Characterization of Mitotic Neurons Derived From Adult Rat Hypothalamus and Brain Stem

JENAFER EVANS,1 COLIN SUMNERS,1 JENNIFER MOORE,1 MATTHEW J. HUENTELMAN,1 JIE DENG,2 CRAIG H. GELBAND,1 AND GERRY SHAW2

1Department of Physiology and the McKnight Brain Institute and 2Department of Neuroscience, University of Florida College of Medicine, Gainesville, Florida 32610

Received 2 February 2001; accepted in final form 20 August 2001

Evans, Jenafer, Colin Sumners, Jennifer Moore, Matthew J. Huentelman, Jie Deng, Craig H. Gelband, and Gerry Shaw. Characterization of mitotic neurons derived from adult rat hypothalamus and brain stem. J Neurophysiol 87: 1076–1085, 2002; 10.1152/jn.00088.2001. Embryonic or neonatal rat neurons retain plasticity and are readily grown in tissue culture, but neurons of the adult brain were thought to be terminally differentiated and therefore difficult to culture. Recent studies, however, suggest that it may be possible to culture differentiated neurons from the hippocampus of adult rats. We modified these procedures to grow differentiated neurons from adult rat hypothalamus and brain stem. At day 7 in tissue culture and beyond, the predominant cell types in hypothalamic and brain stem cultures had a stellate morphology and could be subdivided into two distinct groups, one of which stained with antibodies to the immature neuron marker α-internexin, while the other stained with the astrocyte marker GFAP. The α-internexin positive cells were mitotic and grew to form a characteristic two-dimensional cellular network. These α-internexin positive cells coimmunostained for the neuronal markers MAP2, type III β-tubulin, and tau, and also bound tetanus toxin, but were negative for the oligodendrocyte marker GalC and also for the neurofilament triplet proteins NF-L, NF-M, and NF-H, markers of more mature neurons. Patch-clamp analysis of these α-internexin positive cells revealed small Ca2+ currents with a peak current of −0.5 ± 0.1 pA/pF at a membrane potential of −20 mV (n = 5) and half-maximal activation at −30 mV (n = 5). Na+ currents with a peak current density of −154.5 ± 49.8 pA/pF at a membrane potential of −15 mV (n = 5) were also present. We also show that these cells can be frozen and regrown in tissue culture and that they can be efficiently infected by viral vectors. These cells therefore have the immunological and electrophysiological properties of immature mitotic neurons and should be useful in a variety of future studies of neuronal differentiation and function.

INTRODUCTION

As early as 1897, investigators argued for the existence of immature cells in the adult mammalian CNS, which can differentiate into neurons (reviewed by Bjorklund 1999). However, because of a lack of neuronal proliferation following brain lesions, as well as an apparent lack of proliferative neurons in adult brain, it has been generally accepted that neurons of the adult brain are fully differentiated and nonreplacible. This dogma has been reexamined in the light of recent findings, suggesting that there is indeed a small population of neuronal progenitor cells in the adult nervous system (Cameron and McKay 1998; Doetsch et al. 1999; Gage 1998; Gritti et al. 1996; Johansson et al. 1999; Kempermann and Gage 1999; Kempermann et al. 1998; Kuhn et al. 1996; Reynolds and Weiss 1992; Takahashi et al. 1998; Weiss et al. 1996). These cells can be grown and expanded in tissue culture and have great potential in studies of neural function and development. They may also be useful therapeutically, to functionally replace damaged or diseased adult neurons.

Early in mammalian CNS development, neuroepithelial cells express vimentin and nestin intermediate filament proteins (Lendahl et al. 1990; Raju et al. 1981; Shaw 1998). These cells then divide and differentiate to produce migratory cells that become committed to either the neuronal or glial lineages. As development progresses, cells in both lineages cease to express nestin and begin to express differentiation-specific intermediate filament subunits. Glial lineage cells may continue to express vimentin and may also begin to express GFAP, generally taken as a hallmark of mature astrocytes (Raju et al. 1981). In contrast, oligodendrocytes are generally believed to lose vimentin and other intermediate filament subunits but begin to express a variety of myelin-associated proteins. Cells in the neuronal lineage lose nestin and vimentin and begin to express α-internexin during postmitotic development (Kaplan et al. 1990). Expression of the α-internexin protein precedes expression of the neurofilament triplet subunits in the developing embryonic rat brain, so that this protein may be the only neurofilament subunit present (Kaplan et al. 1990). Later the two smaller members of the neurofilament triplet protein family, NF-L and NF-M, are expressed apparently in a coordinated fashion (Carden et al. 1987; Shaw and Weber 1982).

Finally, NF-H is expressed about the time that the maturing cells are forming synapses (Carden et al. 1987; Shaw and Weber 1982). Many mature CNS neurons express NF-L, NF-M, and NF-H, often along with α-internexin. However, in a few subsets of neurons α-internexin is the only neurofilament subunit ever expressed and these cells continue to express this intermediate filament protein alone in the mature brain (Chien et al. 1996). The presence of α-internexin protein alone is therefore not a unique marker of developing neurons, although, since this protein has been found only in the neuronal lineage,
high-quality α-internexin antibodies are likely to be useful markers of early neuronal differentiation as well as of certain mature neurons. Here we describe the production and characterization of such antibodies and describe how they can be used to define an interesting class of mitotic neuronal cell we have discovered in our tissue cultures.

The discovery of these cells was serendipitous, since we were initially interested in culturing mature neurons. Primary cultured neurons provide a convenient model to study cellular processes of the brain and are usually derived from embryonic or neonatal brain tissue. Neurons in the adult brain have made complex terminal connections and severing these processes, while preparing cells for tissue culture, usually results in neuronal death. However, Brewer has developed a procedure for culturing neurons from adult rat hippocampus and has demonstrated that the cells retain neuronal characteristics for 6 days in culture (Brewer 1997). The hippocampus, functioning in learning and memory, retains plasticity into adulthood, so that neurons in the hippocampus may be in a less static state than neurons of less plastic brain areas. However, it is possible that similar neurons might be cultured from other regions of the brain, and here we used Brewer’s method to derive cells from the adult rat hypothalamus and brain stem, areas that are associated with autonomic functions, such as cardiovascular control, of particular interest to us. We obtained some cells that appear to be derived from adult neurons, as expected, but also noted an interesting class of stellate mitotic cell, which we characterize in some detail here.

We show that by both immunocytochemical and electrophysiological criteria these stellate cells express an immature neuronal phenotype, which may be related to neuronal progenitor cells described by other investigators (Brewer 1999; Johe et al. 1996; Kuhn et al. 1997; Palmer et al. 1999; Shihabuddin et al. 1997). We also show that these cells stain with several well-accepted markers of neurons and that they do not stain with antibodies to well-accepted glial markers. In addition our new antibodies to the neurofilament subunit α-internexin protein are a reliable marker for these cells, allowing their identification in future studies. Finally, we show that these cells may be expanded, stored frozen, and infected with lentiviral expression vectors, setting the stage for a variety of future studies.

METHODS

Preparation of cultures

Cultured adult neurons were prepared as described by Brewer et al., with exceptions noted (Brewer 1997). Fifteen-week or older Sprague–Dawley female rats or Wistar Kyoto male rats were injected with 3 ml of euthanasia solution and decapitated. The brain was rapidly dissected in a cell culture hood into 7 ml of cold Hibernate A supplemented with 2% B27 defined media supplement and 0.5 mM glutamine (Hibernate A/B27). The hypothalamus or brain stem was dissected and the meninges removed in 7 ml of cold Hibernate A/B27. Tissue blocks of hypothalamus or brain stem weighting approximately 100 mg of wet tissue were obtained from each animal and were transferred to a culture dish containing 5.5 ml of cold Hibernate A/B27 and chopped into small pieces using fine dissecting scissors. Tissue pieces in Hibernate A medium were transferred to a centrifuge tube, digested with papain (2 mg/ml in Hibernate A medium), and triturated with a fire-polished glass Pasteur pipette. The suspended cells were added to the top of an Optiprep gradient made with four 1-ml steps of 35, 25, 20, and 15%, and the cell suspension was centrifuged for 15 min at 800 g. Debris was removed and fractions containing cells were diluted into Hibernate A/B27 and centrifuged for 1 min at 200 g. Cell pellets were resuspended in Neurobasal A/B27. A 2-ml aliquot of cell suspension was added to 35 mm Nunc tissue culture dishes coated with poly-d-lysine. Typically fifteen 35-mm dishes were plated per single hypothalamus or brain stem. One hour after plating, the media was replaced with 1 ml of Hibernate A/B27, which was then replaced with Neurobasal A/B27 containing 5 ng/ml fibroblast growth factor 2 (FGF2). Every 3 days, half of the media was removed and replaced with fresh Neurobasal A/B27 containing twice the original concentration of FGF2. Neurobasal A, Hibernate A, B27, FGF2, trypsin, papain, and glutamine were obtained from Gibco Life Technologies (Grand Island, NY). Poly-d-lysine was purchased from Sigma (St. Louis, MO).

Passaging of cultured cells

Media was aspirated from dishes roughly 3 wk after initial plating. Dishes were treated with 0.5 ml warm trypsin solution (1 g/400 ml in Hibernate A at 37°C) per dish for 4 min while gently swirling the dish to loosen cells. Neurobasal A containing 10% serum was added to the dish to quench the trypsin activity, and cells were removed from the dish with a glass Pasteur pipette. Cells were spun at low speed (250 g) for 4 min and the supernatant was discarded. The pellet was diluted into Neurobasal A/B27/glutamine and replated at half the initial density onto poly-d-lysine-coated 35-mm culture dishes. Cultures were permitted to recover for 2 wk before fixing and staining.

Cryopreservation of cultured cells

Cells were trypsinized and pelleted as in the passaging procedure described above. The pellet was resuspended in a minimal amount of Neurobasal A/B27/glutamine medium containing 7.5% sterile dimethylsulfoxide (DMSO). The cells were pipetted into 1-ml cryotubes and frozen slowly in a −70°C freezer. Ten days postfreezing, aliquots were removed from storage and thawed quickly in a 37°C water bath. A 1-ml sample of preserved cells was diluted in 45 ml of Neurobasal A/B27/glutamine and centrifuged at low speed for 5 min. The supernatant was discarded and the pellet resuspended in fresh medium and plated on poly-d-lysine-coated 35-mm dishes.

Infection of cultured cells

The virus used was an HIV-1-derived, replication-incompetent, self-inactivating vector produced by transient transfection of 293T cells (Chang et al. 1999; Iwakuma et al. 1999). Virus was concentrated by ultracentrifugation at 50,000 g for 2.5 hr and resuspended in 1/500th of the original volume in Hank’s balanced salt solution. The pellet was resuspended by orbital shaking at 200 rpm for 5 hr. The titer of the virus was 2 ± 1 × 10⁶ infectious units per ml. Cells were transfected in a minimal amount of media (~1 ml) with 1 μL [−10 multiplicity of infection (MOI)] of concentrated HIV-1 vector pseudotyped with the VSV-G envelope and encoding the gene for enhanced green fluorescent protein (eGFP; for methods review, see Huentelman et al., Methods in Enzymology, in press), expressed from the human elongation factor 1 α promoter. After 24 hr approximately half the media was replaced, and at this time eGFP expression was evident. Half the media was replaced every 3 days for the following 10 days. After 10 days the cells were fixed in 4% paraformaldehyde solution for 10 min, rinsed with PBS, and mounted in Fluoromount-G (Gibco). GFP expression was documented by epifluorescent photography using fluorescein filters.

Immunocytochemistry

Cultures were washed briefly with Dulbecco’s phosphate-buffered saline (PBS) and then fixed for 1 min with PBS containing 0.1%...
Production and characterization of α-internexin antibodies

We generated an α-internexin expression construct from the full-length rat cDNA, which was a kind gift of Dr. Ron Liem (Fliegner et al. 1990). This cDNA was cleaved with the restriction endonuclease *Nla* III, the recognition sequence for which includes the initiator methionine of the α-internexin cDNA, and *Eco* RI, which cleaves beyond the α-internexin coding sequence; the appropriate fragment was directionally cloned into *Sph* I and *Eco* RI cleaved pATH21 eukaryotic expression vector. This vector produces the entire coding sequence of rat α-internexin fused to the C-terminus of *Escherichia coli* Trp-E. Expression of the Trp-E fusion protein was as described previously (Harris et al. 1991). The resulting inclusion bodies were solubilized in 6 M urea and the major approximately 100-kDa fusion protein band was purified by fractionation on a 5% polyacrylamide gel in the Bio-Rad Prep Cell apparatus (Bio-Rad, Richmond, CA). Mice and rabbits were injected with 200–400 μg of the fusion protein to raise polyclonal antisera. Hybridomas were produced by standard procedures and culture supernatants positive on the α-internexin fusion protein alone were then tested on Western blots of various mammalian brain and spinal cord homogenates. Both monoclonal antibodies recognize a recombinant fusion protein containing the C-terminal 164 amino acids of rat α-internexin (see Fig. 1). Both also stain neuronal processes in formalin-fixed and paraffin-embedded sections of human brain, and so can be used for a variety of future immunohistological and immunopathological studies. Both monoclonal antibodies recognize a recombinant fusion protein containing the C-terminal 164 amino acids of rat α-internexin (Harris et al. 1993), which includes the highly conserved coil II sequences that are very similar in all intermediate filament molecules, and also the more variable C-terminal tail sequences, which contain several interesting sequence motifs (Shaw 1998). Based on the conservation of the coil II region between intermediate filament subunits and the contrasting

### TABLE 1. Primary antibodies used for immunocytochemical characterization of adult brain-derived cultures

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity in Mammals</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA2</td>
<td>NF-L</td>
<td>Ascites, 1:500</td>
<td>Chemicon International, Temecula, CA</td>
</tr>
<tr>
<td>3H11</td>
<td>NF-M, extreme C-terminus</td>
<td>Ascites, 1:500</td>
<td>Chemicon</td>
</tr>
<tr>
<td>NE14</td>
<td>Phospho-NF-H, and NF-M</td>
<td>Ascites, 1:500</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>NS2</td>
<td>Nonphosphorylated NF-H</td>
<td>Ascites, 1:500</td>
<td>Sigma</td>
</tr>
<tr>
<td>2E3, ID2</td>
<td>Mammalian α-internexin</td>
<td>Culture supernatant, 1:5</td>
<td>New</td>
</tr>
<tr>
<td>HM2</td>
<td>MAP2</td>
<td>Ascites, 1:100</td>
<td>Sigma</td>
</tr>
<tr>
<td>AF20</td>
<td>MAP2</td>
<td>Ascites, 1:100</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tau-1</td>
<td>Tau</td>
<td>Ascites, 1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tetanus toxin C</td>
<td>Tetanus toxin C fragment</td>
<td>IgG, 5 μg/ml</td>
<td>Boehringer-Mannheim, Indianapolis, IN</td>
</tr>
<tr>
<td>SDL.3D10</td>
<td>Type III β tubulin</td>
<td>Ascites, 1:400</td>
<td>Sigma</td>
</tr>
<tr>
<td>V9</td>
<td>Vimentin</td>
<td>Ascites, 1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>GA5</td>
<td>GFAP</td>
<td>Ascites, 1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Rat 301</td>
<td>Nestin</td>
<td>Ascites, 1:50</td>
<td>Developmental studies hybridoma bank</td>
</tr>
<tr>
<td>ID8</td>
<td>Plectin</td>
<td>Culture supernatant, 1:5</td>
<td>Dr. Gerhard Wiche</td>
</tr>
<tr>
<td>MAB3262</td>
<td>BrdU, iodouridine</td>
<td>Ascites, 1:500</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Polyclonal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R35, R36</td>
<td>Mammalian α-internexin</td>
<td>IgG, 1 μg/ml</td>
<td>New</td>
</tr>
<tr>
<td>GFAP</td>
<td>GFAP</td>
<td>IgG, 1 μg/ml</td>
<td>Dako Immunochemicals, Carpinteria, CA</td>
</tr>
<tr>
<td>GalC</td>
<td>Galactocerebroside (GalC)</td>
<td>Serum, 1:50</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Tween 20 (PBS/Tween) and 10% of a 37% w/w formaldehyde solution (Fisher Scientific, Rockville, MD). Dishes were then washed briefly with PBS/Tween. For all antibody combinations, except those with anti-tetanus toxin or anti-GalC, dishes were then postfixed with −20°C methanol for 1 min, followed by an additional wash with PBS/Tween. 10% Goat serum in PBS/Tween was added to the dish for 30 min at 37°C to block nonspecific binding, followed by a further wash with PBS/Tween. Primary antibodies, diluted in a 1-ml total volume of PBS/Tween, were added to the dish and incubated for 30 min to 1 h at 37°C. Following two 30-min washes with PBS/Tween, the dishes were incubated with secondary antibodies; washed twice, 30 min each time, with PBS/Tween; and mounted with antibleaching medium and a glass coverslip. Primary antibodies were titrated for use to give robust staining and are as described in Table 1. Secondary antibodies were extensively cross-adsorbed goat anti-mouse and goat anti-rabbit IgGs coupled to either ALEXA 594 or ALEXA 488 and were obtained from Molecular Probes (Eugene, OR). These were used at 1:2000 dilution, corresponding to a final concentration of 1 μg/ml. All other chemicals were purchased from Sigma (St. Louis, MO).
enzymes, and DNA ligase were all obtained from Promega (Madison, WI) in all mammalian preparations tested (Fig. 1). The slight differences in SDS-PAGE mobility seen in the different species are as expected from previous studies (Chiu et al. 1989; Kaplan 1990). Bands visible at 55–60 kDa in all of these immunoblots are presumptively proteolytic fragments of α-internexin, as noted by other workers (e.g., Chiu et al. 1989; Kaplan 1990), and are especially prominent in the human protein sample, which was derived several hours postmortem. Immunoblots were performed by standard techniques and the signal was visualized using appropriate chemiluminescence reagents from NEN (Boston, MA). Cyanogen bromide–activated Sepharose 4B was obtained from Sigma and Nla III, Sph I, and Eco RI restriction enzymes, and DNA ligase were all obtained from Promega (Madison, WI).

Current recording and analysis

Membrane currents were measured using the whole-cell patch-clamp technique (Hamill et al. 1981). When filled with internal solution, electrode resistances measured 1–4 MΩ. Voltage-clamp command potentials were applied to the cells and the membrane current was recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Burlingame, CA). The membrane current was digitized on-line (10.0 kHz) with an A/D interface and filtered at 5.0 kHz. All experiments were performed at room temperature. The pipette solution for Na+ currents contained (in mM) 140 CsCl, 2 MgCl2, 10 HEPE, 20 TEA-Cl, 0.2 GTP, and 1 ATP (pH 7.2 with CsOH). The bath solution for Na+ recordings contained (in mM) 140 NaCl, 1 MgCl2, 10 HEPES, and 10 EGTA (pH 7.4 with TEA-OH). The pipette solution for Ca2+ currents recorded contained (in mM) 110 TEA-Cl, 9 HEPES, 4.5 MgCl2, 4 ATP, 0.3 GTP, and 14 phosphocreatine (pH 7.3 with TEA-OH). The bath solution for Ca2+ recordings contained (in mM), 142 TEA-Cl, 10 HEPES, and 10 BaCl2 (pH 7.3 with TEA-OH). Cell capacitance measurements ranged from 9 to 90 pF.

Proliferation assay via BrdU incorporation

Bromodeoxyuridine (BrdU) was added to the culture medium to a final concentration of 30 μg/ml. After 24 h of incubation in the dark at 37°C, cells were collected from the culture plates by treatment with trypsin (0.25 g/l in Hibernate A) for 3 min. Four plates were used for each sample. The cells were centrifuged at 300 g for 3 min. The cell pellet was resuspended in PBS and fixed in 10 ml of ice-cold methanol for 1 min. The cells were collected by centrifugation and resuspended in 25 ml of ice-cold 0.1% Triton X-100/0.1 M HCl. Following a 1-min incubation on ice, cells were again centrifuged and collected. The pellet was resuspended in 1 ml of DNA denaturation buffer (0.15 mM NaCl and 15 μM trisodium citrate dihydrate) using a Pasteur pipette. The cell suspension was heated for 5 min at 90°C, and then placed on ice for 5 min prior to the addition of 10 ml of antibody-diluting buffer (100 ml PBS containing 100 μl Triton X-100 and 1 g BSA) and subsequent centrifugation. The resulting pellet was incubated with 0.005 μg/μl of FITC-labeled anti-BrdU antibody (clone BMC9318; Chemicon International, Temecula, CA) in the dark for 30 min at room temperature prior to addition of 20 ml of antibody-diluting buffer and centrifugation until a pellet formed (about 1500 g). The supernatant was discarded and the pellet was resuspended in 2 ml of PBS containing 20 μg of propidium iodide and 0.2 mg RNase A for 30 min in the dark. The samples were then analyzed using a FACScan (BD Biosciences, San Jose, CA) flow cytometer. The instrument illuminated the cells with a 15-mW argon-ion laser, emitting 488 nm. Signals collected were forward light scatter, side light scatter, green fluorescence emission (515–545 nm), and red fluorescence emission (>650 nm). Data were acquired for 30,000 cells per sample. The resulting computer files were transferred to a Macintosh G3–350 computer running Cell Quest Software (BD Biosciences) or a PC running WinMDI Version 2.8 (courtesy of J. Trotter, Scripps Institute, La Jolla, CA) for routine plotting and percentage calculations. Cell cycle analysis was performed on the Macintosh using ModFit LT, version 2.0 (Verity Software House, Topsham, ME).

For dual staining with α-internexin antibodies and BrdU antibodies, cells were treated with BrdU as above for 36 h, and fixed and stained as detailed in the dual-staining procedure, using the R35 antibody to α-internexin and a FITC-tagged anti-BrdU antibody.

RESULTS

Immediately following the culture procedure only a few adherent cells derived from the adult rat brain stem or hypothalamus were apparent, although there was nonadherent material, which contained live cells as well as debris. However, after 7 days in culture many adherent cells were visible in cultures derived from either brain area. Most of these cells showed a generalized neural morphology with rounded cell bodies and short processes (Fig. 2). Cultures 10 days of age have significantly more adherent cells with longer processes and neuronal and glial–like morphology. By 2 wk after plating a variety of different cell types were seen, including interesting islands of smaller cells with a stellate morphology that appears in clusters over about half of the culture dish. Cells of this type continued to divide and formed continually expanding monolayers for 35 days in culture (data not shown). Since these cells formed a regular, reproducible patterned network we named them network cells. Visual observation made it clear that the network cells we noted in our cultures were dividing (Fig. 2). Further confirmation of this came from flow cytometry of BrdU incorporation and DNA content demonstrated that ≥7% of the cells in 20-day-old brain stem–derived cultures are incorporating BrdU during a 24-h period (Fig. 3, top).

To further characterize the phenotype of this population of proliferating cells, we initially utilized antibodies to intermediate filament proteins, convenient and widely used markers of CNS cell types (Osborn and Weber 1982). Immunocytochemical analysis of the hypothalamus–derived cultures with such

![FIG. 2. Population expansion of cells cultured from adult rat hypothalamus and brain stem photographed in phase-contrast microscopy. Cells cultured from adult rat hypothalamus (top row) or brain stem (bottom row) develop a stellate morphology and form extended monolayer networks as shown in the photomicrographs of live cells. Magnification: ×100.](http://www.jn.org)
antibodies revealed a variety of different neuronal and glial cell types. We noted a few cells, generally no more than 10 per dish, with an obviously neuronal morphology, which were stained with antibodies to the neurofilament subunits of mature neurons NF-L and NF-M (Fig. 4, A and B, green channel). These presumably correspond to the mature neurons described in cultures derived from the adult hippocampus by Brewer (1999). In initial experiments, we also stained these cultures with monoclonal NF-H antibody NE14, as described by Brewer, and noted prominent nuclear staining in essentially all detectable cells (Fig. 4C). As discussed in the following text this staining is probably artifactual, and was not replicated with other NF-H antibodies. For example, NF-H monoclonal antibodies N52 and 5B8, which recognize a different type of NF-H epitope, showed little immunoreactivity in these cultures, though an occasional NF-H positive fiber could be seen, usually associated with the NF-M and NF-L positive cells (not shown). Further immunocytochemical analysis showed that the NF-L and NF-M positive cells coexpress neuronal markers, including tetanus toxin binding sites, MAP-2, tau, and class III β-tubulin, and similar results were found in cultures derived from the adult rat brain stem (data not shown). These relatively rare larger cells therefore have the immunocytochemical properties expected of mature neurons and should be useful for future electrophysiological and molecular biological studies of hypothalamic and brain stem neurons. Possibly optimization of the present culture technique for hypothalamic and/or brain stem neurons would allow us to obtain more cells of this kind.

Our attention was focused on the staining pattern we noted with our new antibodies raised against α-internexin, a neurofilament subunit expressed in developing and certain mature neurons of the CNS. All of these antibodies revealed strong filamentous staining of the islands of network cells (Fig. 4, A–C, red channel), suggesting that such antibodies were good markers for cells of this type in these cultures. Staining cultures exposed to BrdU for 36 h showed that the α-internexin positive cells incorporate BrdU, and are thus proliferating (Fig. 3, bottom). Further double labeling showed that some α-internexin positive cells in the hypothalamus-derived cultures expressed some level of vimentin, an intermediate filament also found in developing neural cells (Fig. 4D). Some cells expressed primarily vimentin (green), some expressed α-internexin (red), and some expressed both proteins (orange). Similar findings were made with nestin (Fig. 4E), a marker for neuronal stem cells (Lendahl et al. 1990). Both of these proteins are found in both developing neurons and glia and so are not useful for cell type classification. Both become developmentally regulated as development progresses, suggesting that different individual network cells are at different stages of development.

Since the network cells somewhat resembled astrocytic and oligodendrogial cells we tested these cultures with markers for these proteins. There was no overlap of α-internexin expression with glial markers, such as glial fibrillary acidic protein (GFAP, green in Fig. 4F) in the islands of network cells, though we occasionally saw individual stellate cells that stained with both GFAP and α-internexin. Staining with an antibody to galactocerebrosidase (GalC; red in Fig. 5A) showed no overlap with α-internexin. There was also no overlap of α-internexin with isolecitin B4, a microglial marker (data not shown). Similar staining patterns were obtained with brain stem–derived cultures (data not shown).

All immunocytochemical data presented above were consistent with the view that network cells were neuronal, and we therefore stained the network cells for markers that are specific for different types of neuronal cell. Interestingly, some of the network cells also showed clear staining with MAP2 antibody (Fig. 5B). The α-internexin positive cells showed clear staining for type III β-tubulin and almost perfect overlap with α-internexin, revealing an orange signal (Fig. 5C). Network cells incubated with tetanus toxin followed by tetanus toxin antibody revealed punctate staining associated with the plasma membrane, shown in green in Fig. 5D. Network cells also stained with antibody to tau (Fig. 5E, orange cells have α-internexin and Tau), although certain glia also stained. Finally, we had access to monoclonal antibody to the intermediate filament associated protein plectin, which proved to stain fibroblastic cells strongly and specifically in these cultures but
not any of the other cell types, including the network cells, a finding that might be useful for researchers requiring a marker of fibroblastic cells (Fig. 5F). We conclude that network cells have several immunocytochemical properties expected of neuronal cells.

The hallmark characteristic of cells committed to a neuronal fate versus other cells of the mature CNS is the functional ability to fire action potentials and release quanta of neurotransmitter. Action potential initiation and propagation require Na⁺ channel expression, while neurotransmitter release requires Ca²⁺ influx through voltage-gated Ca²⁺ channels. Whole-cell voltage clamp of the adult-derived cells revealed the presence of surprisingly tiny inward currents, barely noticeable above the noise, although tail currents characteristic of voltage-gated Ca²⁺ channels were apparent (Fig. 6A). The current-voltage relationship, constructed from average current density elicited during test pulses to increasingly depolarized potentials, peaked at $-0.5 \pm 0.1 \text{ pA/pF}$ at a membrane potential of $-30 \text{ mV}$ and showed the characteristic shape of such relationships for Ca²⁺ current ($n = 5$, Fig. 6B). The small, noisy current was indeed carried by Ca²⁺ channels, as confirmed by testing the current for sensitivity to Cd²⁺, which blocks all voltage-gated Ca²⁺ current (Fig. 6C).

Fast inward currents were recorded in solutions lacking Ca²⁺ and K⁺ ions. These currents, likely to be carried via voltage-gated Na⁺ channels, show a peak current-voltage relationship of $-154.5 \pm 49.8 \text{ pA/pF}$ at a membrane potential of $-10 \text{ mV}$ ($n = 5$, Fig. 7). Similar results were obtained by voltage-clamp recordings from hypthalamic cultures (data not shown). We were unable to detect spontaneous action potentials in network cells in either type of culture, despite the presence of the voltage-gated currents, consistent with an immature neuronal phenotype.

Exploiting the proliferative nature of these cells, the cultures were passaged and split. Postpassage, the cells continued to express α-internexin and maintained their proliferative nature and stellate morphology (Fig. 8A). The cells also survived cryopreservation for $\approx 15$ days (data not shown). Finally, the cells were tested for infectability using a lentiviral vector containing a GFP construct. Efficient expression of GFP was evident in many stellate cells 1 day postinfection, as shown in low-power views of control versus transfected cultures [com-
FIG. 5. Markers indicative of an early neuronal phenotype are expressed in the α-internexin positive network cells. A: cells expressing GalC (Sigma antibody, red) are fewer in number and distinct from α-internexin positive network cells (2E3 antibody, green) showing the absence of a well-accepted oligodendrocyte marker in these cells. B: α-internexin positive cells (R35 antibody, red) coexpress MAP2 (AP20 monoclonal antibody, green) in dendrite-like processes. Hypothalamic cells 18 days in tissue culture. C: β-tubulin III (green), a well-accepted neuronal marker, overlaps with α-internexin expression (R35, red) in network cells in a 26-day hypothalamic culture, producing an orange signal. D: tetanus toxin binding sites (revealed with Boehringer-Mannheim kit, in green) are present on the processes of the α-internexin positive cells (R35 antibody, red). Hypothalamic cultures 29 days in tissue culture. E: microtubule associated protein tau (green) is coexpressed with α-internexin (R35 antibody, red). Some other stellate cells, apparently astrocytes, also show tau expression. Hypothalamic cultures 24 days in culture. F: labeling of network cells with α-internexin antibody (R35, red) compared with plectin monoclonal antibody ID8 (green). The plectin antibody does not recognize network cells but does provide a strong and useful marker for fibroblasts in these cultures. Scale bars: A and B, 50 μM; C–F, 25 μM.

pare Fig. 8B (top) with Fig. 8B (bottom)]. Higher magnification revealed many examples of network cells that strongly expressed GFP (Fig. 8, C and D). GFP expression and was maintained for ≤10 days postinfection.

DISCUSSION

Cultures derived from adult rat hypothalamus and brain stem generated a class of dividing cell that formed a distinctive two-dimensional network that we therefore dubbed network cells. We found that our new α-internexin antibodies were excellent markers for this type of cell. The α-internexin protein has only ever been seen in neuronal cells, so the presence of this protein in network cells is highly suggestive that these cells are part of the neuronal lineage. Significantly, these α-internexin positive cells also stained with antibodies to several other well-accepted neuronal markers, that is, type III β tubulin, MAP2, and tau. Incubation with tetanus toxin and subsequent antibody staining also revealed the presence of membrane-associated tetanus toxin binding sites, another well-accepted neuronal marker. However, the absence of NF-L, NF-M, and NF-H immunoreactivity suggests that these cells are relatively undifferentiated, since these proteins are typically expressed later in development than α-internexin (Fliegner et al. 1990). The ability of these cells to divide is, of course, also suggestive of a relatively early stage of neuronal differentiation. In contrast, these cells lacked GalC and GFAP staining, showing an absence of well-accepted oligodendrocyte and astrocyte markers, again consistent with these cells belonging to the neuronal lineage. The presence of vimentin and nestin is also as expected for early neuronal lineage cells.

The electrophysiological data presented here indicate the expression of Na\(^+\) channels and voltage-gated Ca\(^{2+}\) channels. While the inward currents detected were small, this result might be expected for neurons at an early stage of differentiation. Since neurons generally acquire the ability to generate action potential relatively late in neurogenesis, our inability to detect action potentials in network cells is also not inconsistent with the properties expected of early differentiating neuronal cells. Studies of mitogen-expanded neural precursor cells have also failed to detect action potentials (Liu et al. 1999). In summary, we conclude that the network cells, by several independent criteria, belong to the neuronal lineage but have the
unusual property of retaining the ability to divide in tissue culture.

The network cells are morphologically similar to a class of dividing cells found in similar cultures derived from adult rat hippocampus (described while the present work was in progress), although it remains to be seen whether the cells are identical (Brewer 1999). It will be interesting to see how far it is possible to expand these cells in tissue culture and whether these cells can be induced to differentiate into more mature neuronal phenotypes by appropriate adhesion and growth factor treatments. It is somewhat surprising that these cells are able to divide, as previous studies have suggested that α-internexin is normally expressed postmitotically (Kaplan et al. 1990), as are tau, MAP2, type III β tubulin, and tetanus toxin...
binding sites. Even after 35 days in tissue culture these α-internexin positive cells do not express NF-L or NF-M, the neurofilament subunits characteristic of more mature neurons, suggesting that these cells maintain immature neuronal characteristics over several divisions. Possibly the FGF2 treatment or other aspects of the culture conditions select for an immature neuron type, suppressing terminal differentiation of the cells.

The α-internexin positive cells may resemble cells extracted from the mature CNS by several other groups, starting with the pioneering work of Reynolds and Weiss (1992), and recently including cells from the adult human hippocampus (Roy et al. 2000). These cells generally develop into “neurospheres” in tissue culture under the influence of growth factors such as epidermal growth factor and FGF2. The present procedure, while distinct in many ways from that used to generate these progenitor cells, does use FGF2 and may well also select for the proliferation of cells of this type. We note that a significant difference in the present procedure is the use of a substrate coated with poly-D-lysine, which is highly adhesive. The α-internexin positive network cells may therefore have the same origin as so-called neuronal progenitors currently being studied by many other groups. In support of this conclusion we sometimes observed what appeared to be spontaneous neurospheres in these cultures and that a few cells in these neurospheres stained with α-internexin antibodies (Shaw et al., unpublished observations). The present work therefore adds α-internexin antibodies to the relatively short list of markers useful for studies of neuronal progenitor cells and show that such antibodies are particularly useful for the identification of the network cells.

The origin of the α-internexin positive cells described here is an interesting subject for future study. Perhaps they derive from a mature neuron type that dedifferentiates in tissue culture, or alternately they may derive from a population of stem or progenitor cells such as those described by other workers. It is now generally accepted that there are two regions of relatively active neurogenesis in the adult mammalian brain, that is, the subgranular zone of the dentate gyrus of the hippocampus and the subventricular zone lining the ventricles (Gage 2000). One group has provided evidence that a subpopulation of ciliated ependymal cells in the adult brain can generate colonies in tissue culture that contain both neurons and glia (Johansson et al. 1999), while another has shown that a distinct class of GFAP positive cells can generate both neurons and glia (Doetsch et al. 1999).

A further, more recent study shows that only the nonependymal subventricular zone cells are able to generate both neurons and glia (Chiasson et al. 1999). Our starting material for hypothalamic cultures includes the third ventricle, which is expected to include subventricular zone neuronal progenitor cells. The brain stem cultures were derived from tissue that includes the cerebral aqueduct also lined with ependymal cells and a small subventricular zone. These α-internexin positive cells could therefore be derived from either the ependyma or the subventricular zone. In any case it is apparent that a neuronlike dividing cell type can be harvested from adult brain, identified with α-internexin antibody, and made to divide and differentiate to an early neuronal phenotype. The culture procedure used for these studies provides a cell type useful for studies of neurogenesis relating to brain injury and repair. Since they proliferate and can be split and passaged, it should also be possible to grow clonogenic cultures. If enough cells can be obtained it should be possible to perform cDNA microarray analysis of these cells, to further characterize these cells and to obtain clues about which growth factors they may respond to. Finally, as demonstrated here, the ability to infect these cells and cause them to express exogenous proteins can be exploited in further differentiation studies and also to track the movement and differentiation of these cells in future transplantation studies.

We thank N. Benson at the University of Florida Interdisciplinary Center for Biotechnology Research Flow Cytometry Core for assistance with the flow cytometry analysis. Dr. Gerhard Wiche provided the plectin monoclonal antibody. We thank M. King in the University of Florida Department of Neuroscience for assistance with cell counting and the Center for Structural Biology for use of digital photomicrographic equipment.

This work was supported by American Heart Association Grant 9810149FL (to G. Shaw), National Institutes of Health Grant HL-49130 (to C. Sumners), and National Research Service Award predoctoral fellowship MH-12031 (to J. Evans).

REFERENCES


