Multipotent progenitor cells from the adult human brain: neurophysiological differentiation to mature neurons

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It was long held as an axiom that new neurons are not produced in the adult human brain. More recent studies have identified multipotent cells whose progeny express glial or neuronal markers. This discovery may lead to new therapeutic strategies for CNS disorders by stimulating neurogenesis in vivo or by transplanting multipotent progenitor cells (MPCs) that have been propagated and differentiated in vitro. The clinical application of such approaches will be limited by the ability of these cells to develop into functional neurons. To facilitate an understanding of mechanisms regulating neurogenesis in the adult human brain, we characterized the developmental processes MPCs go through when progressing to a neuron. Human tissue was harvested during temporal lobe resections because of epilepsy, and cells were cultured as neurospheres. Our findings demonstrate that at an early stage, these cells often stain with neuronal markers without possessing any functional neuronal properties. Over a period of 4 weeks in culture, cells go through characteristic steps of morphological and electrophysiological development towards functional neurons; they develop a polarized appearance with multiple dendrites, whereas the membrane potential becomes more negative and the input resistance decreases [from −48 ± 10 mV/557 ± 85 Ω (n = 15) between days 7 and 11 to −59 ± 9 mV/380 ± 79 Ω (n = 9) between days 25 and 38, respectively]. Active membrane properties were first observed on day 7 and consisted of a voltage-gated K⁺-current. Later in the second week the cells developed voltage-gated Ca²⁺-channels and fired small Ca²⁺-driven action potentials. Immature Na⁺-driven action potentials developed from the beginning of the third week, and by the end of the fourth week the cells fired repetitive action potentials with a completely mature waveform generated by the combined action of the voltage-gated ionic channels I₉, I₆ and I₄. After 4 weeks, the newly formed neurons also communicated by the use of GABAergic and glutamatergic synapses. The adult human brain thus harbours MPCs, which have the ability to develop into neurons and in doing this follow characteristic steps of neurogenesis as seen in the developing brain.

Keywords: action potentials; differentiation; human brain stem cells; multipotent precursors; synaptic connections

Abbreviations: 4-AP = 4-aminopyridine; [Ca²⁺] = intracellular Ca²⁺; CNQX = 6-cyano-7-nitroquinoxaline-2,3-dione; FCS = fetal calf serum; L15 = Leibowitz-15 medium; MK-801 = D-2-amino-5-phosphonovaleric acid; MPCs = multipotent progenitor cells; NiCl = nickel chloride; SCs = stem cells; TEA = tetraethylammonium; TCSM = Time Course/Ratiometric Software Module; TTX = tetrodotoxin; VGlut-1 = glutamate transporter-1; VZ = ventricular zone

Introduction

A hallmark of neurons is the ability to generate action potentials, i.e. excitability (Reh, 2002). Neuronal excitability depends on four factors (Hodgkin and Huxley, 1952; Spitzer et al., 2002): (i) a regenerative process that rapidly produces a large signal, (ii) a threshold, so that only a stimulus significantly greater than noise can initiate an action potential, (iii) a limitation of signal duration that returns the membrane potential to its resting state and (iv) a recovery process that enables reexcitation. The development of excitability shows some dissimilarities between different species and CNS regions, but generally consists of a coordinated succession of changes in passive and active electrical membrane properties, particularly the development of a set of voltage-gated sodium, potassium and calcium channels (for a review see Spitzer et al., 2002).

Neuronal precursors start terminal differentiation when they exit the cell cycle. Acquisition of voltage-gated ionic channels is a crucial part of terminal differentiation, as such channels (i) are required for excitability and (ii) play a fundamental role in morphological and functional maturation of individual neurons and neural networks (Zhang and Poo, 2001; Spitzer et al., 2002; Ben-Ari et al., 2004). Thus, in the developing brain, voltage-gated ionic channels have important functions with respect to cell migration (Komuro and Rakic, 1998; Marin and Rubenstein, 2003), rate of neurite outgrowth (Chemin et al., 2002), axonal targeting (Catalano and Shatz, 1998; Dantzker and Callaway, 1998), modification of action potential waveform (Spitzer et al., 2002), neurotransmitter specification (Borodinsky et al., 2004; Spitzer et al., 2004) and neuronal survival (Mennerick and Zorumski, 2000; Salthun-Lassalle et al., 2004).

During embryogenesis, neurons populating the mammalian neocortex arise from repeated divisions of stem cells (SCs). Glutamatergic projection neurons arise mainly in the ventricular zone (VZ) of the pallium and GABAergic interneurons in the subcortical telencephalon (Anderson et al., 1999; Xu et al., 2004). The VZ persists as a locus of cell proliferation in adult animals and generates neuroblasts wandering by the rostral migratory stream to the olfactory bulb (Lois and Alvarez-Buylla, 1994).

Over the last decade it has become quite well established that also in humans the adult VZ generates new cells, some of which display neuronal antigens after differentiation (Arsenijevic et al., 2001; Johansson et al., 1999; Kirschenbaum et al., 1994; Kukekov et al., 1999; Roy et al., 2000). If these cells have the ability to become synaptically integrated neurons, one may start to speculate in new therapeutic strategies against degenerative diseases by transplantation of multipotent progenitor cells (MPCs) that have been propagated in vitro or by stimulating neurogenesis in vivo (Bjorklund, 2000; Langmoen et al., 2003).

We have earlier shown that monoclonal MPCs harvested from the adult human brain and maintained in vitro differentiate into two functionally separate cell types, one with excitable and one with in-excitable membrane (Westerlund et al., 2003). Future development of therapies based on the use of such cells from the adult human brain, will in the end depend on our understanding of the mechanisms governing proliferation, differentiation and migration of these cells.

Owing to the profound effects voltage-gated ionic channels have on neuronal differentiation and maturation, it is of importance to characterize the sequential steps MPCs from the adult human brain go through when differentiating into functional neurons. In this study, we show that at an early stage cells often stain with neuronal markers without having any functional neuronal properties. Over a period of 4 weeks in culture, however, the cells undergo stepwise acquisition of voltage-gated K⁺, Ca²⁺ and Na⁺ channels, and ultimately not only exhibit the distinct membrane properties of mature neurons, but also connect in a synaptic network as evidenced by spontaneous post-synaptic currents blocked by antagonists against glutamate and GABA. Our results thus also indicate that MPCs from the adult human brain develop into cells fulfilling the criteria for being neurons (Reh, 2002), i.e. excitable cells communicating via synapses.

Materials and methods

Cell culture

Biopsies from the ventricular wall were harvested from 21 temporal lobe specimens obtained during neurosurgery in the cases of medical intractable epilepsy. Tissue harvesting was approved by the Norwegian National Committee for Medical Research Ethics. MRI excluded the presence of tumour, and the patients were screened for infectious diseases. The patients ranged in age from 20 to 44 years (median 28 years). The samples were transported from the operating theatre to the lab in Leibowitz-15 medium (L15) (Invitrogen Corp., Carlsbad, CA) and stored at 4°C

The tissue was mechanically separated by a scalp and placed in a medium containing Papain 13.2 U/ml (Sigma, St Louis, MO) for 5 + 5 min. DNAse 200 U/ml (Sigma) was added after 5 min. The dissociated suspension was passed through a 70 μm strainer (BD Biosciences) and stored at 4°C. The cultures were supplemented with bFGF and EGF twice a week, and additional DMEM/F12 was added once a week (Westerlund et al., 2003). The neurospheres were cultured for 3–6 weeks, passaged with Papain/DNAse before the centre became necrotic, and resuspended in 50 : 50 fresh and condensed neurosphere medium. To ensure strict clonal conditions, single cells were manually isolated with a micromanipulator (Eppendorf, Westbury, NY) and cultured after passage. Differentiation of single cells from neurospheres was induced by adding 2% fetal calf serum (FCS), removal of mitogens and plating on laminin-coated (20 ng/ml) glass bottom dishes (WillCo Wells BV, Amsterdam, The Netherlands) and 4-well glass slides (Nunc, Roskilde, Denmark).

Immunocytochemistry

Immunostaining of cell cultures was performed as previously described (Johansson et al., 1999; Westerlund et al., 2003), with
the following primary antibodies and dilutions (rb: rabbit, ms: mouse, gp: guinea pig, goat: gt): glutamate transporter-1 (VGlut-1) (gp, 1: 10 000, Chemicon, Temecula, CA), MAP-2 (ms, 1: 200, gift from Prof. P. Morgan, University of Wales), GABA receptors (Sigma). The cells were stimulated by pressure or nitrogen (N2) to block the voltage-gated Ca\(^{2+}\)-channels, 500 μM of 4-aminopyridine (4-AP) and 5 mM of tetrodotoxin (TTX) to block NMDA receptors and 15 μM of D-2-amino-5-phosphonovaleric acid (MK-801) to block NMDA receptors and 10 μM of bicuculline to block GABAB receptors (Sigma). The cells were stimulated by pressure puff application of 60 mM of KCl for 10 s.

**Statistics**

The results are presented as mean ± SEM. Differences were tested with independent-sample t-tests and considered significant when P < 0.05.

**Results**

**Isolation of MPCs**

Repeated cell divisions where a single cell ultimately forms a cluster of cells (neurosphere) is shown in Fig. 1A. Biopsies from 21 patients were dissociated into individual cells and grown under conditions promoting neurosphere formation. Primary neurospheres consisting of ~300 cells were formed after 2–6 weeks in culture in 18 of the 21 samples. Self-renewal capacity was confirmed up to passage 3 (i.e. tertiary spheres) by dissociating spheres into single cells and transferring them to separate wells of a 24-well plate (n = 10) (Fig. 1A, upper left). Of the 240 cells harvested from 10 patients 19 (8%) formed quaternary neurospheres (Fig. 1A). This is comparable with what others have observed after the second passage earlier (Johansson et al., 1999). Attempts on propagating beyond quaternary spheres were in most cases quite unproductive, as the number of newly formed spheres decreased considerably. All the same, multipotency was conserved throughout passages as immunocytochemical staining of progeny of tertiary neurospheres was positive for immature neuronal (doublecortin), neuronal (MAP-2), astrocytic (GFAP) and oligodendroglial (RIP and O4) markers on days 15 of differentiation in all samples tested (n = 5) (Fig. 1B–D).

**Development of glial and neuronal morphology**

We then followed the development of cells positive for either the glial fibrillary protein GFAP (Fig. 2A, left panel), or the neuronal microtubule-associated protein MAP-2 (Fig. 2A,
right panel). This was done by studying cells from secondary and tertiary neurospheres from day 5 (D5) of differentiation to day 28 (D28). During differentiation, GFAP-positive cells were either multipolar (Fig. 2A, D10 left) or more spongiform in appearance resembling protoplasmic astrocytes (Bushong et al., 2004) (Fig. 2, D15 left). MAP-2 positive cells were initially monopolar or bipolar, but later developed multiple extensions, some with local swellings resembling dendritic spines (Yuste and Bonhoeffer, 2004) (Fig. 2, D15 right). On day 15, 68% of the differentiated cells stained for...
Active membrane properties

The ability to fire APs was tested in current-clamp mode in cells differentiating from secondary and tertiary neurospheres. A total of 14 cells were recorded from during the first week of differentiation. All of these had a completely passive membrane (Fig. 2B, D5). The first active membrane response observed consisted of an apparent small depolarization, 'depolarizing hump', evoked by positive current pulses (Fig. 2B, D7). Cells recorded during the second week of differentiation (n = 15) showed either a 'depolarizing hump', or a small action potential (Fig. 2B, D10). The former was first observed on day 7, and the latter in the second week.

During the third week, the neuron-like cells developed a more distinct action potential (Fig. 2B, D15) and eventually repetitive firing (Fig. 2B, D20). The action potentials were still broad, and of relatively low amplitude and could not clearly be distinguished from action potentials sometimes seen in developing glial cells (Sontheimer et al., 1992). Between days 25 and 28 most neuron-like cells developed the short-lasting, low-threshold, high-amplitude APs characteristic of mature neurons (Fig. 4B); of 17 tested cells, APs could be elicited in 13, whereas only 3 showed 'depolarizing humps' and one failed to show any active membrane properties.

There was a considerable variation in the rate of development. In general, differentiation was faster in more populated areas of the cell cultures, and cells with mature APs were often found in distinct clusters (data not shown). Importantly, differentiated progeny of quaternary spheres were also able to develop into neuron-like cells with mature, repetitive APs (Fig. 2B, D28) (n = 3).

**Table I** Changes in membrane properties during neuronal differentiation of MPCs harvested from the adult human brain

<table>
<thead>
<tr>
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<th>D7–11 (n = 15)</th>
<th>D15–18 (n = 10)</th>
<th>D25–28 (n = 9)</th>
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<tr>
<td><strong>Passive membrane properties</strong></td>
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<tr>
<td>Resting membrane potential (mV)</td>
<td>−48 ± 10</td>
<td>−59 ± 9*</td>
<td>−59 ± 9*</td>
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<tr>
<td>Membrane input resistance (MΩ)</td>
<td>557 ± 85</td>
<td>380 ± 79*</td>
<td>24 ± 7</td>
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<tr>
<td>Membrane time constant (ms)</td>
<td>31 ± 11</td>
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<tr>
<td><strong>Active membrane properties</strong></td>
<td></td>
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<tr>
<td>Action potential threshold (mV)</td>
<td>−34 ± 5</td>
<td>−43 ± 5*</td>
<td>−43 ± 5*</td>
</tr>
<tr>
<td>Action potential Na⁺ (nA)</td>
<td>7.3 ± 2.3</td>
<td>3.2 ± 0.4*</td>
<td>3.2 ± 0.4*</td>
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<tr>
<td>1/2 width (ms)</td>
<td>1.0 ± 0.2</td>
<td>2.6 ± 0.3*</td>
<td>2.6 ± 0.3*</td>
</tr>
<tr>
<td>I_{Na} (nA)</td>
<td>0.5 ± 0.2</td>
<td>1.7 ± 0.4*</td>
<td>1.7 ± 0.4*</td>
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<tr>
<td>I_{K} (nA)</td>
<td>0.3 ± 0.1</td>
<td>1.3 ± 0.2*</td>
<td>1.3 ± 0.2*</td>
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The measurements on D25–28 are based only on cells firing repetitive action potentials. *P < 0.05 compared with the earlier stage of differentiation.
From the beginning of the third week, a sharper waveform was elicited at a more negative membrane potential (Fig. 2B, D15 and Fig. 4A, left). This potential was blocked by TTX (Fig. 4A, right). Voltage-clamp recordings revealed an inward current that was activated between $-65$ and $-50$ mV (Fig. 4C upper, $n = 10$) and blocked by TTX (not shown), i.e. a voltage-gated Na$^+$-current (Henderson et al., 1974). The current-voltage plot yielded a bell-shaped curve. The current increased in amplitude with differentiation until the cells had developed mature firing properties (Fig. 4C) (Table 1), reflecting an increase in sodium channel density with maturation.

Repetitive, mature APs were seen between days 25 and 28. Utilizing various blockers of voltage-gated ionic channels we were able to isolate the underlying ionic currents (Fig. 5). In voltage-clamp, depolarizing pulses evoked an initial brief inward current that was activated between $-65$ and $-50$ mV (Fig. 4C upper, $n = 10$) and blocked by TTX (not shown), i.e. a voltage-gated Na$^+$-current (Henderson et al., 1974). The current-voltage plot yielded a bell-shaped curve. The current increased in amplitude with differentiation until the cells had developed mature firing properties (Fig. 4C) (Table 1), reflecting an increase in sodium channel density with maturation.

Synaptic transmission
A prerequisite for the communication between neurons is the formation of synaptic contacts. We first tested whether β-III-tubulin positive cells expressed VGlut-1, which is exclusively expressed in glutamatergic neurons (Ni et al., 1995; Takamori et al., 2000), or GAD-65, which is a rate-limiting enzyme in GABA-synthesis and thus is present in
Fig. 4 (A) On D15, a neuron-like cell showed an overshooting, but immature looking AP (left) that was completely abolished by 0.5 μM TTX (right), indicating Na⁺-dependence. Current pulse: 0–100 pA, 500 ms. (B) Recordings from neuron-like cells during the fourth week of differentiation (left) revealed mature Na⁺-dependent, low-threshold, repetitive APs blocked by TTX (right). Current pulse: 0–100 pA, 300 ms. (C) Voltage-clamp recordings show development of Na⁺-currents during development. The inward currents are plotted as a function of the holding potential (right panel). Scale bars: 5 mV and 25 ms (A and B); 0.5 nA and 5 ms (C).

Fig. 5 (A) Voltage-clamp recordings in a D20 differentiated cell. The protocol consisted of a prepulse from a holding potential of −70 mV to −90 mV for 100 ms, followed by 200 ms steps of 10 mV to +60 mV. (B) A concentration 0.5 μM of TTX was added to block sodium currents. (C) 4-AP (500 μM) blocked the rapid component of the outward current, resembling the Iₖᵢ current. (D) TEA (5 mM) blocked the remaining Iₖc current. (E) The Iₖᵢ current was calculated by subtracting the 4-AP insensitive from the total K⁺-currents. (F) The peak outward currents are plotted as the function of the holding potential. Scale bars: 1 nA and 50 ms.
GABAergic neurons (Martin and Barke, 1998). Seventeen percent of β-III-tubulin positive cells tested during days 25–28 costained with VGlut-1 (Fig. 6A), whereas GAD-65 (Fig. 6B) was found in 24%.

We then examined whether the expression of these markers reflects the presence of functional glutamatergic and GABAergic synapses. During voltage-clamp of neuron-like cells lying in a network (Fig. 6C, D25) we observed brief spontaneous synaptic currents from a holding potential of Vm = 70 mV (Fig. 6D, upper panel). Such spontaneous currents were first detected on day 25, and in 3 of 10 cells tested the currents were blocked by bath application of the glutamate-receptor antagonists CNQX (AMPA/kainate) and MK-801 (NMDA) (D), indicating glutamate-mediated synaptic communication. In another cell at a holding potential of Vm = 30 mV, outward currents were blocked by the GABA A-antagonist bicuculline (E), indicating GABAergic synaptic transmission. Scale bars: 20 µm (A–C); 20 pA and 50 ms (D); 15 pA and 200 ms (E).

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**Discussion**

Using the patch-clamp technique combined with labelling of individual neurones and immunocytochemistry, we show that a self-renewing multipotent cell from the adult human brain proceeds through distinct developmental steps to finally become a mature neuron. Although the pace of maturation differed, our observations may be summarized as follows.

(i) During the first week of differentiation, the cells stained with neuronal markers without exhibiting the functional properties of neurons.

(ii) During the second week, the cells first expressed voltage-gated K⁺-channels that at membrane depolarization resulted in an apparent depolarizing hump, and somewhat later voltage gated Ca²⁺-channels that produced small action potentials.

(iii) Broad, high-threshold, Na⁺-dependant action potentials appeared at the beginning of the third week and gradually evolved into the short-lasting, low-threshold, repetitive action potentials seen in mature cortical neurones.

(iv) By the end of the fourth week, the newly formed neurons exhibited spontaneous GABAergic and glutamatergic post-synaptic currents, indicating that the progeny from a single MPCs had developed into a network of neurons communicating by GABAergic and glutamatergic synapses.

**Functional neurogenesis**

The ventricular wall tissue samples contained precursors that could be propagated in vitro, and cells were able to differentiate into neurons, astrocytes and oligodendrocytes after both 2 and 3 passages. As each neurosphere developed
from a single cell, these observations indicate that the cells fulfil the stem-cell criteria of self-renewal and multipotency (Gage, 2000). The limited replicative ability (4 passages) of the NPCs under our culture conditions is in coherence with other studies of adult human brain progenitors (Johansson et al., 1999; Arsenijevic et al., 2001; Nunes et al., 2003), but contrasts studies on neural progenitors from the foetal brain that have reported extensive self-renewal under similar culture conditions (Piper et al., 2001). As previously suggested (Nunes et al., 2003), the neurosphere-forming cells isolated from the adult human brain thus seem to be a transitional cell type in between SCs and phenotypically committed progenitors.

We next characterized individual cells differentiating after two or more passages. During the first week of development, neuron-like cells had relatively high input resistance and low membrane potential, and showed no signs of active membrane properties. Thereafter, the resting membrane potential gradually increased with development, a phenomenon that has been observed in developing neurons in a variety of experimental preparations (Toda et al., 2000; Wang et al., 2003; Zhou and Hablitz, 1996), although it has been suggested that it may be an artefact owing to the use of patch-clamp electrodes (Zhou and Hablitz, 1996).

The observed decrease in input resistance during development may reflect an increased cell surface area consequent to cell growth. Capacitance also increased, albeit not to a degree that could account for the decrease in input resistance, and as a result the time constant was reduced. The decrease in input resistance is, therefore, most likely a combined effect of growth (increased surface area) and reduced specific resistivity of the cell membrane caused by expression of additional ionic channels (Picken Bahrey and Moody, 2003; Zhou and Hablitz, 1996).

The active current that developed first was not that of a regenerative process characteristic of excitable cells. Although recordings in current-clamp gave the impression of a ‘depolarizing hump’, it was blocked by 4-AP and TEA, and represented an outward K+-current activated by membrane depolarization. It, thus, signifies an early expression of a class of currents terminating the AP, and the waveform gradually changed into the short-lasting, overshooting action potential seen in mature neurons. In the developing nervous system of lower mammals this process has been related to an increased density of sodium and potassium channels (Gao and Ziskind-Conhaim, 1998; Zhou and Hablitz, 1996). Cells were initially only able to fire single action potentials. This may prevent excessive calcium influx during the prolonged action potential seen in an immature neuron (Zhou and Hablitz, 1996). Immature excitability has previously been observed after 2 weeks differentiation of multipotent neural progenitor cells isolated from the adult human subcortical white matter (Nunes et al., 2003). Voltage-gated Na+-channels and immature APs do, however, also occur in developing glial cells (Sontheimer et al., 1992). In the present study, we identified cells with repetitive, overshooting, low-threshold, short duration APs, signifying that the neuron-like cells had developed all the characteristic of mature neuronal excitability (Spitzer et al., 2002), including a recovery process that enables reexcitation.

Mature neurons typically (i) fire short-lasting, repetitive, low-threshold action potentials and (ii) communicate by synapses. These two properties also define the two most important criteria for determining whether an SC has developed into a functional neuron (Reh, 2002). Following 4 weeks of differentiation, we not only observed mature intrinsic neuronal behaviour, but also spontaneous postsynaptic currents that were blocked by antagonists against GABA and glutamate receptors, respectively. These currents require both spontaneous release of transmitter quanta from presynaptic terminals and postsynaptic receptors. Their presence thus indicates that the cells are integrated in a network of neurons communicating by synapses.

The specificity of differentiated neurons

The cortex has two general groups of neurons, excitatory projection neurons and inhibitory interneurons. The former are glutamatergic and the latter mainly GABAergic. During brain development excitatory projection neurons originate from the VZ (Luskin et al., 1988; Takahashi et al.,
1994). Lineage experiments have, however, indicated that interneurons are derived from a separate population of progenitors. Recent studies utilizing topical injections of [3H]thymidine into the embryonic VZ and ganglionic eminence identified the developing striatum as the main source of cortical interneurons (Anderson et al., 2002), and showed that different classes of interneurons originate from distinct regions of the ganglionic eminence (Xu et al., 2004). In the present study we found that the progeny derived from MPCs differentiated into both glutamatergic and GABAergic neurons. This is in keeping with the hypothesis that a part of the GABAergic interneurons of the human cortex originate from the ventricular wall (Letinic et al., 2002), and that new cells in the olfactory bulb express GABA (Winner et al., 2002). Whether cultured MPCs from the human ventricular wall are restricted in differentiation to the cell types found in the cortex is not known. We did, however, observe a number of cells that were both GAD-65 and VGlut1 negative, but still displayed neuronal antigens. These cells may represent neurons with other transmitter specificities, or cells that display neuronal antigens without being true neurons.

### Putative clinical implications

The discovery of MPCs in the adult human brain and the emerging technology for expanding and differentiating these cells in vitro may open new possibilities for the treatment of neurodegenerative diseases either by transplanting cells that have been propagated in vitro or by stimulating recruitment of endogenous MPCs. One fundamental mechanism in this scenario is thought to be neuronal replacement, i.e. the ability of the MPCs to replace the neurons lost through the disease process. A more intimate knowledge of how these cells differentiate and behave will improve the prospects for future therapeutic applications. The present paper, where we have characterized the development from progenitor cell to mature neuron, is a modest first approach to such an essential task.

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### References


Adult human brain progenitors: neuronal development


