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Contents

Factors regulating the differentiation of neural precursors in the forebrain

P. F. Bartlett, L. R. Richards, T. J. Kilpatrick, P. T. Talman, K. A. Bailey*, G. J. Brooker, R. Dutton, S. A. Koblar, V. Nurcombe† M. O. Ford†, S. S. Cheema, V. Likiardopoulos, G. Barrett and M. Murphy

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Abstract. Precursors from the neuroepithelium of the developing cortex and the adult subventricular zone can be cloned *in vitro* after stimulation with fibroblast growth factor 2 (FGF-2), and they have the potential to give rise to both neurons and glia. The generation of neurons from these clones can be stimulated by either a factor derived from an astrocyte precursor line, Ast-1, or FGF-1. We have shown that neuronal differentiation stimulated by FGF-1 can be inhibited by diacylglycerol lipase inhibitor and mimicked by arachidonic acid, suggesting that the neuronal differentiation is signalled through the phospholipase Cγ pathway. The sequential expression of FGF-2, followed by FGF within the developing forebrain neuroepithelium, fits with the different functions that the two FGFs play in precursor regulation. We have shown that the precursor response to FGF-1 is regulated by a heparan sulphate proteoglycan expressed within the developing neuroepithelium. Precursors restricted to the astrocyte cell lineage can be stimulated by epidermal growth factor or FGF-2; however, the differentiation into glial fibrillary acidic protein-positive astrocytes appears to require a cytokine acting through the leukaemia inhibitory factor-β receptor.

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The cerebral cortex is the product of precursor cells contained within the neuroepithelium of the dorsal aspect of the telencephalon. The precursor cells proliferate rapidly and generate the first neurons which migrate to the pial surface to form the preplate, which later becomes the subplate. The preplate neurons have recently assumed increased importance because they may retain the positional information contained within their neuroepithelial precursors that is required for targeting the appropriate thalamic input (Ghosh et al 1990). In contrast to these early neurons, some of the later neuronal progeny of the neuroepithelium (also called the ventricular zone), which migrate primarily in association with radial glia (Rakic 1978) and penetrate the subplate to form the

cortical plate, have been shown to be geographically unaligned with their parent precursor, and they probably lack positional information (for review see O'Leary et al 1994). Once the neuronal populations have been generated, the majority of cell proliferation occurs within the subventricular zone, the progeny of which appear to be predominantly glial cells.

In order to understand the foundation for the structural and cellular development of the cerebral cortex, we need to define the genetic and epigenetic events that regulate neurogenesis and gliogenesis, and characterize the lineage potential of precursors within the germinal zone. Previous studies using retroviral markers have suggested that the majority of precursors within the developing cerebral cortex are restricted to either the glial cell or the neuronal lineage (for review see Luskin 1994). This interpretation, however, is hampered by an inability to label cell precursors at the onset of neurogenesis. This is due to inadequate phenotyping of all the progeny of a single precursor (clone) and not being able to monitor progeny of a single clone over an extended period of time. Our studies have centred on sampling the precursor population at various times during neurogenesis. We have been able to examine the lineage repertoire of individual precursors, and we have gained some insight into the epigenetic factors that influence lineage determination of precursor cells by using defined growth conditions and clonal analysis *in vitro*.

Lineage potential of forebrain precursors

Cloning of precursors from the earliest neuroepithelium

In the mouse, embryonic day (E) 9–E10, is the earliest stage at which one can successfully isolate neuroepithelial cells from the developing telencephalon. At this stage less than 1% of the cells have neuronal markers (Drago et al 1991). Previous studies have shown that proliferation of neuroepithelial cells isolated at this stage can be stimulated by fibroblast growth factor 2 (FGF-2) (Murphy et al 1990), a factor that is expressed by the neuroepithelium during this developmental period (Drago et al 1991, Nurcombe et al 1993). This factor was, therefore, chosen to stimulate precursors plated out at one cell per well. Clones arose at the frequency of about one in 20 cells plated (Kilpatrick & Bartlett 1993) and they consisted of flat epithelioid cells. The clones continued to proliferate over two to three weeks, but contained few if any neurofilament-positive neurons. However, after 10 days many of the clones contained significant numbers of astrocytes, i.e. were glial fibrillary acidic protein (GFAP)-positive. The observation that many of the clones contained a low number of neurons as well as glia confirmed our previous studies using immortalized cell lines from E10, which suggested that some of the precursors were in fact multipotential (Bartlett et al 1988). However, the major finding was that the majority of clonal progeny failed to differentiate, an observation confirmed by the high recloning

rate (up to 85%) of the clonal progeny (Kilpatrick & Bartlett 1993). At this time we had identified Ast-1 as a factor, present in medium conditioned by an astrocyte precursor cell line, that stimulated neuron differentiation in E10 neuroepithelium (Kilpatrick et al 1993). We were able to induce significant neuronal production within one to two days in a significant proportion of clones by application of this conditioned medium (Kilpatrick & Bartlett 1993), providing the first evidence that a discrete second signal was required for neuronal differentiation. Recent evidence suggests that Ast-1 is a transforming growth factor β-like molecule (M. Murphy, unpublished work 1994); however, its precise nature and expression pattern *in vivo* is not yet known. It now appears that a more potent signal for initiating neuronal differentiation is likely to be FGF-1 (see below).

Evidence for early commitment of neuronal precursors within the developing neuroepithelium

Although *in vitro* clonal analysis indicates that many precursors with significant proliferative potential are also bipotential, several studies using retroviral markers have indicated that some cells have restricted lineage capabilities. Recently, we have identified a cell surface marker that is associated with commitment of precursor cells to the neuronal lineage and we have demonstrated that as early as E10, many of the neuroepithelial cells are committed to the neuronal lineage. Unlike glia and virtually all other cell types in the body, neurons have an inability to express major histocompatibility complex (MHC) class I molecules on their surface in response to γ-interferon (Bartlett et al 1989). We, therefore, decided to examine the stage at which this unresponsiveness occurs. At E9, all the neuroepithelial cells can express MHC molecules, but as development of the cortex proceeds, an increasing percentage of the precursor cells lose their ability to be induced (Fig. 1). We have selected the non-inducible cells by cell sorting and we have demonstrated that when they are replated under a variety of conditions, they have the potential to give rise exclusively to neurons (Bailey et al 1994). Thus, it appears as if these precursors are committed to the neuronal lineage. Although the neuronal precursors have the ability to proliferate, this is more restricted than the uncommitted precursors. This may explain the limited size of neuron-only clones labelled by retroviral markers *in situ* in the forebrain (Walsh & Cepko 1992).

Evidence for lineage-committed precursors later in cortical development

To examine the potential of precursors later in development, we have cloned cells from the forebrain at E17, and we have found that FGF-2 stimulates three types of precursors: one that is restricted to producing astrocytes; one that

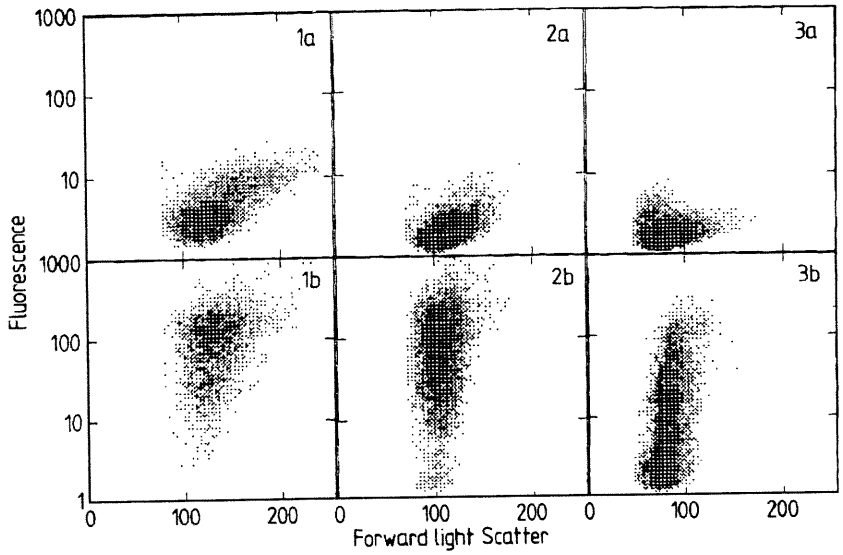


FIG. 1. Neuroepithelial cells taken from the telencephalon at embryonic day (E) 10 (1) E12 (2) and E14 (3) were incubated with γ-interferon for 24 h before being stained by immunofluorescence for major histocompatibility complex class I expression with anti-H-2^k antibodies (b) or with isotype-matched control serum (a). The flow cytometry profiles show an increasing percentage of cells which fail to express the class I antigen as development proceeds. The fluorescence axis is in arbitary units and forward light scatter is a measure of cell size (arbitrary units).

produces predominantly neurons; and one that is similar to the E10 precursors that generate both astrocytes and neurons (Kilpatrick & Bartlett 1995). In contrast to the E10 clones, the differentiation of these clones does not require an exogenous second signal. This indicates that these precursors have already received the appropriate signals *in situ*, and in the case of the multipotential clone, it may reflect the presence of factors produced by other cells within the clone, such as astrocytes. This result confirms the earlier studies with retroviral tracers, but it also indicates that numerous precursors cells capable of generating neurons are present towards the end of neuronal production in the mouse cortex. It was also found that the glial-committed precursor in the forebrain at E17 could be stimulated selectively by epidermal growth factor (EGF) (Kilpatrick & Bartlett 1995).

Presence of multipotential precursors in the adult mouse forebrain

We and another group have described the presence of neuronal precursors within the adult brain (Richards et al 1992, Reynolds & Weiss 1992). We have now localized these precursors to the subventricular zone of the lateral

ventricle, the remnant of the germinative layer that gives rise to the cells of the cerebral cortex. Using the same procedures as for the E10 cells, we have generated epithelioid clones from cells of the subventricular zone by stimulating them with FGF-2 (Fig. 2a). Like the E10 clones, the subventricular zone clones generated with FGF-2 contain GFAP-positive cells but not neurofilament-positive cells (i.e. neurons). Unlike E10 precursors, however, about 50–60% of the subventricular zone clones, when stimulated with FGF-1, contained more than 50 neurons (Fig. 2b) that stained positively for the neuronal marker microtubule-associated protein 2 (MAP-2).

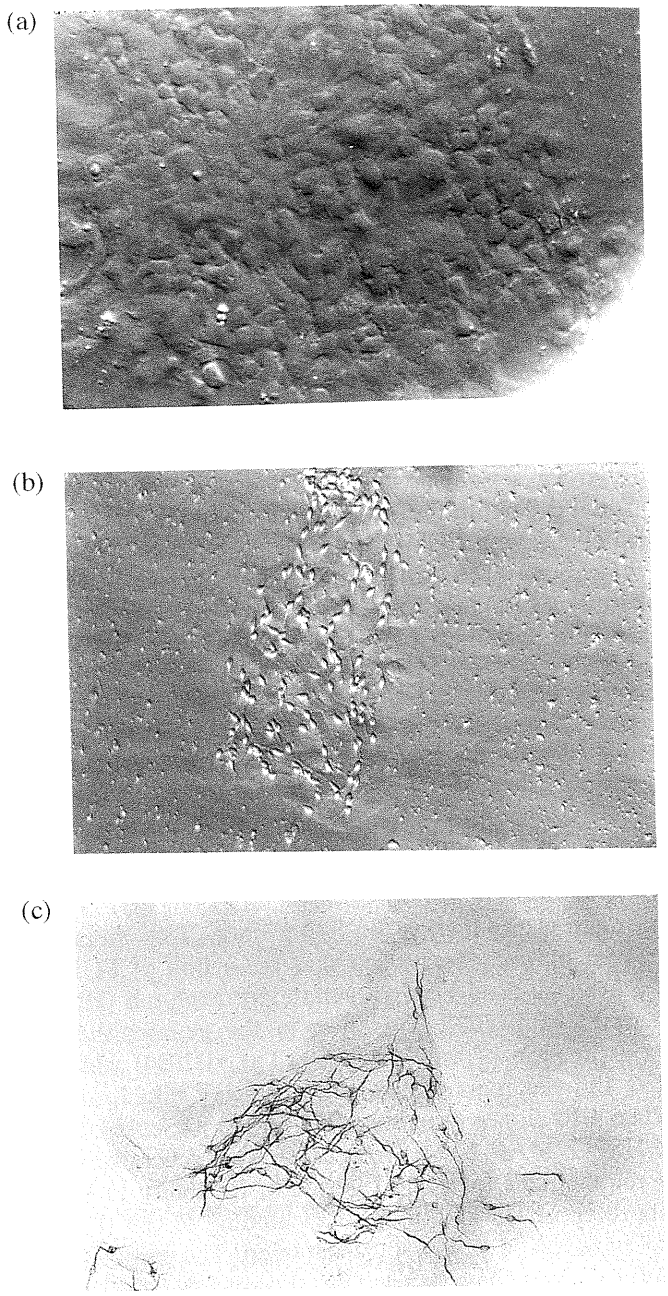
FGF-1 stimulates neuronal differentiation

FGF-1 and FGF-2 stimulate the same precursor

The observation that FGF-1 stimulated subventricular zone clones to produce neurons, whereas FGF-2-stimulated clones contained only glia, can be explained in two ways: either they stimulated different precursors; or FGF-1 induced neuronal differentiation in addition to proliferation. To test this, we first generated clones with FGF-2 and after five days exposed them to FGF-1. We found that about 55% of the switched clones produced neurons of an identical phenotype to that observed in clones generated with FGF-1, indicating that the precursor population stimulated with both factors was probably identical.

Requirement for heparan sulphate proteoglycan for the action of FGF-1

The concept that FGF-1 is the primary stimulator of neuronal differentiation was supported by our previous observations that FGF-1 expression in the neuroepithelium of the telencephalon coincided with the time neurogenesis commenced, i.e. at about E11 (Nurcombe et al 1993). FGF-1, however, failed to stimulate neuron production in E10 clones. This discrepancy may be explained in part by our observation that the action of FGF-1 required the presence of a brain-derived heparan sulfate proteoglycan (HSPG). We found that at the onset of FGF-1 expression in the neuroepithelium, there was concomitant expression of an HSPG (Ford et al 1994) which preferentially bound FGF-1 and was required for neuroepithelial stimulation (Nurcombe et al 1993). When E10 clones were generated in the presence of FGF-1 and the appropriate HSPG, MAP-2-positive neurons were generated in about 60% of clones. The requirement for HSPG is due to its ability to bind and present FGF-1 to its receptor. In addition, direct binding of the HSPG to the FGF receptor may be involved in signal transduction. The corollary of this result is that the adult precursors must express the appropriate HSPG on its cell surface constitutively. Although we have not shown this definitively, neuronal



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differentiation of adult precursors can be prevented by the addition of heparin, which is another FGF-1-presenting molecule. This demonstrates that it is the context in which FGF-1 is presented to its receptor that determines the signalling pathway.

FGF-1 stimulates neuronal differentiation through a second messenger pathway distinct from the proliferative pathway

The binding of FGF to its receptor (of which there are four well characterized types) leads to receptor dimerization and *trans*-autophosphorylation on certain tyrosine residues. This in turn creates binding sites for molecules with SH2 domains, including Src and phospholipase Cγ (PLCγ), and activates several signalling cascades which may be independent or synergistic (Schlessinger & Ullrich 1992). The proliferative signal initiated by FGF is not dependent on PLCγ activation (Peters et al 1992), but recent evidence suggests that activation of this molecule is required for neuronal functions, such as neurite outgrowth (Williams et al 1994). We have investigated whether this pathway (from PLCγ activation to diacylglycerol (DAG) production, the conversion of DAG to arachidonic acid by DAG lipase then finally to Ca^{2+} influx) was involved in stimulating neuronal differentiation by examining the effect of two components of this pathway: DAG lipase inhibitor and arachidonic acid. The addition of DAG lipase inhibitor in the dose range of 5–10 μg/ml to FGF-1-stimulated clones resulted in total inhibition of neuron formation, whereas the total number of clones was not substantially reduced (Fig. 3). This supports the notion that the neuronal differentiation pathway required the production of arachidonic acid. In addition, up to 90% of the clones which could be generated with arachidonic acid at 100 μg/ml were of the neuronal type, again supporting the idea that signalling through this pathway promotes predominantly neuron differentiation. The addition of arachidonic acid to FGF-2-stimulated clones also induced neuronal differentiation similar to that induced with FGF-1.

EGF stimulates non-neuronal precursors

As mentioned previously, EGF-stimulated precursors appeared to give rise only to clones containing astrocytes. This was also true for the adult precursors. In order to examine whether this reflected a different precursor

FIG. 2. Photomicrographs of clones generated from the adult subventricular zone stimulated with fibroblast growth factor 2 (FGF-2) (a) or FGF-1 (b). Note the phase bright neurons on top of the monolayers (b) which stain positively for microtubule-associated protein 2 expression using immunoperoxidase staining (c).

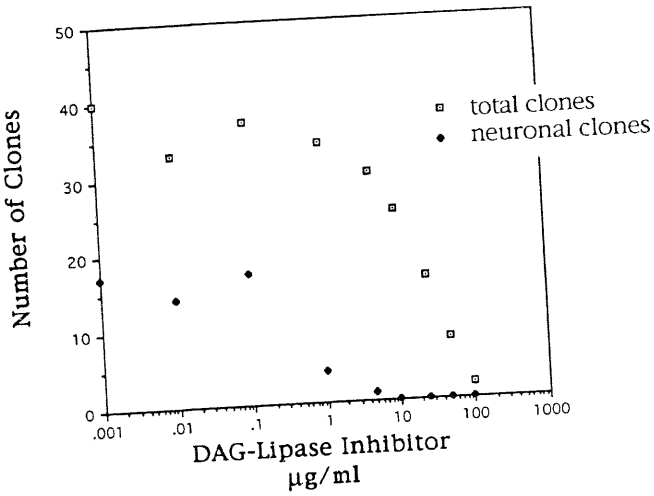

FIG. 3. Effect of diaclyglycerol (DAG) lipase inhibitor on the number of clones generated in the presence of fibroblast growth factor 1.

population or merely a failure to produce neurons, we added both arachidonic acid and FGF-1 to these cultures. However, none of the clones produced neurons. Clones generated with EGF under serum-free conditions are called neurospheres. These also failed to generate neurons under these conditions, which was in contrast to results reported previously by Reynolds & Weiss (1992). A possible explanation for this is that the previous study was not carried out at the clonal level. EGF supports the survival of mature neurons *in vitro* suggesting that these surviving neurons have been incorporated into the neurospheres. It is also possible that the conditions we have used are not conducive to the neuronal differentiation of EGF-stimulated precursors.

Astrocyte differentiation requires a factor acting through the leukaemic inhibitory factor β receptor

The above studies suggest that the production of astrocytes from their precursors does not require discrete epigenetic signalling apart from that required to stimulate cell division. Another explanation, however, could be that astrocyte production, as measured by GFAP expression, which does not occur until between seven and eight days after plating in an E10 clone (previous studies by Abney et al [1981] have shown the temporal expression of GFAP *in vitro* matches that *in vivo*), is due to the production of endogenous factors within the clone. Recent experiments tend to support this latter contention because E10 neuroepithelial cells cultured *in vitro* can be prevented from differentiating into astrocytes by antibodies which bind to, and block ligand binding to, the

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leukaemic inhibitory factor β (LIF-β) receptor (L. R. Richards, M. Murphy & P. F. Bartlett, unpublished results 1994). In addition, mice bearing a mutation in this receptor appear to have an astrocyte deficiency (Ware et al 1995) and we have recently shown that E10 neuroepithelial cells obtained from these mice fail to differentiate into astrocytes when plated *in vitro* (S. Koblar, C. B. Ware, R. Dutton, M. Murphy & P. F. Bartlett, unpublished results 1994). LIF and ciliary neurotrophic factor (CNTF), which both act through this receptor, have been shown to influence astrocyte production *in vitro*. However, mice with mutations in either of the genes coding for these ligands appear to have normal astrocyte production. Thus, other members of the family, like oncostatin M or an as yet unidentified ligand, may be responsible for regulating astrocyte differentiation because recent evidence suggests another CNTF-like molecule may be present during embryogenesis (G. Yancopoulos, personal communication).

Conclusions

The results of *in vitro* clonal analysis have demonstrated that the precursor cells which give rise to the cerebral cortex require a number of growth factors to regulate their proliferation and differentiation. It now appears that FGF-1, in association with a specific HSPG, stimulates neuronal differentiation through the PLCγ pathway, and a molecule acting through the LIF-β receptor regulates glial differentiation. A multipotential precursor persists throughout development, presumably by self renewal, and is also present in the adult subventricular zone, in addition to precursors that are committed to the neuronal or glial pathway. EGF is involved predominantly in stimulating precursors that are committed to the glial pathway. It should now be possible to examine the mechanisms regulating precursor differentiation into the different subtypes of neurons found in the cortex by clonal analysis and the use of defined growth factors. Ultimately, it should also facilitate an understanding of how epigenetic factors and genetic factors interact to regulate early cortical development.

Acknowledgements

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DISCUSSION

Walsh: Do the leukaemic inhibitory factor (LIF) knockout mice have astrocytes that either do not have glial fibrillary acidic protein (GFAP) or do not have astrocytes at all?

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Bartlett: That's an interesting point, which depends on your definition of an astrocyte. Unfortunately, LIF receptor knockout mice die at birth, as do the ciliary neurotrophic factor (CNTF) receptor and the gp130 knockout mice, so it is impossible to answer the question unequivocally. As far as we can tell, however, newborn mice do not contain GFAP-positive cells in the brain. But, the first GFAP-positive cell does not appear until embryonic day (E) 17, so development may be delayed rather than abrogated. We haven't looked at other markers. The number of neurons looks normal, although some degeneration of the neuronal population is apparent.

Ghosh: Is there a LIF knockout mouse that does not die at birth?

Bartlett: The LIF knockout mice do not die at birth, nor do the CNTF knockout mice. We have some evidence (S. S. Cheema & P. Bartlett, unpublished observations) that LIF knockout mice also have a reduction in the number of GFAP-positive cells (about 30–40%). If one looked at histological sections stained with anti-GFAP antibody, one wouldn't pick it up unless one did a detailed cell count. What we're looking at with the LIF-β receptor mutant is the cumulative effect of the loss of LIF, CNTF, oncostatin M and possibly another CNTF-like ligand (G. Yancopoulis, personal communication).

J. Price: Why don't they generate oligodendrocytes?

Bartlett: It is possible that stimulation with fibroblast growth factor (FGF) causes clones to remain in a more primitive state. Alternatively, it may do the opposite, as Jack Price suggested in the discussion following his presentation (Price et al 1995, this volume), i.e. it may promote the proliferation of an astrocyte neuronal precursor which looks like, but is different to, an epithelial cell. We should use other stimulants, like platelet-derived growth factor (PDGF), to attempt to stimulate oligodendrocyte production.

J. Price: Have you tried stimulating with just serum?

Bartlett: It doesn't work with single cells.

Parnavelas: What is the age of the embryos that these cells are taken from?

Bartlett: E10.

Parnavelas: Is it possible that oligodendrocyte precursors are not present at that stage?

Bartlett: We showed many years ago (Abney et al 1981) that oligodendrocytes can be generated *in vitro* from single cell suspensions of rat brains taken from as early as E10.

Parnavelas: We have looked at over 300 neuronal and glial clones in adult rats following retroviral injections in the cerebral ventricles of embryos between E14 and E20 (Parnavelas et al 1995, this volume). We found few oligodendrocyte clones in those samples. In contrast, when we injected retroviruses in the subventricular zone in postnatal rats, we found many oligodendrocyte clones throughout the cortical white and grey matter.

Bartlett: Another step, possibly another environmental signal, seems to be required for the generation of oligodendrocytes. If we looked at clones

generated from E12 or E14, we may see oligodendrocytes but they may be derived from the subventricular zone.

LaMantia: Factors are present at this age that determine whether these cells become neurons or not. Cortical neurons are not generated until four or five days after this, so are factors present in the cortical rudiment's neuroepithelium that keep the cells in a precursor state?

Bartlett: Yes, and it is possible that FGF-2 is that factor. We have to remove the stimulus of FGF-2 in order for differentiation to occur in the proliferating neuroepithelial cells. One way of initiating differentiation of precursor cells is for them to move away from the ventricular zone—the area that has the highest levels of heparan sulfate proteoglycan (HSPG) and FGF-2. We understand how the presentation of both those molecules is important in the right context, but we don't understand how the cell actually gets fed one or the other.

LaMantia: FGF-3 is expressed dorsally in the telencephalon at E10. Where are FGF-1 and FGF-2 expressed?

Bartlett: FGF-2 is expressed throughout the neuroepithelium of the telencephalon at E10. FGF-1 is not expressed until E11 or E12, but again it is fairly widespread throughout the neuroepithelium.

Rubenstein: There is evidence that in the forebrain, oligodendocytes may not come from a cortical source but may migrate to the subventricular zone later on (Timsit et al 1995). Is it possible that you don't observe oligodendrocytes when you make your dissections at E10 or E12 because they simply aren't there at that stage?

Bartlett: This is an interesting proposition, although recent results using cortical tissue from E12 rats (Davis & Temple 1994) suggest that clones can generate all three principal cell types (neurons, astrocytes and oligodendrocytes), so it is unlikely that this is the explanation. These experiments were done under serum-free conditions with conditioned medium from neuronal cells. It is possible that stimulation with FGF is the primary reason why we're not generating oligodendrocytes.

Innocenti: What is left of the old hypothesis that microglia and neuroglia share the same origin (reviewed in Fujita et al 1981)?

Bartlett: There is no evidence to suggest that neuroepithelial cells have the potential to become microglial cells. All the studies to date, using cell-surface markers to follow microglial precursor cells, show that they migrate in.

J. Price: I agree only with the proviso that microglial precursor cells migrate early. For example, if you culture the E16 ventricular zone, microglial cells appear in the cultures because they have already moved into the brain by that stage.

Bartlett: Progenitors of microglia are circulating in the blood at E11–E12, at a time when the thymus is also being invaded by dendritic precursors. Presumably, this is the time when they invade the brain as well. Their presence

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at the commencement of neurogenesis has made interpretations problematic. There is no evidence of microglia in cultures obtained from E10 neuroepithelium.

Innocenti: However, the possibility that at least the ramified form of microglia is of neuroectodermal origin has only recently been advocated and discussed (reviewed by Ling & Wong 1993). I was curious why the people who described the clones of cortical cells did not mention whether or not they saw microglia. Perhaps this would imply that microglia were not of neuroectodermal origin.

Levitt: This is only a minor controversy. There is clearly a population that does migrate, but that doesn't mean to say that there isn't a population that's intrinsic to the neuroepithelium. Almost all the evidence suggests that microglia are formed from monocytes migrating from the periphery (Ling & Wong 1993). Even after injury in embryogenesis or early in development, the cell that responds is the peripheral monocyte and not the intrinsic microglia (Milligan et al 1991).

Is your neuroepithelium generating a glial cell type and a neuronal cell type at the same time?

Bartlett: Yes, a clone can generate both cell types.

Levitt: But this does not mean that *in vivo* the epithelium generates both cell types at the same time as the wall expands. We showed that there are large numbers of radial glial cells at the beginning of neurogenesis in the monkey, although I don't know if this is true for the rat (Levitt & Rakic 1980, Levitt et al 1983).

LaMantia: There are not many radial glial cells present at the stage that Perry Bartlett is looking at (E10 in the mouse). Instead, the columnar cells may just be epithelial cells.

Levitt: We tend to use conventional markers to decide whether a cell is present. If stained cells are observed in a species in which the conventional marker works early, then one can be confident that they are present. However, if stained cells are not observed, they may still be present. The important question is perhaps, if one assumes an early coexistence of neurons and glia, what is behind the simultaneous expansion of the glial cell population and the neuronal population?

Bartlett: The generation of neurons and astrocytes are temporally different both *in vitro* and *in vivo*. Neurons are generated virtually within a day of plating E10 neuroepithelium, whereas the GFAP-positive population is not apparent until about one week after plating (equivalent to E17, the time at which GFAP first appears in the mouse). The clones show a similar temporal difference.

Levitt: Are the glial cells making intermediate filaments?

Bartlett: Yes. GFAP is present, but not until the clone is at least a week old. One question I would like to raise concerns the origin of radial glial cells. How

do we identify radial glial cells *in vitro* and what is their relationship to a stem cell? Leber et al (1990) suggest that radial glial cells and stem cells may be the same cell. The studies on radial glial cells in the chick also support that interpretation (Alvarez-Buylla et al 1990), but I would like to hear Pasko Rakic comment on what is known about the generation of radial glial cells.

Rakic: It is probably a question of the timing of cellular events, which is different in different species. For example, there is an early commitment to glial and neuronal lineages in the monkey and human cerebrum. In both species glial cells express vimentin and GFAP early. In rats the same type of cells may be present but they don't express GFAP. People using GFAP came to the conclusion that radial glial cells appear in the rat only after birth. However, these cells are actually there before birth. Therefore, these markers are not good enough to detect cell differences.

Bartlett: Do we have any good radial glial markers? It seems to me that most of the radial glial markers also mark neuroepithelial cells.

Rakic: Then they are not good markers!

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General discussion II

Teleology for tangential migration

Daw: I would like to bring up the subject of the teleology for tangential migration, in terms of the final organization of the cortex. Pasko Rakic (1972) proposed that the teleology for radial migration is related to the columnar system. One could propose, for example, that tangential migration in the proliferative zone might be related to areas that are topographically connected, and that the migration which takes place later on within the cortical plate might be related to columns that have similar properties. Alternatively, one could propose that tangential migration is simply Brownian motion, and that it doesn't matter where the neurons end up tangentially, in terms of the final organization of the cortex.

Walsh: There are two different ways of looking at this. One is an argument similar to the proposal by Dennis O'Leary (1989) that it may be evolutionarily advantageous to have a cortex which is specified primarily by its afferents, as opposed to being specified by intrinsic patterns of cell division. The advantages for that may be that a simple mutation in a homeobox gene may, for example, change the limb of an animal from a paw to a fin. The brain will, therefore, have to deal with different peripheral inputs over a relatively short number of generations. Consequently, the greater the extent to which the functional organization of the cortex is regulated by its afferents, the more flexibility the cortex has to adapt to a changed periphery.

This is simply an argument for having a relatively uninformed cortex that is specified by its afferents and that will allow the cortex and the periphery to evolve independently. The cortex, for example, could enlarge by simply adding more cells, but it could still maintain its functional organization with the periphery because that functional organization can be dictated and inscribed by the peripheral afferents to the cortex.

There's a second evolutionary argument for why tangential dispersion may be selected. Somatic mutation must be a common event during the development of an organism. DNA polymerases have an error rate of at least one in every 10^9 bases and the size of the eukaryotic genome is about 10^9 bp. This means that every mitotic division may involve mutation. Bernards & Gusella (1994) suggested that there are cells in everyone's body that carry mutations for any given gene. If a somatic mutation occurred in a neural gene in a dividing cell, the mutation would be inherited in its progeny. In the absence of tangential migration, the resulting clone would then produce a focus of cells

that had a defective neural function. On the other hand, if you just sprinkled the abnormal cells over the cortex they might have less functional impact.

Bonhoeffer: But there is no advantage for cells to follow a pattern rather than being dispersed throughout the cortex by Brownian motion.

Walsh: The idea of cells following a pattern may not mean a plan to disperse cells, but may mean that cells have to wait a certain amount of time before they can divide again. The cells may actively disperse from each other or it may just be difficult to keep them together in an epithelial sheet.

Bonhoeffer: But then you're back to teleology.

O'Leary: If one assumes that the progenitors start off at different points in the epithelium, then a scattered distribution of cells will be generated, although within a given clone there will be periodicity.

J. Price: It is a mistake to assume that the scattered dispersion of cells contradicts the idea of a proto-map, even though this is probably not what Pasko Rakic had in mind when he proposed that model (Rakic 1988). A certain degree of dispersion can be accommodated within the idea of areal specification in the ventricular zone. For example, if just 10% of the cells, which would have been missed in everyone's analyses, stayed put in an absolute radial fashion, they could provide the scaffold for a proto-map.

Walsh: Microenvironmental differences may occur throughout the cortex and would also not be inconsistent with tangential clonal dispersion.

LaMantia: Also, cells that are specified and that migrate can still carry their positional information with them. An example of this is the migration of neural crest cells.

Blakemore: According to your evidence, progenitor cells are multipotential while they're migrating, so it is difficult to imagine how they could constitute a stable, committed proto-map at that stage. Only the static progenitors that give rise to local clusters appear to be more or less committed in the type of cell that they generate. Therefore, if there is a proto-map, it must surely be represented by the immobile progenitors that give rise to local clusters, which may lie in the subventricular zone. It is possible that the microenvironment in that layer determines their commitment.

J. Price: Do you mean commitment to cell type?

Blakemore: Yes.

J. Price: Why is this relevant?

Blakemore: Because some of the areal differences in the neocortex are defined as differences in the proportions and laminar distributions of different cell types.

J. Price: The process whereby ventricular zone cells acquire positional identity and that by which cells become allocated to a particular type could be totally orthogonal to one another.

Molnár: We're talking about the cortex as though it was a completely liquid tissue during development, but there are planar sheets in the developing cortex

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with a constant topography; for example, the bottom of layer VI and the subplate. These are stable sheets with topographies that are defined from embryonic day (E) 15–16 in the rodent. The deployment of thalamocortical projections is complete by this stage, and there is an initial matching between the different thalamic nuclei within the sheet that establishes a relatively constant map at the peak of the cell generation. Consequently, even if there is a widespread dispersion of the cortical plate cells, there is a constant stable sheet onto which the afferents can be deployed, lined up and sorted out.

Bolz: This is a chicken and egg situation because the stable sheet needs to recognize something to organize itself.

Blakemore: Zoltán Molnár, are you suggesting that the stable sheets formed by the lowest layers of the cortex can influence the migration of subsequent generations of neurons?

Molnár: I am suggesting that it is possible. Different growth factors or surface molecules may be involved. The regulation of cell divisions in the ventricular and subventricular zones by the ingrowing thalamic axons is an uncharted territory. One can only speculate about the possible ways of regulation. Voltage-sensitive dyes (Crair et al 1993, Higashi et al 1993) or current source–density analysis techniques (K. Toyama, T. Kurotani, N. Yamamoto & Z. Molnár, unpublished results 1993) can be used to show that the activation patterns elicited by thalamic stimulation do not reach the subventricular zone. It is possible that the signals are too small to be detected, even if one had more sensitive techniques. We still have to find the way that thalamic fibres exert their influence, if any, on the dividing cells in the ventricular zone. Radial glia would be an ideal substrate to transmit information from thalamic afferents down to the mitotic factory in the ventricular zone.

Krubitzer: It is possible that the events that we observe during development are not relevant to the final function of the organism, and that some of these events represent ways of moving around obstacles, which are laid down early in evolution and may constrain or channel future development, rather than being necessary steps that ultimately generate a particular function.

Blakemore: If the pattern of lateral migration were similar in absolute dimensions in the monkey and in the mouse, then the consequences would be different. In the mouse, clones can be distributed over virtually the whole of the neocortex, contributing to somatosensory, motor, olfactory and visual systems. Similar lateral dispersion, in absolute distance, in the monkey would only spread the clone across a small fraction of a single cortical field.

Kennedy: Yes, but it takes 10 times longer in the monkey to do the same thing. If you had the same migration rates, you would end up with the same relative degree of scatter.

Rakic: Menezes & Luskin (1994) have shown recently that some cells migrate from the proliferative subventricular zone of the telencephalon to the olfactory

bulb. Apparently, these cells continue to divide as they move perpendicularly to the radial glial fibres. However, just because they migrate laterally does not mean that they are not committed. In fact, they are immunoreactive to cell class-specific antibodies and their movement is restricted. Therefore, lateral migration in the telencephalic wall may actually signify cell commitment rather than lack of it. If a similar population of cells exist in primates, they would have to migrate at least 10 times further than those in the mouse. Likewise, if some cells in the monkey, as suggested by Chris Walsh for the mouse, migrate from the temporal or parietal lobes to the frontal lobe, the total length of their journey in the middle of gestation must be measured in centimetres rather than in millimetres. The cell size in both species is approximately the same, so this would pose a considerable transportation problem in a large and partially convoluted cerebrum. The problem of distance and pathfinding in humans would be even greater. If, however, in primates this long distance allocation of postmitotic cells does not exist, this would signify to me that the dispersed cells are irrelevant for the specification of neocortex. They may represent a class of neurons that subserve the same function in all areas or brain structures, unlike neuronal classes which migrate strictly radially and may carry gradient molecules or other signals important for regional specificity.

Kennedy: The distance in the monkey is 10 times greater than the mouse. That's fine because the time period is 10 times longer—60 days for the monkey and six days for the rat. If they are migrating at the same rate in both species, they would migrate the same equivalent distance in terms of cortical space.

Rakic: But cells don't migrate for 60 days in the monkey.

Bonhoeffer: What do we actually mean by the term committed? Do we mean sensory as opposed to motor neurons, visual as opposed to auditory neurons or neurons in area 17 as opposed to area 18?

Blakemore: That's an important point. Some of the most important features of the differentiation of an area probably involve the connections and relationships between neurons, established after they're in position. The neurons themselves may not differ very much in their genetic expression. For example, a layer V pyramidal cell in area 18 may not be very different initially from one in area 3.

Bonhoeffer: There is also evidence that some sensory areas can be turned into other ones (Sur et al 1988). Consequently, how stringent should we be with a definition of commitment?

Blakemore: It seems clear that the kind of commitment we've been talking about—commitment of a progenitor to produce a certain type of glial cell or pyramidal neuron, rather than non-pyramidal, is only the first step in establishing the ultimate fate of cells. An entirely different form of commitment might determine many aspects of the internal connectivity of distinct areas, and hence their cytoarchitecture and their computational function.

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J. Price: It's possible that the periodicity of dispersion is not that important. We studied pyramidal neurons in the pyramidal areas of the hippocampus (Grove et al 1992). The clones here were regularly spaced, 150 μm on average, so we were able to trace them with regard to the hippocampal fields. We found that they don't respect the boundaries between fields, but march straight through the presubiculum, the subiculum, CA1, CA2 and CA3.

Bonhoeffer: What about the dentate gyrus?

J. Price: Our evidence suggested that clones included CA4 neurons and dentate gyrus neurons, but we obtained few examples of these, so I can't be completely certain. I can't explain why we see equal spacing in the hippocampus or the neocortex, but I doubt that this has any functional consequence.

Daw: Could the periodicity have anything to do with the possibility that there are integral velocities of migration?

Walsh: That did not occur to me. I have no results that it has or that it hasn't.

Rubenstein: Do you think that the timing of the cell cycle is involved?

Walsh: The timing of the cell cycle has to be important because the cells have to wait a certain amount of time before they can divide again. But Nigel Daw's question was whether some cells migrate twice as fast as others per cell cycle and I don't know the answer to that.

Parnavelas: In what percentage of your clones do you see periodicity?

Walsh: In all the clones that we analysed, fewer that 1% of sibling cells are spaced 1–1.5 mm apart (Reid et al 1995). That is most remarkable because there are large numbers of siblings that are either spaced 0–0.5 mm or 2–3 mm apart, but there is a striking lack of sibling cells spaced 1–1.5 mm apart.

Rakic: The developing cerebellum may be an instructive model system for understanding the relationship between cell lineages and connectivity in other areas. In the cerebellar rhombic lip, which is a small mitotically active centre situated on each side of the rhombencephalon, cells proliferate and then migrate in two directions: one population leads to the pons, and the other forms the external granular layer of the cerebellar hemispheres. The pontine cohort eventually generates neurons of the pontine grey nucleus, whereas the external granular layer generates granule cells of the cerebellar cortex. The interesting point is that the cells of the pons later form long axons that enter the cerebellar hemisphere and find their way to the cortex where they contact the granule cells. Thus, the clonally related cells that originate from the same progenitor pool, and later have a different history, eventually become connected to each other.

Rubenstein: Gray & Sanes (1991) used retroviral labelling on the tectum of the chick. They showed that radial migration is not random—the cells migrate to the subventricular layer, then some migrate longitudinally and others migrate radially.

LaMantia: That's a very specific cell class which takes a specific migratory route.

Rubenstein: It is possible that the cortex is so complicated that some of the tangential movement we're observing may not be random but may be part of a directed migration.

Boncinelli: Cells may move freely within compartments, but may get trapped at their boundaries. Therefore, it is possible that migration does fit within the proto-map hypothesis.

Walsh: The existence of compartments would have been tested for by analysing the distances between non-sibling cells. If compartments were present, there may be places in the cortex where cells divide preferentially. Such 'hot-spots' of cell division would be an alternative explanation for the preferential spacing between sibling cells because a cell divides in one hot-spot, then goes to another at a set distance, and so on. However, if there were compartments or hot-spots for cell division, then there would be a similar periodic spacing between sibling cells and between non-sibling cells. We did not see this periodic spacing between non-sibling cells (Reid et al 1995) which argues against the existence of compartments, although we can't rule it out entirely.

Blakemore: Also, if there are specific zones where migratory progenitors tend to divide hence where non-migrating progenitors accumulate, one would expect the cortex to be thicker above those zones, but in fact it is fairly uniform in thickness.

LaMantia: Chris Walsh, have you looked at the intermediate stages of cortical neuroblast migration to see whether there are cells that migrate radially then disperse tangentially? It is possible to use [^{3}H]thymidine labelling to show that cells migrate radially from the ventricular zone at the place where they undergo their final division. If it is the ventricular cells that are migrating tangentially, they could be displaced randomly if they were constrained in the lateral dimension. However, the known migrations that are tangential are also directed in some way (Domesick & Morest 1977, Gray & Sanes 1991).

Walsh: It's difficult to do that experiment because the clones are not coherent, and so it is difficult to infer mechanisms of migration. The retroviral method is not the optimum method for studying migration because you can only infer patterns of migration based on the patterns of dispersion. However, if we inject at E15 and analyse at E21, we observe that about a third of the dispersed clones have dispersed sibling cells within the ventricular zone (Walsh & Cepko 1993). The presence of dispersed cells within the proliferative zones before radial migration occurs is strong evidence that some of the progenitor cells themselves disperse.

Rubenstein: Are you sure they're not in the subventricular zone?

Walsh: The material was not always counter stained so it may be difficult to determine whether they're in the ventricular zone or subventricular zone. However, they were in the proliferative zones.

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About another third of the clones have one cell in the proliferative zone in one place, and one cell in the intermediate zone of the cortex in another place (Walsh & Cepko 1993). How did they separate like this? It may be the same mechanism, but it is possible that one cell left the proliferative zone and migrated non-radially through the intermediate zone.

The last third of the clones contained cells within the cortical plate that are dispersed widely. The retroviral method cannot determine how these cells migrated apart. We can, therefore, be confident that there's dispersion in the proliferative zone, but we can't say what other mechanisms may also be operating.

Bartlett: This argument can be turned around the other way. There are many areas where progenitors are moving in relation to the rostrocaudal axis. We know that boundary formation, even in the hindbrain, is probably not due to clonal restriction, but is due to the regulated expression of certain genes either side of the boundary. Do we really have to explain why dispersion occurs? We're really interested in the other mechanisms, which involve gene regulation, that form these functional boundaries.

LaMantia: There are two studies (Fraser et al 1990, Wingate & Lumsden 1994) that show that there is a restriction of cell movement in the hindbrain which obeys rhombomere boundaries.

Bartlett: That's not entirely true. Fraser et al (1990) are saying that even after the boundaries are formed in the hindbrain, there is still a lot of dispersion across these boundaries.

LaMantia: They claimed that the numbers of cells transgressing rhombomere boundaries was between 5 and 10%.

Rubenstein: That may be similar to what we're seeing here.

Bartlett: The boundaries of expression of the *Hox* genes aren't initially well-defined, they're fuzzy and then they sharpen up probably because of differential gene regulation.

J. Price: David Wilkinson (unpublished observations) also sees this with *Kroz-20* expression. Expression is initially fuzzy, but by the time the rhombomere boundaries are morphologically apparent, the pattern of gene expression has sharpened up.

Boncinelli: Gene expression produces boundaries, and boundaries constrain the movement, so it depends on exactly when you ask the question.

J. Price: Exactly. Dispersion occurs within the rhombomeres, but the boundaries stop dispersion between rhombomeres.

Molnár: I agree that boundaries exist in the developing and adult cerebral cortex. These boundaries may be rigid or modifiable. The boundaries of different areas in the adult cerebral cortex can shift, and there is a spectacular plasticity in the system (Merzenich et al 1983, Kaas 1991). There are signs of movement of these cytoarchitectonic areas well after the completion of cell generation, migration and differentiation.

Differentiation factors in culture

Ghosh: We have taken cells from the rat cortex at E13 or E14, dissociated them and looked at differentiation over time in culture (Ghosh & Greenberg 1995). At the time of plating in serum-free medium, all of the cells are nestin positive. It is difficult to get these single dissociated cells to divide without serum. However, clusters of cells in serum-free medium will continue to divide. When we added fibroblast growth factor 2 (FGF-2) to the serum-free medium, we found that it is a potent mitogen for these cells. However, we also found that differentiated neurons appear in these cultures over time in the presence of FGF-2. This suggests that in the presence of a proliferative signal, signals that regulate neuronal differentiation can continue to function. We were interested in the possibility that endogenous factors made by these cells play a role in mediating their differentiation. In our hands the best candidate for the differentiating factor is neurotrophic factor 3 (NT-3) because when we added anti-NT-3 antiserum to these cells, there was a significant reduction in the number of microtubule-associated protein 2-positive cells in each cluster. This is not a survival effect because the anti-NT-3 antiserum does not have this effect on cultures of postmitotic neurons. Also, there is no reduction in the total cell number in these clusters in the presence of anti-NT-3. NT-3 probably causes the cells to leave the cell cycle because if you add excess NT-3, there is a reduction in the number of bromodeoxyuridine-positive cells. This is consistent with the finding that during development, there's a loss of FGF responsiveness in some cells. It is not known whether NT-3 regulates this step, but NT-3 does seem to be a good candidate for a neuronal differentiation factor.

We have also studied the generation of oligodendrocytes in culture. We have found that neurons are generated only in the first few days in culture. They are not generated after about five or six days. However, there is a pool of cells that continues to divide and undergoes a morphological transformation after about 10 to 12 days in culture. These cells stain with anti-galactocerebroside antibodies suggesting that they have adopted an oligodendrocyte-like fate.

I would like to point out that, although these cell culture experiments are useful for trying to identify differentiation factors, there are probably a lot of factors that influence differentiation. Consequently, the danger is that cell culture experiments will give us an over-simplified view.

Bartlett: I have two points. FGF-2 is expressed at the right time in development, but I'm not sure that NT-3 is expressed at the right time, although something else could be acting through the TrkC receptor. Secondly, there is a vast difference between culturing a single cell and culturing a clump of cells. We had the same result three or four years ago (Murphy et al 1990). Many of the cells become neuronal in the presence of FGF-2. We first thought that the difference between proliferation and differentiation was just a matter

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of FGF dose, but it's not. A secondary message is involved. Transforming growth factor β-like molecules seem to be able to induce neuronal differentiation, but in our hands FGF-1 is far more effective. FGF-1 can induce a robust single-celled clone to make 200–500 neurons. This is not to say that other factors are not involved. These factors may induce the differentiation of the neuronal subtypes and may be responsible for the apparent homogeneity of the progeny of individual precursors. NT-3 may well play a part in the selection of these subtypes.

Ghosh: These experiments have been done in culture, so it's difficult to know whether this is exactly what's happening *in vivo*.

Bartlett: NT-3 might also be involved in neurite outgrowth. What you might be measuring is a secondary differentiation event.

Ghosh: NT-3 has an influence on the cell proliferation, which suggests that it can act on precursors while they are still dividing.

Jones: We looked at the expression of nerve growth factor, brain-derived neurotrophic factor (BDNF), NT-3 and NT4/5 in the frontal cortex of fetal monkeys and rats. We found that their expression was turned on after lamination is fully formed in the cortex and at a time when process formation is probably at its maximum, including the outgrowth of axons and the finding of their targets (Huntley et al 1992). For example, in the monkey, BDNF expression is turned on at about E100. I can't rule out that these, or other members of the neurotrophin family, are not operating earlier, but in terms of what we see *in vivo*, NT-3 is not expressed at the correct time.

Ghosh: But there is published evidence that both NT-3 and the TrkC receptor are expressed in the embryonic cortex (Maissonpierre et al 1990, Tessarollo et al 1993) and, therefore, could play a role in neuronal differentiation. Nevertheless, it is worth considering that an unidentified molecule related to NT-3 may be involved in regulating neurogenesis *in vivo*.

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