Immunocytochemical and Physiological Characterization of a Population of Cultured Human Neural Precursors

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Piper, David R., Tahmina Mujtaba, Mahendra S. Rao, and Mary T. Lucero. Immunocytochemical and physiological characterization of a population of cultured human neural precursors. J Neurophysiol 84: 534–548, 2000. Human neural precursor cells (HNPC) have recently become commercially available. In an effort to determine the usefulness of these cells for in vitro studies, we have grown cultured HNPCs (cHNPCs) according to the supplier specifications. Here we report our characterization of cHNPCs under nondifferentiating and differentiating growth conditions and make a comparison to primary HNPCs (pHNPCs) obtained at the same developmental time point from a different commercial supplier. We found that under nondifferentiating conditions, cHNPCs expressed nestin, divided rapidly, expressed few markers of differentiated cells, and displayed both 4-aminopyridine (4-AP)-sensitive and delayed-rectifier type K+ currents. No inward currents were observed. On changing to differentiating culture conditions, cHNPCs expressed nestin, divided rapidly, expressed few markers of differentiated cells, and displayed both 4-aminopyridine (4-AP)-sensitive and delayed-rectifier type K+ currents. The inward currents included TTX-sensitive and -resistant Na+ densities were indistinguishable from those in undifferentiated cells. Neurotransmitters acetylcholine and glutamate. The outward current voltage-dependent currents, and responded to the application of the markers, did not divide, expressed inward and outward time- and ating culture conditions, a majority of the cells expressed neuronal inferences. No inward currents were observed. On changing to differenti- rents, sustained Ca2+ currents. Comparison of the properties of differentiated cells from cHNPCs with neurons obtained from primary fetal cultures (pHNPCs) revealed two major differences: the differentiated cHNPCs did not express embryonic neural cell adhesion molecule (E-NCAM) immunoreactivity but did co-express GFAP immunoreactivity. The co-expression of neuronal and glial markers was likely due to the growth of cells in serum containing medium as the pHNPCs that were never exposed to serum did express E-NCAM and did not co-express glial fibrillary acidic protein (GFAP). The relevance of these results is discussed and compared with results from other neuronal progenitor populations and cultured human neuronal cells.

INTRODUCTION

Pluripotent stem cells in the CNS can give rise to glia and neurons either directly or through intermediate precursors (Kalyani et al. 1999; Kilpatrick and Bartlett 1993; Price and Thurlow 1988; Price et al. 1992; Reynolds and Weiss 1996; Reynolds et al. 1992; Temple and Davis 1994; Williams and Price 1995) and include the neuroepithelial (NEP) cells identified in the developing spinal cord of day E10.5 rats and E9.0 mice. These NEP cells divide in vitro and in vivo and have been shown to give rise to lineally restricted, intermediate precursor cells in both mass and clonal cultures (Kalyani et al. 1997; Mujtaba et al. 1998). Two classes of restricted precursors have been shown to differentiate directly from multipotent stem cells: a neuron restricted precursor (NRP) and a glial restricted precursor (GRP or oligosphere). These intermediate precursor cells can be isolated from E13.5 rat and E12.0 mouse in vivo or derived from NEP cells in vitro (Kalyani et al. 1998, 1999; Mayer-Proschel et al. 1997; Rao et al. 1998). GRPs may further differentiate into oligodendrocytes or astrocytes but not neurons. Conversely the NRPs may generate multiple kinds of neurons but not oligodendrocytes or astrocytes. NRP cells are characterized by E-NCAM/2F7 immunoreactivity and the absence of A2B5 immunoreactivity while GRP cells are charac- terized by the expression of A2B5 and the absence of 2F7 or E-NCAM immunoreactivity (Kalyani et al. 1998, 1999; Mayer-Proschel et al. 1997; Rao and Mayer-Proschel 1997; Rao et al. 1998). A great deal of evidence has been collected to support this model of lineally restricted, neural development; however, it represents but one of several views in the current literature (for a discussion on alternative models see Kalyani and Rao 1998).

Several lines of evidence indicate that human neural develop- ment may mirror rodent neural development through a sig- nificant period of embryogenesis (Herschkowitz 1988; Mrzljak et al. 1990). By week six of embryological development, the human neural tube has differentiated into an outer mantle layer, and an inner proliferative zone and some neurons have already differentially differentiated. Neurogenesis and gliogenesis proceed over the next several weeks and most, though not all, neuronal proliferation is completed by 8–10 wk of gestation. Gliogenesis proceeds for longer periods, and multipotent stem cells, GRPs and NRPs can be isolated from embryos at 10–18 wk of gestation (Chalmers-Redman et al. 1997; Kalyani et al. 1998; Li et al. 1999; Quinn et al. 1999; Svendsen et al. 1997; Tohyama et al. 1991). The antigens expressed by human pre-cursor cells, as well as their growth factor responses, appear similar to those described in rodent cells (Li et al. 1999; Scolding et al. 1999; Thal et al. 1992). Several groups have isolated human stem cells (Carpenter et al. 1999; Johansson et al. 1999; Vescovi et al. 1999; Yandava et al. 1999). Li et al. (1999) showed that human primary neuronal cell cultures con- tained dividing neuron restricted precursors that expressed E-NCAM and could differentiate into relatively mature neu-
rons. Precursor cells could be immortalized and neuronal re-
stricted precursor cell lines generated. The electrophysiological
properties of any of these precursor cells and the differentiated
population were not described, and to date only morphological
and antigenic characteristics have been examined.

To test the properties of human neural precursor cells
(HNPCs) we examined two commercial sources of HNPCs
isolated at overlapping gestational ages. Here we report the
mitotic, antigenic and electrophysiological phenotypes of cells
from one source (Clonexpress) in a set of baseline culture
conditions. This heterogeneous population, which has been
cultured and passaged multiple times in the presence of serum
cell. Cells were maintained at 37°C in 5% CO2 -95% air. The basal,
dishes (Corning) coated with fibronectin at a dilution of 5,000 cells/
day. Plates were rinsed with medium once before plating the cells.
Comparison of the electrophysiological properties of neuronal
precursor cells described from other species and spatiotempo-
ral points in development (Feldman et al. 1996; Luskin et al.
1997) as well as glia and developing neurons in acute and
primary cultures (Black and Waxman 1996; Sontheimer et al.
1992) reveal both similarities and differences. We found that
nestin-immunoreactive cells that incorporate 5-bromodeoxy-
uridine (BrDU) at high rates are present in these human
neural cell cultures. These cells can differentiate into postmi-
totic cells that exhibit neuronal morphologies; express multiple
neuronal-specific markers, including β-III tubulin, MAP-2, and
neurofilament and a repertoire of voltage- and neurotransmit-
ter-gated ion channels but do not fire action potentials. These
results show that heterogenous cHNPCs can be directed to
differentiate into a neuronally restricted lineage that appears
arrested at an early stage.

METHODS

Substrate preparation

Poly-L-lysine (PLL, Sigma, St. Louis, MO) was dissolved in
distilled water (13.3 µg/ml) and applied to tissue culture plates for an
hour. Excess PLL was withdrawn and the plates were allowed to air
dry. Plates were rinsed with medium once before plating the cells.

Human fetal cell cultures

The cHNPCs (Clonexpress, Gaithersberg, MD) are provided as live
suspension cells. The cells are obtained from the CNS of 12- to 18-wk
embryos. They can be maintained as undifferentiated cultures by
plating on uncoated plastic tissue culture dishes (Corning, Corning,
NY) in a chemically defined basal medium (provided by Clonexpress)
that consisted of DMEM/F12 medium 1:1 (DMEM/F12) supplemented with additives, basic fibro-
blast growth factor (bFGF) and epidermal growth factor (EGF).

Growth of cHNPCs cells

Undifferentiated cHNPCs were maintained in medium (Clonexpres-
press) that consisted of DMEM/F12 + 10% FBS (fetal bovine serum)
+ neuronal cell supplement (NCS, containing bFGF and
EGF). NCS was a proprietary supplement provided by Clonexpress
and contained insulin, transferrin and an undefined set of additives.

Cells will survive for short periods in the absence of serum but could not
be passaged or maintained for 7–10 days without serum supple-
mentation as undifferentiated cells.

For neuronal differentiation, cells were plated on PLL-coated
dishes at a density of 1–2 × 10^3 cells/cm^2 as recommended by the
supplier. The differentiation medium consisted of N2 supplement
(GIBCO, Grand Island, NY) to replace serum; NCS (contains bFGF
and EGF); and dibutyl cyclic AMP (D6cAMP, 100 µM/ml, Sigma),
nerve growth factor (NGF, 50 ng/ml, Upstate, Lake Placid, NY), or
bone morphogenic protein (BMP-2, 10 ng/ml, Creative Biomolecules,
Boston, MA), which was added every 2 days. The cells were allowed
to mature for ≥4 days in differentiation medium prior to physiological
or immunocytochemical analysis of cells. Under differentiating
conditions, the cells could be maintained for 3–4 wk provided that the
medium was changed every 72 h.

Immunocytochemistry

Staining procedures were as described previously (Rao and Mayer-
prosche 1997). Staining for cell surface markers such as embryonic
neural cell adhesion molecule (E-NCAM) was done in cultures of
living cells. To stain cells with antibodies against internal antigens,
cultures were fixed with 2–4% formaldehyde for 30 min at room
temperature. In general, dishes were incubated with the primary
antibody for one hour, followed by incubation with an appropriate
secondary antibody for an additional hour. Double-labeling exper-
iments were performed by simultaneously incubating cells in appro-
priate combinations of primary antibodies followed by noncrossreac-
tive secondary antibodies. Negative controls with omission of primary
or secondary antibodies were run simultaneously. The 4',6 di-
amidino-2-phenylindole (DAPI) histochemistry was performed as de-
scribed previously (Kalyani et al. 1997). DAPI staining was generally
done after all other antibody staining had been completed. Stain-
ing was visualized under phase optics using special dichroic filters
(Omega) that isolate the appropriate excitation and emission wave-
lenghts associated with each marker and thereby reduce any marker to
marker bleedthrough to low background levels, though they also
reduce overall fluorescence intensity. We also routinely stain with
only one marker to test for bleedthrough across the filter sets used.

E-NCAM antibody was a hybridoma supernatant obtained from
Developmental Hybridoma Studies Bank (DHSB, University of Iowa,
Iowa City, IA). Neuron-specific β-III tubulin and microtubule asso-
ciated protein 2 (MAP-2) antibodies were obtained from Sigma. An
anti-nestin polyclonal used in some double-labeling experiments was a
kind gift of Dr. Keith Cauley (Signal Pharmaceuticals, San Diego,
CA). The A2B5 antibody (Eisenbarth et al. 1979) was obtained from
American Type Culture Collection (ATCC, Manassas, VA) and used
to label glial precursor cells as described previously (Rao et al. 1998).
O4 and Gal-C antibodies that recognize specific glycoprotein epitopes
expressed by oligodendrocytes were obtained from DHSB and used as
described previously (Rao et al. 1998). BrDU (Sigma) was used to
determine the number of S-phase cells. Mouse and rat monoclonal
anti-BrDU antibodies were obtained from Boehringer Mannheim
(Indianapolis, IN). All secondary antibodies were purchased from
either Jackson ImmunoResearch Laboratories (West Grove, PA) or
Southern Biotechnology Associates (Birmingham, AL).

Electrophysiology

Current- and voltage-clamp recordings were made using the whole
cell patch-clamp technique (Hamill et al. 1981). Electrodyes were
pulled from thick-walled borosilicate glass on a Flaming/Brown P-87
pipette puller (Sutter Instruments, Novato, CA), to resistances of
~3–4 MΩ. An Axopatch 200B amplifier was used to control electrode
potentials or inject current, a TL-1 DMA interface to convert A/D
values at 5–50 kHz, filtered at 2–10 kHz, and pClamp 5.5 software to
control both the amplifier and the converter (Axon Instruments, Foster
Cells were plated on 12-mm glass coverslips as described in the preceding text. Coverslips were placed in an acrylic chamber on an Olympus IMT-2 microscope and perfused at 1.0–1.8 ml/min. The external bath solution, rat Ringer (RR), consisted of (in mM) 140 NaCl, 3 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose. External solutions were set to pH 7.4 with NaOH. Bath ground was established with a 3 M KCl/Agar bridge. External test solutions were made up in RR and included 300 nM tetrodotoxin (TTX), 5 mM 4-AP, and 500 μM acetylcholine (Ach). Test solutions were applied through square glass tubes, which could be rotated into or out of position with respect to the cell using a galvanometer.

The internal pipette solutions varied. To study Na⁺ currents in isolation, either Cs⁺ was used to replace internal K⁺ or internal tetraethylammonium (TEA) was used to block K channels. The CsF internal solution consisted of (in mM) 125 CsF, 15 CsCl, 11 EGTA, and 10 HEPES. The TEA internal solution consisted of (in mM) 125 potassium tetramethylammonium-n-oxide (KTMAO), 15 KCl, 5 MgCl₂, 11 EGTA, 10 HEPES, and 10 TEA. To measure K⁺-selective currents, we used a KF internal solution that consisted of (in mM) 125 KF, 15 KCl, 11 EGTA, and 10 HEPES. Some K⁺-selective currents and action potentials were recorded using a KASP solution, which consisted of (in mM) 50 KF, 75 KAspartate, 15 NaCl, 11 EGTA, and 10 HEPES. All internal solutions were set to pH 7.2 with CsOH, tetramethylammonium hydroxide or KOH as appropriate. Liquid junction potentials (LJP's) were calculated with Axoscope (Axon Instruments), and data were corrected appropriately. The calculated potentials for all internal solutions with RR as the external solution were: CsF = 9.1 mV, KF = 8.4 mV, TEA = 10.0 mV, KASP = 12.1 mV.

Passive membrane properties such as cell capacitance (Cₘ), input resistance (Rᵢ), and series resistance (Rₛ) were calculated from current records obtained by averaging 24 records stepping from −70 to −100 mV. Cₘ was calculated by integrating the charge under the current transient resulting from the −10 mV step and dividing it by −10 mV. Rᵢ was calculated by fitting a single exponential to the falling phase of the capacitance transient to obtain the RC time constant, τᵣᵢ. Rₛ was then calculated as

\[ Rₛ = \tauᵣᵢ/Cₘ \]

The Rₛ values were used to correct the command voltages off-line by

\[ Vₘ = V_{command} - V_{series}; \quad Vₘ = V_{command} - (Iₒ*₮Rₛ) \]

Where Vₘ = actual membrane voltage; V_{command} = applied pipette potential; V_{series} = voltage across series resistance; Iₒ = measured membrane current; and Rₛ = calculated series resistance.

Activation parameters were obtained from current-voltage (I-V) relationships by voltage-clamping the cell membrane to −80 or −100 mV, stepping to test voltages between −80 and +80 mV, in 10-mV increments, for either 10 or 100 ms, and returning to the initial holding potential. Linear leak currents were subtracted using a P/4 protocol (Armstrong and Bezanilla 1973). Peak currents elicited by the voltage step were measured in either the inward or outward direction and plotted against the test voltage, which was corrected off-line for series resistance and LJP errors. Average activation curves were constructed by averaging the fitted values because the actual test voltages varied with Rₛ from cell to cell. Estimates of activation parameters were obtained by fitting the I-V relationships to the following form of the Boltzmann equation

\[ I_{test} = g(V)(Vₘ - V_{rev}) \]

\[ g(V) = G_{max}*(1/1 + \exp((V_{rev} - V_{test})/k)) \]

Where, I_{test} = the current flowing through the channels; V_{test} = the test voltage; V_{rev} = the Nernst reversal potential of the activated currents; g(V) = the activation parameter approximated by a Boltzmann function; where, G_{max} = the maximal whole-cell conductance, V_{half} = the voltage at which the conductance is half-maximally activated and k, the inverse slope factor, represents the dependency of g(V) on voltage.

Steady-state inactivation data were acquired by holding the membrane at −120 mV to allow full channel recovery from inactivation, stepping to a prepulse potential between −120 and −25 mV for 500 ms (3 s for K⁺ currents) and then stepping to 0 mV (50 or 60 mV for K⁺ currents) to measure the available current. Percent available current was calculated by normalizing the currents obtained at each potential by the maximum current response, I_{max}. The distribution I_{test}/I_{max} versus voltage could then be approximated by a Boltzmann equation

\[ I_{test}/I_{max} = 1/(1 + \exp((V_{test} - V_{half})/k)) \]

Where, I_{test} = current elicited by a voltage step, V_{test}; I_{max} = maximal current activated; V_{half} = the voltage at which half of the maximal current is available, and k is the inverse slope factor.

**Intracellular Ca²⁺ measurements**

Calcium-imaging experiments were performed on chHNPCs maintained under differentiating conditions as described above. Cells were loaded with 5 μM fura-2 AM and 80 μg/ml Pluronic F127 (Gryniewicz et al. 1985) in RR for 30 min at 23°C in the dark. The cells were then washed three times with RR, and the fura-2 AM was allowed to de-esterify for 30 min. Changes in the ratio of fluorescence emission intensity at 520 nm by excitation at 340/380 nm were measured and correlated to changes in intracellular calcium, [Ca²⁺], using a simple two-point calibration scheme

\[ [Ca^{2+}] = Kₐ/[R(R_{Hi}-R)/R_{Hi}]\]

Where R = measured ratio; Kₐ = 225 mM, the dissociation constant for Ca²⁺ and fura-2; R_{Hi} = ratio for 0 mM Ca²⁺ standard; R_{Hi} = ratio for 10 mM Ca²⁺ standard; Denominator = denominator intensity for 0 Ca²⁺ standard; and Denominator = denominator for 10 mM Ca²⁺ standard (Gryniewicz et al. 1985).

A Zeiss-Attofluor imaging system (Atto Instruments, Rockville, MD) was used to acquire and analyze the data, which were sampled at 1 Hz. Cells were constantly perfused at 1–1.5 ml/min with RR. Neurotransmitters (500 μM) were made fresh in RR and delivered by bath exchange using a small volume loop injector (200 μl) located ~250 μl upstream from the bath. A response to neurotransmitter was defined as a minimum 10% transient rise over the baseline fluorescence ratio within 60 s from the time of loop insertion. The dead time from loop to bath inport was ~10–15 s. Neurotransmitters examined included: γ-amino-butyric acid (GABA), glycine (G), dopamine (DA), glutamate (E), and ACh. In addition, a 50 mM K⁺ RR solution was used to depolarize the cells and test for Ca²⁺ influx through voltage-gated channels (45 mM NaCl in RR replaced by 45 mM KCl). Ascorbic acid (500 μM) was added to dopamine solutions to prevent oxidation. Control applications of 500 μM ascorbic acid had no effect (Fig. 9). The pH of all test solutions was adjusted to 7.4 with 1 M NaOH.

**RESULTS**

**Cell Division, morphology, and immunoactivity**

Fetal human CNS cells are now available from two commercial sources: Clonetics (pHNPCs) and Clonexpress (cHNPCs). Clonetics cells have been shown to be a mixed population that can generate neurons and astrocytes (Svendsen et al. 1997). Clonetics cells have been shown to be a mixed population that can generate neurons and astrocytes (Svendsen et al. 1997). Clonexpress cells are derived from similar ages (cHNPCs). Clonetics cells have been shown to be a mixed population that can generate neurons and astrocytes (Svendsen et al. 1997). Clonexpress cells have been shown to be a mixed population that can generate neurons and astrocytes (Svendsen et al. 1997).
development, we obtained cHNPCs, maintained them in culture by supplier’s protocols, and examined their properties under nondifferentiating and differentiating culture conditions.

Unlike previously described pHNPCs (Li et al. 1999; Svendsen et al. 1997), the cHNPCs obtained from Clonexpress consisted of a relatively undifferentiated population. All cells expressed nestin and many were dividing in culture as shown by BRdU incorporation (Fig. 1, 30–40%, n = 5 independent experiments). Morphologically, cells appeared flat with few processes, and they formed tight clusters. Cells could be maintained in an undifferentiated condition for multiple passages (≥5). At both early and late passages, <5% of the cells expressed GFAP or β-III tubulin immunoreactivity and no cells that expressed either A2B5 (Eisenbarth et al. 1979) or other markers of oligodendrocyte differentiation were detected (data not shown).

When cells were replated into differentiation conditions (growth in DMEM/F12 with NCS containing EGF and bFGF, replacement of serum with N2 supplement, and addition of either DbcAMP, NGF, or BMP-2) cells altered their morphology and a substantial number (>80%, n = 5 independent experiments) appeared phase bright and generated processes of
greater than two cell diameters (Fig. 1). Immunocytochemistry showed that both β-III tubulin and GFAP immunoreactive cells could be detected in culture and occasional A2B5 reactive cells were seen. No Gal-C or O4 immunoreactive cells (oligodendrocytes and oligodendrocyte precursors) were detected in any culture condition (data not shown). β-III tubulin immunoreactive cells expressed other neuronal markers such as neurofilament (NF) and MAP-2 but did not incorporate BrDU when cells were differentiated. DbcAMP, NGF, and BMP-2 all arrested mitosis and enhanced neurite outgrowth (Fig. 1 and data not shown), but in no condition did we observe fully mature neurons as assessed by cell size, cell aggregation, and neurite length.

**Passive membrane properties and overall ionic currents**

We measured the passive membrane properties of 66 cells with typical neuronal morphologies (Fig. 1B, maturing) and from 6 cells without processes (Fig. 1A, immature). The maturing cells had $C_m = 32.1 \pm 2.1 \text{pF}$, $R_m = 13.6 \pm 1.4 \text{MΩ}$, and $R_T = 810 \pm 127 \text{MΩ}$; the immature cells had $C_m = 16.6 \pm 2.7 \text{pF}$, $R_m = 9.1 \pm 1.7 \text{MΩ}$, and $R_T = 867 \pm 290 \text{MΩ}$ (± SE unless noted). Comparison of these values by Student’s $t$-test revealed a significant difference in $C_m$ for the two cell types ($P < 0.03$), presumably due to neurite outgrowth in the maturing cells, but not for either $R_m$ or $R_T$ ($P > 0.8$ and $P > 0.3$), suggesting similar levels of resting conductances and comparable levels of electrical access to the cell membrane in the two cell types. Figure 2, A and B, shows representative currents from an immature and a maturing, multipolar, neuronal cell obtained by holding the membrane potential at $-80 \text{ mV}$ and stepping to test potentials between $-60$ and $+80 \text{ mV}$ in 10-mV increments. While both cell types express significant time- and voltage-dependent outward currents, none of the immature cells expressed any inward currents. In contrast, 53 of 64 maturing cells (83%) exhibited recognizable time- and voltage-dependent inward currents. These data suggest that as the immature cells stop dividing and begin differentiating, they concurrently begin expressing Na channels without a significant change in the density of K channel expression. Resting membrane potentials ($V_{\text{rest}}$) were measured from 17 maturing, neuronal cells using the KF ($n = 7$) or KAsp ($n = 10$) internal solutions, ranging from $-17.1$ to $-56.5 \text{ mV}$ and averaged $-34.3 \pm 3.0 \text{ mV}$.

**Outward currents—potassium**

The outward currents appeared to be a mixture of several outward, presumably K$^+$-selective, currents ($I_K$) in both the immature and maturing neuronal cells. The transient outward current obvious in the records (Fig. 2, A and B) resembles the A-type, 4-AP-sensitive current, $I_{K(\text{A})}$, described in neurons and many other cell types (Connor and Stevens 1971; Rudy 1988). Figure 2, C and D, displays currents recorded from the same two cells in the presence of 5 mM 4-AP. 4-AP eliminated the fast outward current and revealed a slowly activating outward current that resembled a delayed-rectifier type of K$^+$ current, $I_{K(DR)}$. Figure 2, E and F, represents the 4-AP-sensitive currents obtained by subtracting the current records in the presence of 5 mM 4-AP from those in the absence of 5 mM 4-AP. Every cell examined displayed some outward current except for two neuronal cells, (6/6 immature, 34/36 maturing neuronal). We obtained the outward current densities by dividing the peak outward current at $+50 \text{ mV}$ of each cell by its capacitance. In the immature cells, the outward current density averaged $36.6 \pm 13.4 \text{ pA/µF}$ ($n = 6$) and did not differ from the maturing neuronal cells, $28.3 \text{ pA/µF} \pm 3.5 \text{ pA}$ ($n = 34$, Student’s $t$-test, $P > 0.4$). These data demonstrate that as the neuronal cells begin to differentiate, the density of K channels in the plasma membrane remains relatively constant. We further characterized the kinetics of the 4-AP-sensitive current and the steady-state properties of both the 4-AP-sensitive and -insensitive components of the outward current.

**4-AP-sensitive K$^+$ channel kinetics**

The time to half-peak ($t_{1/2}$) and inactivation time constant ($\tau_i$) of $I_{K(\text{A})}$ were measured or fit by single exponential distributions, respectively, from 4-AP-subtracted currents recorded from the immature ($n = 5$) or maturing ($n = 4$) cells. Since there was no significant difference for either $t_{1/2}$ or $\tau_i$ between the two cell types, the distributions were averaged. Figure 3A shows the time to half-peak versus voltage for both cell morphologies from a sample of nine cells. Fitting a single exponential function to this distribution (or distributions obtained from either the immature or maturing neuronal cell types separately, from $-20$ to $80 \text{ mV}$) revealed that $t_{1/2}$ gets faster with respect to voltage and follows a $\tau = 14.0 \pm 0.8 \text{ mV}$ (---). Figure 3B plots $\tau_i$ versus voltage for the same group of cells. Inactivation kinetics were relatively voltage independent and the mean time constant across the voltage range shown ($0–80 \text{ mV}$) was $\tau_i = 18.1 \pm 0.9 \text{ ms}$. The similarity in kinetics between the two cell populations suggests that both the immature and maturing neuronal cell types express the same 4-AP-sensitive K channel. Kinetic analysis was not performed on $I_{K(DR)}$ as we did not isolate it from other currents including possible Ca$^{2+}$-activated K$^+$ currents.

**K$^+$ channel steady-state inactivation and activation**

Steady-state activation and inactivation parameters were obtained as described in METHODS. $I_{K(\text{A})}$ generally activates and inactivates faster and at more hyperpolarized potentials than $I_{K(DR)}$ (Connor and Stevens 1971). Figure 4A shows the raw current traces obtained by the steady-state inactivation protocols shown in the inset from a maturing neuronal cell expressing both $I_{K(\text{A})}$ and $I_{K(DR)}$. The majority of $I_{K(\text{A})}$ is inactivated by $-60 \text{ mV}$, revealing $I_{K(DR)}$, which begins to inactivate near $-70 \text{ mV}$. Figure 4B plots the average $I_{\text{max}}$ versus prepulse voltage for seven cells that expressed a 4-AP-sensitive, transient current. Fitting a Boltzmann distribution to the $I_{K(\text{A})}$ availability data yielded a $V_{\text{half}} = -80.3 \pm 0.5 \text{ mV}$. For $I_{K(DR)}$, a fit to the availability data from three cells yielded $V_{\text{half}} = -42.1 \pm 0.8 \text{ mV}$. Activation parameters were fit to either 4-AP-subtracted currents, $I_{K(\text{A})}$, or 4-AP-resistant currents, $I_{K(DR)}$, as described in METHODS. The fitted values were averaged and plotted with the inactivation data in Fig. 4, B and C: $I_{K(\text{A})}$, $V_{\text{half}} = -18.9 \pm 5.2 \text{ mV}$, and $I_{K(DR)}$, $V_{\text{half}} = 10.4 \pm 7.6 \text{ mV}$ ($n = 7$). For both currents, a small “window current” (centered around $-57 \text{ mV}$ for $I_{K(\text{A})}$ and $-22 \text{ mV}$ for $I_{K(DR)}$ can be seen where the activation and availability curves overlap.
suggesting that around these voltages, steady-state activation of the particular $K_{\text{1}}$ channel can occur and partially contribute to $V_{\text{rest}}$.

Inward currents

POTASSIUM. In several of the cells studied, a nonsaturating, inward current artifact was observed, caused by the P/4 subtraction protocol activating nonlinear conductances at hyperpolarized potentials (data not shown). The currents in Fig. 5A were activated by the hyperpolarizing voltage step protocol shown in the inset. The $I$-$V$ relationships for the peak currents (○, taken from the 1st 20 ms after the test voltage step) and the stationary currents (○, measured at 900 ms after the test voltage step) are shown in Fig. 5B. The kinetics and voltage dependence of these hyperpolarization-activated currents resemble those of an inwardly rectifying potassium channel, $I_{K_{\text{IRP}}}$, but we did not characterize these currents pharmacologically. Other currents that may activate in these voltage ranges include the hyperpolarization activated nonselective cation channel, $I_{\text{h}}$, and the human

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**FIG. 2.** Gross neuronal function correlates with gross neuronal structure. A and B: currents recorded from a voltage-clamped round, immature cell (A) or a multipolar, maturing cell (B) with neuronal morphology. C and D: currents recorded from the same cells in the presence of 5 mM 4-aminopyridine (4-AP), reveal a delayed-rectifier like $K^{+}$ current ($I_{K_{\text{DR}}}$). E and F: currents obtained by subtracting the currents recorded in the presence (A and B) from those obtained in absence (C and D) of 5 mM 4-AP represent the 4-AP-sensitive component of this mixed $K^{+}$ current. *Insets:* I-$V$ relationships where the total outward current is plotted with squares; the 4-AP-sensitive, $I_{A}$-like, ○; and the 4-AP-insensitive current, presumably $I_{K_{\text{DR}}}$, □. Immature, round cells, presumably mitotic, have outward $K^{+}$ currents but not inward $Na^{+}$ currents (6/6). Maturing cells with neuronal morphologies have both outward $K^{+}$ currents and inward $Na^{+}$ currents. Solutions: KF internal/rat Ringer (RR) external ±5 mM 4-AP.
ether-a-go-go-related gene product, HERG. $I_h$, however, typically activates over an order of magnitude more slowly, and HERG channels would typically be closed at holding potentials of $-80$ mV where many of the P/4 subtraction protocols activated this current.

**CALCIUM AND SODIUM.** In our recordings, only the maturing neuronal cells expressed inward time- and voltage-dependent

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**Fig. 3.** K channel kinetics. **A:** time to half-peak of the 4-AP-sensitive current is weakly voltage dependent. Time to half-peak was measured at voltages ranging from $-20$ to $80$ mV, averaged, plotted against membrane voltage, and fit to a single exponential function yielding a $\tau = 14.0 \pm 0.8$ ms. **B:** inactivation kinetics of the 4-AP-sensitive current are voltage independent. The inactivation time constant, $\tau_i$, was fit by a single exponential function to the decay of the 4-AP-sensitive current at voltages ranging from $0$ to $80$ mV, averaged, and plotted against voltage. The mean $\tau_i$ for all voltages was $18.1 \pm 0.9$ ms ($n = 9$). Solutions: KF internal/RR external ±5 mM 4-AP.

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**Fig. 4.** K channel steady-state inactivation and activation properties. **A:** raw currents were elicited from a maturing neuronal cell by the protocol shown in the inset. Currents are an expanded view of the 20-ms test pulse following the 3-s prepulse to various potentials. The transient $I_{K(A)}$ current inactivates at more hyperpolarized potentials than $I_{K(DR)}$. The availability of $I_{K(A)}$ was calculated as described in METHODS for each of 7 cells. The values were averaged at each voltage and plotted in ○ with error bars. The line through the data points is a fit to a Boltzmann function yielding a $V_{1/2} = -80.3 \pm 0.5$ mV and a slope = $8.5 \pm 0.5$ ($n = 7$). —, average activation curve for $I_{K(A)}$, obtained from 4-AP-subtracted currents, where $V_{1/2} = -18.9 \pm 5.2$ mV and slope = $20.5 \pm 1.5$ ($n = 7$). **B:** availability and activation curves for $I_{K(DR)}$. Same analysis as in **B** for voltages between $-60$ and $0$ mV. A fit of the mean availability values to the Boltzmann equation yielded a $V_{1/2} = -42.1 \pm 0.8$ mV and slope = $8.2 \pm 0.8$. —, mean of the fits to activation curves derived from currents in the presence of 4-AP, $V_{1/2} = 10.4 \pm 7.6$ mV and slope = $20.3 \pm 1.3$ ($n = 7$). Solutions: KF/KAsp internal/RR external ±5 mM 4-AP.
inward currents that resembled Na\textsuperscript{+} pharmacologically. Of 64 cells, 53 displayed fast, transient Ca\textsuperscript{2+} slowly activating, sustained inward currents that resembled neuronal morphologies (not round). Some cells showed currents so all subsequent recordings were made from cells that expressed I\textsubscript{Na} that was only blocked 20–50% by TTX and 3 of the 26 cells expressed I\textsubscript{NaG} that was completely insensitive or resistant to 300 nM TTX (TTX-R). Figure 6A shows TTX-subtracted currents recorded from a cell that expressed TTX-S currents; application of 300 nM TTX blocked all of the I\textsubscript{Na} in this cell. Figure 6B shows currents recorded from a cell that expressed TTX-R currents; 300 nM TTX blocked almost none (13%) of the I\textsubscript{Na} in this cell. We fit the I-V relationships (with peak I\textsubscript{Na} > |150 pA|) from the TTX-S and TTX-R currents to a modified form of the Boltzmann equation (see METHODS) and found that the half voltage for the TTX-S and TTX-R I\textsubscript{Na} differed significantly: \(V_{1/2}(\text{TTX-S}) = -25.4 \pm 3.2\) mV (\(n = 10\)), \(V_{1/2}(\text{TTX-R}) = -34.9 \pm 1.9\) mV (\(n = 7\), \(P < 0.0004\), Student’s t-test). TTX-R sodium currents have been reported in developing dorsal root ganglion neurons (Caffrey et al. 1992; Elliott and Elliott 1993) but show a depolarizing shift in the voltage dependence of activation and are considerably slower to activate and inactivate. Another TTX-R sodium current was first described in glia (Black et al. 1992; Sontheimer and Waxman 1992; Sontheimer et al. 1992), and termed I\textsubscript{Na(G)} for glial sodium current. Two different genes have been identified that may encode this current, Na-G and NaCh6 (Gautron et al. 1997). I\textsubscript{Na(G)} differs from I\textsubscript{TTX-R} in dorsal root ganglion cells by displaying a hyperpolarizing shift in both the steady-state activation and inactivation curves compared with the classic neuronal TTX-S I\textsubscript{Na} and by exhibiting little difference in the kinetics of either activation or inactivation. We examined both the kinetic and steady-state properties of the TTX-S and TTX-R currents to see how they compared with other Na\textsuperscript{+} currents.

**Sodium channel pharmacology and activation**

We studied 26 of the 53 cells further using a CsF or TEA containing pipette solution and 300 nM TTX to isolate the current from the accompanying I\textsubscript{G} and I\textsubscript{Ca}. TTX has been used as a specific blocker of Na channels (Henderson et al. 1974), and 19 of 26 cells tested (77%) expressed I\textsubscript{Na}, that was blocked by 300 nM TTX (TTX-S). Interestingly, 4 of the 26 cells expressed I\textsubscript{Na} that was only blocked 20–50% by TTX and 3 of the 26 cells expressed I\textsubscript{NaG} that was completely insensitive or resistant to 300 nM TTX (TTX-R). Figure 6A shows TTX-subtracted currents recorded from a cell that expressed TTX-S currents; application of 300 nM TTX blocked all of the I\textsubscript{Na} in this cell. Figure 6B shows currents recorded from a cell that expressed TTX-R currents; 300 nM TTX blocked almost none (13%) of the I\textsubscript{Na} in this cell. We fit the I-V relationships (with peak I\textsubscript{Na} > |150 pA|) from the TTX-S and TTX-R currents to a modified form of the Boltzmann equation (see METHODS) and found that the half voltage for the TTX-S and TTX-R I\textsubscript{Na} differed significantly: \(V_{1/2}(\text{TTX-S}) = -25.4 \pm 3.2\) mV (\(n = 10\)), \(V_{1/2}(\text{TTX-R}) = -34.9 \pm 1.9\) mV (\(n = 7\), \(P < 0.0004\), Student’s t-test). TTX-R sodium currents have been reported in developing dorsal root ganglion neurons (Caffrey et al. 1992; Elliott and Elliott 1993) but show a depolarizing shift in the voltage dependence of activation and are considerably slower to activate and inactivate. Another TTX-R sodium current was first described in glia (Black et al. 1992; Sontheimer and Waxman 1992; Sontheimer et al. 1992), and termed I\textsubscript{Na(G)} for glial sodium current. Two different genes have been identified that may encode this current, Na-G and NaCh6 (Gautron et al. 1997). I\textsubscript{Na(G)} differs from I\textsubscript{TTX-R} in dorsal root ganglion cells by displaying a hyperpolarizing shift in both the steady-state activation and inactivation curves compared with the classic neuronal TTX-S I\textsubscript{Na} and by exhibiting little difference in the kinetics of either activation or inactivation. We examined both the kinetic and steady-state properties of the TTX-S and TTX-R currents to see how they compared with other Na\textsuperscript{+} currents.

**Sodium channel kinetics**

The time to half-peak and the decay phase kinetics of the TTX-S and TTX-R currents were measured. Single exponential fits to the decay phase of the currents provided the inactivation time constant, \(\tau_h\). No significant difference was found between \(I_{\text{TTX-S}}\) or \(I_{\text{TTX-R}}\) for either \(t_{1/2}\) or \(\tau_h\) at any voltage (Student’s t-test, 0.5 < \(P < 1\) and 0.08 < \(P < 0.9\), respectively, \(n = 3\)). Figure 7A shows that the times to half-peak for \(I_{\text{TTX-S}}\) and \(I_{\text{TTX-R}}\) decrease with voltage and both could be fit by a single exponential function between −20 and 80 mV, yielding a \(\tau_{\text{TTX-S}} = 28.7 \pm 2.3\) mV that reached a baseline value of 0.48 ms and a \(\tau_{\text{TTX-R}} = 35.3 \pm 2.1\) mV that reached a baseline value of 0.46 ms. Figure 7B plots \(\tau_h\) versus voltage where the mean (−10 to 80 mV) for \(I_{\text{TTX-S}}\) was \(\tau_{h(\text{TTX-S})} = 0.56 \pm 0.03\) ms and for \(I_{\text{TTX-R}}\) was \(\tau_{h(\text{TTX-R})} = 0.66 \pm 0.008\)
ms (Student’s t-test, \( P > 0.16 \)). These data demonstrate that the activation and inactivation kinetics of both the TTX-S and TTX-R Na\(^+\) currents are similar to each other and to the analogous currents described by Sontheimer et al. (1992).

**Sodium channel availability**

We measured the steady-state availability of sodium channels using a typical prepulse protocol to various test potentials between \(-120\) and \(-30\) mV to inactivate the channels before stepping to a depolarized potential (0 mV) to measure the fraction of current left available. Figure 8A shows the TTX-subtracted currents evoked in this manner for a cell expressing TTX-S currents while Fig. 8B shows the currents evoked in the presence of TTX for a cell expressing TTX-R currents. Figure 8C plots \( I_{\text{max}} \) versus prepulse voltage for cells with only TTX-S currents (circles) and for cells with only TTX-R currents (triangles). The availability curve for each current was fit by a Boltzmann equation (see **METHODS**) to estimate the half-point of inactivation and \( V_{\text{half(TTX-S)}} = -60.9 \pm 1.0 \) mV (\( n = 4 \)) while \( V_{\text{half(TTX-R)}} = -89.1 \pm 1.0 \) mV (\( n = 3 \)). Similar to the activation curves, the \( I_{\text{TTX-R}} \) availability curves are significantly shifted to hyperpolarized potentials when compared with \( I_{\text{TTX-S}} \). Since most of the cells we studied showed lower \( I_{\text{Na}} \) densities than typical mature neurons (Feldman et al. 1996) and 7/26 (27%) expressed TTX-R currents, we expect that this neuronal cell population represents intermediate progenitors or precursors to fully mature neurons.

**Intracellular calcium changes in response to neurotransmitters**

To ascertain whether or not the neuronal cells could respond to neurotransmitters like mature neurons, we used fura-2 imaging techniques to monitor \([Ca^{2+}]_i\) in response to application of 500 \( \mu \)M GABA, G, DA, ascorbic acid, E, ACh, and elevated extracellular potassium (50 mM) (Fig. 9). Of the 68 cells tested, 34 responded to E (50%), 68 responded to ACh (100%), and 3 responded to 50 mM extracellular K\(^+\) (−4%). None of the 68 responded to GABA, G, DA, or ascorbic acid.

**Electrophysiological response to ACh**

We also measured the currents elicited by application of 500 \( \mu \)M ACh from two cells in voltage-clamp mode while simultaneously monitoring a group of cells under visual control using fura-red dye. Like the fura-2-loaded cells, most of the cells under visual control exhibited an increase in \([Ca^{2+}]_i\) in response to ACh. Of the two cells we recorded from electrically, both exhibited inward currents of about \(-130 \) pA in response to 300-ms applications of ACh. Figure 9B shows the responses of one of the cells to a 300- and a 900-ms application of 500 \( \mu \)M ACh. In cells with \( R_i \sim 800 \) MΩ, these currents could provide ample depolarization to evoke action potentials, providing the Na channel density is high enough to support regenerative firing behavior (\( V = IR, 80 \) mV = 100 pA*800 MΩ).

**Action potentials**

While the neuronal cells expressed sodium currents, the densities of these currents do not compare to those found in mature neurons (Feldman et al. 1996). To test whether or not the sodium current could support action potential generation, we used whole-cell current-clamp to inject small, depolarizing currents for 1–200 ms from various resting potentials (set by constant current injections) and measured the resulting changes in membrane potential. Of the nine cells tested, none fired even weak action potentials (data not shown). These results suggest that the failure of 65/68 cells to increase \([Ca^{2+}]_i\) in response to elevated extracellular potassium arises, at least in part, from inadequate densities of available sodium current, and the inability to generate action potentials in response to membrane depolarization.
While the cHNPCs grown in differentiating conditions expressed multiple neuronal markers and displayed electrophysiological properties characteristic of neurons, they nevertheless did not appear to mature completely in culture. A possible reason for the failure to mature may be exposure to serum. The cHNPCs are grown in serum containing medium and appear to require serum for their survival. Switching cHNPCs to serum-free medium prior to initiating differentiation is not possible, as cells did not survive the change in medium (data not shown). Serum, however, has been shown to inhibit neuronal differentiation and alter growth characteristics (Kilpatrick and Bartlett 1993). Likewise, transformed glial precursor cells will undergo a mesenchymal transformation and no longer differentiate into oligodendrocytes (Noble and Mayer-Proschel 1997). To test if exposure to serum may have altered the properties of cHNPCs, we examined the expression of E-NCAM and GFAP by β-III tubulin immunoreactive cHNPC-derived cells. Cells were grown in Clonexpress-supplied medium, were allowed to differentiate as previously described, and were immunostained for co-expression of β-III tubulin with E-NCAM and GFAP. As can be seen in Fig. 10, β-III tubulin cells derived from cHNPCs did not exhibit E-NCAM immunoreactivity. The failure to detect E-NCAM immunoreactivity was likely due to culture conditions, as first-passage human fetal cultures (pHNPCs) derived at the same developmental age, cultured in the absence of serum, co-expressed β-III tubulin and E-NCAM (Fig. 10).

Exposure to serum also appeared to alter GFAP immunoreactivity. We noted that β-III tubulin immunoreactive cells that

Co-expression of GFAP

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These neurite outgrowths were correlated with an increase in two, or multiple directions and begin to resemble neurons. Cells send out long processes, over two cell diameters, in one, forced to stop dividing and differentiate. The differentiating cells send out flat, round morphology with nestin immunoreactivity. These neurite outgrowths were correlated with an increase in two, or multiple directions and begin to resemble neurons. We used external TTX, a common and specific Na channel blocker, to study the TTX-subtracted currents, which should represent the Na\(^+\) current specifically. Surprisingly, we noted that seven cells expressed some I\(_{\text{Na}}\) that was not blocked by 300 nM TTX. The TTX-S and -R currents were compared and found for nestin, GalC, or O4, suggesting the relative absence of oligodendrocyte precursors, oligodendrocytes, astrocyte precursors, and type 2 astrocytes. Thus the exchange of growth factors induces the proliferating human CNS cells to become a postmitotic population of cells that resemble neurons in morphology and antigenicity (β-III tubulin\(^+\), NF\(^+\), MAP-2\(^+\)), but also display the glial antigenic marker GFAP. We went on to describe the functional phenotype of these maturing, neuronal cells by studying their functional properties using whole-cell patch-clamp and fura-2 imaging techniques.

**K\(^+\) currents**

The presence of outward K\(^+\) currents in both the round, immature, mitotic, nestin\(^-\) cells and the neuronal, postmitotic, β-III tubulin\(^+\) cells was not surprising since most cell types have been shown to express K channels (Rudy 1988). Both cell types had at least two kinds of K\(^+\) currents, a delayed-rectifier type K\(^+\) current and a transient, A-type, 4-AP-sensitive K\(^+\) current. These currents had similar steady-state and kinetic properties in both cell types and resembled those described in neurons, glia, and the precursor cells that give rise to either of these neural cell types (Barres et al. 1990; Feldman et al. 1996; Ritchie 1991; Sontheimer 1994; Stewart et al. 1999; Swanson et al. 1990; Verma Kurvari et al. 1997). The presence of K\(^+\) currents in the dividing population of cells contrasts with the observations of Feldman et al. (1996). They reported that dividing, EGF-dependent cells in neurospheres, derived from the subventricular zone (SVZ) of postnatal rat (P0–P3), do not express K channels until they begin differentiating following attachment to a suitable substrate. Similar to our observations, Luskin et al. (1999), recorded several K\(^+\) currents from dividing, β-III tubulin\(^+\), neuronal progenitor cells, obtained directly from the SVZ of postnatal rat (P0–P1). Our data indicate that the human, EGF/bFGF-dependent, mitotic, neuronal progenitors express at least two K\(^+\) currents, as do their progeny, similar to the SVZ cells. Although we expected to find K\(^+\) currents expressed in the nestin\(^-\) cells, we imagined that the postmitotic, neuronal cells would express a higher density of current, which was not the case. Instead, the main difference in the repertoire of ion channel expression between the two cell types was the absence of inward current in the mitotic, nestin\(^-\) cells. These inward currents include an inwardly rectifying K\(^+\) current, a slowly activating and inactivating Ca\(^{2+}\) current, and a fast activating and inactivating Na\(^+\) current. We next concentrated on describing the I\(_{\text{Na}}\) expressed by the postmitotic, β-III tubulin\(^+\), maturing neuronal cells.

**Na\(^+\) currents**

Developing human CNS cells obtained from Clonexpress (12–18 wk gestation) could be maintained in a mitotically active state when grown in medium supplemented with serum, bFGF, and EGF. These cells were without processes and displayed a flat, round morphology with nestin immunoreactivity. By replacing serum with N2 supplement and adding DbcAMP, NGF, or BMP-2, we found that the cells could be forced to stop dividing and differentiate. The differentiating cells sent out long processes, over two cell diameters, in one, two, or multiple directions and begin to resemble neurons. These neurite outgrowths were correlated with an increase in whole-cell capacitance. In addition, the differentiating cells lost nestin immunoreactivity and many displayed β-III tubulin, NF, MAP-2, or GFAP immunoreactivity. Only a small amount of staining was found for A2B5 and no staining was found for nestin, GalC, or O4, suggesting the relative absence of oligodendrocyte precursors, oligodendrocytes, astrocyte precursors, and type 2 astrocytes. Thus the exchange of growth factors induces the proliferating human CNS cells to become a postmitotic population of cells that resemble neurons in morphology and antigenicity (β-III tubulin\(^+\), NF\(^+\), MAP-2\(^+\)), but also display the glial antigenic marker GFAP. We went on to describe the functional phenotype of these maturing, neuronal cells by studying their functional properties using whole-cell patch-clamp and fura-2 imaging techniques.

**DISCUSSION**

Morphology, mitotic activity, and immunoreactivity

Developing human CNS cells obtained from Clonexpress (12–18 wk gestation) could be maintained in a mitotically active state when grown in medium supplemented with serum, bFGF, and EGF. These cells were without processes and displayed a flat, round morphology with nestin immunoreactivity. By replacing serum with N2 supplement and adding DbcAMP, NGF, or BMP-2, we found that the cells could be forced to stop dividing and differentiate. The differentiating cells sent out long processes, over two cell diameters, in one, two, or multiple directions and begin to resemble neurons. These neurite outgrowths were correlated with an increase in whole-cell capacitance. In addition, the differentiating cells lost nestin immunoreactivity and many displayed β-III tubulin, NF, MAP-2, or GFAP immunoreactivity. Only a small amount of staining was found for A2B5 and no staining was found for nestin, GalC, or O4, suggesting the relative absence of oligodendrocyte precursors, oligodendrocytes, astrocyte precursors, and type 2 astrocytes. Thus the exchange of growth factors induces the proliferating human CNS cells to become a postmitotic population of cells that resemble neurons in morphology and antigenicity (β-III tubulin\(^+\), NF\(^+\), MAP-2\(^+\)), but also display the glial antigenic marker GFAP. We went on to describe the functional phenotype of these maturing, neuronal cells by studying their functional properties using whole-cell patch-clamp and fura-2 imaging techniques.

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We used external TTX, a common and specific Na channel blocker, to study the TTX-subtracted currents, which should represent the Na\(^+\) current specifically. Surprisingly, we noted that seven cells expressed some I\(_{\text{Na}}\) that was not blocked by 300 nM TTX. The TTX-S and -R currents were compared and
Primary human cells

found to differ in steady-state activation and inactivation properties but not kinetics. The TTX-S $I_{\text{Na}}$ resembled the classical neuronal $I_{\text{Na}}$ in that it was half-maximally activated at $-25$ mV and half inactivated at $-52$ mV. Like the neuronal $I_{\text{Na}}$, the TTX-S current turned on and off quickly ($\tau_h$) and was sensitive to nanomolar concentrations of TTX. The TTX-R $I_{\text{Na}}$ steady-state parameters were shifted in the hyperpolarized direction compared with the TTX-S so that TTX-R was half-maximally activated at $-35$ mV and half-maximally inactivated at $-80$ mV; similar to the TTX-R current, $I_{\text{Na(G)}}$, described in astrocytes, Schwann cells, and some neurons (Barres et al. 1989; Howe and Ritchie 1990; Sontheimer and Waxman 1992). Like the currents described by Sontheimer et al. (1992), but not the other groups, the kinetics of the TTX-R current recorded from these neuronal precursor cells were fast, essentially identical to those of the TTX-S current. While the expression of Na channels suggests a neuronal phenotype, the densities of the currents in these cells ($\sim 9 \text{ pA/pF at peak}$) were lower than those found in mature neurons and more comparable with those found in cultured glial and neural progenitor cells (Feldman et al. 1996). Although Luskin et al. (1999) did not report Na current densities, they did note that 28/32 cells tested had $I_{\text{Na}}$.  

Clonexpress

FIG. 10. “Neuronal” neuron restricted precursors (NRPs) crossreact with GFAP. Primary human precursor cells (A, A′, B, and B′) and cells obtained from Clonexpress (C, C′, D, and D′) were grown in parallel and the expression of embryonic neural cell adhesion molecule (E-NCAM, A and C), β-III tubulin (B and D), and GFAP (B′ and D′) was analyzed. Primary human cells expressed E-NCAM immunoreactivity (red, A′), but no E-NCAM immunoreactivity was seen in Clonexpress cells (C) under any culture condition tested. β-III tubulin immunoreactive cells present in human primary cell culture do not co-express GFAP (compare B and B′: top left arrow shows a cell stained for β-III tubulin but not GFAP and bottom right arrow shows a cell stained for GFAP but not stained above background for β-III tubulin); however, Clonexpress cells co-express β-III tubulin and GFAP (compare D and D′: both arrows show co-localization of β-III tubulin and GFAP).
but 11/12 tested could not fire action potentials, suggesting a low density of Na\(^+\) current. The \(I_{\text{Na}}\) recorded by Feldman et al. (1996) from EGF-dependent neurosphere-derived cells, which differentiated in the presence of EGF approached mature, neuronal densities (~50 ± 35 pA/pF); while \(I_{\text{Na}}\) recorded from cells that differentiated in the presence of bFGF (9.4 ± 3.7 pA/pF) remained closer to the values that we observed. They also noted a decrease in K channel density when bFGF was included in the culture medium, suggesting that bFGF may generally inhibit ion channel expression in these culture systems. Several reports have described the expression of Na channels in developing neurons and have shown that several Na channel isoforms are up-regulated as neurons mature, including both the “glial,” \(I_{\text{Na,G}}\) (TTX-R) and the “neuronal,” rat brain IIA (TTX-S), isoforms (Feltz et al. 1997). The chNPC derived, maturing, neuronal cells may resemble their in vivo cousins in that they may first express a low density of Na channels in early differentiated states, followed by a sharp rise in the density of Na channels in terminally differentiated states. For reasons unknown, we may have arrested the differentiation process at an early step, where early neuronal markers and fundamental channel expression have been turned on but not amplified or fine-tuned. In cell cultures, transcriptional regulation can be influenced dramatically by serum, growth factors, or co-culture with other cell types. In particular, the expression pattern of ion channels in both neurons and glia appears highly dependent on whether or not these two cell types are cultured together (MacDonald et al. 1996; Maxwell et al. 1996; Thio et al. 1993), and only additional studies will allow us to understand the factors necessary to promote both proliferation of progenitor cells and complete differentiation and maturation of progeny. This work provides a structural and functional baseline to compare with neurons induced to differentiate under different culture conditions: various growth factors, co-culture with glia, glial or neuronal conditioned medium, explants, or transplants.

**Neurotransmitter responses**

Having established that the neuronal cells express ion channels typical of neurons, we next examined their response profile to a set of neurotransmitters. Fura-2 Ca\(^{2+}\) imaging demonstrated that all of the maturing neuronal cells responded to ACh, a majority to E, a few to elevated extracellular potassium, and that none responded to GABA, G, DA, or ascorbic acid. The lack of response to ascorbic acid is expected as a negative control. Because GABA and glycine receptor ion channels are selective for chloride ions, intracellular Ca\(^{2+}\) responses to GABA or glycine are not expected even in the presence of these receptors unless the reversal potential for chloride depolarizes the cell in response to receptor activation, leading to an opening of Ca\(^{2+}\) channels. Likewise, if dopamine receptors are present, they may act on signaling mechanisms that are uncoupled from both membrane depolarization and Ca\(^{2+}\) fluxes and so the lack of response to dopamine does not rule out surface expression of certain dopamine receptor subtypes. The responses to glutamate were invariably smaller, ~10% of baseline, and slower, 30- to 40-s latency, than the responses to ACh, ~200% of baseline, ~10–20 s latency (see Fig. 9A, latencies uncorrected for dead time). These results differ from those obtained when similar cells were examined from rat and mouse (Kalyani et al. 1998; Mujtaba et al. 1999). In both the rat and mouse, NRP cells gave rise to a postmitotic population of E-NCAM\(^+\) cells with neuronal morphologies and antigenticities. The differentiated rat cells responded to both ACh and E with similar high-frequency and magnitude, some cells responding to either or both. A substantial fraction responded to DA and elevated extracellular potassium while a few responded to GABA and G (Kalyani et al. 1998). The differentiated mouse cells also displayed a high-frequency and magnitude of response to both ACh and E, with only a small fraction responding to DA, and few responding to elevated extracellular potassium (Mujtaba et al. 1999). Together, these data suggest that the population of human, differentiated neuronal cells is less heterogeneous than those of the rat or mouse, and perhaps less mature. In particular, the failure of the human (and mouse) cells to respond to elevated extracellular potassium suggests any or all of the following mechanisms: cells may not set \(V_{\text{rst}}\) via K channels, cells may lack voltage-dependent Ca channels, or cells may lack a sufficient density of available Na channels to fire an action potential.

**Action potentials**

To test whether Na channel density was sufficient to generate action potentials, we examined several of the maturing neuronal cells using current-clamp to inject depolarizing currents and monitor the membrane potential response. In nine cells tested by injecting currents from 10 to 200 pA for 1 to 200 ms, we were unable to elicit an action potential. In some cases, the decay of the membrane depolarization appeared slower than the onset and suggested that a Na\(^+\) or Ca\(^{2+}\) conductance may be sustaining the response slightly, but the density of such depolarizing influences was simply not enough to fire a classic, regenerative, neuronal action potential. These results are similar to those obtained by Luskin et al. (1999), who showed that despite a recognizable sodium current in the dividing SVZ cells, the SVZ cells were also unable to fire action potentials in response to depolarizing current injections. So despite a neuronal complement of ion channels and receptors, these differentiating cells have not developed the proper balance of channels to support action potential generation. Future work examining perturbations in the culture conditions will be necessary to determine what factors are necessary to allow or promote full maturation of human neurons in culture.

**Significance of staining patterns**

Although bFGF has been reported to enhance neuronal proliferation and differentiation in some culture systems the opposite has been reported in others (Feldman et al. 1996; Kuhn et al. 1997; Murphy et al. 1990; Vescovi et al. 1993). In particular, the work of Feldman et al. (1996) should be noted in which the EGF-dependent neuronal precursors appeared “stunted” in the differentiation process if cultured in the presence of bFGF instead of EGF. In addition, these authors reported that many of the β-III tubulin\(^+\) cells in the presence of bFGF expressed GFAP. Several other groups have observed either permissive or inhibitory effects on differentiation that were related to the presence of serum in the culture medium (Kilpatrick and Bartlett 1993). Double labeling of the differentiating Clonexpress cells for β-III tubulin\(^+\) and GFAP con-
firmed co-expression of these markers on single cells and revealed that β-III tubulin+ cells did not stain for ENCAM. A different population of human precursor cells, isolated from overlapping gestational ages (Li et al. 1999) was shown to express ENCAM and not to co-express GFAP but was never exposed to serum. These data underscore the direct effect of culture conditions have on the developing phenotypes of differentiating neuronal cells.

Collectively, these data show that a self-renewing population of cells can be isolated from human developing CNS that can give rise to both neurons and glia. External factors play a critical role in shaping the fates of these cells as they divide and differentiate. The replacement of serum with N2 supplement and addition of NGF, DbcAMP, or BMP-2 arrests mitosis and initiates a process of neural differentiation that restricts some of the cells to what may be an astrocytic fate while most of them take on neuronal phenotypes. The maturing neuronal phenotype is characterized by a lack or slow rate of cell division; β-III tubulin, NF, and MAP-2 immunoreactivity; expression of K, Ca, and Na channels typical of neuronal tissue; functional responses to ACh and glutamate; an inability to fire action potentials; and co-expression of GFAP. When primary human precursor cells were grown and differentiated in the absence of serum the cells did not co-express β-III tubulin and GFAP. We suggest that the presence or absence of an undetermined factor arrests the full maturation of these developing neurons in culture. Future studies will be aimed at understanding the nature of these factors, their interactions with these developing cells and the expression patterns they dictate.

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