rent records were filtered at 1 kHz with an 8-pole Bessel filter and acquired digitally with CCURRENT and analyzed by CQUANT (software written by K.B.). Recordings were performed at room temperature (22° to 23°C) or at 11°C, with a Peltier plate (Cambion).
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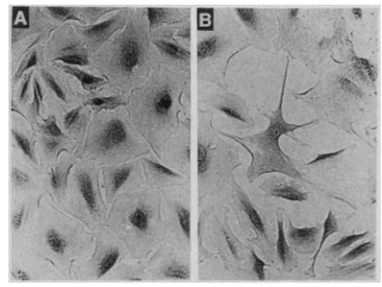


**Fig. 1.** (**A** and **B**) Morphology of undifferentiated HCN-1A cells (*10*), which appear polygonal with only occasional short processes (B). Magnification ×400.

# Human Cortical Neuronal Cell Line: Establishment from a Patient with Unilateral Megalencephaly

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**A cell line has been established in continuous culture of human cerebral cortical neurons obtained from a patient with unilateral megalencephaly, a disorder associated with continued proliferation of immature neuronal cells. When differentiated in the presence of nerve growth factor, 1-isobutyl-3-methylxanthine, and dibutyryl adenosine 3′,5′-monophosphate (cAMP), the cells display mature neuronal morphology with numerous long, extensively branched processes with spines and varicosities. The cells stain positively for neurofilament protein and neuron-specific enolase (selective neuronal markers) but are negative for glial markers, such as glial fibrillary acidic protein, S-100, and myelin basic protein. The cells also stain positively for the neurotransmitters γ-aminobutyric acid (GABA), glutamate, somatostatin, cholecystokinin-8, and vasoactive intestinal polypeptide. These cells may facilitate characterization of neurons in the human central nervous system.**

THE GREAT HETEROGENEITY OF THE central nervous system (CNS) has precluded extensive molecular characterization of individual types of CNS neurons, which might be overcome through the use of continuous cultures. Techniques previously utilized to establish cell lines can have limitations. Primary cultures are heterogeneous and have limited life-spans, whereas cell lines derived from malignant tumors and somatic cell hybrids may differ from the mature neuronal phenotype. Here we have utilized cerebral cortical tissue from a patient with unilateral megalencephaly, a low-grade proliferation and migration disorder of neurons, to establish a human cortical neuronal cell line.

We obtained cerebral cortical tissue from an 18-month-old female undergoing hemispherectomy for intractable seizures (*1*). Dissociated cells from gray matter were immediately plated in medium containing serum. After 21 days all cells had died except for two small foci of growth, which were cloned and designated HCN-1 and HCN-4. These cells have been passaged more than 20 times in the course of 19 months with no significant changes in morphology or growth characteristics (*2*).

Although both of these cell lines originated from small foci of growth in culture, to ensure clonality we subcloned them; one of these attempts yielded a subclone from HCN-1 cells, designated HCN-1A. Both the parental and subcloned line have identical growth and staining characteristics, and therefore all further comments are restricted to the HCN-1A cell line. HCN-1A cells appeared epithelioid (Fig. 1, A and B) and rarely extended short processes (Fig. 1B). Cells grew to confluence but were contact-inhibited beyond this density. Doubling time was approximately 72 hours. Karyotype analysis demonstrated a chromosomal number of 46 ± 2 (*3*).

We examined the influence of various agents on the growth and morphology of HCN-1A cells. Nerve growth factor (NGF), insulin, dexamethasone, the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), ascorbic acid, dibutyryl cAMP, 1-isobutyl-3-methyl xanthine (IBMX), and retinoic acid were examined alone and in various combinations. The most mature morphology, with considerably slowed growth (a doubling time of 120 hours), occurred in cells grown with a mixture of IBMX, NGF, and dibutyryl cAMP (*4*). The undifferentiated HCN-1A cells were generally flat and polygonal with occasional short, unbranched processes. By contrast, the differentiated cells displayed round cell bodies with numerous, long and extensively branched processes with spines and varicosities. The differentiated cells were either bipolar or multipolar (Fig. 2). One hundred percent of cells differentiated over a 3-day period. Withdrawal of the differentiating agents caused retraction of processes, but cellular division remained slow with a doubling time of more than 120 hours.

To further characterize the cells, we stained them with antibodies to neurofila-

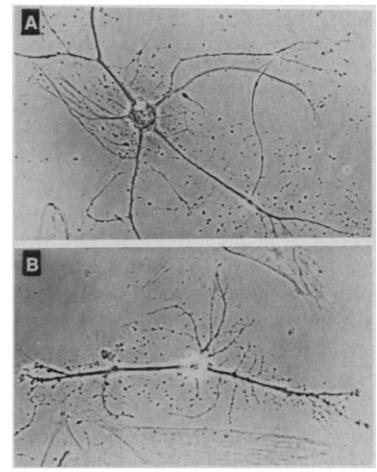


**Fig. 2.** Morphology of differentiated HCN-1A cells (*4*). Whereas undifferentiated cells were flat and polygonal, differentiated cells demonstrated multipolar (**A**) or bipolar (**B**) morphologies. All cells had spines and varicosities along their processes.

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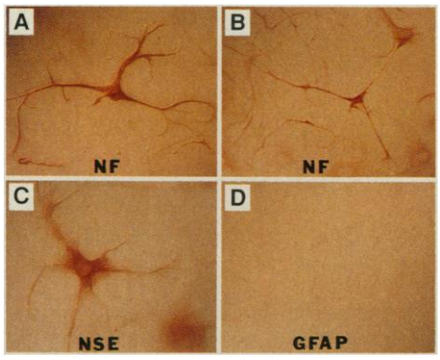


**Fig. 3.** Immunocytochemical staining of differentiated HCN-1A cells (*11*). (**A** and **B**) Cells stain positively for neurofilament protein (NF) with three different monoclonal antibodies. The results of staining with two of these (from Labsystems and Sternberger-Meyer) are shown (**C** and **D**). HCN-1A cells also stain positively for NSE, but are negative for GFAP.

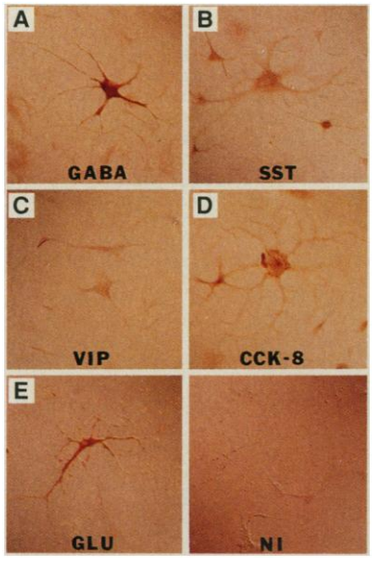


**Fig. 4.** Immunocytochemical staining of differentiated HCN-1A cells for neurotransmitters (*12*). HCN-1A cells were grown, fixed, and stained as described (*4, 11*). HCN-1A cells stain positively with antibodies (Incstar) against (**A**) GABA at 1:500 dilution, (**B**) SST at 1:500 dilution, (**C**) VIP at 1:500 dilution, (**D**) CCK-8 at 1:500 dilution, and (**E**) glutamate (GLU) at 1:250 dilution; nonimmune serum (NI) was negative.

ment protein (which occurs in neurons), neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP) (which occurs exclusively in astrocytes), vimentin, myelin basic protein (MBP) (which occurs exclusively in oligodendrocytes), S-100, and tubulin (Fig. 3). As with the undifferentiated HCN-1A cells, 100% of the differentiated HCN-1A cells stained positively for neurofilament protein. They also all stained positively for NSE but were negative for GFAP. Additionally, the cells were positive for vimentin and tubulin. Undifferentiated and differentiated cells were negative for S-100 and MBP, both of which occur in glia. To confirm the association of neurofilament proteins, NSE, vimentin, and tubulin with the mature cells, we conducted protein immunoblot analysis (*5*). Both in control rat brain and HCN-1A cells the neurofilament antibodies recognized the high molecular weight (200 and 168 kD) species of neurofilament protein. Immunoblots for NSE, vimentin, and tubulin also showed identity of protein bands in rat brain and HCN-1A cells, whereas S-100 and MBP were present in rat brain but not in HCN-1A cells.

Expression of neurotransmitters indicates differentiation of CNS neurons. We analyzed the neurotransmitter content of differentiated HCN-1A cells by immunohistochemical examination, using antisera for a variety of neurotransmitters and neurotransmitter-related enzymes (Fig. 4). The cells all stained positively for somatostatin (SST), GABA, glutamate, cholecystokinin-8 (CCK-8), and vasoactive intestinal polypeptide (VIP). In all instances staining was blocked by preincubating the antiserum with the appropriate antigen (50 μg/ml). No staining of the cells was apparent with antisera to tyrosine hydroxylase, phenylethanolamine-*N*-methyltransferase (PNMT), dopamine β-hydroxylase (DBH), serotonin, choline acetyltransferase, or nonimmune serum. In the undifferentiated state, staining for VIP, somatostatin, and CCK-8 was lighter; staining for glutamate was restricted to the perinuclear region. Therefore differentiation with IBMX, NGF, and dibutyryl cAMP causes biochemical as well as morphological changes.

The HCN-1A cells are positive for neuronal markers and do not stain for glial or other nonneuronal markers. Neuron-specific enolase in the brain is exclusively associated with neurons (*6*). Neurofilament protein is associated with neurons, so that its presence in HCN-1A cells indicates a neuronal origin of the cells. The morphology of differentiated HCN-1A cells with spines and varicosities also suggests a mature neuronal phenotype.

The HCN-1A cells stain positively for SST, GABA, glutamate, CCK-8, and VIP, five neurotransmitters that occur in particularly high density in the cerebral cortex, from which the HCN-1A cells are derived. By contrast, the catecholamine marker enzymes are not present in these cells, consistent with the low density of catecholamines in the cerebral cortex. There is some variation in staining intensity for neurotransmitters from cell to cell as in neural crest cells (*7*). The existence of five distinct neurotransmitter candidates in various amounts within a single clonal cell line may be indicative of the pluripotentiality of these cells. Two or more neurotransmitters are often expressed by normal cortical neurons, whereas as many as five neurotransmitters in the same neuron have been observed only rarely (*8*).

The HCN-1 cells appear to be clonal, because continuous passage does not significantly alter cellular morphology, growth, or staining characteristics. Additionally, the growth and morphologic properties of HCN-1A are indistinguishable from those of the parent cell line.

The unique pathology associated with unilateral megalencephaly may have allowed the establishment of this cell line. In this condition, there is continued proliferation of immature neuronal cells (*9*). Whatever factors account for this continued proliferation may have permitted the maintenance of these cells in continuous culture. At the same time, the cells are not as primitive as cells in many CNS tumors that have been used for tissue culture.

As a continuous cell line, HCN-1A cells can be carried for numerous passages. They should be of use to investigators for study of biochemical, physiological, and pharmacological factors that regulate the function of human CNS neurons.

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10. For light microscopy, HCN-1A cells were quickly rinsed three times with phosphate-buffered saline (PBS) at 37°C, fixed for 15 min with 4% (w/v) paraformaldehyde, and mounted with PBS.
11. HCN-1A cells were quickly rinsed three times with PBS at 37°C, permeabilized with 0.1% Triton X-100 for 15 min, fixed with 4% paraformaldehyde for 15 min, and rinsed three times with PBS. Immunocytochemical staining was performed (*12*) with Vector (Burlingame, CA) ABC kits; the chromogen used was 3-amino-9-ethyl carbazole, (Biomeda, Fos-

ter City, CA). Primary antisera used were monoclonal antibody to rat neurofilament (Labsystems, Helsinki, Finland) at 1:75 dilution (Fig. 3A), monoclonal antibody to rat neurofilament antibody SMI 33 (Sternberger-Meyer, Jarrettsville, MD) at 1:2000 dilution (Fig. 3B); rabbit antibody to NSE (Incstar, Stillwater, MN) at a dilution of 1:2 (Fig. 3C); and rabbit antibody to GFAP (DAKO-Patts, Santa Barbara, CA) at 1:800 dilution (Fig. 3D). There was negligible nonspecific staining where immune primary serum was deleted or replaced by nonimmune serum. In addition, specificity of staining for each antibody was checked with rat brain control slides and by protein immunoblotting (*5*). Cells are fixed for immunohistochemistry when they are about 50% differentiated because, if all cells are allowed to differentiate, they tend to lift off the slides during fixation.

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# Identity of Inositol 1,2-Cyclic Phosphate 2-Phosphohydrolase with Lipocortin III

THEODORA S. ROSS, JONATHAN F. TAIT, PHILIP W. MAJERUS

**The amino acid sequences of three fragments of cyanogen bromide–digested human placental inositol 1,2-cyclic phosphate 2-phosphohydrolase, an enzyme of the phosphatidylinositol signaling pathway, are identical to sequences within lipocortin III, a member of a family of homologous calcium- and phospholipid-binding proteins that do not have defined physiological functions. Lipocortin III has also been previously identified as placental anticoagulant protein III (PAP III) and calcimedin 35$_\alpha$. Antibodies to PAP III detected PAP III and inositol 1,2-cyclic phosphate 2-phosphohydrolase with identical reactivity on immunoblotting. In addition, inositol 1,2-cyclic phosphate 2-phosphohydrolase was stimulated by the same acidic phospholipids that bind lipocortins.**

THE TURNOVER OF INOSITOL PHOSpholipids produces messenger molecules, including inositol phosphates, diacylglycerol, and eicosanoids, in response to a variety of extracellular effectors (*1*). Phosphatidylinositol (PI)-specific phospholipase C hydrolyzes PI, phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to produce both cyclic and noncyclic inositol phosphates (*2*). Inositol 1,2-cyclic phosphate is derived directly from phospholipase C cleavage of PI or indirectly via phospholipase C cleavage of PIP and $PIP_2$, with subsequent enzymatic removal of the phosphates from positions 4 and 5 of the inositol ring (*2, 3*). The existence of inositol cyclic phosphate in vivo has been shown in a variety of tissues (*4*), and carbachol stimulation of pancreatic minilobules leads to a 20- to 400-fold increase in inositol 1,2-cyclic phosphate (*5*), as well as an increase in cyclic inositol 1,2,4-bisphosphate and cyclic inositol 1,2,4,5-trisphosphate (*4, 5*). Inositol cyclic phosphates accumulate in stimulated cells for longer periods than the inositol phosphates (*4*), suggesting their importance in evoking sustained or delayed responses, including stimulation of cell growth. Cyclic inositol 1,2-phosphate 2-phosphohydrolase cleaves the cyclic bond of inositol 1,2-cyclic phosphate to form inositol 1-phosphate. Because all inositol cyclic phosphates are metabolized through this reaction, it is likely that this enzyme regulates the cellular concentrations of these molecules. This enzyme is widely distributed (*6*) and has been isolated and characterized from human placenta (*7*).

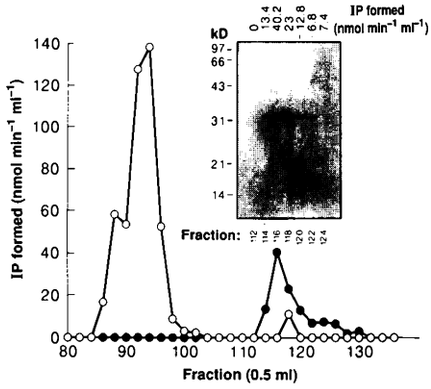
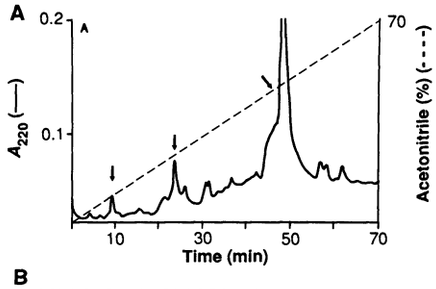



**Fig. 1.** Inositol 1,2-cyclic phosphate phosphohydrolase activity after gel filtration in the presence (closed circles) or absence (open circles) of 100 m*M* NaCl. (**Inset**) An SDS–12% polyacrylamide gel showing the correlation of a 33-kD band with enzyme activity from the final gel filtration step in the presence of NaCl. The column is a Biosil-TSK 250 size-exclusion (three columns in series, 7.5 mm by 600 mm each) column and was equilibrated with 20 m*M* 2-[*N*-morpholino]ethanesulfonic acid (MES) (*p*H 6.5) in the absence or presence of 100 m*M* NaCl. Activity was eluted with a flow rate of 0.5 ml/min of the same buffer. All assays were done in the presence of alkaline phosphatase as described (7). IP, inositol 1-phosphate.




**B**

MASIWVGHRQTVRDYPDFSPSVDAEAIQKAIRG
IGTDEKMLISILTERSNAQRQLIVKEYQAAYGK
ELKDDLKGDLSGHFEHLMVALVTPPAVFDAKQL
KKSMKGAGTNEDALIEILTTRTSRQMKDISQAY
YTVYKKSLGDDISSETSGDFRKALLTLADGRRD
ESLKVDEHLAKQDAQILYKAGENRWGTDEDKFT
EILCLRSFPQLKLTFDEYRNISQKDIVDSIKGE
LSGHFEDLLLAIVNCVRNTPAFLAERLHRALKG
IGTDEFTLNRIMVSRSEIDLLDIRTEFKKHYGY
SLYSAIKSDTSGCYEITLLKICGGDD

**Fig. 2.** Partial amino acid sequence of inositol 1,2-cyclic phosphate 2-phosphohydrolase is identical to lipocortin III amino acid sequence. (**A**) Purified phosphohydrolase (~12 μg, 340 pmol) was lyophilized and treated with CNBr (~1 mg) in 70% trifluoroacetic acid (TFA) for 24 hours at 23°C in the dark under an $N_2$ atmosphere. The sample was then dried under $N_2$, degassed, dissolved in 50% TFA, and injected onto a reversed-phase 300 column (2.1 mm by 30 mm) on an Applied Biosystems microbore HPLC. A linear gradient of 0 to 90% acetonitrile in 0.1% TFA at a flow rate of 0.1 ml/min over 90 min was used to elute the peptides. Peaks were detected by absorbance at a wavelength of 220 nm ($A_{220}$). The arrows indicate peaks that yielded amino acid sequence. (**B**) The predicted amino acid sequence of lipocortin III and the sequence obtained on an Applied Biosystems gas-phase sequencer from the CNBr fragments of inositol cyclic 1,2-phosphate 2-phosphohydrolase. The sequenced peptides are underlined and numbered. Peptide 3 was sequenced twice from two different preparations. The yield of amino acids ranged from 60 to 100 pmols. The single-letter code is used for amino acids (*20*).

For the current study, we modified our previous procedure for the isolation of the phosphohydrolase (*7*) and obtained material that gave a single band after SDS–polyacrylamide gel electrophoresis (SDS-PAGE) as before, but with higher specific activity (5 to 20 versus 2 μmol min$^{-1}$ mg$^{-1}$). We attribute this difference to preservation of activity by the more speedy purification (*8*). When the enzyme is subjected to chromatography by gel filtration in the absence of NaCl, the peak of activity is heterogeneous and elutes at a position corresponding to a molecular size of ~55 kD; however, after the addition of 100 m*M* NaCl to the buffer, the enzyme elutes at a position correspond-

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