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Intrinsic programs of patterned cell lineages in isolated vertebrate CNS ventricular zone cells

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SUMMARY

Using long-term, time-lapse video-microscopy, we investigated how single progenitor cells isolated from the early embryonic cerebral cortex produce neurons and glia over time. Clones of 10 cells or less were produced by short symmetric or asymmetric division patterns, commonly terminating in a 'pair progenitor' for two morphologically identical neurons. Larger trees were composites of these short sub-lineages: more prolific neuroblasts underwent repeated asymmetric divisions, each producing a minor neuroblast that typically made ≤10 progeny, and a sister cell capable of generating more progeny. Particular division patterns were seen repeatedly. In contrast, glioblasts underwent a prolonged series of symmetric divisions. These patterned lineage trees were generated from isolated cells growing on plastic, suggesting they are largely intrinsically programmed. Our data demonstrate for the first time that CNS progenitor cells have stereotyped division patterns, and suggest that as in invertebrates, these may play a role in neural development.

Key words: Cell lineage, Neuron, Glia, Cerebral cortex, Mouse

INTRODUCTION

The mammalian cerebral cortex arises from a simple, one-cell thick sheet of germinal neuroepithelial cells that undergoes massive cell division and generates, at precise times, the diverse neurons and glia of the mature cortex (Jacobson, 1991). Clonal studies have demonstrated that the cortical neuroepithelium contains intrinsically different types of progenitor cells (reviewed by Kilpatrick et al., 1995; Temple and Qian, 1996). It is not clear how these are regulated so that they produce appropriate numbers and types of differentiated neural progeny during cortical histogenesis.

The simplicity and accessibility of the developing invertebrate nervous system have allowed, in some species, a full description of how neurons and glia are generated during development. In grasshopper and *Drosophila sp.* the nervous system arises from an array of neuroblasts that are precisely organized and individually recognizable (Poulson, 1950; Bate, 1976; Doe et al., 1985; Hartenstein et al., 1987; Udolph et al., 1993; Bossing et al., 1996). Neuroblasts are stem cells that divide asymmetrically (an asymmetric division is one that generates two different daughters) to produce another neuroblast and a precursor called a ganglion mother cell that divides once to give two differentiated progeny – usually neurons, but occasionally two glia or one neuron and one glia (Udolph et al., 1993). While individual neuroblasts generate different ganglion mother cells that produce specific types of progeny, their basic lineage tree is the same. Reconstruction of neural lineage trees in the nematode *C. elegans* and the leech also reveal predominantly asymmetric division patterns that are largely predictable, for a given identified precursor (Sulston and Horvitz, 1977; Zackson, 1984).

Predictable division patterns appear to be important in designating neural cell fate. For example, in *C. elegans*, a number of mutations that alter neural lineage trees also alter the types of progeny that they generate (Sternberg and Horvitz, 1984; Finney and Ruvken, 1990; Guenther and Garriga, 1996). In *Drosophila*, mutations in genes such as *numb* and *prospero* that are key factors for asymmetric neuroblast divisions, lead to alterations in neural cell fate (Doe et al., 1991; Rhyu et al., 1994; Spana and Doe, 1995).

Exactly how cell division patterns are linked to fate specification is not completely clear, but unequal partitioning of fate-determining molecules between daughter cells appears to be of key importance. For example, the arthropod neuroblast contains prospero protein and mRNA, which are both asymmetrically distributed into the ganglion mother cells when the neuroblast divides (Doe et al., 1991; Hirata, et al., 1995; Li et al., 1997). There the protein translocates into the nucleus and effects transcription of neural-specific genes. In some cases, environmental mechanisms interact with the asymmetric distribution of cell-intrinsic factors. For example, in the *Drosophila* sensory organ precursor, asymmetric segregation of numb leads to asymmetry in Notch expression, so that cells

are biased towards receiving particular environmental input that can influence cell fate (Jan and Jan, 1995).

For the vertebrate CNS, it is not known if predictable division patterns occur. In the cerebral cortex, studies of growth rates have suggested that before neurogenesis cell divisions are largely symmetric, changing to largely asymmetric once neurogenesis begins (Caviness et al., 1995; Rakic, 1995). In support of this, observations of single divisions of ventricular zone cells in slices of ferret cortex reveal that at early times most cell divisions are vertical with respect to the ventricular zone surface, generating two daughter cells placed side-by-side that appear to have similar fates, while later in development divisions are largely in the horizontal plane, producing two daughters that sit on top of each other in the ventricular zone and appear to have different fates (Chenn and McConnell, 1995). However, no studies to date have allowed us to obtain continuous information over a long period of time to allow us to follow progenitor cells through multiple division cycles to their final fate in vivo. Hence it is not clear whether vertebrate neural lineage trees are patterned or random.

In vertebrates, the complexity and inaccessibility of the developing CNS have delayed our ability to follow neuroblasts continuously in vivo. However, the fact that in *Drosophila* the same basic division pattern seen in vivo is maintained in vitro (Seecof et al., 1973; Huff et al., 1989; Luer and Technau, 1992) suggests that lineages obtained from mammalian cells dividing in culture may shed light on those in vivo. Hence, we have used a model culture system in which single neuroblasts from the early cortical ventricular zone develop into clones, to study mammalian neural lineages, with the help of continuous, long-term, time-lapse video recording. Because single cells are grown in isolation, this culture system also provides clues about the cell-intrinsic information that the initial, isolated cells carry. This study has demonstrated for the first time that there are stereotyped patterns within vertebrate CNS lineages, and furthermore that they occur from isolated cells, suggesting that critical components of the lineage trees are cell-intrinsic.

MATERIALS AND METHODS

Preparation of single cell suspension of cortical ventricular zone cells

E10.5-E14 (plug date is considered day 0.5) mouse cerebral cortices were dissected, harvested in hibernation medium (Kawamoto and Barrett, 1986) and dissociated enzymatically using papain, with a method based on that of McLeish and Townes-Anderson (1988). Cerebral cortices were placed into 5 ml prewarmed, filtered dissociation solution containing DMEM, 1 mM glutamine, 1 mM sodium pyruvate, 1 mM N-acetyl-cysteine (Sigma), and 7 unit/ml Papain (Worthington). 15 μl of 4 mg/ml DNase (Sigma) were added, and 95% O_2 and 5% CO_2 was bubbled through the suspension for 30 seconds. The tube was placed on a shaker at a 30° angle and gently shaken for 30 minutes at low speed (15 rpm/minute) at room temperature with brief agitation after 15 minutes. The tissues were then rinsed 3 times with DMEM with gentle centrifugation. After the final wash, the tissue was dissociated by trituration using a plastic transfer pipette 2-3 times, and a polished Pasteur pipette 1-2 times, then allowed to settle for 10-15 minutes before collecting the top fraction containing >95% single cells.

Clonal cell culture

Single cortical ventricular zone cells were plated at clonal density into poly-l-lysine coated microwells in Terasaki plates. Each well contained 11-12 μl of 'basal' serum-free culture medium (DMEM, B27, N2, sodium pyruvate, glutamine, and 1 mM N-acetyl-cysteine supplemented with FGF2). Cultures were maintained in a tissue culture incubator at 35°C, 6% CO_2 with 100% humidity.

Time-lapse video recording and analysis of isolated cortical ventricular zone cells

Cells were plated at clonal density into poly-l-lysine-coated Terasaki wells containing 11-12 μl of basal culture medium with 10 ng/ml FGF2. Immediately after plating, or up to 48 hours afterwards, a time-lapse video recording of the cells was made. Terasaki plates containing selected cells were placed in an incubated, humidified chamber maintained at 35°C with 6% CO_2. Images were captured using an inverted Olympus microscope linked to a CCD camera and recorded with a Panasonic time-lapse tape deck. The video images were replayed and the fate of each cell followed to generate the lineage tree. After recording, clones were fixed and stained with cell-type-specific markers – β-tubulin III (Sigma) and neurofilament protein (clone 2H3 Developmental hybridoma studies bank) for neurons, GFAP (Dako) for astrocytes, and O4 and O1 for oligodendrocyte lineage cells (Sommer and Schachner, 1981). In this way the progeny of the initial progenitor cell could be identified.

Immunostaining of clonally derived cortical neural cells

Live cultures were incubated for 20 minutes at 37°C with the O4 antibody, then washed with room temperature calcium-magnesium-containing PBS (CMPBS) and fixed with 4% paraformaldehyde for 30 minutes prior to incubating with secondary antibody. Fixed cultures were permeated by exposure to methanol at –20°C for 5 minutes. Cells were subsequently stained using a monoclonal antibody against β-tubulin isoform III (1:100 dilution; Sigma) and/or a polyclonal antiserum against glial fibrillary acidic protein (GFAP) (1:400 dilution; Dako) to identify neurons and/or astrocytes respectively. Briefly, fixed cultures were blocked by incubation in CMPBS with 10% normal goat serum (NGS) for 30 minutes, then incubated with primary antibody for either 30 minutes at room temperature (GFAP) or overnight at 4°C (β-tubulin III). Cells were then washed 4 times with CMPBS, and blocked with 10% NGS before incubating with secondary antibodies (1:75-100, diluted in CMPBS with 5% NGS) for 30 minutes. Fluorescein- (FITC) or rhodamine-conjugated goat-anti-mouse IgG and IgM (Cappel) were used to detect β-tubulin III and O4; FITC or rhodamine-conjugated anti-rabbit IgGs (Cappel) were used to detect GFAP staining. Finally, stained cells were washed with CMPBS three times and mounted in PBS containing 25 mM NaI to reduce fading, before examination with a Zeiss Axiovert 35 fluorescence microscope.

Analysis of pair progenitor progeny

Single isolated E14 cortical cells were plated at clonal density in DMEM, N2, B27 medium containing 10 ng/ml FGF2 and cultured for 14 days. Cells were fixed with 4% paraformaldehyde and stained with β-tubulin III antibody (1:500 dilution; Sigma), followed by biotinylated secondary antibody (1:100 dilution; Vector), visualized with the VIP substrate kit (Vector) and examined with a Zeiss Axiovert 35 inverted microscope. This staining procedure reveals fine details of neuronal morphology.

RESULTS

At E10.5 the murine cerebral cortex consists of a single layer of ventricular zone cells, the vast majority of which are still dividing (Bayer and Altman, 1991). Differentiated neurons first

appear around day 12-13, peaking in production in the second half of gestation and declining towards birth. We showed previously that the majority of progenitor cells present in the early (around E10.5-E13) murine cortical ventricular zone generated purely neuronal clones, whether they were grown in complex growth conditions (Davis and Temple, 1994) or in simple, serum-free medium containing the mitogen fibroblast growth factor 2 (FGF2; Qian et al., 1997). This suggests that the majority of early cells are restricted neuroblasts, consistent with in vivo clonal data (Luskin et al., 1988; Walsh and Cepko, 1988; Reid et al., 1995, 1997). With increasing embryonic age, restricted glioblasts become more prevalent, although they remain a minor population until perinatal ages (Kilpatrick et al., 1995; Levison and Goldman, 1997; Qian et al., unpublished). In this study we have examined the generation of neurons and glia in clones growing in serum-free medium containing FGF2, selecting later ages (around E13) to examine glioblasts, which will be described in a later section.

Early cortical neuroblasts are heterogeneous in the numbers of progeny they generate

Mouse E10.5-13 cortices were dissociated, and single cells plated at clonal density into Terasaki wells. Over 80% of plated cells survived the first 24 hours in culture, and their fates were recorded. Approximately 63% of plated cells differentiated into single neurons, while the remainder generated clones of ≥2 cells. After 10-14 days in culture, most clones had largely ceased division and contained mainly differentiated progeny. They were then fixed and stained for the three major CNS cell types – using β-tubulin III for neurons, GFAP for astrocytes and O4 for oligodendrocyte lineage cells. The percentage of neuron-only clones was similar to that described previously in complex growth media and in vivo at slightly later ages (Davis and Temple, 1994; reviewed by Kilpatrick et al., 1995). We found that neuroblasts from these young embryos were heterogeneous, as shown in Fig. 1 for E10.5. E10.5 cells were grown in three different concentrations of FGF2 – 0.1, 1 or 10 ng/ml, and the number of neurons generated after 10-14 days in culture were compared. When growing under these standardized culture conditions at clonal density, some progenitor cells gave only 1 or 2 neurons, while others gave up to 100. There was no significant difference in the number of neurons generated per clone over this 100-fold range of FGF2 concentration. However, in the absence of FGF2, the cells divided very little, most differentiating as single neurons (Qian et al., 1997). These observations revealed that early cortical neuroblasts, even at one age, are intrinsically heterogeneous in the number of progeny they can generate.

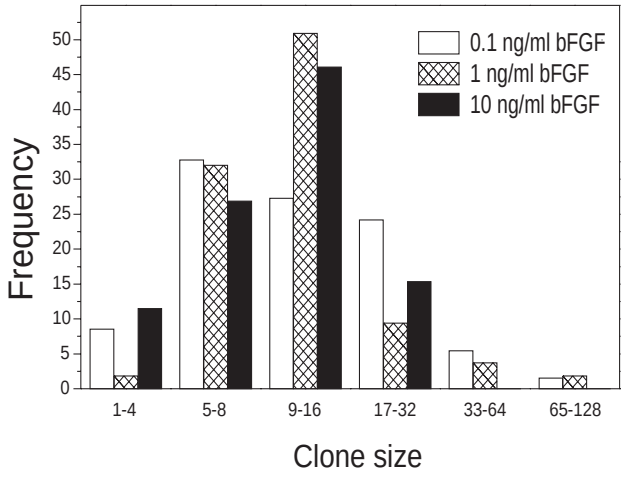

Fig. 1. E10.5 cortical neuroblasts are heterogeneous. E10.5 cortical cells were dissociated and plated at clonal density in basal serum-free medium supplemented with 0.1, 1.0 or 10 ng/ml FGF2. Clone growth was monitored by microscopic examination. After 10-14 days in culture almost all the neuroblast clones had ceased proliferating. Clones were then fixed and stained using cell-type-specific markers (O4, GFAP and β-tubulin III) and the numbers of neurons in neuroblast clones was examined. Clone size varied widely, from two to approximately 100 neurons. There was no significant difference in the numbers of neurons generated per clone in the different FGF2 concentrations used.

Short lineages: simple symmetric or asymmetric patterns

In order to examine how cortical neuroblasts generate progeny over time, we used time-lapse video microscopy. Embryonic murine cortices were dissociated and plated at clonal density. Immediately after plating, or up to 48 hours later, cells were selected for time-lapse recording, and videotaped for up to 14 days. Clones were then fixed and stained with neural cell-type-specific markers to identify the types of cells in the clones. The recorded time-lapse information was analyzed to reconstruct the lineage trees of individual cells, and then matched with the immunohistochemical data to determine progeny fate (see Fig. 2). By combining these two sources of information, we were able to reconstruct the lineage trees of individual recorded cells.

We reconstructed the lineage trees for 87 clones. Sixty-nine of these generated 10 progeny or less, and these are illustrated in Fig. 3. Fifty of these exhibited simple lineages yielding 2, 4 or 8 progeny that, as shown in Fig. 3A-C, are symmetric in appearance. (In this case, we are using the term symmetric to describe the division pattern, without including information about cell type.) In contrast, the remaining nineteen clones in this group produced progeny via asymmetric lineages, shown in Fig. 3D-N. (Here the divisions are clearly asymmetric because one daughter divides more than the other does, i.e. they have different fates.) As shown in Fig. 3, the number of asymmetric divisions that neuroblasts underwent varied; up to 3 were observed in these small clones. Many of the lineages contained a characteristic asymmetric motif – a neuroblast divides to generate one daughter that undergoes a further asymmetric division and one daughter (a cell we have called the 'pair progenitor') that produces two post-mitotic neurons (see Fig. 3N). The terminal precursor was usually, (83% of the time), a pair progenitor cell, the remaining 17% being precursors that did not divide, and generated a single neuron. In some cases, the production of single and pair progenitor cells appears patterned – for example the clones shown in Fig. 3J-M have the same basic pattern: pair, pair, single, pair.

Of the 230 progeny produced in the clones shown in Fig. 3, 188 (81.7%) were identified (based on immunohistochemical staining with neural-specific markers and their morphology), and all were neurons. 42 progeny could not be identified definitively (for example, because they had not differentiated when recording ceased, or, in the case of eight progeny, because they

died before the clone was stained). Hence, we conclude that these simple symmetric and asymmetric lineages are predominantly associated with neuron generation, although it is possible (given that not all progeny were identified) that some types of glia also arise via this division mode. Data gathered from E10.5-E13 animals were pooled in this section because small clones were observed at all ages. There were no obvious age-related differences in lineage trees amongst the small clones. In contrast, the large clones, which will be discussed in the next section, were observed largely from E10.5 animals.

Complex neuroblast lineages are composites of simple lineages

Eleven clones generated ≥10 neuronal progeny, and these had more complex lineage trees, which are illustrated in Fig. 4A-K. In these cases all the identified progeny were found to be neurons. Most of the small number of progeny that could not be identified were later-born cells (see Fig. 4), suggesting that they had not yet differentiated.

The most prominent feature of these larger neuroblast clones is that they are predominantly asymmetric. Typically, the original neuroblast undergoes a series of asymmetric divisions, each of which generates two cells with different division potential. The cell that produces the smaller number of progeny we call the minor neuroblast, and in the clones we observed the minor cell usually gave ≤10 progeny – only two examples produced more progeny (11 and 12 neurons). Minor neuroblasts exhibit the same simple lineage patterns seen for the neuroblasts generating small clones (illustrated in Fig. 3). For example, the clone illustrated in Fig. 4J is a combination of the simple symmetric lineages seen in Fig. 3A,B and the simple asymmetric lineages seen in Fig. 3I,N. Again, the immediate neuronal precursor is usually a pair progenitor giving two neurons, but a precursor for one neuron is occasionally observed. As noted in the simple lineages, in some cases there are patterns to the production of these two types of terminal neuron precursor cells. A prominent pattern is the 'pair, single, pair' 5 cell unit, seen in its simple form in Fig. 3H, and repeatedly as a minor neuroblast sub-lineage in complex lineages – twice in clones shown in Fig. 4B,D,I and once in Fig. 4G. Another common motif is the 'pair, pair, single, pair' 7 cell unit noted in Fig. 3J,K,L,M, and also seen as a minor neuroblast sub-lineage in the complex clones shown in Fig. 4C,K. We also noted that the end of some lineages e.g. Fig. 4A,C,E,I exhibit the same 7 cell pattern. Finally, a particular division pattern is sometimes repeated within a single lineage tree. For example, in Fig. 4H the more prolific neuroblast of the first cell division produces two offspring with complex but nearly identical lineages (indicated

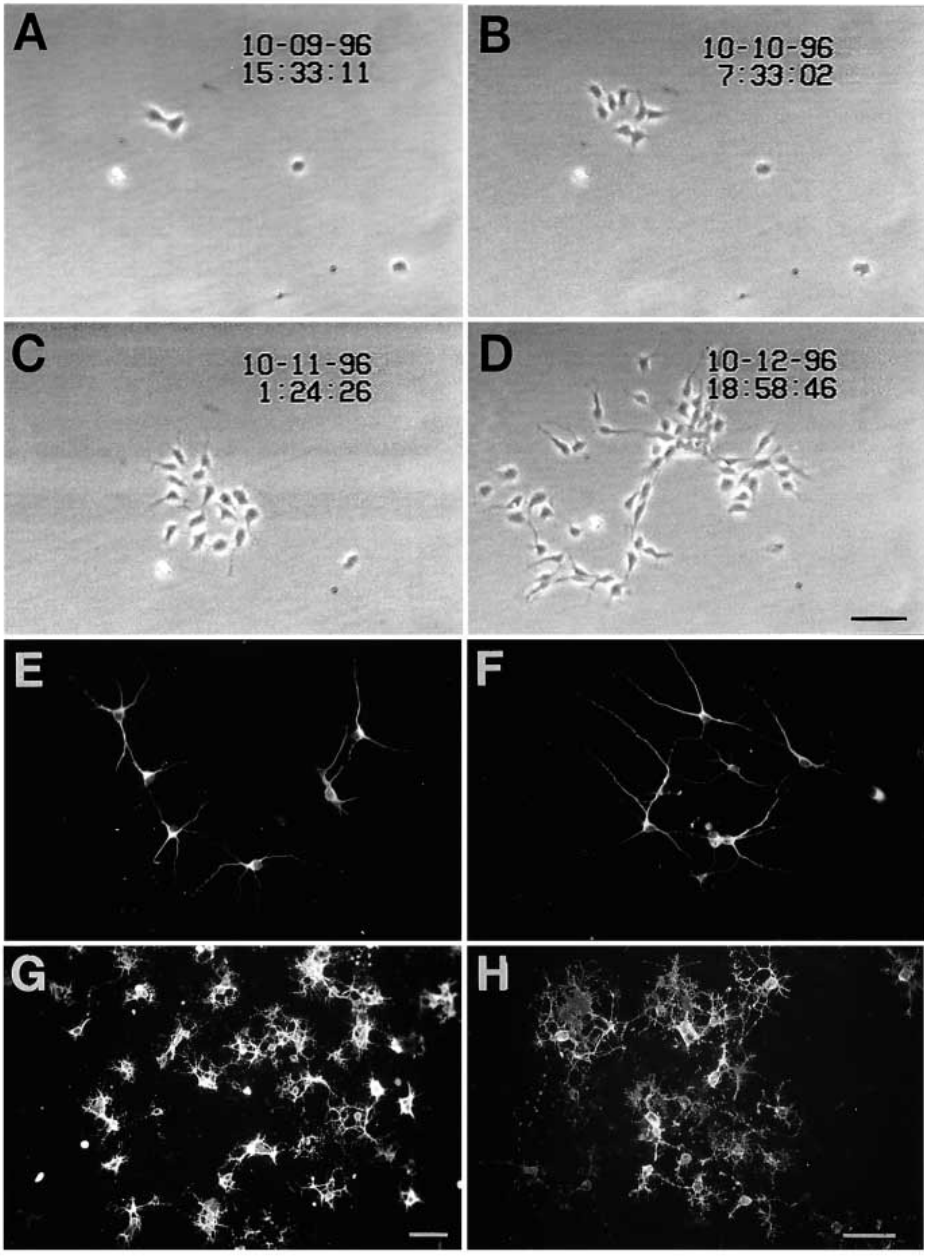

Fig. 2. Time-lapse video microscopy and immunohistochemistry of clones derived from single murine cortical ventricular zone cells. E10.5-13 cortical cells were dissociated and plated at clonal density in DMEM-B27-N2 plus 10 ng/ml FGF2. After 12 hours, a pair of sister cells was selected for time-lapse microscopy and recorded continuously for 8 days prior to fixation and staining with neural cell-type-specific markers. (A-D) Four still images taken from a video printer illustrating stages of clonal growth over the first 5 days in culture (the selected cell was a symmetrically dividing, glial progenitor). (E-F) Two examples of clones stained with an antibody to β-tubulin III that identifies neuronal lineage cells. (G-H) Two examples of clones stained with the O4 antibody that labels oligodendrocyte lineage cells. Bars, 50 μm.

by i and ii in the figure). Interestingly, one of these sub-lineages is repeated in clone 4D (indicated by ii). It is tempting to speculate that the observation of repeated patterns reflects predictable elements within the lineage trees.

In some cases, as shown in Fig. 4I,J, there is a trend that successive asymmetric divisions of the initial, primary neuroblast generate progenitors that make progressively fewer progeny, until at the end there is a 'tail' of repeated pair progenitors – like the lineage shown in Fig. 3N. However, this is not seen in all cases – for example in four of the lineages shown in Fig. 4, the first division generates a single neuron, while subsequent divisions generate minor neuroblasts producing more cells. Nevertheless, in all the cases we followed, the production of neurons eventually ceased, despite the fact that cultures are re-fed with fresh medium and mitogen every 2-3 days, suggesting that there is a limit to the number of divisions that the progenitor cells can undergo. Most neuroblast clones generate the majority of their progeny over the first 4 days in culture, and cease division by day 10 in culture.

The similarity between individual complex neuroblast clones, the fact that they are composites of simpler lineages that we have observed from other isolated cells, and the observation of repeated patterns within and between lineage trees reveals that vertebrate CNS neuroblasts exhibit a surprising degree of order in the way they generate their progeny over time.

Pair progenitor cells generate similar progeny

The most common asymmetric division motif seen in the neuroblast lineages involves the production of a pair progenitor cell that gives rise to two neurons. We examined the progeny of individual pair progenitors to find out whether they were similar or different. Embryonic day 14 cortical cells (an age that we have found to be rich in pair progenitors) were plated at clonal density, grown for 14 days to allow pair progenitor cells to generate differentiated progeny, then fixed and stained with β-tubulin III to reveal neuron morphology (Fig. 5). The cultured cortical neurons were heterogeneous, exhibiting a wide range of morphologies as reported previously (Kriegstein and Dichter, 1983). However, when we compared the morphologies of neurons within the same pair, we found they were remarkably similar. Of the 167 neuron pairs examined, 80.2% had essentially identical morphologies, based on soma size, shape and process outgrowth characteristics, and many were mirror-images.

Glial progenitor cells undergo prolonged symmetric divisions

Seven of the clones we examined, mostly from E13 cortices, were found to arise by a different mechanism than those described above. In these cases, the plated progenitor cells divided symmetrically a large number of times to generate clones of hundreds of cells (Figs 2A-D, 4L). Examination of the types of cells in these clones showed that none were neurons; almost all the cells were identified and all were glia. As described previously for clones growing in this defined serum-free medium with 10 ng/ml FGF2, over 90% of the glia present were oligodendrocytes (Fig. 2G,H); additional factors are required for astrocyte production (Qian et al., 1997). We conclude that restricted glial progenitor cells undergo a different mode of cell division from restricted neuroblasts, and that the lineage trees of these two basic progenitor cell types are distinct.

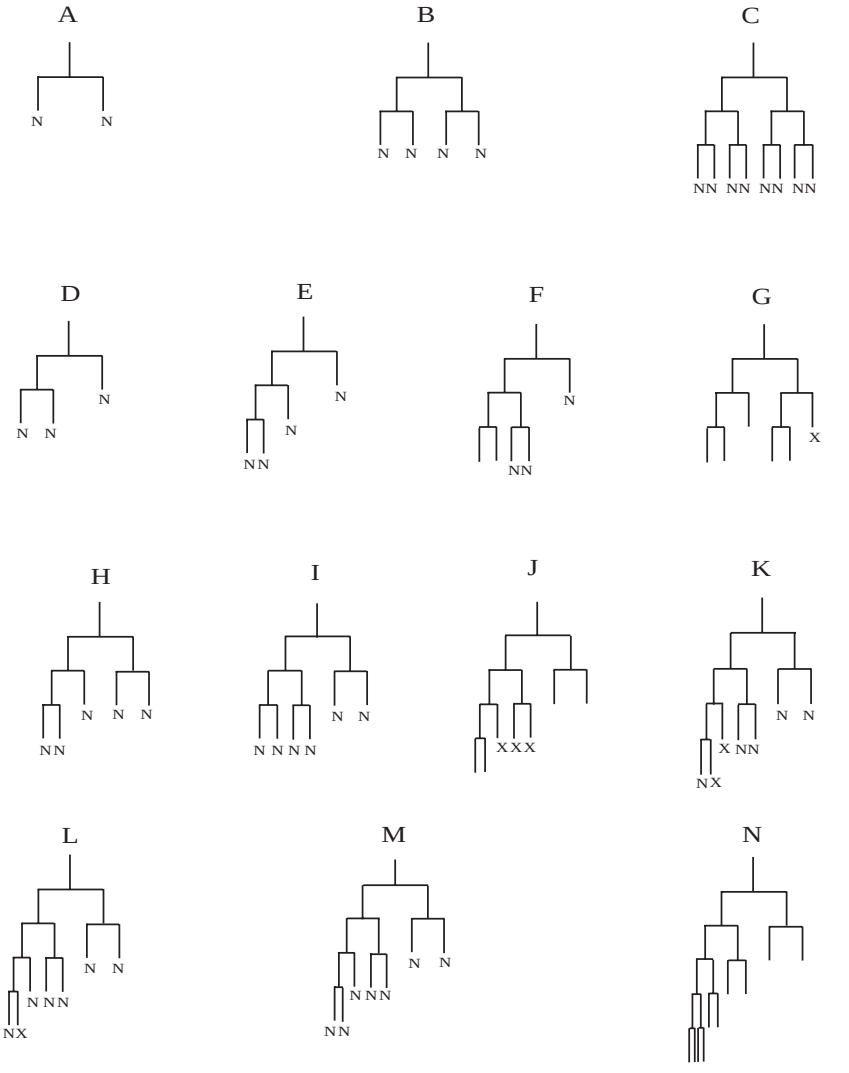

Fig. 3. Short symmetric and asymmetric lineages generate neuronal progeny. Embryonic cortical cells were plated at clonal density and continuously monitored by time-lapse microscopy for up to 14 days. Time-lapse tapes were analyzed to generate a lineage tree for each progenitor cell. The lineages of cortical ventricular zone cells that generated 10 or fewer progeny were compiled. (A-C) Short, symmetric lineages producing 2, 4 or 8 progeny. (D-N) Asymmetric lineages generating 3-10 progeny. Note that frequently a progenitor divides to generate one daughter (a 'pair progenitor') that produces two differentiated progeny and another that undergoes another asymmetric division. We recorded 39 examples of A, ten of B, two of C, four of D, two of F, G and H, three of I and one of E, J-N. All of the progeny identified in these lineages were neuronal. N, neuron; X, dead cell.

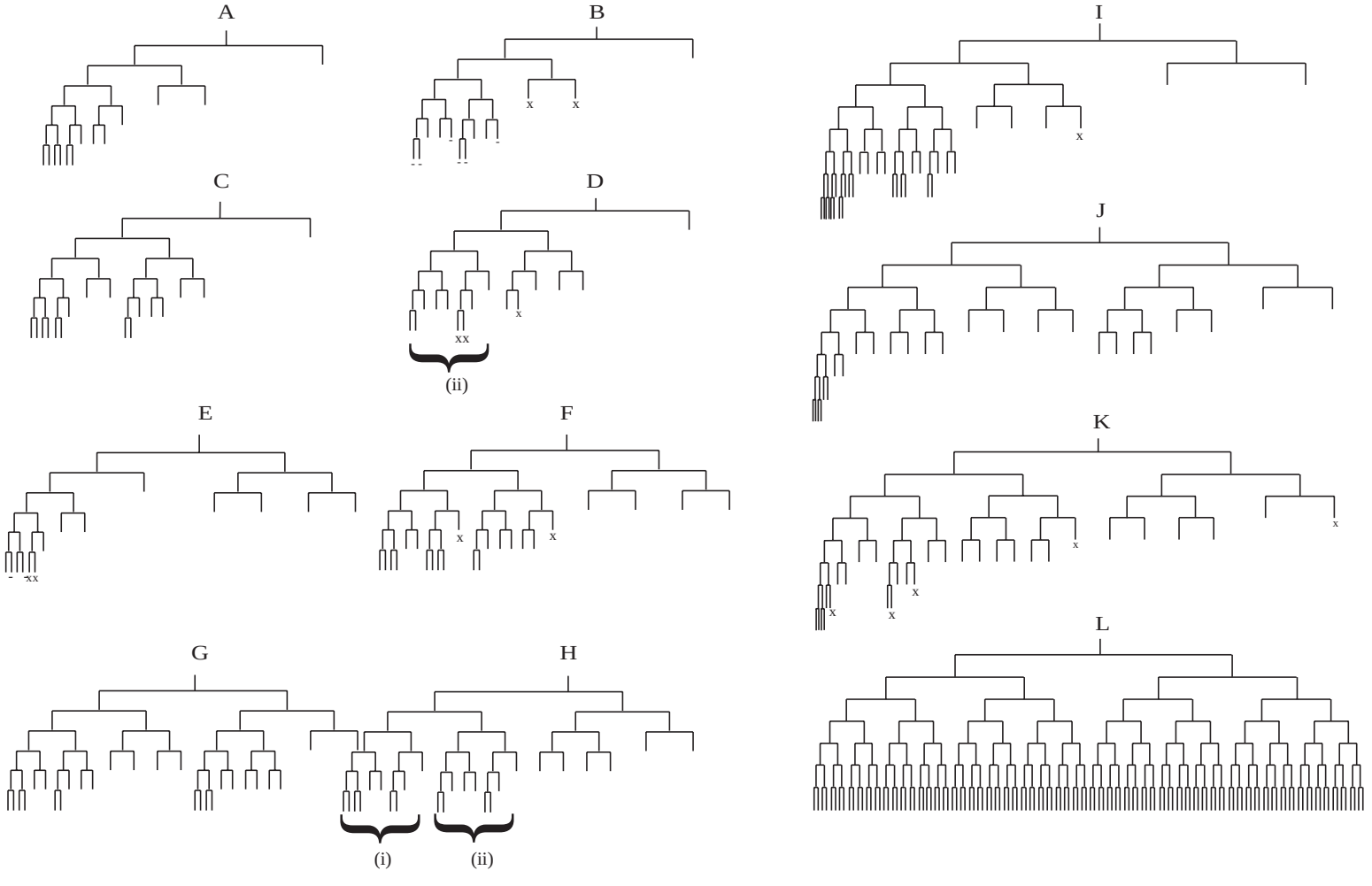

Fig. 4. Large clones: composite neuronal lineages and prolonged, symmetric glial lineages. Early embryonic cortical cells were plated at clonal density in serum-free medium, and followed under time-lapse video microscopy for up to 14 days. Seventeen of the recorded progenitor cells generated clones of more than ten progeny. (A-K) Large clones of neurons. These exhibited complex asymmetric lineage trees in which the initial primary neuroblast undergoes repeated asymmetric divisions, at each typically generating a secondary neuroblast that produces ≤10 progeny. These complex lineages are largely composites of lineages described in Fig. 3. For example, the lineage illustrated in J (a cell that generated 26 progeny of which 18 were identified as neurons and the remainder either died or had not differentiated at the time of staining) is composed of elements seen in Fig. 3A,B,I, and the final divisions making up the 'tail' of the lineage tree, are like the lineage shown in Fig. 3N. (L) Seven of the cells divided symmetrically and rapidly to generate large clones containing hundreds of glial progeny. The first seven divisions of one of these clones is illustrated. This clone continued to divide beyond the lineage shown, eventually producing approximately 300 progeny. On staining, using cell-type-specific markers, 290 of the progeny were found to be oligodendrocytes, 4 were astrocytes, none were neurons and 6 did not stain with any markers, suggesting that they had not yet differentiated. G, glia; no marking, neuron; –, a cell that did not stain for neuronal or glial markers. These negative cells are usually at the end of lineages, and are thought to have not differentiated at the time of staining; X, dead cell; (i) and (ii), parts of lineages referred to in text.

DISCUSSION

We have used long-term time-lapse recording to examine how single cortical ventricular zone cells generate progeny over time. Our data indicate that there are patterns within vertebrate progenitor cell lineage trees, even for progenitor cells growing in isolation, showing a degree of organization to the temporal production of neural cells that was not previously appreciated.

Neuroblast heterogeneity – proliferative potential

The fact that neuroblasts isolated from the early ventricular zone generate different numbers of progeny even when exposed to the same culture conditions, demonstrates that there is cell-intrinsic heterogeneity in the starting population. We suggest that the cerebral cortex contains intrinsically different neuroblasts that vary in the number of neurons they can generate, and possibly also the neuron types. Retroviral lineage studies of cortical progenitor cells in vivo show that at slightly later ages to those studied here, single neuroblasts generate clones of variable sizes, up to approximately 9 cells, with 2 neuron clones being particularly common (Luskin et al., 1988; Walsh and Cepko, 1988; Price et al., 1991; Parnavelas et al., 1991, 1995). Perhaps these clones represent the products of the different types of neuroblasts that we have observed in clonal studies in vitro, e.g. minor neuroblasts and pair progenitor cells (given the later time the in vivo studies were conducted we might expect a smaller average clone size than those we observe at E10.5 (Luskin et al., 1988; Qian et al., unpublished). Interestingly, another in vivo study indicates that many CNS neurons arise from an immediate precursor that generates a pair of neurons (Brittis et al., 1995). This is consistent with our hypothesis that the pair progenitor cell is a common neuron precursor that exists in vivo.

The mechanisms controlling the numbers of neurons made by a particular neuroblast are currently unknown. Perhaps clone size is controlled by the gradual reduction or accumulation of a cell-based factor, similar to the mechanism regulating oligodendrocyte number in the CNS (reviewed by Raff, 1996). Given that the cortical neuroblasts, unlike oligodendroblasts, undergo largely asymmetric divisions, factors involved in stem cell self-renewal, similar to PIE-1 which regulates self-renewal in the *C. elegans* germ line (Mello et al., 1996;

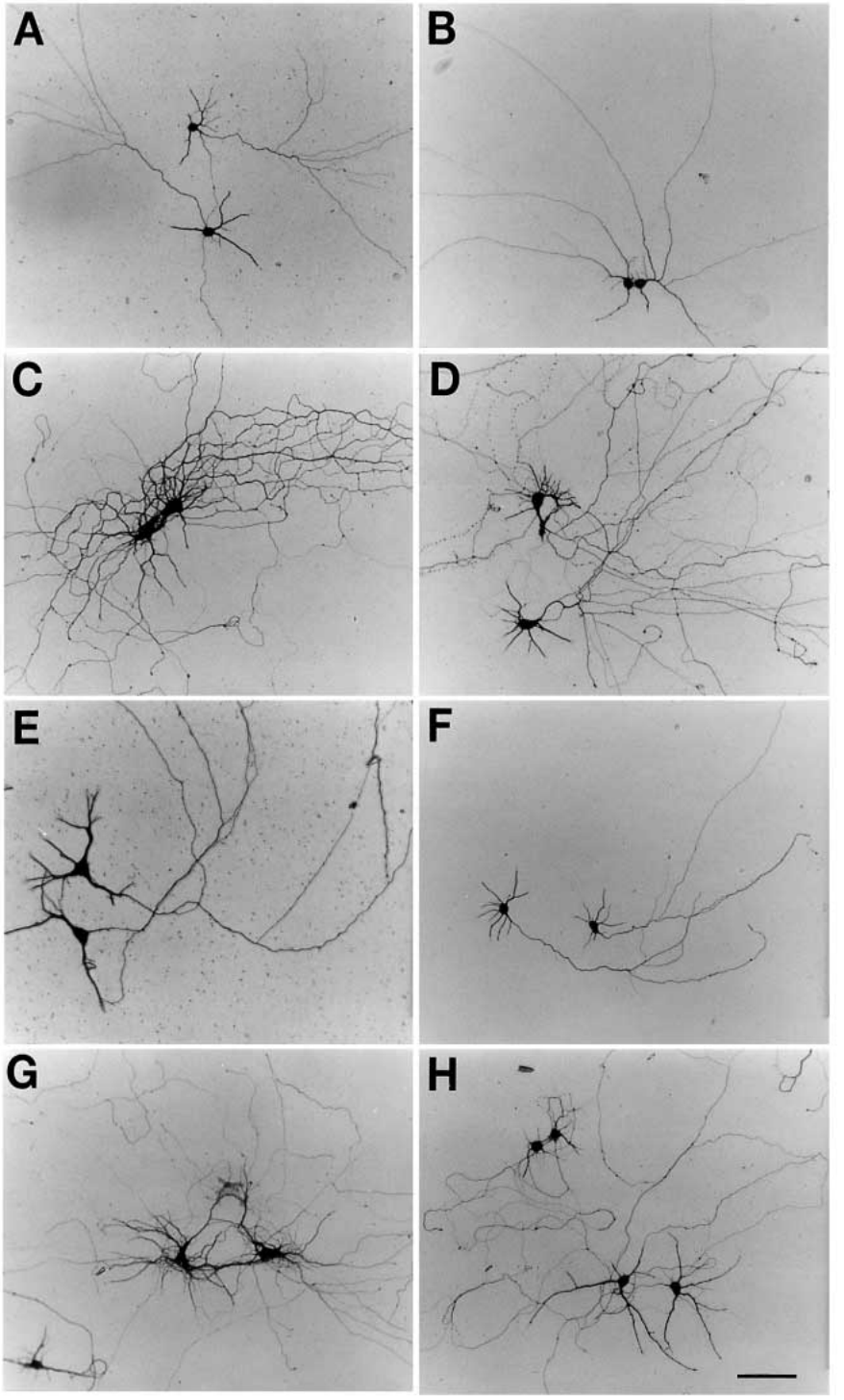

Fig. 5. Pair progenitors generate neuronal progeny that are morphologically alike. Embryonic day 14 cortical cells were dissociated and plated at clonal density in 10 ng/ml FGF2, allowed to develop for 14 days then fixed and stained for β-tubulin III to reveal neuron morphology. The morphology of neuron pairs was analyzed, based on cell soma size, shape, axon branching pattern and dendrite growth pattern. There was a wide array of neuronal morphologies, however the two progeny of a given pair progenitor were remarkably similar. (A-G) Seven examples of pairs of neurons illustrating the variety in neuronal morphologies and the similarity of sister cells. Note that some of the neuron pairs are mirror images. (H) A clone containing two pairs of neurons, illustrating that pair progenitor cells within the same clone can generate different types of progeny: the daughters within each pair are similar, but comparison between the two pairs shows different morphologies. Bar, 50 μm (A,B,D,F,H) and 31 μm (C,E,G).

Seydoux et al., 1996), might be involved in determining the shape and length of neuroblast lineage trees.

Common elements in vertebrate and invertebrate lineages

The data presented here for mammalian CNS lineages provide an interesting parallel to the division patterns of invertebrate neural progenitor cells. For example, the 5-cell lineage (pair, single, pair) shown in Fig. 3H and seen repeatedly in complex lineages e.g. in Fig. 4B,I, is identical to the lineage observed for neuroblasts derived from progenitor cells P1-P12 in the developing ventral nerve cord of the hermaphrodite *C. elegans* (Sulston and Horvitz, 1977). The division pattern: pair, pair, single, pair, shown in Fig. 3J-M and in the clones shown in Fig. 4C,K is also generated by the P3, P8 and P12 neural progenitors in the hermaphrodite *C. elegans.* The longer neural lineages shown in Fig. 4 are very similar to the postembryonic V lineages in *C. elegans*, which are also involved in generating neural structures. For example, V5 produces first a single hypodermal cell, then a group of 5 cells via the pair, single, pair motif – in which one of the last daughter cells dies – and then 10 more cells via an asymmetric tree. This has a striking resemblance to the cortical lineage shown in Fig. 4D. Other similarities emerge on comparison of cortical with arthropod lineages. The repeated production of pair progenitors seen in a number of cortical lineages e.g. in Fig. 3N and the 'tail' that terminates the lineages shown in Fig. 4J,K follows the same pattern as the arthropod neuroblast lineage, in which asymmetric divisions also produce a progenitor for two neurons – the ganglion mother cell (Doe and Skeath, 1996). This raises the interesting possibility that the pair progenitor cell we see in cortical cultures is in some way analogous to the arthropod ganglion mother cell. Furthermore, the observation that cortical glia are produced by symmetric lineages may have a parallel in arthropods, where some glioblasts divide symmetrically (Jacobs et al., 1989). These similarities suggest that features of neural lineage trees may be evolutionarily conserved from invertebrates through mammalian species, and further that the molecular mechanisms underlying neural lineage construction may be conserved. It will be interesting to examine the role of proteins homologous to those involved in directing asymmetric neuroblast divisions in *Drosophila*, e.g. numb, miranda, prospero, inscuteable and staufen, in vertebrate CNS lineages (Knoblich, 1997).

The role of lineage in vertebrate CNS development

One interesting question raised by these studies is what role predictable division patterns might play in vertebrate CNS development. In invertebrates, asymmetric lineage trees are intimately involved in generating diverse cell fates, and perhaps they have a similar role in the vertebrate cerebral cortex. For example, neuroblasts could generate intrinsically different neuronal progenitors at successive asymmetric divisions, and hence regulate the types of neurons generated over time. Alternatively, it is also possible that neuroblasts generated at different points in the lineage tree may be initially equivalent, but that they become different due to exposure to environmental factors. In this case, the asymmetric lineage tree would play a more indirect role in generating diverse cell types – by producing neural precursors at different times during development. Progenitors produced at different points in the lineage tree would undergo their last cell division cycle (a critical period in cortical neuron fate determination; McConnell and Kaznowski, 1991) at different times, and in this way be exposed to different fate-determining environmental signals.

In invertebrate systems, particular division patterns are used repeatedly to generate multiple groups of interacting cells that behave as a functional unit – e.g. sensory bristles in *Drosophila* (reviewed by Ghysen et al., 1993), sensory rays and postderids in *C. elegans* (Sulston and Horvitz, 1977; Sulston and White, 1980; Sulston et al., 1983). This suggests the intriguing possibility that some of the repeated patterns seen in cortical lineages, e.g. the pair, single, pair 5 neuron motif, or the 'tail' of pair progenitors, are used to generate a functionally linked group of cells. It will be interesting to test the hypothesis that predictable lineages are used to generate particular types of cortical neurons by examining the identity of clonal progeny.

The neuroblast lineage tree may also play a role in regulating neuron number. The neuroblasts we followed produced most of their progeny during the first few days in culture, and then gradually ceased division so that virtually all the neurons were born by day 10 of culture. If cells in vivo have similar lineages, this basic trend might explain the normal limit of neurogenesis (Bayer and Altman, 1991).

While asymmetric neuroblast lineage trees might be intimately associated with generating neuron diversity, symmetric lineage trees provide a means of amplifying a particular cortical cell type. This has been suggested to occur in the hindbrain where some progenitors generate groups of 8 cells of one type: perhaps these arise via a symmetric cell division mode (Lumsden et al., 1994). The fact that pair progenitors generate two similar progeny is consistent with this hypothesis, and studies comparing the morphologies of 4 and 8 neuron clones generated by symmetric lineages are underway. Similarly, a prolonged series of symmetric divisions, seen here for cortical glioblasts that generate predominantly oligodendrocytes, and also described previously for optic nerve oligodendrocyte progenitor cells (Raff et al., 1988; Raff, 1989), could be needed to amplify the relatively rare glioblasts present in the early cerebral cortex and produce the large numbers of glia needed to myelinate forebrain tracts.

Hence, as in invertebrates, vertebrate neural lineage patterns may have a significant role in key aspects of neural development, e.g. in cell fate determination, regulation of cell number and in the generation of cytoarchitecture, e.g. by organizing the formation of cortical layers. Previous studies have provided strong evidence that environment plays a critical role in these processes in vivo (McConnell and Kaznowski, 1991, Kilpatrick et al., 1995, Hirotsune et al., 1995; D'Arcangelo et al., 1995). Perhaps, as in invertebrates, specific division patterns produce intrinsic differences in progenitor cells that provide a framework on which environmental signals can act (Jan and Jan, 1995).

Lineage patterns are programmed in isolated cells

While the mechanisms underlying asymmetric divisions are being unfolded at the molecular level, we still don't understand whether the key factors that direct asymmetry are cell-intrinsic or environmental. Observation of asymmetric divisions has usually been carried out in vivo where the normal germinal environment could provide essential cues to drive the

asymmetry. In invertebrates, asymmetric neuroblast divisions can occur normally in vitro, albeit in multi-cell cultures (Seecof et al., 1973; Huff et al., 1989; Luer and Technau, 1992), suggesting that key underlying mechanisms are independent of the normal three-dimensional germinal environment. Our data suggest the same is true for vertebrate neural cells. In addition, in our case, single cells are grown at clonal density on poly-l-lysine coated plastic, hence preventing the initial plated cells from encountering environment-based cues that might still be present in multi-cell cultures. As a clone develops, some progeny do contact each other, and while it is possible that they reconstruct asymmetric cues, it seems unlikely because the clone is usually sparse, essentially two-dimensional, and there are no obvious organizational features that could direct asymmetric divisions. Furthermore, we have observed later-born progeny that divide asymmetrically without cell contact. Based on these observations, we suggest that the information for asymmetric divisions can be programmed in individual, isolated cortical progenitor cells, although it may rely on permissive environmental input (provided through intra-clonal interactions and the culture medium) to be expressed.

Pair progenitor cells generate similar progeny

Frequently, the immediate precursor generated during asymmetric cortical neuroblast divisions was a precursor for two neurons. Pair progenitor cells usually generate two very complex but nearly identical cell types. This implies that the information for these morphological patterns was programmed in the pair progenitor before it underwent its final division (it appears highly unlikely that environmental cues could have directed identical, complex cell shapes independently on two separate cells). A similar result has been demonstrated for cells from a neuroblast cell line – sister cells were found to be more alike than non-sister cells, and as with the pair progenitor cells, most sisters were found to have either identical or mirror-image morphologies (Solomon, 1979). Interestingly, arthropod ganglion mother cells also generate two apparently equivalent cells in vivo, however, the environment can later act on the daughter cells to specify different fates (Kuwada and Goodman, 1985). Hence, while pair progenitor cells generate morphologically similar cells in clonal culture, they may not be committed to doing so in vivo.

Currently, our studies are limited to an in vitro system, and it is possible that the normal germinal environment might alter the progenitor division patterns we see. As yet, no continuous lineage studies have been carried out in vivo. However, there are clues that the lineages we see in vitro may be related to those in vivo. For example, retrovirally labeled cortical clones of neurons generated in vivo are similar in size to minor neuroblast clones we see in vitro (Luskin et al., 1988; Walsh and Cepko, 1988). Examination of retrovirally labeled clones indicate that neuronal clones appear to develop from short symmetric or asymmetric divisions (Kornack and Rakic, 1995; Reid et al., 1995), perhaps similar to the minor neuroblast lineages we see in vitro.

In conclusion, continuous observation of the development of cortical clones has revealed that CNS neuroblasts exhibit patterns within their lineage trees, and indicates that division mode is a defining characteristic of progenitor cell type. These data also suggest that, as in invertebrates, predictable division patterns have a critical role in determining key aspects of CNS development.

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