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A self-renewing multipotential stem cell in embryonic rat cerebral cortex

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NEUROECTODERM cells in the cortical ventricular zone generate many diverse cell types, maintain the ventricular zone during embryonic life and create another germinal layer, the subventricular zone, which persists into adulthood[1,2]. In other vertebrate tissues, including skin, intestine, blood and neural crest, stem cells are important in maintaining a germinal population and generating differentiated progeny[3–6]. By following the fates of single ventricular zone cells in culture, we show here that self-renewing, multipotential stem cells are present in the embryonic rat cerebral cortex. Forty per cent of these stem cells produced all three principal cell types of the central nervous system: neurons, astrocytes and oligodendrocytes. Stem cells constituted about 7% of cortical clones; in contrast, over 80% consisted of small numbers of neurons or glia. We suggest that multipotential stem cells may be the ancestors of other cortical progenitor cells that exhibit more limited proliferation and more restricted repertoires of progeny fates.

We analysed the development of 464 clones (Table 1) derived from embryonic day-12 (E12) and E14 cerebral cortices, which consist almost entirely of ventricular zone cells[1,2]. The majority (~80%) were small clones of neurons, defined by morphology and staining for neurofilament protein (Fig. 1*a*, *b* and Table 1). About 2% of single cells gave solely astrocytic or solely oligodendrocytic clones, judged by staining with antibodies against glial fibrillary acidic protein, which labels astrocytes (Fig. 1*c* and *d*) or with antibodies O4 and O1, which label oligodendrocytes[7] (Table 1). In contrast, about 7% of single cells continued to divide for weeks and produced hundreds of progeny. We examined the differentiated cell types present in these large clones and found that ~40% contained neurons, astrocytes and oligodendrocytes, revealing a common precursor for the three major classes of cells from the central nervous system (CNS) (Fig. 2). The remainder appeared to contain only neurons and astrocytes or only astrocytes and oligodendrocytes.

We investigated whether the cortical cells that generated large, multiple-type clones were stem cells. A defining characteristic of stem cells is their ability to renew themselves[3,4,8]. To test for self-renewal, we grew putative stem cell clones until they contained 20–30 cells (~4 days of culture), dissociated them and subcloned individual progeny into culture wells. If the initial cell had undergone self-renewal, some of the progeny would behave as stem cells, generating large, diverse clones. We found that this was the case in all eight clones that were subcloned. For example, for one 26-cell clone, ten subcloned progeny survived, seven generated clones of 1–5 cells, one gave 16 astrocytes, and two generated hundreds of progeny of multiple cell types (Fig. 3). This indicates that the initial cell was probably a stem cell capable of self-renewal.

The frequency of progenitor cell types was similar at E12 and E14; 76% of E12 and 81% of E14 cortical ventricular zone cells differentiated into one or two neurons. The frequency of stem cells at E12 and E14 was $7.4 \pm 7.5\%$ versus $7.3 \pm 1.5\%$ respec-

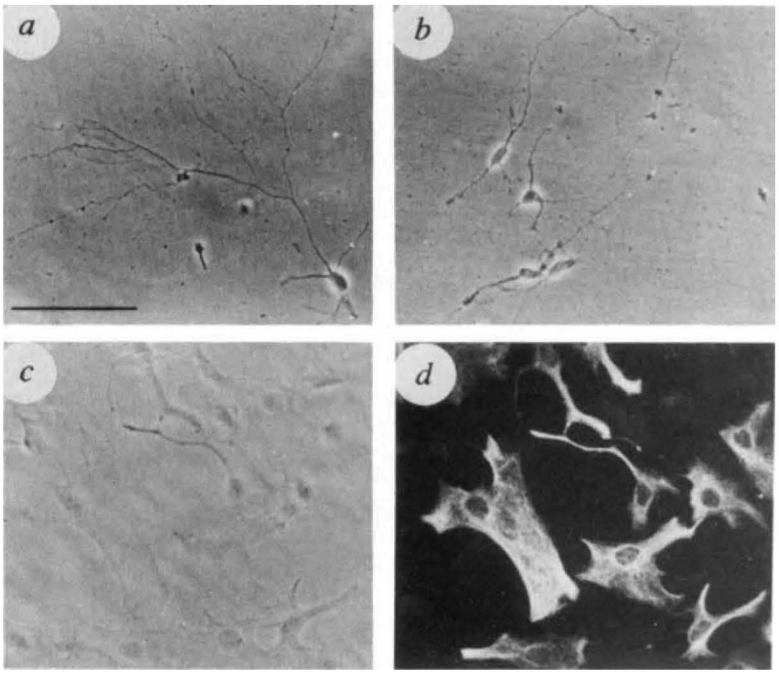

FIG. 1 Three of the clonal types. *a*, Phase micrograph of a single neuron, the most common product of the isolated ventricular zone cells. *b*, Larger neuron-only clone consisting of four progeny. *c*, Phase micrograph of a clone consisting solely of astrocytes. *d*, Astrocyte clone evident after staining with antiserum against GFAP, visualized with a fluorescein-labelled anti-rabbit antibody (Tago). Scale bar, 100 μm. Single E12–E14 cortical cells were mechanically dissociated in hibernation medium[27] and plated as one cell per Terasaki well by micromanipulation[19]. Culture medium was a serum-free medium (DMEM B27 plus N2 (Gibco)) which had been conditioned by cortical astrocytes and meningeal cells[28]. Culture wells contained sonicated membrane homogenates from C6 cells at 0.01 mg protein per ml. The C6 membrane supports division of isolated embryonic cortical ventricular zone cells[29]. Under these culture conditions about 85% of single E12–E14 cortical cells survived the first 24 h of culture, as judged by the presence of a live cell or its progeny in the culture wells.

TABLE 1 Frequency of clonal types from single cells cultured from E12 and E14 cerebral cortex

Age		Clone composition						
		Single neuron	Pairs of neurons	>2 neurons	Astrocytes or oligodendrocytes	Mixed neurons and non-neuronal* (<100 cells)	Stem cells†	Total clones
E12	Total of 3 replications	114	48	9	3	22	27	223
	As a percentage of 3 replications	50±4.3%	26±6.9%	6.4±4%	1.9±0.8%	9.2±1.6%	7.4±7.5%	
E14	Total of 3 replications	143	53	8	4	17	16	241
	As a percentage of 3 replications	59.6±14.2%	21±8.8%	2.6±3%	1.3±0.6%	8±4.6%	7.3±1.2%	

See legend to Fig. 1 for conditions. Cells were classified using morphological criteria and cell-type-specific markers. Markers included antibodies O1 and O4 (Boehringer–Mannheim), both of which stain the surface of oligodendrocytes[7], antibody RT97 (Developmental Studies Hybridoma Bank) against neurofilament protein for neurons, and an antiserum to glial fibrillary acidic protein (GFAP) (Dako) for astrocytes.

* Average size of mixed clones was 25 for E14 and 29 for E12 cortex.

† Stem cell clones consisted of hundreds of cells of different neural phenotypes.

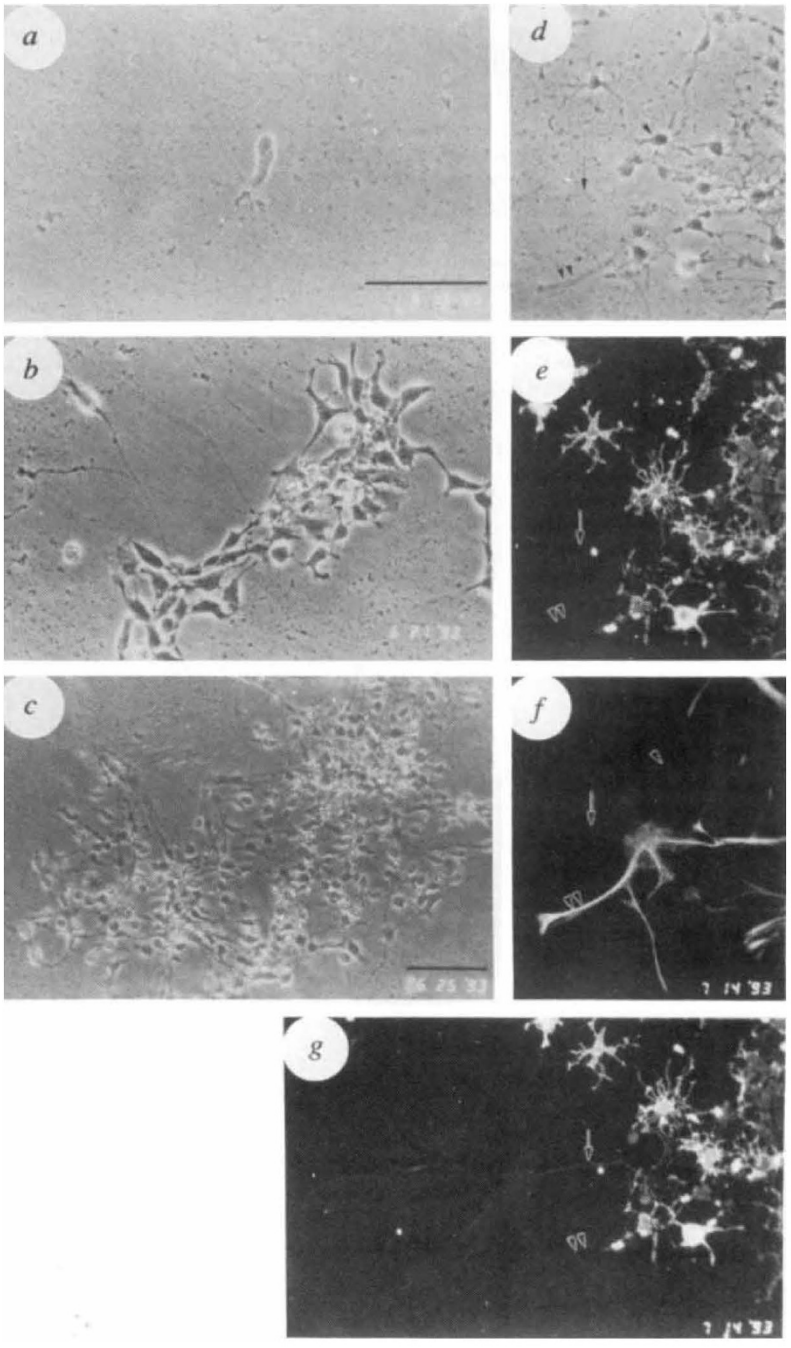

FIG. 2 A multipotent stem cell that generated neurons, astrocytes and oligodendrocytes. *a*, Two progeny of the original single cell after 1 day of culture; *b*, same clone after 6 days of culture. Note the increase in cell number and the differentiation of neuron-like cells. *c*, Further growth and differentiation of the clone by 10 days *in vitro*. *d*, Phase micrograph of a section of the culture at 20 days showing the morphology of cells subsequently stained with antibody markers to CNS cell types. The cell markers were used sequentially and never overlapped in a single cell. *e*, The clone after staining with O4 monoclonal antibody, visualized with a rhodamine-conjugated anti-mouse antibody (Tago). Cells with the morphology of oligodendrocytes are clearly labelled with O4. *f*, Clone after fixation, permeabilization and staining with an antiserum to GFAP, visualized with a fluorescein-conjugated, anti-rabbit antibody. GFAP-positive astrocytes are labelled. *g*, Clone after staining with a monoclonal antibody to neurofilament protein (RT97, visualized with a rhodamine-conjugated anti-mouse antibody). Fine processes of neurons are labelled; these are shown (arrows) to be present in the phase micrograph (*d*), to be negative for O4 (*e*) and GFAP (*f*). Note that in *g* the O4-labelled cells are also visible with rhodamine labelling from the first round of cell-surface staining. Scale bar, 100 μm.

tively (Table 1). After six days of culture, E12 stem cell clones were significantly larger (mean, 65.2 cells) than E14 stem cell clones (mean, 33.3 cells) ($P<0.05$). The underlying reason for the faster growth rate of E12 stem cells, possibly decreased cell-cycle time, reduced cell differentiation or reduced cell death, remains to be determined.

We compared clones in single-cell culture to those reported using retroviral vector labelling of murine cerebral cortex at a similar developmental age and found a close correlation between the composition of neuronal clones. For example, the average size of multicellular neuronal clones was 3.23 ($n=57$) for E12 versus 2.36 for E14. Retroviral labelling of E12–E14 mouse cerebral cortex yielded 83% purely neuronal clones, averaging 3.5 cells for injections made at E15 and 2.0 cells at E17 (ref. 10). But in the case of glial clones, we found a much lower percentage (2%) of purely astrocytic or oligodendroglial clones than reported from retroviral labelling studies at similar ages *in vivo* (12–40%)[9,12]. There may be selection against the development of immature glia in our culture system, although some can divide and differentiate. Alternatively this difference could be explained if some glial progeny of ventricular zone cells *in vivo* migrated through the developing cortex, creating additional subclones, as suggested from patterns of retrovirally labelled glial clones *in vivo*.[13,14].

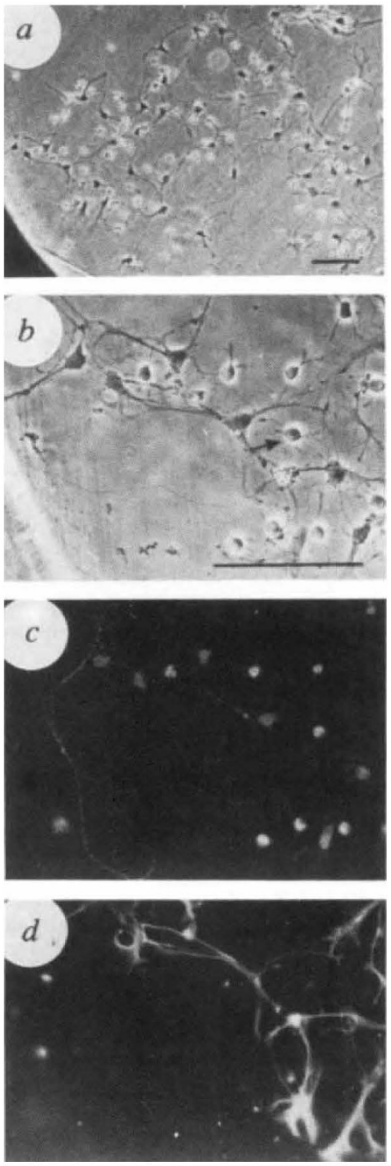

FIG. 3 The growth of one of the progeny of a stem cell after subcloning. *a*, Low-power phase micrograph of a portion of culture at 14 days to show that the subcloned cell produced numerous progeny. *b*, Phase micrograph showing the variety of morphologies present in this clone: oligodendroglial-like cells are clearly visible (single arrow). *c*, On staining with RT97, neurofilament-positive processes are seen in this region of the clone. *d*, GFAP-positive astrocytes are also present. A single cell from E14 ventricular zone was plated into a Terasaki well and grown under conditions described for Fig. 1. After 4 days the clone consisted of 26 cells and these were removed from the well by 0.5% trypsin plus 1 mM EDTA and replated as single cells in Terasaki wells under the same culture conditions as the initial single cell. Scale bar, 100 μm.

The most notable conflict between the two sets of data is the fact that a multipotential stem cell has not been found by retroviral lineage tracing *in vivo*. Although occasional retrovirally labelled clones containing neurons and glia have been seen *in vivo*[9,10,11,15], they are significantly smaller than stem cell clones *in vitro*. Perhaps ventricular zone cells acquire stem cell properties and generate large clones under these culture conditions (which include glial membranes and conditioned medium) although they are constrained to yield small clones *in vivo*. Alternatively, stem cells may not have been scored *in vivo*: the occasional observation of a large, multicell-type, widespread (due to cell migration[10,16]) clone might have been difficult to define as the product of a single infection[9,16].

Because most retrovirally labelled clones *in vivo* consist of one cell type[9,10,11], even of one neuronal subtype[12], it has been suggested that most cortical progenitor cells are restricted in their fates[12,14]. We also observed mainly single-type clones, even in conditions supporting a multipotential fate, and in over 95% of neuron-only clones, all the progeny appeared to differentiate before cell death occurred. Our data therefore support (but do not prove) the idea that these progenitor cells were restricted.

The coexistence of a minor stem cell population among a larger population of apparently more restricted progenitor cells is reminiscent of the blood system[5]. By analogy with haemopoiesis, we propose that multipotential stem cells form the foundation of the cerebral cortex: self-renewing to produce and maintain cortical germinal layers, and dividing asymmetrically to generate different restricted progenitor cells that amplify individual cortical cell types, probably through symmetric divisions. As neurogenesis precedes gliogenesis *in vivo*, we suggest that the restricted progenitor cells generated early in development are predominantly neuronal, and the later ones predominantly glial. How the restriction of cortical ventricular zone cells from multipotency to unipotency occurs (perhaps through oligopotent intermediates[17]) remains to be seen. Interestingly, we did not observe any neuron–oligodendrocyte stem cell clones, although small neuron–oligodendrocyte clones have been described in retroviral studies[15], which perhaps represents a restriction in the development of stem cells.

Thus, as in neural crest[6,18], there may be a haemopoietic-like mechanism operating in the cerebral cortex. Is this model relevant to other regions of the CNS? Rare, large, neuron–glial clones have been described in cultures of septal region[19] and striatum treated with epidermal growth factor[20], and the fact that similar cells have been extracted from adult striatum[21] suggests self-renewal. In chick hindbrain, most clones are single-type, suggesting the presence of restricted progenitor cells[22]. Developing murine retina and chick spinal cord contain multipotential cells, but lack progenitors restricted to give one class of retinal or spinal cell[23–26]. It appears then, that different regions of the CNS may use different strategies to generate cell diversity, although further studies may reveal common mechanisms. □

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Neurotrophin-6 is a new member of the nerve growth factor family

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DURING vertebrate development, many neurons depend for survival and differentiation on their target cells[1–3]. The best documented mediator of such a retrograde trophic action is the neurotrophin nerve growth factor (NGF)[1]. NGF and the other known members of the neurotrophin family, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) are conserved as distinct genes over large evolutionary distances[4–6]. Here we report the cloning of neurotrophin-6 (NT-6), a new member of this family from the teleost fish *Xiphophorus*. NT-6 distinguishes itself from the other known neurotrophins in that it is not found as a soluble protein in the medium of producing cells. The addition of heparin (but not chondroitin) effects the release of NT-6 from cell surface and extracellular matrix molecules. Recombinant purified NT-6 has a spectrum of actions similar to NGF on chick sympathetic and sensory neurons, albeit with a lower potency. NT-6 is expressed in the embryonic valvulla cerebelli; expression persists in some adult tissues. The interaction of NT-6 with heparin-binding molecules may modulate its action in the nervous system.

Neurotrophin-6 was cloned from a genomic library of the platyfish *Xiphophorus maculatus* during our attempts to clone the fish NGF gene[4]. Overlapping genomic clones (Fig. 1*a*) were identified that hybridized to the mouse NGF probe under low-stringency hybridization conditions. DNA sequencing of the hybridizing region predicted a single long open reading frame of 858 base pairs encoding a polypeptide related to the known neurotrophins. Sequence alignments (Table 1) to the other known neurotrophin sequences in fish, including the complete NGF and BDNF sequences of *Xiphophorus*[4], a partial NT-3 sequence of salmon[6], and the sequences of neurotrophins from other vertebrate species suggested that it did not represent a homologous molecule in *Xiphophorus* of any known neurotrophin but rather a new member of the neurotrophin gene family. Accordingly, this factor was termed neurotrophin-6 (NT-6).

TABLE 1 Amino-acid identity in per cent of fish NT-6 with other members of the nerve growth factor protein family

NGF, fish	61	NT-3, fish	52
NGF, human	56	NT-3, human	46
BDNF, fish	48	NT-4, frog	39
BDNF, human	48	NT-4/5, human	38

Note that for salmon NT-3 only 44 amino acids are known.

The deduced structure of the NT-6 precursor of 286 amino acids (predicted M_r of 31,424) is in accordance with the features of all known neurotrophins (Fig. 1*b*). (1) A hydrophobic domain at the N terminus with the characteristics of a signal peptide (amino acids 1–19). (2) A pro-region (amino acids 20–142) containing basic motifs (R-X-K/R-R at residues 63–66 and 139–142), necessary for proteolytic cleavage of precursor proteins into their mature, secreted forms[7]. (3) An amino-acid sequence at the carboxy half of the precursor starting at lysine 143 which encodes the mature NT-6 of 143 residues (M_r 15,968, p*I* 10.8; see also below the data on the recombinant protein). (4) Thirty-four residues, including the six cysteines conserved in all neurotrophins known so far, are also conserved in NT-6 suggesting that NT-6 shares a common tertiary structure with other neurotrophins; these residues are thought to be involved in the correct folding of the neurotrophins on the basis of the X-ray diffraction structure of NGF[8]. However, a distinguishing feature of NT-6 within the neurotrophin family is the presence of a 22 residue insert between the second and third conserved cysteine containing domain (Fig. 1*b*). This segment contains six basic and eight bend-inducing glycine[9] residues.

Analysis of NT-6 gene expression during embryonic development by northern blotting revealed a transcript of 1.4 kilobases (kb) from organogenesis onwards, in 8-day-old fish and continuing expression of this transcript in adult brain (Fig. 2*a*). NT-6 was also expressed in adult gill, liver and eye with weak expression in skin, spleen, heart and skeletal muscle (Fig. 2*a*). *In situ* hybridization in consecutive serial sections of embryos revealed expression in the valvula cerebelli, a rostral protrusion of the teleostean cerebellum under the midbrain tectum[10] (Fig. 2*b–d*).

Recombinant NT-6 was obtained from a rabbit kidney cell line (RK_{13}) infected with a vaccinia virus expression vector that contained the fish NT-6 gene inserted in its genome (Fig. 3*a*), and from insect cells infected with a baculovirus expression vector containing the NT-6 gene, respectively. Purified NT-6 from both preparations showed survival activity on embryonic chick neurons prepared from different ganglia. NT-6 promotes the survival of sympathetic and sensory dorsal root ganglion (DRG) neurons to the same extent as NGF, albeit with a much lower specific activity (Fig. 3*b*). It had no survival effect on ciliary and nodose neurons (Fig. 3*b*), which also do not respond to NGF. Thus, the spectrum of responsive neurons is similar to that of NGF. A monoclonal antibody that inhibited the activity of NGF showed no inhibition of the survival activity of NT-6 (Fig. 3*c*). The high concentrations of NT-6 needed (50 ng ml^{-1} for half-maximal survival) to obtain neuronal survival with chick

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