and pattern formation in the nervous system: the possible versus the actual. Neuron, 30, 19– 35.

Belachew, S., Chittajallu, R., Aguirre, A.A., Yuan, X., Kirby, M., Anderson, S. & Gallo, V. (2003) Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. J. Cell Biol., 161, 169– 186.

Carpenter, M.K., Cui, X., Hu, Z.Y., Jackson, J., Sherman, S., Seiger, A. & Wahlberg, L.U. (1999) In vitro expansion of a multipotent population of human neural progenitor cells. Exp. Neurol., 158, 265– 278.

Carpenter, M.K., Winkler, C., Fricker, R., Emerich, D.F., Wong, S.C., Greco, C., Chen, E.Y., Chu, Y., Kordower, J.H., Messing, A., Bjorklund, A. & Hammang, J.P. (1997) Generation and transplantation of EGF-responsive neural stem cells derived from GFAP-hNGF transgenic mice. Exp. Neurol., 148, 187– 204.

Chipperfield, H., Bedi, K.S., Cool, S.M. & Nurcombe, V. (2002) Heparan sulfates isolated from adult neural progenitor cells can direct phenotypic maturation. Int. J. Dev. Biol., 46, 661– 670.

Cho, T., Bae, J.H., Choi, H.B., Kim, S.S., McLarnon, J.G., Suh-Kim, H., Kim, S.U. & Min, C.K. (2002) Human neural stem cells: electrophysiological properties of voltage-gated ion channels. Neuroreport, 13, 1447– 1452.

Filippov, V., Kronenberg, G., Pivneva, T., Reuter, K., Steiner, B., Wang, L.-P., Yamaguchi, M., Kettenmann, H. & Kempermann, G. (2003) Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrical and morphological characteristics of astrocytes. Mol. Cell. Neurosci., 23, 373– 382.

Fumagalli, M., Brambilla, R., D'Ambrosi, N., Volonté, C., Matteoli, M., Verderio, C. & Abbracchio, M.P. (2003) Nucleotide-mediated calcium signaling in rat cortical astrocytes: Role of P2X and P2Y receptors. Glia, 43, 218– 230.

Gage, F.H. (2002) Neurogenesis in the adult brain. J. Neurosci., 22, 612– 613.

Gage, F.H., Coates, P.W., Palmer, T.D., Kuhn, H.G., Fisher, L.J., Suhonen, J.O., Peterson, D.A., Suhr, S.T. & Ray, J. (1995) Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc. Natl. Acad. Sci. USA, 92, 11879– 11883.

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem., 260, 3440– 3450.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F.J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch., 391, 85– 100.

Hansen, M.A., Balcar, V.J., Barden, J.A. & Bennett, M.R. (1998) The distribution of single P2x1-receptor clusters on smooth muscle cells in relation to nerve varicosities in the rat urinary bladder. J. Neurocytol., 27, 529– 539.

Hansen, M.A., Dutton, J.L., Balcar, V.J., Barden, J.A. & Bennett, M.R. (1999) P2X (purinergic) receptor distributions in rat blood vessels. J. Auton. Nerv. Syst., 75, 147– 155.

Hogg, R.C., Chipperfield, H., Whyte, K.A., Nurcombe, V. & Adams, D.J. (2002) Electrical properties of isolated neural stem cells from the adult rat hippocampus change upon differentiation. J. Physiol. (Lond.), 543, 20P.

Hurelbrink, C.B., Armstrong, R.J., Dunnett, S.B., Rosser, A.E. & Barker, R.A. (2002) Neural cells from primary human striatal xenografts migrate extensively in the adult rat CNS. Eur. J. Neurosci., 15, 1255– 1266.

Koshimizu, T.A., Van Goor, F., Tomic, M., Wong, A.O., Tanoue, A., Tsujimoto, G. & Stojilkovic, S.S. (2000) Characterization of calcium signaling by purinergic receptor-channels expressed in excitable cells. Mol. Pharmacol., 58, 936– 945.

Mistry, S.K., Keefer, E.W., Cunningham, B.A., Edelman, G.M. & Crossin, K.L. (2002) Cultured rat hippocampal neural progenitors generate spontaneously active neural networks. Proc. Natl. Acad. Sci. USA, 99, 1621– 1626.

Nguyen, L., Malgrange, B., Belachew, S., Rogister, B., Rocher, V., Moonen, G. & Rigo, J.M. (2002) Functional glycine receptors are expressed by postnatal nestin-positive neural stem/progenitor cells. Eur. J. Neurosci., 15, 1299– 1305.

Nurcombe, V., Ford, M.D., Wildschut, J.A. & Bartlett, P.F. (1993) Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan. Science, 260, 103– 106.

Ornitz, D.M. (2000) FGFs, heparan sulfate and FGFRs: complex interactions essential for development. Bioessays, 22, 108– 112.

Palmer, T.D., Markakis, E.A., Willhoite, A.R., Safar, F. & Gage, F.H. (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J. Neurosci., 19, 8487– 8497.

Piper, D.R., Mujtaba, T., Rao, M.S. & Lucero, M.T. (2000) Immunocytochemical and physiological characterization of a population of cultured human neural precursors. J. Neurophysiol., 84, 534– 548.

Poo, M.M. (2001) Neurotrophins as synaptic modulators. Nat. Rev. Neurosci., 2, 24– 32.

Qian, X., Davis, A.A., Goderie, S.K. & Temple, S. (1997) FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. Neuron, 18, 81– 93.

Qian, X., Shen, Q., Goderie, S.K., He, W., Capela, A., Davis, A.A. & Temple, S. (2000) Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron, 28, 69– 80.

Reynolds, B.A., Tetzlaff, W. & Weiss, S. (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J. Neurosci., 12, 4565– 4574.

Richards, L.J., Kilpatrick, T.J. & Bartlett, P.F. (1992) De novo generation of neuronal cells from the adult mouse brain. Proc. Natl. Acad. Sci. USA, 89, 8591– 8595.

Rietze, R.L., Valcanis, H., Brooker, G.F., Thomas, T., Voss, A.K. & Bartlett, P.F. (2001) Purification of a pluripotent neural stem cell from the adult mouse brain. Nature, 412, 736– 739.

Robinson, R.B. & Siegelbaum, S.A. (2003) Hyperpolarization-activated cation currents: From molecules to physiological function. Annu. Rev. Physiol., 65, 453– 480.

Sah, D.W., Ray, J. & Gage, F.H. (1997) Regulation of voltage- and ligand-gated currents in rat hippocampal progenitor cells in vitro. J. Neurobiol., 32, 95– 110.

Song, H.J., Stevens, C.F. & Gage, F.H. (2002) Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. Nature Neurosci., 5, 438– 445.

Standen, N.B. & Stanfield, P.R. (1978) A potential- and time-dependent blockade of inward rectification in frog skeletal muscle fibres by barium and strontium ions. J. Physiol. (Lond.), 280, 169– 191.

Suhonen, J.O., Peterson, D.A., Ray, J. & Gage, F.H. (1996) Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. Nature, 383, 624– 627.

Svendsen, C.N., ter Borg, M.G., Armstrong, R.J., Rosser, A.E., Chandran, S., Ostenfeld, T. & Caldwell, M.A. (1998) A new method for the rapid and long term growth of human neural precursor cells. J. Neurosci. Meth., 85, 141– 152.

Takahashi, M., Palmer, T.D., Takahashi, J. & Gage, F.H. (1998) Widespread integration and survival of adult-derived neural progenitor cells in the developing optic retina. Mol. Cell Neurosci., 12, 340– 348.

Thompson, S.M. & Wong, R.K. (1991) Development of calcium current subtypes in isolated rat hippocampal pyramidal cells. J. Physiol. (Lond.), 439, 671– 689.

Toda, H., Takahashi, J., Mizoguchi, A., Koyano, K. & Hashimoto, N. (2000) Neurons generated from adult rat hippocampal stem cells form functional glutamatergic and GABAergic synapses in vitro. Exp. Neurol., 165, 66– 76.

Virginio, C., MacKenzie, A., North, R.A. & Surprenant, A. (1999) Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X7 receptor. J. Physiol. (Lond.), 519, 335– 346.

Wu, P., Tarasenko, Y.I., Gu, Y., Huang, L.Y., Coggeshall, R.E. & , Y. (2002) Region-specific generation of cholinergic neurons from fetal human neural stem cells grafted in adult rat. Nature Neurosci., 5, 1271– 1278.