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The Brain Invaders: Widespread Neuronal Dispersion in Developing and Adult Mammalian Brain

Hynek Wichterle

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**THE BRAIN INVADERS: WIDESPREAD NEURONAL
DISPERSION IN DEVELOPING AND ADULT MAMMALIAN BRAIN**

Hynek Wichterle

A thesis presented to the faculty of
The Rockefeller University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

New York, 2000

To my grandfather

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I was very fortunate to have Arturo Alvarez-Buylla as a mentor. His passion for science was a constant source of inspiration, optimism and insight during my five-year crusade. Arturo taught me that even the greatest mysteries of nature will yield their secrets if we use the right kind of tools. And if there is no right tool, we just have to design and build it. I appreciate the freedom and moral support, which I received to pursue topics and directions, which I found interesting and exciting. I hope that in my future career I will be able to stand up to the rigorous, creative, and conceptual approach to science which I experienced in Arturo's lab.

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ABBREVIATIONS

| | |
|------|--|
| DiI | 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate |
| GFAP | glial acidic fibrillary protein |
| LGE | lateral ganglionic eminence |
| LV | lateral ventricle |
| MGE | medial ganglionic eminence |
| NSE | neuron specific enolase |
| SVZ | subventricular zone |

ABSTRACT

Cell migration is the principal determinant of the final brain cytoarchitecture. However, we have only limited knowledge about the migratory pathways and mechanisms utilized during brain development. In my thesis I present new and unexpected findings about neuronal migration in the developing forebrain. I analyzed and characterized a novel type of neuronal migration, so called tangential chain migration that was first identified in the subventricular zone (SVZ) in the adult mouse brain. I developed an in vitro migration assay using Matrigel as a substrate, which allowed me to analyze dynamics and mechanism of SVZ cell translocation. I demonstrated that neurons migrating in chains move rapidly along each other in the absence of glia, implying that chain migration is different from previously described radial migration.

Using the in vitro assay I discovered two regions in the embryonic mouse ventral forebrain containing large numbers of tangentially migrating neurons. I studied migratory potential of these embryonic cells after transplantation into the adult brain. I showed that cells from the medial ganglionic eminence (MGE) demonstrate a novel migratory behavior. MGE cells disperse through the adult brain tissue and differentiate into GABAergic neurons. I showed that MGE cells could disperse even in experimentally lesioned adult brain. Thus, the unique migratory behavior of MGE cells might open new clinical approaches to the repair of damaged brain.

Finally, I show that widespread migration of MGE cells reflects their normal behavior in the developing brain. I analyzed migratory potential of MGE cells in vitro and by ultrasound guided transplantation in vivo. These experiments revealed large-scale directional migration of MGE cells into the developing neocortex in embryonic mouse brain. I propose that a ventro-dorsal permissive gradient detected in the developing forebrain might be the principal mechanism guiding MGE cell migration dorsally.

In summary, I revealed a population of embryonic neuronal precursors with unprecedented capacity to migrate long distances both in the developing and adult mammalian brain.

CHAPTER 1: INTRODUCTION

Watching a sped up movie of frog development, I am always amazed at how dynamic the transition from a single round cell to a little tadpole is. Coordinated cell movements transforming the embryo during gastrulation and neurulation are without question the “Academy award winners” of developmental biology.

None of these processes could have been fully appreciated before the advent of time-lapse cinematography. Fast-forwarding through ontogenesis and then rewinding back to the fertilized egg makes me always wonder how all these semi-chaotic and dynamic processes are orchestrated to “reproduce” the complex organism with such a high fidelity - a frog, mouse or human which is in first approximation identical to any other frog, mouse or human.

The morphogenetic processes forming a developing embryo are played out on smaller scale in each individual organ. Cells multiply, mold, migrate, differentiate, and die as they endow each part of our body with a specific shape and function. Detailed knowledge of individual cells’ behaviors with respect to their environment is necessary for better understanding of the structure and function of our organism.

Studying cell migration is a special case in this grand endeavor. Structural and histological organization of tissues, organs and organisms depends on cell movements. The central nervous system, where most if not all cells migrate at some point in their life, is a prime example. The exquisite arrangement of individual brain cells in respect to each other and the complexity of their connections makes this organ unique. The brain function—from simple reflex and conditioned behavior all the way to cognition,

introspection and emotion—is emergent property of the intricate brain structure, which is in turn predetermined by migration of neurons and their axons during development.

Thus, figuring out how is the brain assembled during ontogenesis can be considered an important step on our journey towards understanding how this organ works in the adult.

In my thesis I present new and unexpected findings about neuronal migration in the developing cerebrum, the most anterior region of the brain which is thought to be the site of higher cognitive functions. I analyze and characterize a novel type of neuronal migration that was first identified in the adult brain (Lois and Alvarez-Buylla, 1994; Lois et al., 1996). I show that this type of migration plays an important role not only in postnatal and adult animals but also during early brain development. I identify two populations of embryonic neural precursors utilizing this type of migration. I focus my analysis on the “Brain Invaders”, a unique pool of cells which possess unparalleled capacity to migrate through embryonic and adult brain tissue.

Neuronal migration during brain development

The bulk of neural cell production is limited to highly specialized layers in the developing brain—the ventricular and subventricular zones (Fig. 1A). As its name suggests, the ventricular zone is the inner most layer of cells lining brain ventricles. As cells in the ventricular zone expand and the brain grows, a secondary proliferative layer—the subventricular zone (SVZ) forms adjacent to the ventricular zone. Although an SVZ seems to be present in most parts of developing central nervous system (CNS), it is most prominent in the ventral telencephalon in regions called ganglionic eminences. The rapid cell proliferation in the ventricular zone in most brain regions ceases at the end of embryonic development when this layer transforms into a terminally differentiated

ependymal layer. It is of particular interest that a mitotically active SVZ persists in the adult forebrain in the lateral wall of the lateral ventricle (Fig. 1B) (Smart, 1961).

The compartmentalization of proliferating neuroblasts close to brain ventricles implies that young cells have to move to the sites of their final residence, which can be several millimeters away from their place of birth (His, 1889; Altman, 1969; Rakic, 1972; Ramón y Cajal, S.1995; Doetsch and Alvarez-Buylla, 1996). It seems that all neurons in the CNS have to pass through this migratory phase. Before I continue with the introduction I will briefly summarize some of the different methods which were used to experimentally study cell migration.

Methods to study cell migration

The initial inference of cell migration during development was based on detailed histological studies (His, 1889; Ramón y Cajal, S.1995). This approach yielded only very limited and rough information about the scope and pathways of cell movements. In order to get a more refined picture of cell migration, scientists developed methods to directly observe cell movements using time-lapse microscopy or indirectly trace groups of cells during development by cell labeling or cell transplantation.

Direct observation

Soon after moving pictures became part of our daily lives, they also found their way to research laboratories. The ability of cameras to capture events which are either too fast or too slow for our eyes to notice, provided scientists with a powerful tool to study cell behavior. Eucaryotic cell migration which is generally too slow to be studied in real time was extensively analyzed using time-lapse microscopy. Time-lapse

microscopy accelerates slow biological events by playing back in regular speed (30 frames per second) pictures captured with much lower frequency (e.g. 5 frames per minute). Time-lapse microscopy was initially employed to study unicellular organisms and cell movements on the surface of developing multicellular embryos. Since many migrating cells are hidden from direct observation it was necessary to develop culture systems allowing their observation in vitro. Using different culture conditions it was possible to directly analyze migration of several types of neural cells during development (neural crest cells (Davis, 1980), radially (Edmondson and Hatten, 1987) and tangentially (Wichterle et al., 1997) migrating neurons).

Cell labeling

The culture systems used to study cell migration in vitro cannot reproduce all conditions found in the living organism. Therefore, it is necessary to confirm data collected in vitro by indirect analysis of cell migration in vivo. The most direct approach to trace migrating cells in the organism is the focal injection of dye (e.g. lipophilic dye DiI or PKH26). This approach is very simple and effective for labeling larger numbers of cells at the injection site.

Drawback of dye injection is fairly diffuse labeling, which makes targeting of discreet regions in a developing organism difficult. Noninvasive selective labeling of large populations of cells was made possible by the introduction of mitotic labels (3H-Thymidine, BrdU), which are incorporated into the DNA of all dividing cells during the S-phase. This technique was widely used to study cell migration during brain development (Angevine and Sidman, 1961; Altman, 1969). Since neuronal proliferation is confined to a narrow region in the developing brain, this method was used to study the

movement of cohorts of labeled cells migrating from the germinal layers to the unlabeled brain parenchyma. This technique was particularly helpful for the demonstration of the inside-out pattern of cortical development (see section on “Radial migration”).

The main problem with both the regular dyes and mitotic labels is that these tracers are diluted and disappear from cells which undergo several rounds of cell division. To overcome this limitation scientists started to use replication incompetent retroviruses carrying a marker gene (e.g. beta-galactosidase (LacZ) or alkaline phosphatase (AP)) for cell labeling. When retrovirus is injected into the brain it infects neural cells and retroviral cDNA integrates into the genome of infected dividing cells. This incorporation is stable and therefore transmitted without dilution to the progeny of the infected cell. Retroviruses were originally used to study cell lineages in the developing brain (Price et al., 1987). Since young neurons often migrate long distances before they terminally differentiate, it was necessary to find a way to determine which of the dispersed labeled cells are clonally related. This was achieved by the development of a large retroviral library containing viruses carrying thousands of unique genetic markers. Use of this library was not only vital to cell lineage studies, it also revealed the unexpected scale of tangential neuronal dispersion in the developing neocortex (Walsh and Cepko, 1992; Walsh and Cepko, 1993).

Recently it became possible to efficiently label different cell populations in the embryo by genetic manipulations. One possible method is to make transgenic mice which express cre recombinase under a control of cell specific promoter. Crossing this mouse with an animal in which reporter gene expression is activated by cre driven recombination results in the reporter gene expression in all cells which expressed cre at

any time during development, as well as in all their descendants (Zinyk et al., 1998; Yamauchi et al., 1999). Alternatively, it is possible to produce mice in which the cell specific expression of cre is regulated by an inducible element. This then allows labeling of the selected cell population at a specific time in development (Tsujita et al., 1999).

Cell transplantation

Cell transplantation is mainly used to study the potential and commitment of selected tissue in the context of different regions (heterotopic transplantation) or different developmental times (heterochronic transplantation). Donor cells have to be labeled before transplantation, so it is possible to identify them later in the host tissue.

Transgenic mice carrying a marker gene are often used as donor animals. For my studies I used Act::hPAP mice which express human placental alkaline phosphatase marker gene in all cells (Deprimo et al., 1996) and NSE::LacZ mice which express LacZ marker gene in all neurons (Forss-Petter et al., 1990). Besides transgenic mice, transplants of male tissue into females (recognized by Y chromosome specific antigens) or cross species grafts (mice to rats, or quail to chicken) were used to trace grafted cells (Harvey et al., 1992; Le Douarin, 1993; Campbell et al., 1995). It is also possible to use any one of the labeling techniques discussed above to label dissected cells in vitro prior to the transplantation. Besides studying cell potential and commitment, homotopic transplantation was also used to study cell migration in the developing (Le Douarin, 1980; Wingate and Hatten, 1999) and adult nervous system (Lois and Alvarez-Buylla, 1994; Jankovski and Sotelo, 1996).

Mechanism of neuronal migration

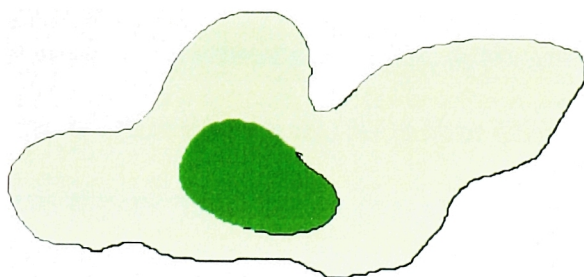
Neuronal migration is a specific type of cell translocation. Although on the subcellular level it might share many molecular mechanisms with other types of cell migration, macroscopically it is markedly different from amoeboid movement of macrophages or lamellopodial migration of fibroblasts (Abercrombie et al., 1977) (Fig. 2A,B). All migrating neurons are either unipolar with a long leading process or bipolar with a thin trailing process opposite to the leading process (Fig. 2C) (Schmechel and Rakic, 1979; Kishi, 1987). Leading processes of some migrating neurons are tipped with a growth cone, which might be similar to growth cones of migrating axons. Neuronal migration can be viewed as three separate processes: 1) extension of the leading process, 2) translocation of the nucleus and surrounding cytoplasm through the leading process, and 3) retraction of the trailing process left behind the cell. There are two main categories of neuronal migration sharing all three steps described above – the radial migration (Hatten and Mason, 1990; Rakic, 1990) and tangential migration (O'Rourke et al., 1992; Fishell et al., 1993; Wichterle et al., 1997). Other scientists have proposed a third type of neuronal migration called nuclear translocation. Cells migrating by nuclear translocation utilize only steps 2 and 3 without active extension of leading process or growth cone movement (Berry and Rogers, 1965).

Nuclear translocation

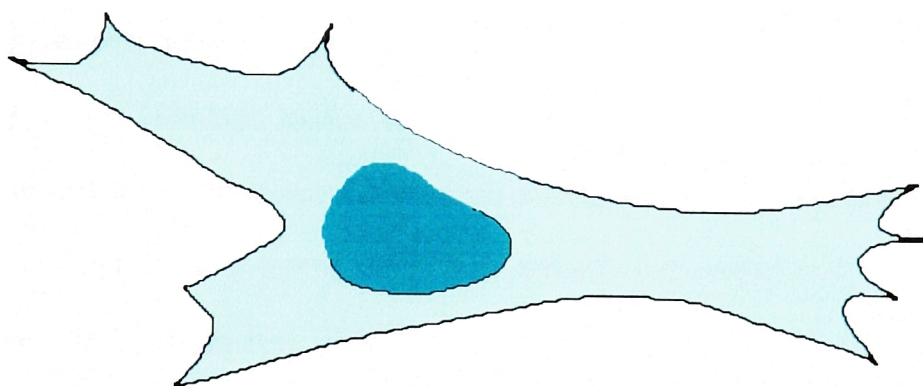
Since nuclear translocation seems to play only a marginal role during forebrain development I will cover it just briefly. Cells migrating by nuclear translocation do not necessarily need growth cones for their movement. It has been observed that during brain formation many cells become elongated stretching between the ventricular and pial

Figure 2: Different shapes of migrating cells. Cells migrating loosely attached to the substrate (e.g. macrophages) retain rounded shape. Cells extend pseudopodia during this type of amoeboid migration (A). Flattened cells moving closely attached to the substrate (fibroblasts) extend broad lamellopodium at the leading edge, which sometimes extends into narrow filopodia (B). There are many focal adhesion contacts formed between the moving cell and the substrate. Neuronal precursors migrate by extension of a leading process and cell body translocation. The leading process is tipped with a growth cone consisting of multiple active filopodia.

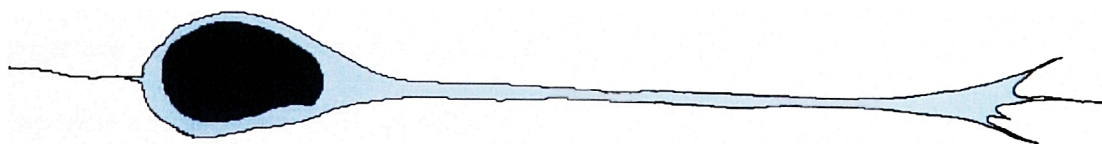
A



B



C



surface of the brain. It has also been noticed that these fibers have little bumps which could correspond to migrating cell bodies. This led to a hypothesis stating that the nuclei of radial cells which reside close to the ventricular surface might divide without subsequent cytokinesis. One daughter nucleus was thought to remain close to the ventricle, while the other was thought to translocate through the radial fiber to more superficial layers of the brain, where the cell would undergo cytokinesis and differentiate into neuron (Berry and Rogers, 1965). Ultrastructural reconstruction of the little bumps several years later showed that those are not nuclei inside the radial fiber but very closely attached migrating neurons (Rakic, 1972). This finding disproved the strong version of nuclear translocation hypothesis and replaced it with now widely accepted radial migration hypothesis. However, nuclear translocation is still considered a possible form of migration in special circumstances (for example during transformation of radial glia into astrocytes (Voigt, 1989) or during radial movement of some cerebellar or cortical neurons (Morest, 1970; Hager et al., 1995)).

Radial migration

Cerebral cortex on cross-section has a characteristic layered appearance. Discovery that later born neurons migrate passed the earlier born cells and settle close to the surface of the developing brain (so called inside-out development of cortical plate) (Angevine and Sidman, 1961) posed an interesting question. How do young neurons migrate across very heterogeneous landscapes in the developing cortex? How do they bridge the distance from cell rich germinal zones, crossing the cell sparse intermediate zone then migrating again through cell dense regions of the cortical plate? As was mentioned above, ultrastructural reconstruction revealed migrating young neurons closely

attached to fibers of radial cells spanning the thickness of the developing neocortex (Rakic, 1971a). Based on this finding it has been proposed that the radial cells, which are believed to be of glial origin, serve as guides for migration of newborn neurons. Radially migrating young neurons have a bipolar shape and they do not possess the typical elaborate growth cone.

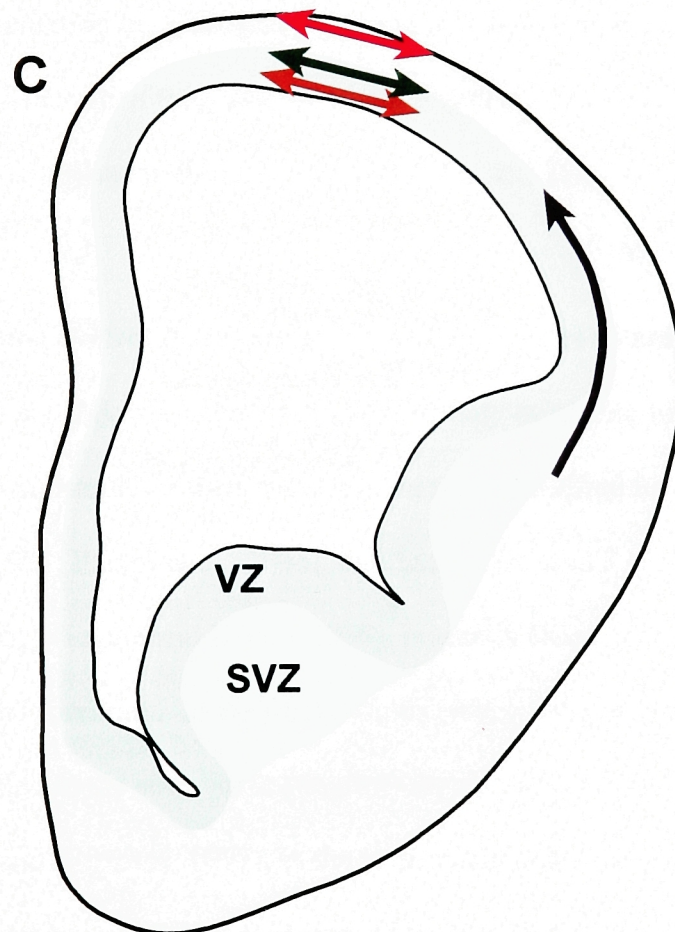
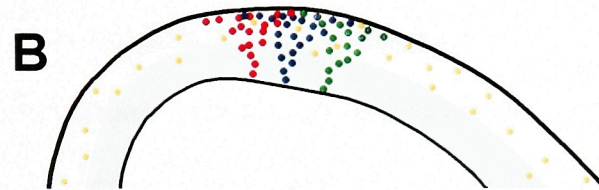
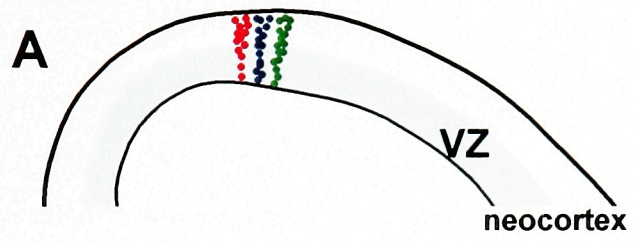
The hypothesis that cortical neuronal precursors slide along radial fibers like little “monorails” was confirmed by direct observation of radial migration in cultures of cerebellar granule neurons (Edmondson and Hatten, 1987). It was shown that cerebellar granule neurons, which are mainly produced postnatally, migrate radially from the surface of the cerebellum into the internal granule layer. Migrating cerebellar neuronal precursors use fibers of Bergmann glia (analogous to radial glia) for their translocation (Rakic, 1971b). Purified cerebellar granule neurons co-cultured with cerebellar astrocytes not only attach to the glial fibers but also actively migrate along glial processes, as was revealed by time-lapse microscopy (Edmondson and Hatten, 1987). These in vitro experiments not only provided the first direct evidence for radial migration, they also allowed analysis of cell body dynamics during translocation along glial fibers and led to identification of molecules involved in radial migration (Rivas and Hatten, 1995; Zheng et al., 1996; Rio et al., 1997; Komuro and Rakic, 1998; Bix and Clark, 1998).

It has been known for a long time that the neocortex is divided into functionally and anatomically distinct regions. More recently it was found that individual cortical regions (e.g. visual, auditory, sensory, motor cortex) are subdivided into smaller functionally distinct segments in which neurons with similar activity are organized in

radial columns (Mountcastle, 1957; Hubel and Wiesel, 1962). Discovery of extensive radial migration during the formation of cortical plate offered an explanation for the development of this intriguing columnar organization of cortical areas. The so-called radial unit hypothesis postulates that the planar diversity in the cortex arises long before cortical neurons are born. It proposes that the early cortical ventricular zone is a mosaic of distinct neuronal precursors giving birth to the regionally distinct neurons of the future cortex (Rakic, 1988) (Fig. 3A). This protomap is maintained during later development, as restricted radial migration of young neurons does not allow extensive tangential intermixing of neighboring neuronal populations. Thus, dividing cells in the ventricular zone and their progeny remain confined in restricted radial columns across all layers of future neocortex. It is believed that this topographic arrangement is maintained not only within the cortical plate but also by efferent axons of principal cortical neurons projecting to other brain areas.

Although the "radial unit hypothesis" offered a simple explanation for anatomical and functional organization of the neocortex, the story might be more complex. For one, it became clear that clonally related columns are much broader than narrow functional columns in the cortex. Their shape is conical rather than columnar fanning out towards the surface of the brain (Fig. 3B). These findings point to some tangential dispersion of radially migrating cells and intermixing of neighboring clones (see also section on "Tangential migration in cortex") (Walsh and Cepko, 1988; Tan and Breen, 1993). It is likely that radial migration lays down the initial cortical framework subdividing the brain into broader phenotypically distinct areas. The functional identity of individual cortical

Figure 3: Different modes of neuronal migration during cortical development. Radial unit hypothesis proposes that migrating neurons remain confined in narrow columns. It is assumed that neighboring neuronal clones (red, blue and green) do not intermix (A). Studies of clonally related cells in the developing cortex revealed two general types of clones: radial conical clones and broadly dispersed clones. It is assumed that radially migrating cells can switch to tangential migration and disperse a little before they finally settle. In this case cells from neighboring clones do intermix (B, red, blue and green). Another type of clones contained cells which were dispersed across most of the developing cortex (B, yellow). Tangential dispersion of neuronal precursors is thought to happen in ventricular and subventricular zones (C, orange arrow), intermediate zone (C, green arrow) and close to the surface of the neocortex in the subpial layer (C, red arrow). Recently it was proposed that some tangentially migrating cells in cortex originate in ganglionic eminences (C, black arrow).



columns might be refined later in the development based on functional connections and interactions among cortical neurons (O'Leary, 1989; O'Leary et al., 1994).

Tangential migration

Radial migration as an important player in cortical development was inferred more than one hundred years ago (His, 1889) and it returned to the spotlight of modern neurobiology when its mechanisms were dissected, described and experimentally validated (Rakic, 1971a; Edmondson and Hatten, 1987). In contrast, neuronal migration perpendicular to the orientation of radial glia (so called tangential migration) remained on the margin of interest. The role of tangential neuronal migration in cerebral cortical development remained unnoticed until fairly recently (Price and Thurlow, 1988; Walsh and Cepko, 1988).

It is now becoming evident that many neurons in most if not all brain regions of the developing CNS do not follow a radial path. Tangentially migrating neurons which do not use radial glia as substrate for their translocation were identified in the spinal cord (Ono and Kawamura, 1989; Phelps et al., 1996), hindbrain (Ono and Kawamura, 1990; Marin and Puelles, 1995; Wingate and Hatten, 1999), midbrain (Kawano et al., 1995; Ohyama et al., 1998) and forebrain. In the forebrain, migrating cells with tangential orientation were identified in the embryonic gangliothalamic pathway (Rakic and Sidman, 1969; Letinic and Kostovic, 1997), in the ventricular, subventricular, intermediate and marginal layers of fetal neocortex (Fig. 3C) (Walsh and Cepko, 1988; Gadisseux et al., 1992; O'Rourke et al., 1992; Fishell et al., 1993; Menezes and Luskin, 1994; DeDiego et al., 1994; O'Rourke et al., 1995; Szele and Cepko, 1996) and in subventricular zone of lateral ventricles in postnatal and adult animals (Luskin, 1993;

Lois and Alvarez-Buylla, 1994). In the following paragraphs I will discuss tangential migration in the forebrain in more detail.

Gangliothalamic body

Gangliothalamic body has been identified as a pathway connecting the ventral forebrain and thalamus in human embryonic brains. This structure was first identified in fixed histological sections (Rakic and Sidman, 1969; Letinic and Kostovic, 1997). Based on the shape of cells and orientation of their leading processes it has been proposed that cells originating in ganglionic eminence in the ventral forebrain cross the telencephalon/diencephalon boundary and migrate into the thalamus. It is thought that these cells differentiate into pulvinar neurons (Rakic and Sidman, 1969). A similar pathway was recently identified in primate brain, but it seems that it is absent in other vertebrates (Letinic and Kostovic, 1997).

Tangential migration in cortex

When clonal analysis of cell lineages in the developing brain was made possible by retroviral labeling techniques, one of the first tasks was to test the "radial unit hypothesis" (Price and Thurlow, 1988; Walsh and Cepko, 1988). It came as a surprise that tangential migration is actually quite prevalent in the embryonic cortex. Although some clones were confined to a single column as expected, many other were tangentially dispersed across most of the cortex ending up in multiple radial columns (Walsh and Cepko, 1992). Thus, it has been proposed that there are two populations of neuronal precursors in the ventricular zone. One of these populations is thought to give rise to radially migrating cells which behave according to the "radial unit hypothesis". The

second population contains ventricular zone stem cells migrating tangentially through the ventricular zone and shedding individual radially migrating neurons along the way (Walsh and Reid, 1995; Reid et al., 1995). These two general patterns of neuronal migration were also demonstrated in chimeric mice carrying LacZ marker on the X chromosome. Since one X chromosome is randomly inactivated early in embryogenesis, it is possible to analyze the entire lineage of cells with active X chromosome carrying the LacZ marker. Analysis of chimeric mice with only few brain cells derived from X-LacZ animals revealed two types of cell arrangement. Isolated columns containing labeled cells were observed in some animals, as would be expected for radially migrating cells, while in others LacZ expressing cells were found dispersed evenly throughout most of the developing cortex (Tan and Breen, 1993; Tan et al., 1995). Analysis of neuronal phenotype in radial and tangential clones revealed that tangentially dispersed cells differentiate preferentially into GABAergic cells, while the radial clones contain mainly glutamatergic neurons (Tan et al., 1998).

Although the presence of radial columns in the chimeric animals and after retroviral labeling suggested that radial migration plays a role in the cortical development, it did not confirm the strong version of the "radial unit hypothesis". Radial clones did not have a strictly columnar organization, rather they looked like funnels with the narrow end close to the ventricle and quite wide opening close to the brain surface (Fig. 3B) (Tan and Breen, 1993). It was suggested that radial glia are not perfectly parallel which might contribute to some neuronal dispersion (Misson et al., 1991). Radially migrating cells were also observed to switch to tangential migration and disperse somewhat before reaching their final destination (O'Rourke et al., 1992; O'Rourke et al.,

1995). Functional columns in the adult cortex as revealed by electrophysiology and optical imaging of the surface of the brain are narrower than individual funnels (Hubel and Wiesel, 1962; Ts'o et al., 1990). It is therefore unlikely that functional units in the adult neocortex are defined purely by the confinement of genetically prespecified neural cells to radial columns during development as was originally suggested by the "radial unit hypothesis".

The tangential movement of cells in the ventricular zone has been demonstrated in vitro by direct observation of fluorescently labeled ventricular zone cells (Fishell et al., 1993; O'Rourke et al., 1997). Similar in vitro experiments revealed another population of tangentially migrating cells in the subventricular or lower intermediate zone¹ (Walsh and Cepko, 1988; O'Rourke et al., 1992). Immunohistochemical staining revealed that tangentially oriented cells in this layer express the early neuronal marker Tuj1 (neuronal beta-tubulin) (Menezes and Luskin, 1994; O'Rourke et al., 1997), neurotransmitter GABA (Van Eden et al., 1989; DeDiego et al., 1994) and transcription factor Lhx6 (Lavdas et al., 1999).

When tangentially oriented SVZ cells were first observed it was assumed that they originate in the cortical ventricular zone. Cells born in the ventricular zone were thought to migrate into the subventricular zone where they continued to divide and dispersed tangentially before they entered the cortical plate (O'Rourke et al., 1992; Fishell et al., 1993; Walsh and Cepko, 1993; O'Rourke et al., 1997). Subsequent analysis however suggested that the origin of these tangentially oriented cells might be

¹ The distinction between the lower intermediate and subventricular zone is not very clear in the developing neocortex. In my thesis I refer to the cellular layer in between the ventricular zone and the cell sparse intermediate zone as the subventricular zone.

extracortical, that they might migrate into neocortex from the ganglionic eminence of the ventral forebrain (Fig. 3C) (DeDiego et al., 1994; De Carlos et al., 1996).

The unorthodox idea that the cortex is invaded by alien cells from another brain region has been supported by observations of tangential cell migration from ganglionic eminence to neocortex in embryonic cultures in vitro (De Carlos et al., 1996; Tamamaki et al., 1997; Anderson et al., 1997a; Lavdas et al., 1999; Wichterle et al., 1999). The best evidence so far for the extracortical hypothesis came from the analysis of *Dlx1/2* double knockout mice (Anderson et al., 1997a). *Dlx 1* and *2* are transcriptional factors expressed mainly in the subventricular zone of the ganglionic eminences. Analysis of the double knockout animals revealed marked reduction in cortical GABAergic cells. It has been proposed that the missing cortical GABAergic cells are derived from ganglionic eminence SVZ cells, which in mutant animals failed to migrate into the neocortex (Anderson et al., 1997b).

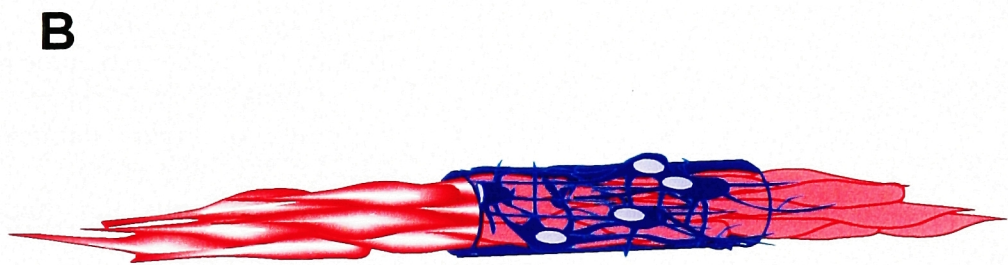
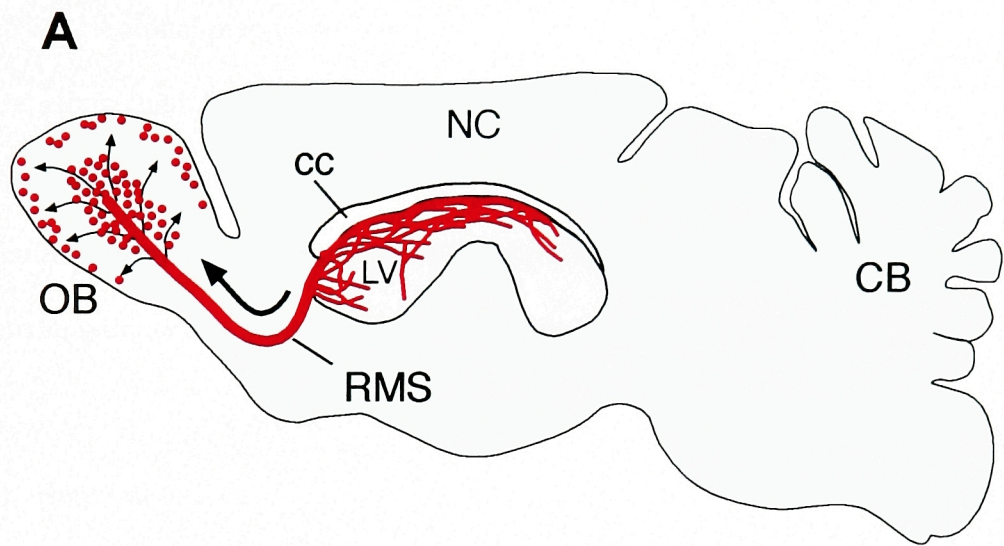
The last population of tangentially migrating neurons in developing cortex has been detected in the most superficial layer. These cells, called subpial granule cells were first identified in the marginal zone of human embryonic cortex. Search for the source of subpial granule cells by serial section analysis led to a proposal that they originate in the SVZ next to the developing olfactory bulb. SVZ cells were thought to migrate anteriorly and laterally to reach the surface of the brain approximately at the level of developing olfactory peduncle. From this point they were thought to tangentially disperse covering the entire surface of the neocortex (Gadisseux et al., 1992).

SVZ of lateral ventricle

As has been mentioned above, cell proliferation persists in the adult SVZ (Smart, 1961). The fate of cells born in postnatal SVZ remained a mystery for a long time. Since neurons were believed to be born only during embryonic and early postnatal life, it was assumed that proliferating SVZ cells either gave rise to glial cells (Privat and Leblond, 1972) or that newly produced cells underwent cell death (Morshead and Van der Kooy, 1992). Altman proposed a different hypothesis. He observed that new neurons are added to the adult rat olfactory bulb and he suggested that these neurons originate in the SVZ of lateral ventricles and migrate along a restricted tangential pathway (called rostral migratory stream) to the olfactory bulb (Altman, 1969) (Fig. 4A). This long distance migration was later confirmed by focal labeling and microtransplantation of labeled SVZ cells in postnatal (Luskin, 1993) and adult animals (Lois and Alvarez-Buylla, 1994).

Analysis of developmental processes in the embryonic brain is confounded by the presence of many intermixed neural cell types belonging to different neural lineages, which are proliferating, migrating, differentiating and dying at the same time and place. The persistence of neurogenesis and neuronal migration in the adult SVZ provided a unique system to study temporally isolated developmental events. Besides the surprising identification of an SVZ astrocyte as a neural stem cell in the adult brain (Doetsch et al., 1999), this system proved to be invaluable for the study of tangential migration. Silver impregnation and ultrastructural analysis of the postnatal SVZ revealed that neuroblasts migrate in this region in a direction perpendicular to the orientation of radial glia (Kishi et al., 1990). Thus, the postnatal SVZ seems to generate a population of tangentially migrating neurons. It was shown that migrating neuronal precursors are arranged in tight

Figure 4: Chain migration of neuronal precursors in the adult SVZ. A) Schematic sagittal view of the adult brain. Neuronal precursors are born in walls of lateral ventricle (light gray) and migrate along a network of chains (red lines) to the olfactory bulb (OB). There they differentiate into granule and periglomerular neurons (red dots). B) Model of a chain migration. Migrating neuronal precursors (red cells) are surrounded by a glial tube (blue cells). Figure A was modified with permission from (Doetsch and Alvarez-Buylla, 1996). Figure B was reprinted with permission from (Lois et al., 1996).



clusters resembling strings or chains of cells, which are surrounded by a layer of specialized astrocytes (Lois et al., 1996) (Fig. 4B). A continuous network of neuronal chains extends throughout the SVZ from the back of the lateral ventricle all the way to the olfactory bulb spanning a distance of several millimeters (Doetsch and Alvarez-Buylla, 1996). Neuronal precursors born in the lateral wall of the lateral ventricle migrate first along chain tributaries, joining in larger chains and finally coalescing into the rostral migratory stream leading them into the olfactory bulb (Fig. 4A). Inside the olfactory bulb neurons disperse radially (still in the absence of radial glia) to differentiate into neurons in granule and periglomerular layers (Bayer, 1983).

As we can see, there are many different neurons utilizing tangential migration (Fig.3C,4). Here I described at least three spatially or temporally distinct pools of tangentially migrating cells in the developing forebrain. It is intriguing that all of these cells are at some point characterized as subventricular zone cells. Is the SVZ the source for all tangentially migrating cells, or is the SVZ a product of tangential migration? Are all tangentially migrating cells the same? Do they originate in a common embryonic brain region? Do they utilize the same substrates for migration? Do they differentiate into a specific neuronal subtype? Why is the seemingly perfect radial cortical organization disturbed by tangential migration?

In this thesis I do my best to answer some of the above questions. I know that the picture is far from complete, that each answer just opens many more questions, but that is the inevitable process of science, which will eventually lead to better understanding of one of the most complex and fascinating organs of our body.

Organization of thesis

In the first part of my thesis, I describe the development of a new assay which for the first time allowed the study of tangential migration in vitro. I used this culture system in combination with time-lapse microscopy to analyze the migratory behavior of postnatal SVZ neuronal precursors. I describe the dynamics of tangential migration and dissect individual steps of cell translocation, focusing on aspects which differentiate tangential and radial migration. I show that SVZ cells migrate homotypically along each other (so called chain migration) in the absence of glia or any other cell type. I also show that tangential migration is the fastest neuronal translocation so far described – it is 4 times faster than radial migration.

In the second part of the thesis, I use the in vitro system to search for embryonic neuronal precursors capable of tangential migration. Out of all screened parts of the embryonic CNS I found only two regions in the embryonic ventral forebrain (lateral and medial ganglionic eminences (LGE, MGE)) which are rich sources of tangentially migrating neurons in my assay. I compare the behavior of LGE and MGE cells by transplantation into the adult brain. I show that LGE cells grafted into the adult SVZ behave similarly to resident SVZ cells and migrate to the olfactory bulb, where they differentiate into granule and periglomerular neurons. In contrast, MGE cells revealed a novel, unexpected potential. They are not able to follow the migratory route to the olfactory bulb. Instead, MGE cells disperse in all directions from the site of transplantation and differentiate into neurons. I show that MGE cells give rise to a specific population of inhibitory GABAergic neurons with deep nuclear invaginations.

In the third part of the thesis, I study the behavior of ganglionic eminence cells in their normal environment in the embryonic brain. First, I address their migratory capacity in embryonic brain slices. I show that the MGE cells migrate in a directional manner towards the neocortex. I show that differential permissiveness of embryonic brain tissue could guide MGE cell migration to the neocortex and suggest that cortical axonal fibers are the best substrate for MGE cell migration in vitro. I also show that LGE cells do not migrate long distances in the embryonic brain slices. I propose as a hypothesis that the MGE and not the LGE is the primary source of neuronal precursors which invade the developing cortex and give rise to the population of GABAergic neurons missing in the *Dlx1/2* knockout mice (Anderson et al., 1997a). This hypothesis is in conflict with previously published results suggesting that LGE is the source of neurons migrating dorsally (De Carlos et al., 1996; Tamamaki et al., 1997; Anderson et al., 1997a). To resolve this issue and defer the criticism that in vitro observations might not reflect normal behavior of MGE cells, I studied the migratory potential of MGE cells directly in vivo by ultrasound guided transplantation in embryonic brain. I confirmed my hypothesis by showing that MGE cells in vivo massively invade cortex. First they migrate through intermediate zone, possibly along cortical axons, and later they move radially to settle in the marginal zone. LGE cells in contrast do not migrate to the cortex, rather they disperse within the developing striatum. Similarly as in vitro, MGE cells do not migrate ventrally to the diencephalon.

In the fourth part of the thesis, I discuss potential therapeutic use of MGE cells. I show that MGE cells can migrate through multiple adult brain regions as well as through experimentally lesioned adult brain tissue. I show that dispersed MGE cells integrate

functionally into the host brain. Although migration and integration of MGE cells in damaged brain are excellent, the potential of MGE cells seems to be restricted. MGE cells grafted into different adult brain regions give rise to a similar type of inhibitory stellate aspiny neurons. I conclude that MGE cells might be used as a source of neurons for treatment of specific types of neurologic conditions, where GABAergic neurons are lost or where they could modulate function of damaged circuits (e.g. Huntington's disease, some types of epilepsy). Alternatively, the understanding of the mechanisms underlying widespread migration of MGE cells in the adult brain could allow us to modify other types of neuronal precursors and help them to integrate better in damaged brain.

I conclude my thesis with thoughts on the role of tangential migration in the developing central nervous system. I propose that there is a limit to how many different developmental events can occur in one place and time. I suggest that tangential migration is the way to overcome this limit and ensure sufficient complexity for the developing brain. If one brain region cannot produce all the necessary cell types at the time they are needed, missing cells have to be imported from neighboring regions.

CHAPTER 2: MECHANISM OF TANGENTIAL MIGRATION IN POSTNATAL SVZ

As I mentioned in the introduction, tangential neuronal migration plays an important role in the development of most brain regions. However, the mechanism and the guidance of tangential neuronal migration are poorly understood. In order to study the behavior of tangentially migrating neurons I developed a culture system in which I could follow the migration of postnatal SVZ cells using time-lapse microscopy. Similar in vitro studies were instrumental in the demonstration of neuronal translocation along radial glial fibers (Edmondson and Hatten, 1987; Komuro and Rakic, 1995; Komuro and Rakic, 1996) and have helped in the identification of some of the molecules involved in radial migration (Rakic and Komuro, 1995; Zheng et al., 1996; Rio et al., 1997; Komuro and Rakic, 1998; Bix and Clark, 1998).

The postnatal SVZ of the lateral ventricles is a rich source of tangentially migrating neuronal precursors (Luskin, 1993; Lois and Alvarez-Buylla, 1994). Young neurons migrate 3-8 mm through the SVZ (Doetsch and Alvarez-Buylla, 1996) and along its anterior extension called the rostral migratory stream, to reach the olfactory bulb where they differentiate into granule and periglomerular neurons (Fig.4A). Detailed immunohistochemical and ultrastructural analysis revealed that migrating neuronal precursors in the SVZ and rostral migratory stream do not follow radial glia. Instead, they are associated with each other forming elongated cell aggregates resembling chains, which are surrounded by a tube-like structures formed by specialized astrocytes (Fig.4B)(Lois et al., 1996). Based on the organization of migrating SVZ neurons in the adult brain this type of tangential migration is also called chain migration.

The inference that neuronal precursors in the SVZ migrate associated with each other without radial glial or axonal guidance derives from serial ultrastructural reconstruction of the rostral migratory stream. It is, however, not known how cells move within chains and whether they require the ensheathing astrocytes for their translocation. In one possible model, tangential migration might be a modified form of radial migration. Migrating neuronal precursors might migrate along astrocytic tubes similarly as radially migrating neurons move along radial glia (Rakic, 1971a). Alternatively, astrocytes form a supportive or protective layer, but neurons do not use them for translocation. The latter possibility would suggest that mechanism of tangential migration is different from radial migration. In order to distinguish between the two possibilities I analyzed the interaction of astrocytes and neurons during SVZ migration in vitro. I show that SVZ cells in chains migrate long distances at high speeds by sliding along each other in the absence of glial cells.

Assay for tangential migration

To establish optimal conditions for studying chain migration in vitro, I cultured SVZ explants from 5 day old mice (P5) on different substrates. I found that SVZ cells became organized as chains in Matrigel (Fig. 5A), a three dimensional extracellular matrix gel composed of collagen IV, laminin, heparan sulfate proteoglycans and entactin/nidogen (Kleinman et al., 1982). In some cases, individual long chains extending from an explant could be observed, while in others, cells formed a web of interconnected chains. Individual cells in chains were tightly apposed to each other. These chains were 1 to 5 cells wide and up to 600 μm long. Chains did not form on plastic, or on plastic coated with laminin (Gibco), poly-D-lysine (Gibco), fibronectin

Figure 5: Explants from 5 day old mouse brain cultured on different substrates.

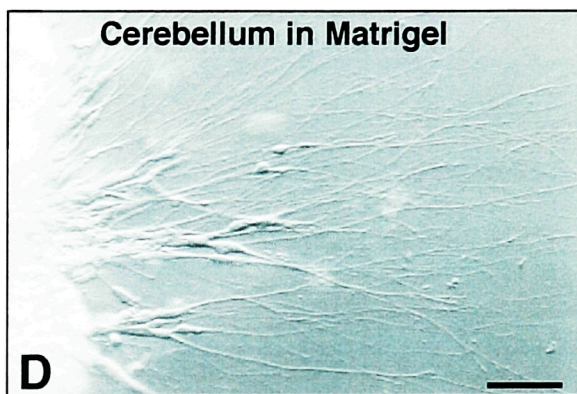
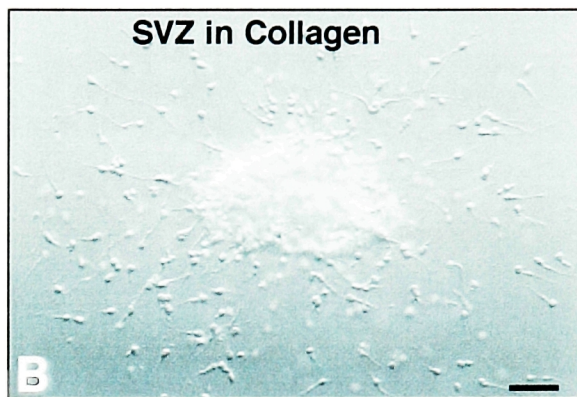
A: SVZ explants embedded in matrigel formed a web of long interconnected chains around the explant.

B: SVZ cells cultured inside a gel of collagen type I migrated away from the explant mostly as individual cells. Some chains were formed under this condition, but they were loose and transient with individual cells becoming detached and migrating on their own.

C: Explants of striatum without SVZ did not result in a cell outgrowth or the chain formation, when cultured in matrigel.

D: Cells from cerebellar explants cultured in matrigel migrated attached to long fibers.

A and B are 24 hours in culture, C and D are 48 hours in culture. Scale bar = 50 μ m.



(Gibco), or type IV collagen (Collaborative research). The organization of cells in a gel prepared from type I collagen was similar to that described previously (Hu and Rutishauser, 1996; Hu et al., 1996). Migrating cells formed transient contacts with other cells and occasionally assembled into loose chains. However, in contrast to Matrigel, the majority of cells in collagen type I gel migrated as individual cells (Fig. 5B). After 5 days in Matrigel, the chains began to fall apart and cells either died or some differentiated into neurons. Since the culture condition might affect behavior of neuronal precursors in vitro, I chose the period as close as possible to the time of dissection for my analysis. The subsequent experiments were done in Matrigel within the first 3 days after explantation.

Chains of migrating cells were observed in cultures of SVZ isolated from P1, P3, P5, P7, P10, P14, P20 and 2 month old mice. An extensive and dense web of chains formed around SVZ explants isolated from P3-P10 mice, a period when the SVZ is largest and a time corresponding to the peak of neurogenesis in olfactory bulb interneurons (Hinds, 1968; Rosselli-Austin and Altman, 1995). For this reason, in most of the following experiments, I used explants of SVZ from P5 mice. In order to study whether chain formation is characteristic of SVZ cells or whether cells from other areas of the postnatal brain could also form chains, I prepared cultures of different postnatal (P5) mouse brain regions (striatum, medial septum, cortex, hippocampus, medial hypothalamus and habenula). Very few cells emerged from these explants and these cells were not organized as chains (Fig. 5C). Since the majority of neurogenesis and cell migration in these regions is completed at the time of birth, I also studied explants isolated from brain regions known to contain migrating cells. Two prominent sites of

Figure 6: Morphological and immunocytochemical characterization of cells in chains. SVZ explants from P5 mice in matrigel. A, C, E and G shows DIC images; the corresponding fluorescent photomicrographs are shown on the right in B, D, F and H. All pictures have the same orientation with the distal part of chains to the right.

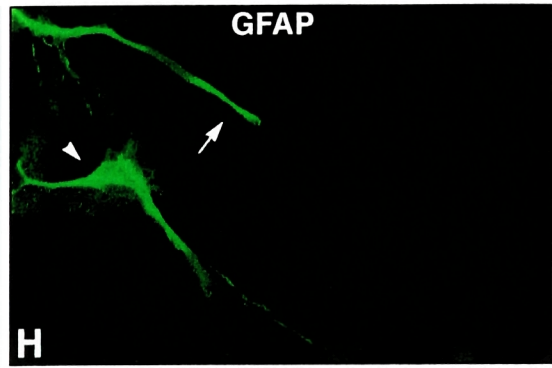
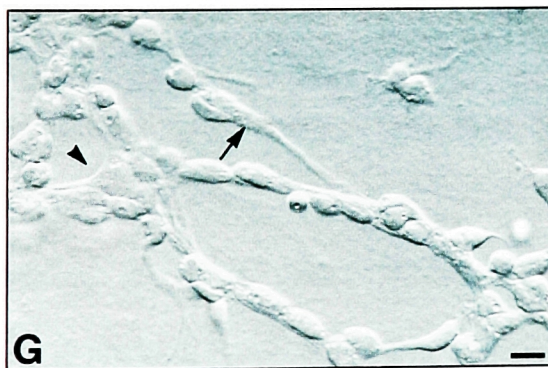
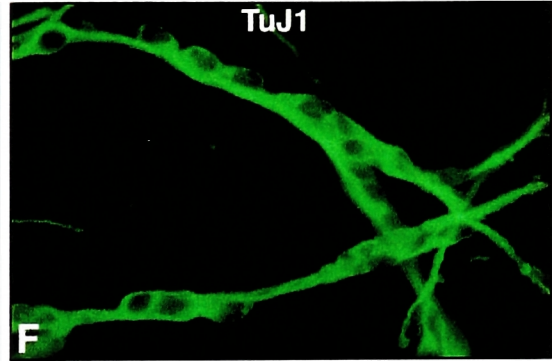
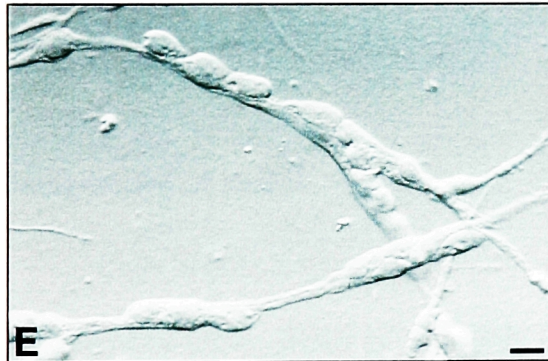
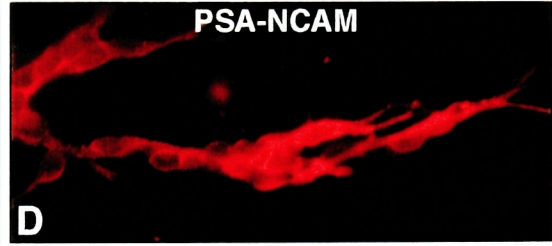
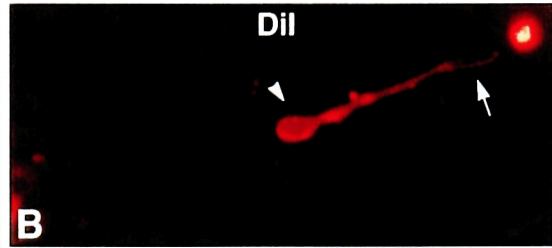
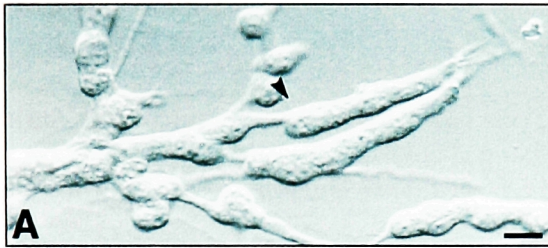
A & B: The morphology of individual cells in tightly packed chains was visualized by small crystals of lipophilic dye DiI added to the explant. An individual labeled cell (arrowhead) in a chain shows the elongated morphology with a leading process and a growth cone with long filopodia (arrow, B) that characterize migrating cells in the rostral migratory stream. (the bright spot in the top-right corner is a DiI crystal)

C & D: Immunolabeling with antibodies against PSA-NCAM revealed that cells in chains were positive for this antigen.

E & F: Cells in chains were also immunoreactive for the early neuronal marker TuJ1.

G & H: Cells in the chains were immunonegative for GFAP. GFAP positive processes extended from explants to the proximal segments of chains (arrow). Occasionally a GFAP positive astrocyte was observed associated to a chain (arrowhead). More distal parts of chains were devoid of GFAP immunoreactive processes.

A-F were 24 hours in culture, G&H was 48 hours in culture. Scale bar = 10µm



neuronal migration are the embryonic cortex (Angevine and Sidman, 1961) and the postnatal cerebellum (Rakic, 1985). Explants from embryonic day 15 (E15) cortical ventricular zone or P5 cerebellar external granule layer in Matrigel did not result in chain formation. Instead, long fibers with closely attached cells (Fig. 5D) resembling radially migrating neuronal precursors (Edmondson and Hatten, 1987; Komuro and Rakic, 1996), grew from cortical and cerebellar explants. Time-lapse video recording of cells along fibers from cerebellar explants revealed a migration similar to that previously described (Edmondson and Hatten, 1987) (data not shown). These results suggest that chain migration is a characteristic feature of SVZ cells and that Matrigel provides a substrate conducive to chain formation in vitro.

Composition of chains

Cells in chains were very closely attached to each other. Using differential interference contrast (DIC) microscopy I was able to distinguish individual cell nuclei, but it was generally not possible to discern the contour and shape of the entire cell. In order to visualize the morphology of individual cells in chains, crystals of a lipophilic dye DiI were mixed with explants so that only a small proportion of migrating cells were labeled. DiI labeled cells in chains had an elongated morphology with a leading process 20-40 μm long and occasionally, a thin trailing process (Fig. 6A,B). The tip of the leading process widened into a conical structure with multiple filopodia resembling growth cones of migrating axons² (arrow, Fig. 6B). This cell morphology was very

² I will refer to the structure at the tip of the leading process of migrating SVZ cells as a growth cone. This terminology was introduced by Ramon y Cajal and adopted by others (Kishi, 1987). Although morphologically they are very similar, it is possible that growth cones at the tip of migrating cells differ on subcellular level from axonal growth cones.

Figure 7: Ultrastructure of cells in chains (24 hrs in vitro).

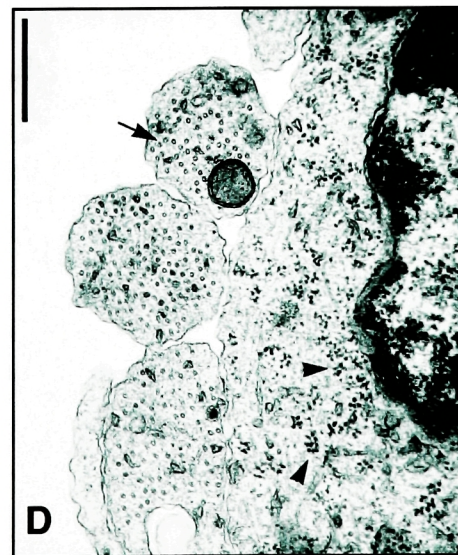
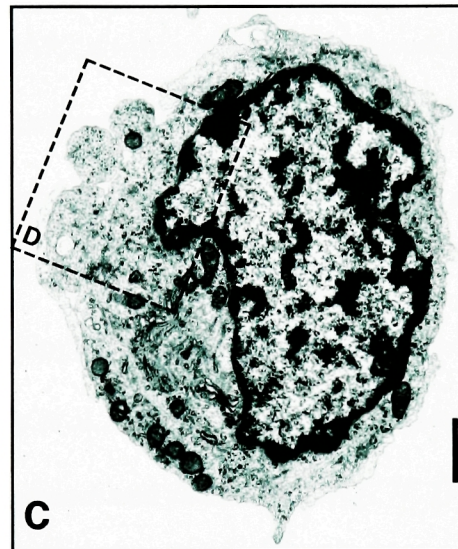
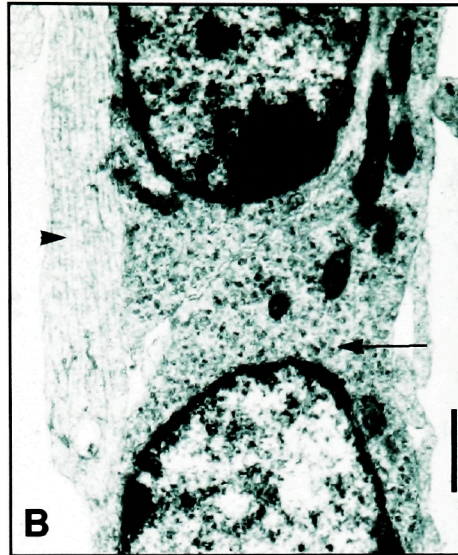
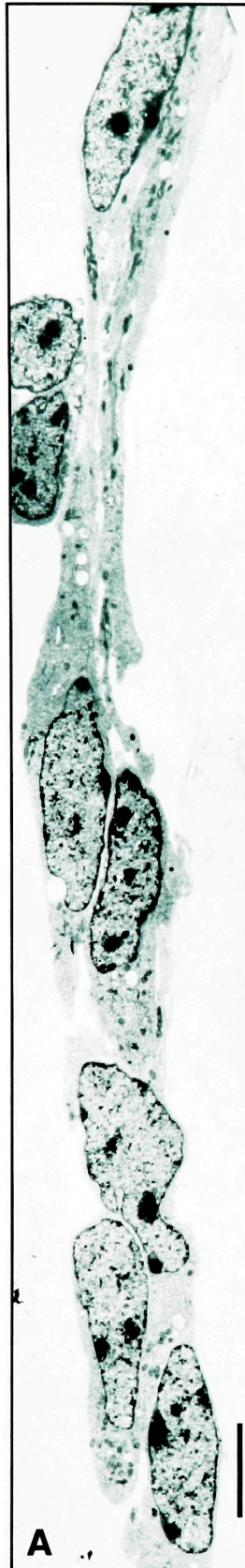
A: Chains were composed of electron-dense spindle-shaped cells with elongated nuclei containing sparse heterochromatin and several nucleoli. Individual cells were closely attached to each other. No axons or glial processes were encountered in the chains.

Scale bar = 5 μ m

B: Higher magnification of portion of longitudinally cut chain showing association of 2 cells and a leading process. Notice the parallel arrays of microtubules (arrowhead) in the leading process. The cytoplasm is rich in polyribosomes (arrow) with scant rough endoplasmic reticulum. Scale bar = 1 μ m

C: Transversal section of a chain showed close association of multiple leading processes with a cell body of another cell. Scale bar = 1 μ m

D: Higher magnification of the transverse section showing the array of microtubules in the leading processes (arrow). The cytoplasm of cells close to the nuclei is rich in polyribosomes (arrowheads). Scale bar = 0.5 μ m



similar, if not identical, to that described in vivo for migrating cells along the rostral migratory stream (Kishi, 1987).

In order to verify that these cells corresponded to the migrating neuronal precursors observed in vivo, the chains were studied by immunocytochemistry and electron microscopy. Cells in the chains were immunopositive for PSA-NCAM (Rougon et al., 1986) (Fig. 6C,D) and TuJ1, a neuron specific beta tubulin (Lee et al., 1990; Moody et al., 1996) (Fig. 6E,F), two molecules present in the migrating neuronal precursors in vivo (Doetsch and Alvarez-Buylla, 1996). Chains did not contain any vimentin positive radial glial fibers or MAP2 positive neurites. Electron microscopy revealed, that cells in chains were electrondense with elongated nuclei containing multiple nucleoli (Fig. 7). Cells had many polyribosomes, bundles of microtubules in leading processes (Fig. 7B) and pinocytic vesicles. This morphology was very similar to that described for type A cells, the migrating neuronal precursors in the rostral migratory stream (Jankovski and Sotelo, 1996; Lois et al., 1996). Transverse sections of chains showed a close apposition of leading processes and cell bodies (Fig. 7C,D). Electron microscopy did not reveal any glial fibers associated with studied chains.

Neuronal migration in the absence of astrocytes

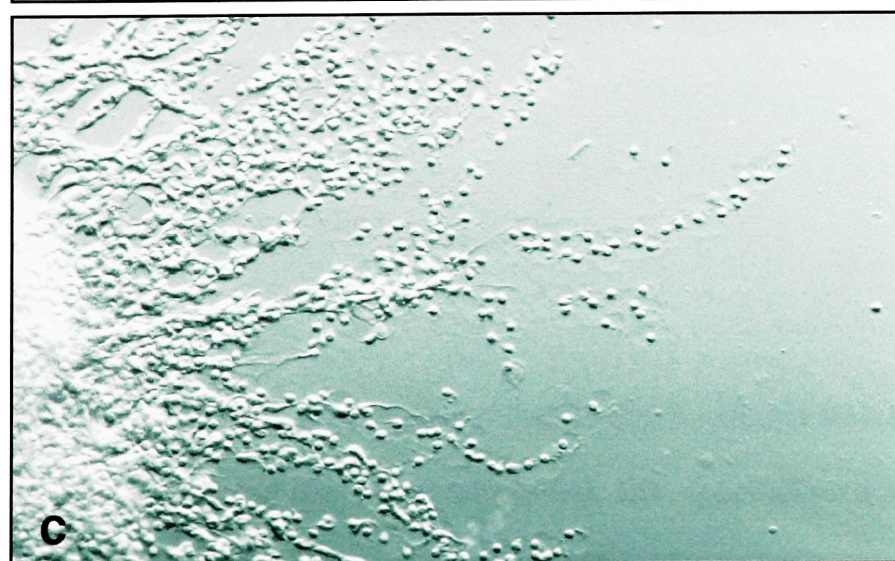
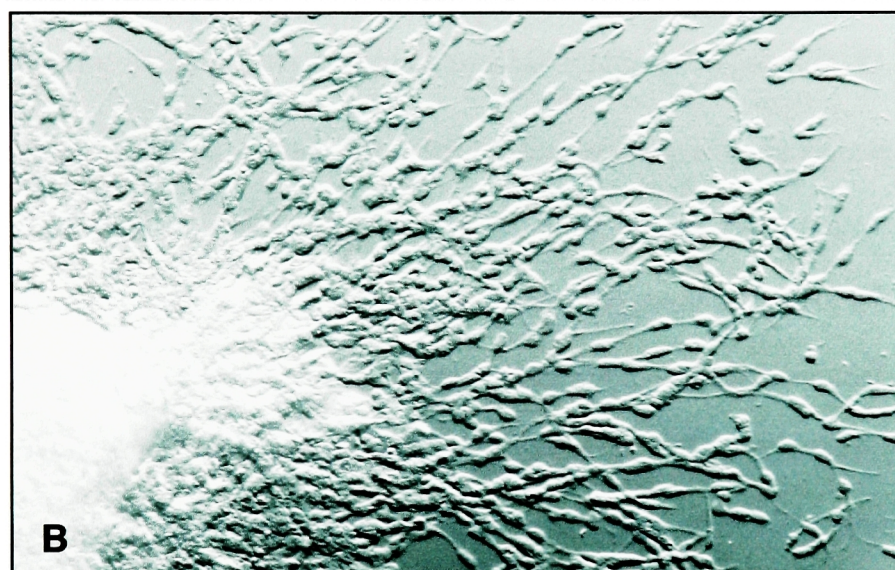
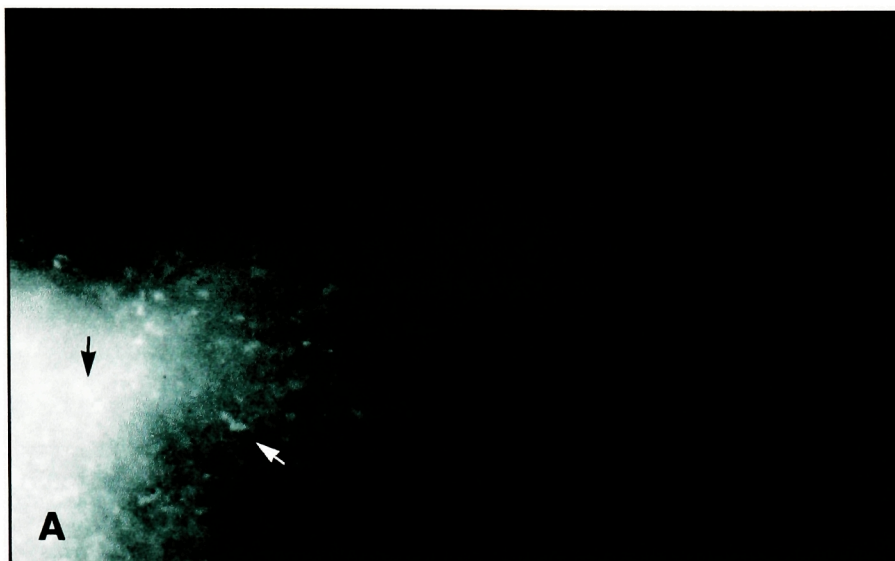
Although an extensive outgrowth of chains was observed around SVZ explants after 24 hours in culture, no glial fibrillary acidic protein (GFAP) immunoreactive cells were observed in the chains. A few chains in which many actively migrating cells were observed were subsequently stained with anti-GFAP antibodies. No GFAP immunoreactive cells were detected in these chains, confirming that extensive movement of cells in chains occurs in the absence of astrocytes. However, beginning at two days

Figure 8: Chains form normally after complement mediated lysis of astrocytes (48 hrs in vitro).

A & B: GFAP positive cells were eliminated from cultures by complement mediated lysis of astrocytes with antibody 7B11. This treatment results in no GFAP positive cells or processes on chains. Some GFAP-positive cell bodies remained inside the explant (arrows), but no GFAP positive fibers were extending from these cells (A). Under these conditions chains formed normally (B).

C: In contrast, when cultures were preincubated with anti-PSA-NCAM antibodies, cells in chains were lysed by complement. Ghosts and rounded-up cell nuclei remain where the chains had extended before.

Scale bar = 50 μ m.



after explantation, a few GFAP positive processes extended from explants (arrows, Fig. 6G,H) and occasionally GFAP positive cells were found associated with proximal segments of chains (arrowhead, Fig. 6G,H). The number and length of GFAP immunoreactive fibers progressively increased with time in culture, but the distal segments of the chains were completely devoid of GFAP immunoreactivity even after five days in culture. An extensive network of chains formed even in cultures in which astrocytes were eliminated by treatment with 7B11 antibody recognizing an early astrocytic surface antigen (Szigeti and Miller, 1993) and a complement mediated lysis, suggesting that the astrocytes are not required for chain formation (Fig. 8).

The glial cells are either not required for chain migration or signals normally provided by these cells were substituted by the three dimensional culture substrate. Glial cells in vivo may play a role in isolating the migrating precursors from the surrounding brain parenchyma or could provide factors for the survival and directional migration of young neurons. It is interesting that GFAP positive cells and processes appearing in cultures at later stages were usually attached to the chains of neuronal precursors, suggesting that this in vitro system may allow future investigations of the role of the tube-like glial meshwork observed in vivo.

Dynamics of tangential migration

Behavior of cell bodies

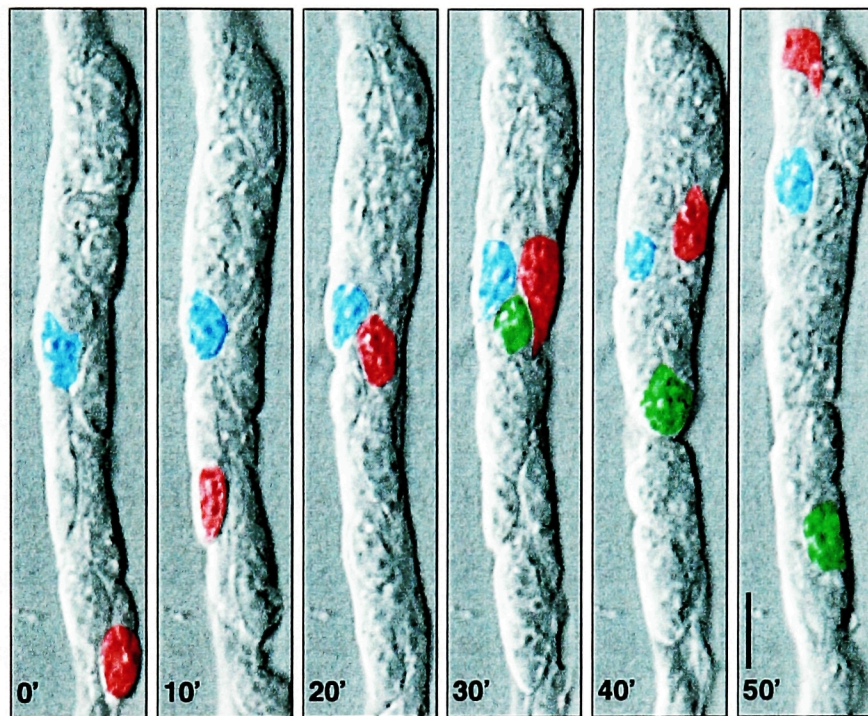
In order to determine the behavior of cells in chains, cultures were studied by time lapse DIC videomicroscopy (see CD-ROM movies). DIC optics allowed me to visualize individual cell bodies within one focal plane of the three dimensional cultures. Since

cells deep in the Matrigel frequently move in and out of the focal plane I focused on recording chains that formed at the interface of the gel and the coverslip surface. Comparison of these chains with chains found deep in the Matrigel did not reveal any differences in cell morphology or behavior. Cells in chains were highly motile. Individual cells passed neighboring cells as they migrated through a chain (Fig. 9, see also CD-ROM movies). Even in very compact chains, individual cells migrated by literally sliding over their neighbors. Migration was bi-directional with cells moving in opposite directions along the same chain (Fig. 10). This suggests that chain migration results from individual cells sliding over each other, rather than from a cooperative motion of cohorts of cells.

Chain migration *in vivo* is highly directional. The majority of cells moving in the rostral migratory stream are polarized with their leading process oriented towards the olfactory bulb (Kishi, 1987; Luskin, 1993; Lois and Alvarez-Buylla, 1994). Cells in culture, however, migrated bi-directionally along the chains. A cell moving in one direction may become stationary, change the orientation of the leading process and resume migration in the opposite direction. Similar bi-directional migration has also been observed during glial-guided migration *in vitro* (Edmondson and Hatten, 1987). The mechanism that establishes and/or maintains a specific polarity of cells *in vivo* remains to be determined. It was recently suggested that a chemorepellent substance secreted from caudal septum may guide the rostral migration of SVZ precursors (Hu and Rutishauser, 1996). However, under the present culture conditions I did not observe any preferential direction of migration when SVZ explants were co-cultured close (100-300 μ m distance) to an explant of caudal or rostral septum, olfactory bulb, striatum, or

Figure 10: Time-lapse series showing bidirectional movement in a compact chain (see also CD-ROM movies).

Images were recorded every 10 minutes and three cells are highlighted in color (red, green and blue). The red cell moves rapidly away from the explant (towards the top), passing by the slowly moving blue cell. The green cell, that moved into focal plane at 30 min., migrated towards the explant (bottom of the image). Scale bar = 10 μ m



cortex (results not shown). The fact that cells continue to migrate in vitro in the absence of directional cues suggests that directional signals are not required for cell translocation.

Migration of cells in chains consisted of cycles of rapid advancement interspersed with stationary periods (Fig. 11 and Table 1). Similar saltatory migratory behavior has been observed in neuronal precursors migrating along glial fibers (Edmondson and Hatten, 1987; Komuro and Rakic, 1996). Recently, Komuro and Rakic showed that increases in cytosolic calcium in these cells coincide with periods of cell body translocation (Komuro and Rakic, 1996). Based on the analogous saltatory behavior and almost identical duration of migratory periods (~4 minutes) (Table 1), it is plausible that a similar calcium mediated mechanism may be employed for cell body translocation during chain migration. Transient increases in intracellular calcium may trigger nucleus movement through an active mechanism involving molecular motors, or it may regulate the assembly and disassembly of microtubules in the leading process resulting in a nucleus translocation (Rakic et al., 1996).

To study the displacement of individual cells, the position of the cell body of 22 moving cells (cells that changed position within a 15 minute sample period) was recorded at 1 minute intervals. The average velocity of these migrating cells was 122 $\mu\text{m/hr}$ and the average peak speed during active translocation was 302 $\mu\text{m/hr}$. Changes of cell body position as a function of time is shown in Figure 11 for 4 representative cells. Three of these cells migrated throughout the 45 minute period with only short stationary periods, while the fourth cell became stationary at 4 minutes and resumed its migration at 27 minutes. The average duration of each step was 4.1 minute in which cells moved on average 19.4 μm , almost twice the soma length (91 steps analyzed from 22 cells). Taking

Figure 11: Saltatory movement of cell body during migration.

For this analysis cell position was recorded in 1 minute intervals and plotted as a function of time. The straight lines indicate the mean \pm SD for 22 cells. The migratory profile of 4 representative cells (a, b, c and d) shows the characteristic saltatory movement of these cells. Cells a, b and c are considered to be migrating cells (stationary periods shorter than 15 min.), while the fourth cell (d) became stationary at 4 minutes and resumed migration at 27 minutes.

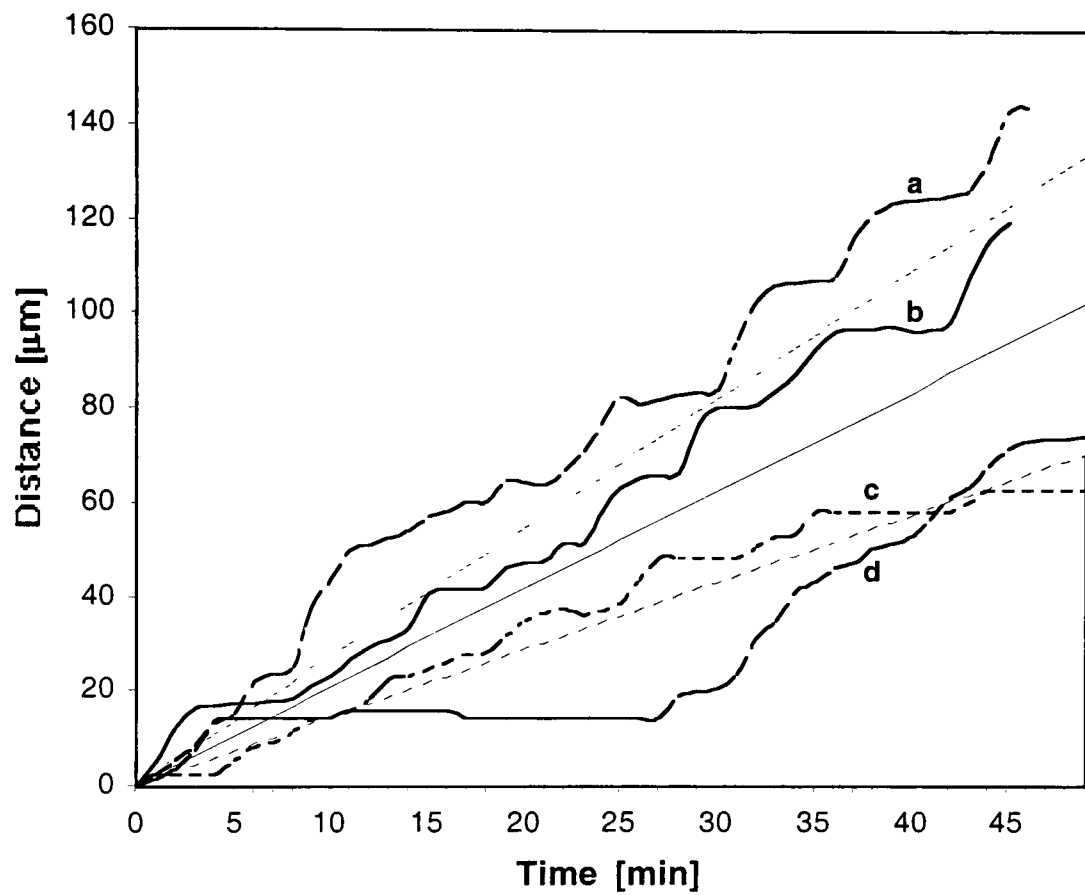


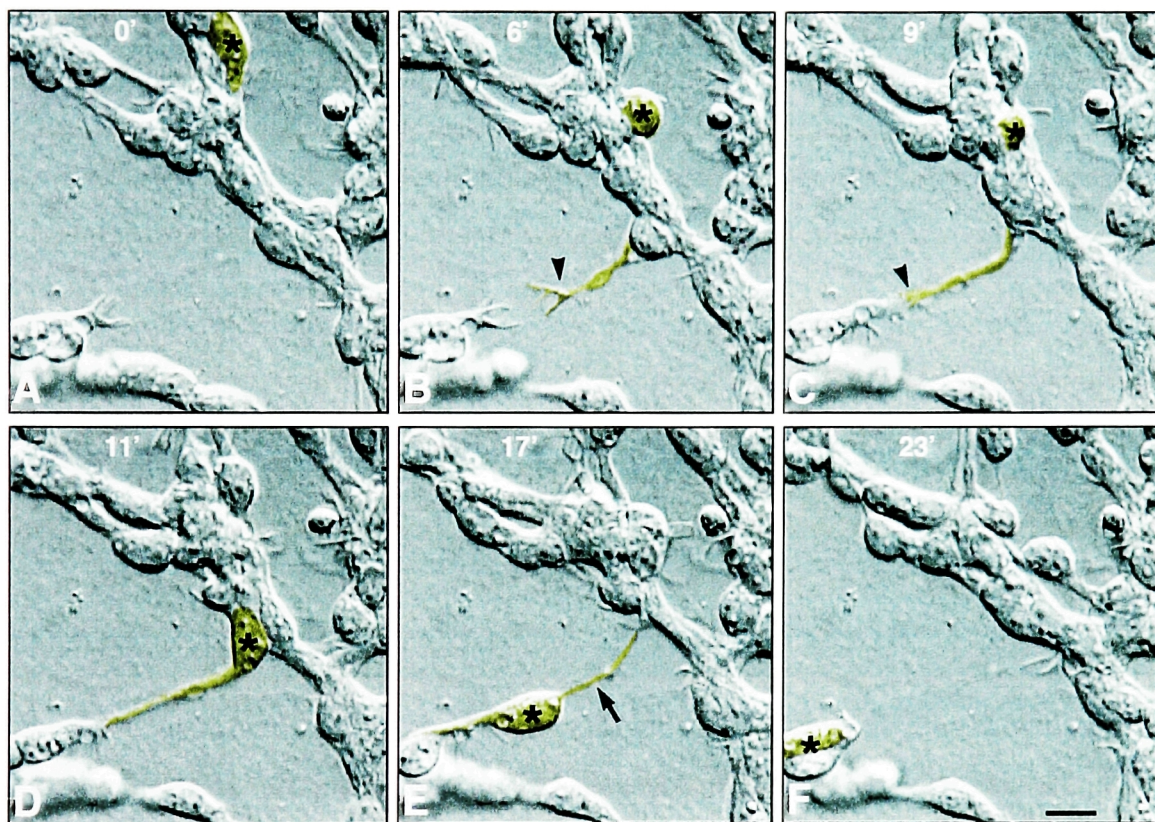
Figure 12: Time-lapse study of cell behavior during translocation (see also CD-ROM movies)

This figure shows individual steps of cell body (star) translocation and behavior of growth cone and leading process during cell movement between two parallel chains.

A&B: The cell first extended the leading process with a highly motile growth cone (arrowhead) towards the second chain.

C: A contact with a cell in the second chain was established (arrowhead).

D-F: Cell body moved between the two chains. Notice the thin trailing process (arrow, E) that was rapidly retracted. Scale bar = 10 μ m



into account only the moving cells (those that moved within the 15 minute period), the duration of pauses was on average 2.2 min.

In order to find out how many cells are migrating and how many are stationary I analyzed all cells in one field in three independent cultures (885 cells studied) during 15 minutes periods at 22, 24, 25, 26, 27, 28 and 32 hours after explantation. Cells which did not move within these 15 min. were considered stationary. The proportion of stationary to moving cells at these different times was very similar. There were $49.1 \pm 6.4\%$ stationary cells, $38.4 \pm 8.0\%$ of cells moving away from explants and $12.5 \pm 2.4\%$ of cells moving towards explants. The speed of cell flow away from explants was determined in 15 chains to be $23.6 \pm 9.2 \mu\text{m/hr}$.

The estimated speeds of chain migration in vivo (Luskin and Boone, 1994; Lois and Alvarez-Buylla, 1994) and in vitro (present results) are three to six times faster compared to cells moving along glial processes (Edmondson and Hatten, 1987; Jacobson, M.1991) (Table 1). The tight apposition of migrating cells and glial fibers through lamellar extensions and interstitial junctions required for radial migration, may also result in higher resistance to displacement. In contrast, the dynamic nature of contacts formed between cells during chain migration (allowing cells to constantly change their partners) might result in faster, less restrained cell movement. The chain migration described here is also faster than tangential migration within the developing ventricular zone ($10\text{--}100 \mu\text{m/hr}$) (Fishell et al., 1993) or tangential migration in the developing intermediate zone of the cerebral cortex ($24 \mu\text{m/hr}$) (O'Rourke et al., 1992). Chain migration is the fastest known neuronal migration.

Figure 13: Correlation of cell body speed and leading process contraction.

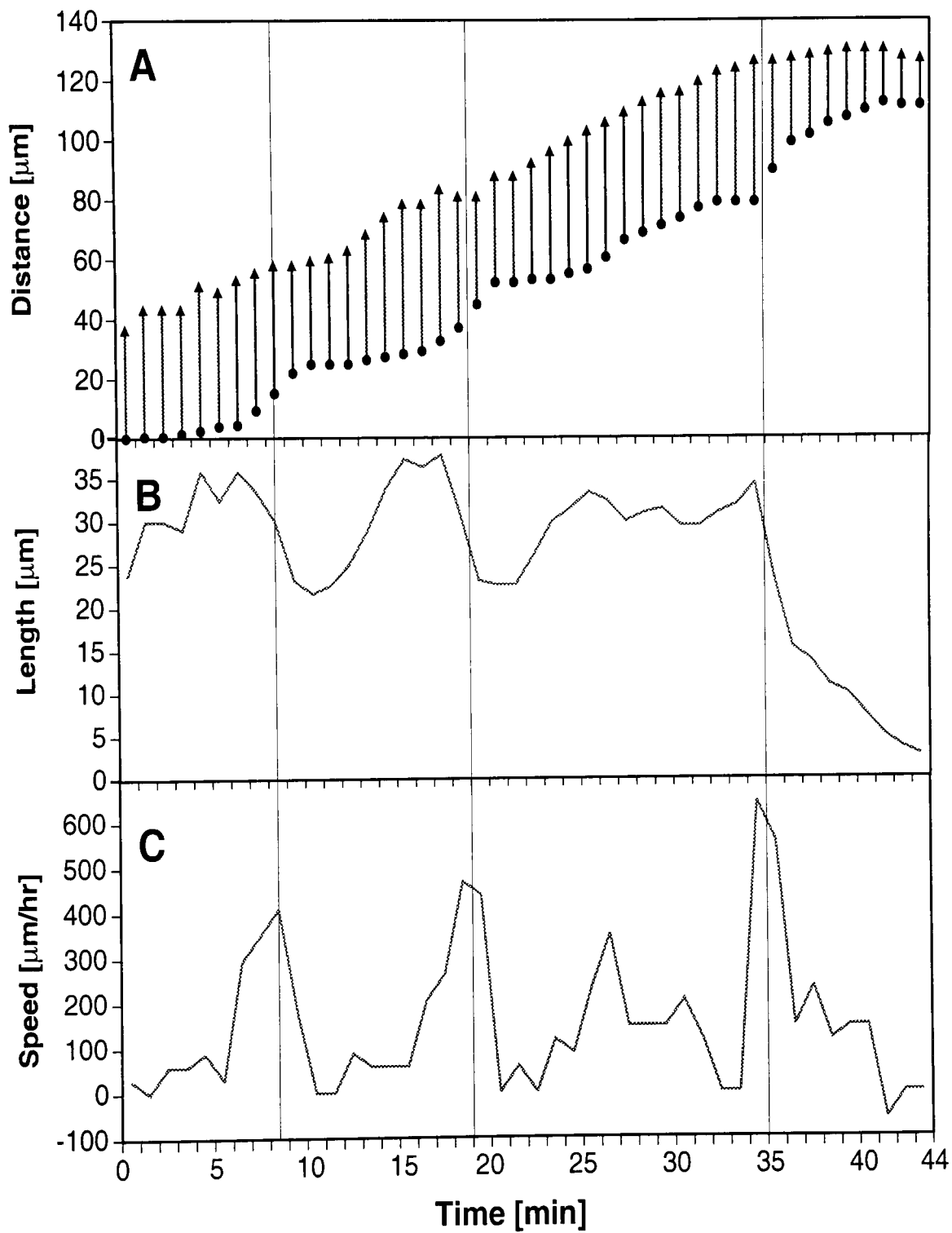
Behavior of a cell, for which I was able to trace cell body as well as growth cone position, was studied in one minute intervals during a 44 minute period.

A: The cell body (circle) and growth cone (arrow head) positions as a function of time.

B: The leading process length during the cell migration was calculated from cell body and growth cone positions.

C: Speed of cell body translocation was calculated from distances between two consecutive cell body positions.

Notice that periods of highest speed of cell body translocation correspond to periods of leading process contraction.

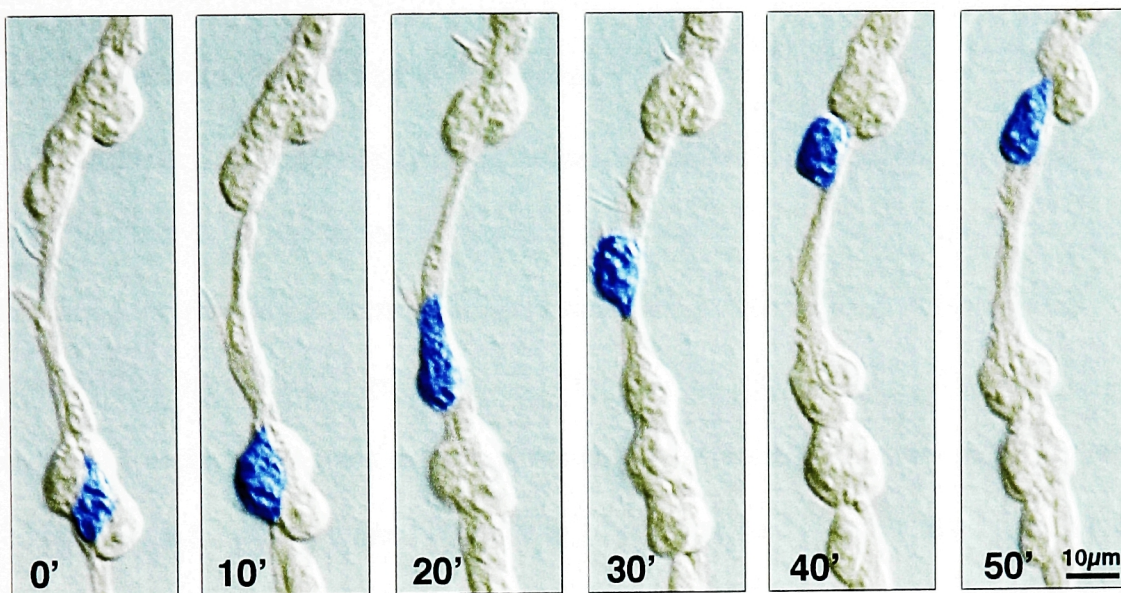


Behavior of leading processes

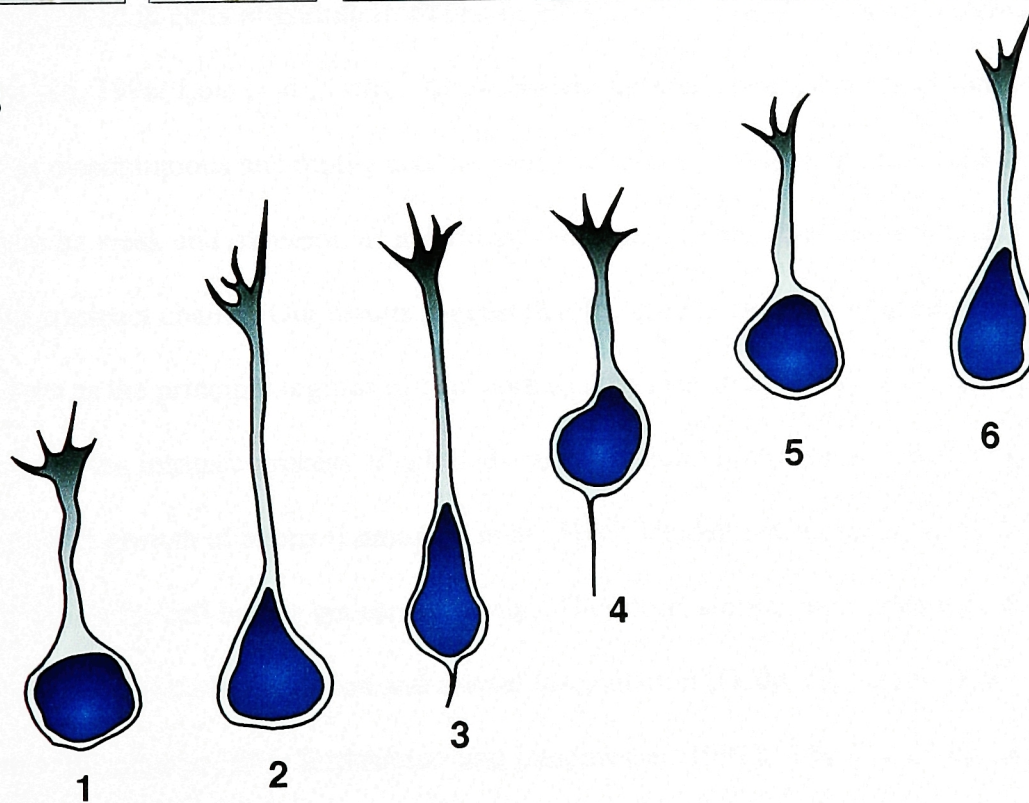
The behavior of the growth cone and leading process during cell migration was difficult to analyze because cells in chains were usually tightly packed. However, on a few occasions the leading process was clearly visible. This occurred in cultures in which chains formed a spread meshwork, or when cells moved from one chain to an adjacent one (Fig. 12, see jumping cell in CD-ROM movies). The dynamics of cell body translocation in these cases were similar to that of cells in compact chains, but allowed me to analyze in more detail the individual steps underlying chain migration (Fig. 14). Similar behavior was observed in 5 other cells that were analyzed in this detail. Initially, the cell body was stationary, but the growth cone was extremely active as if exploring the territory by rapid extensions and retractions of long filopodia and short lamellae. The leading process could repeatedly extend and partially retract without a net change in the cell body position (Fig. 13). This exploratory behavior lasted from a few minutes to several hours. Judging from the constancy in the maximum lengths of the leading process during several cycles of cell body translocation (Fig. 13), there seemed to be a limiting length that the leading process can attain. This maximal length ($\sim 40\ \mu\text{m}$) is similar to that observed in vivo (Kishi, 1987; Lois and Alvarez-Buylla, 1994). Cell body translocation is generally initiated at the moment when the leading process is maximally extended, and the movement of the nucleus correlates with a rapid shortening of the leading process (Fig. 12D,E & 13). Occasionally, a thin trailing process is left behind the cell (arrowhead, Fig. 12E) which rapidly retracts (Fig. 12F). Cell body translocation is followed by another cycle of exploratory behavior during which the cell can again extend the leading process and proceed in migration. Alternatively, the cell can retract the

Figure 14: Model of cell translocation during chain migration. Stationary cell first extends leading process (1,2). When the leading process reaches certain length the cells body starts to translocate into the leading process (2-4). A thin trailing process is usually left behind the cell (3,4) which rapidly retracts (5). Cell body becomes stationary while the leading process is actively extending starting another step (6). Growth cone is extremely active during all stages of cell translocation.

A



B



leading process, become stationary, or form a new leading process in a different direction changing the orientation of its migration (data not shown).

Comparison of tangential and radial migration

Although some aspects of chain migration and glial-guided radial migration may be similar, there also appear to be important differences. A cell migrating along a glial fiber has a long trailing process and a lamellar extension on the leading process that wraps around the guiding fiber (Rakic, 1972). A specialized interstitial junction (Gregory et al., 1988), which is thought to be important for cell body displacement and guidance, forms between the cell body and the guiding fiber. In contrast, none of these structures were observed in cells migrating in chains in vitro (present results) or in vivo (Jankovski and Sotelo, 1996; Lois et al., 1996). The substrate for chain migration (other migrating cells) is discontinuous and motile and the adhesive forces among individual cells are likely to be weak and transient, as individual cells could easily slide past each other even in very compact chains. Our results suggest that the growth cones of migrating cells might act as the principal regions of firm contacts with substrate, serving as stable points supporting the intrinsic process of cell body translocation. In this sense, chain migration is more like growth of axons (Lamoreux et al., 1989; Heidemann and Buxbaum, 1994), but in which the cell bodies get carried along. Therefore, similar molecules may be involved in both chain formation and axonal fasciculation (Dodd and Jessell, 1988; Walsh and Doherty, 1991; Rutishauser and Landmesser, 1991). The in vitro culture system developed here will allow further characterization of the molecular mechanisms underlying cell-cell interaction and cell translocation during chain migration.

Table 1: Comparison of parameters measured for chain and glial-guided migration in vitro.

| | n | Chain\pmSD | Radial\pmSD* | Chain/Radial |
|--|----------|--------------------------------|----------------------------------|---------------------|
| Soma length [μm] | 20 | 11.6 \pm 2.1 | 12 \pm 1.5 | 1.0 |
| Soma width [μm] | 20 | 5.6 \pm 1.8 | 6 \pm 1 | 0.9 |
| Leading process length [μm] | 26 | 29.3 \pm 4.9 | 19 \pm 3.5 | 1.5 |
| Duration of step [min] | 93 | 4.1 \pm 1.7 | 4.8 \pm 1.6 | 0.9 |
| Duration of pause [min] | 91 | 2.2 \pm 2.6 | 4.3 \pm 2.1 | 0.5 |
| Step length [μm] | 58 | 19.4 \pm 7.0 | 4 \pm 2 | 4.8 |
| Maximum speed [$\mu\text{m/hr}$] | 93 | 301.5 \pm 134.7 | 56 \pm 26 | 5.4 |
| Average cell speed [$\mu\text{m/hr}$] | 22 | 121.7 \pm 37.3 | 33 \pm 20 | 3.7 |

*Data published in (Edmondson and Hatten, 1987)

In summary, I demonstrated that young neurons in the SVZ can migrate along each other. I characterized this new form of neuronal migration and showed that it is distinct from previously described glial-guided migration. Glial-guided migration serves to transport young neurons through a biochemically and anatomically discontinuous environment; e.g. from the germinal layer across the cell-poor and cell-rich brain parenchyma to the target region. The nature of chain migration on the other hand suggests that it is well suited for rapid transport of a large number of neurons across fairly uniform, cell dense regions independent of radial glia. This opens the possibility that chain migration is not only utilized in the postnatal and adult brain, but may play an important role in cell dispersion during prenatal brain development. Several reports described tangential neuronal migration through embryonic germinal layers (Rakic and Sidman, 1969; Fishell et al., 1993) and developing cortex (Gadisseux et al., 1992; Menezes and Luskin, 1994; O'Rourke et al., 1995; O'Rourke et al., 1995; De Carlos et al., 1996). In the following chapter I analyze the embryonic brain in search for neuroblasts capable of similar homotypic chain migration in vitro as described here for postnatal SVZ cells.

CHAPTER 3: SOURCES OF CHAIN MIGRATING NEURONS IN THE EMBRYONIC BRAIN

In the previous chapter I described a novel form of tangential migration called chain migration, which is used by postnatal SVZ cells. This set of experiments raised many questions for me. The most intriguing of them was, whether chain migration evolved only to transport neurons from the SVZ to the olfactory bulb or whether is this form of cell movement used more widely, perhaps by some migrating neurons in the embryonic brain? I used the same assay I developed for studying postnatal chain migration to find out if similarly moving neuronal precursors reside in some of the embryonic CNS regions known to contain tangentially migrating neurons.

Identification of embryonic regions containing tangentially migrating neurons

Using the Matrigel migration assay, I identified only two regions in the embryonic mouse brain which contain neuronal precursors capable of extensive migration in Matrigel. These regions called medial and lateral ganglionic eminences (MGE and LGE) are primordia for developing basal ganglia in the ventral forebrain (Fig. 15A). The embryonic ganglionic eminences are largest reservoirs of SVZ cells in the embryo. They develop in mediolateral walls of lateral ventricles, in a location analogous to that of the postnatal SVZ. Cells originating in the LGE and MGE are classically thought to contribute neurons to developing striatum and pallidum, respectively (Smart and Sturrock, 1979; Fentress et al., 1981). Molecular studies indicate that SVZ cells in the MGE and LGE become specified early during forebrain development. Several genes have been identified (Nkx2.1, Gbx2 and Lhx3, 6, 7) which are specifically expressed by

MGE cells, but not by LGE (Bulfone et al., 1993; Price, 1993; Matsumoto et al., 1996; Grigoriou et al., 1998). This suggests that the two forebrain regions contain distinct pools of neuronal precursors.

Indeed, when I compared cell migration in Matrigel cultures, I found that neuronal precursors from neocortex, LGE and MGE behave differently. Long fascicles of fibers grew from cortical explants, but only very few migrating cells penetrated the matrix (Fig. 15B). In contrast, cells from LGE and MGE explants migrated through Matrigel arranged as chains similar to those observed in cultures of postnatal SVZ (Wichterle et al., 1997) (Fig. 15C). Surprisingly, MGE cells migrated significantly more extensively than LGE cells. While neuronal precursors from LGE migrated $117 \pm 18 \mu\text{m}$ ($n=32$) in one day, MGE cells migrated 2.4 times farther ($280 \pm 54 \mu\text{m}$ ($n=38$)) from the edge of similar size explants (Fig. 15D). Comparable extensive migration was observed when MGE was dissected from other embryonic stages in which MGE is identifiable (E12, 13, 15 and 16). Since LGE and MGE are approximately the same size and clearly demarcated between embryonic day 13 and 14, I used this age in most of the following studies³. None of the other embryonic brain regions tested (hypothalamus, thalamus, superior colliculus, ventral midbrain, rhombic lip, spinal cord) contained cells with similar migratory capacity. I concluded that ganglionic eminences and especially MGE contain neuronal precursors with a unique capacity to penetrate the reconstituted extracellular matrix (i.e. Matrigel) in vitro.

³ Wherever I refer to the embryonic brain without specifying the age of the animal, I mean E13-E14 embryos.

Figure 15: Migration of embryonic neuronal precursors in vitro.

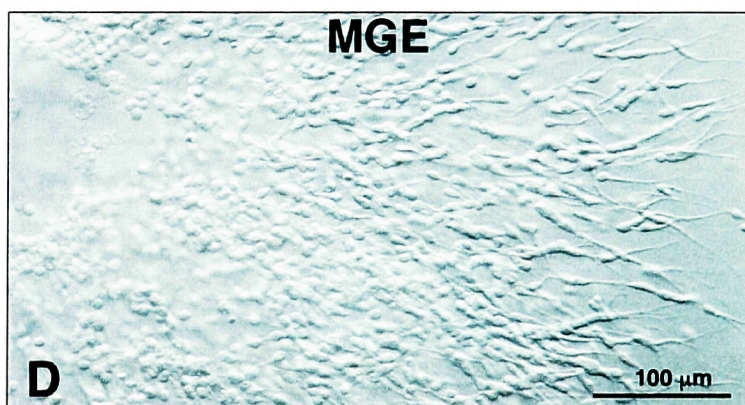
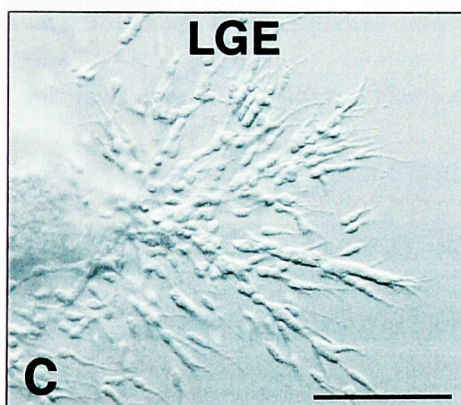
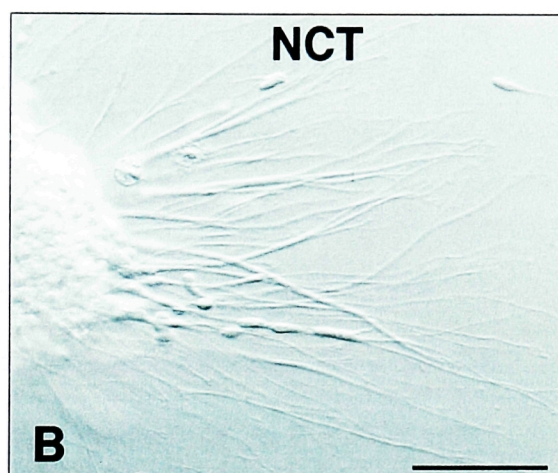
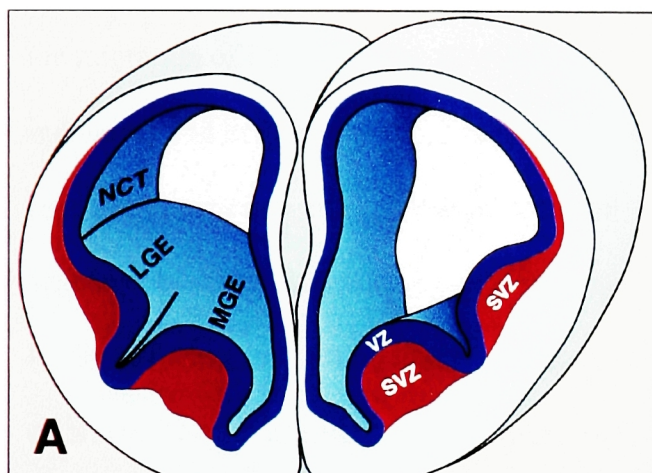
A, Schematic coronal section through 14-day-old embryonic forebrain. Anterior faces the reader, dorsal is to the top. Subventricular (SVZ) and ventricular zones were dissected from medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE) and neocortex.

B-D, Differential interference contrast micrographs of embryonic explants cultured for 24 hours embedded in matrigel. Scale bar = 100 μ m

B, Only a few cells migrated from cortical explants. Most of these cells migrated along fibers that extended from the explant (arrowheads), with rare cells migrating individually (arrow).

C, From LGE explants, cells migrated mostly along each other and frequently formed chains (arrow).

D, Extensive migration of cells from MGE explants. Cells were arranged in loose chains or migrated individually forming a meshwork of cells surrounding the explant (star).



Dynamics of MGE cell migration in vitro

MGE cells migrate more extensively in Matrigel than LGE or postnatal SVZ cells. Is this due to different dynamics of cell translocation or is it merely a reflection of the higher capacity of MGE cells to penetrate Matrigel? To address this issue I studied the migration of MGE cells by time-lapse microscopy and compared movement of individual cell bodies with those of postnatal SVZ cells. The dynamics of MGE cell migration are very similar to that of postnatal SVZ cells. MGE cell movement is saltatory, bi-directional with growth cones actively exploring the environment and leading process contracting during cell body translocation. The average speed of MGE cells was 107 $\mu\text{m/hr}$ and maximum speed during the cell translocation was 313 $\mu\text{m/hr}$, which is almost identical to speeds measured for postnatal SVZ cells migration (122 respectively 302 $\mu\text{m/hr}$). These results suggest that the dynamics of MGE cell migration are not inherently different from movement of LGE (91 respectively 311 $\mu\text{m/hr}$) or postnatal SVZ cells (Table 2). As mentioned above, the speed of SVZ or MGE cell migration along established chains in Matrigel is very similar ($\sim 110 \mu\text{m/hour}$). In contrast, the progression of MGE cells at the tips of the chains (the rate of SVZ cells penetration into the Matrigel) is much higher for MGE cells (280 $\mu\text{m/day}$) compared to LGE (120 $\mu\text{m/day}$) or postnatal SVZ cells (85 $\mu\text{m/day}$). Thus, the rate-limiting step in cell migration through Matrigel is the ability of cells to penetrate the gel. The fact that MGE cells migrate more extensively through Matrigel while the dynamics of their movement are identical to that of LGE or SVZ cells indicates that MGE cells have a superior capacity to penetrate and invade Matrigel.

Table 2: Migration parameters of SVZ, LGE, and MGE cells in vitro.

| | SVZ | MGE | LGE |
|---|---------------|---------------|---------------|
| Cell speed [$\mu\text{m/hr}$] | 122 \pm 37 | 107 \pm 27 | 91 \pm 30 |
| Peak speed [$\mu\text{m/hr}$] | 302 \pm 134 | 313 \pm 111 | 311 \pm 149 |
| Penetration into Matrigel [$\mu\text{m/day}$] | 84 \pm 46 | 280 \pm 54 | 117 \pm 18 |

Differential potential of LGE and MGE cells transplanted into the adult brain

LGE and MGE cells are capable of similar migration through Matrigel like postnatal SVZ cells. To address whether the migratory potential of these three cell types is identical, I studied the behavior of LGE and MGE cells after transplantation into the adult SVZ. If all cells capable of chain migration in Matrigel belong to the same category of neuronal precursors, transplanted LGE and MGE cells should migrate from the adult SVZ to the olfactory bulb exactly like endogenous SVZ cells do. To follow neurons derived from embryonic grafts I transplanted germinal layers dissected from transgenic embryos (E14) carrying the LacZ reporter gene under the control of neuron specific promoter (NSE::LacZ) (Forss-Petter et al., 1990). This allowed me to identify graft-derived neurons (stained blue with X-Gal) within the host brain.

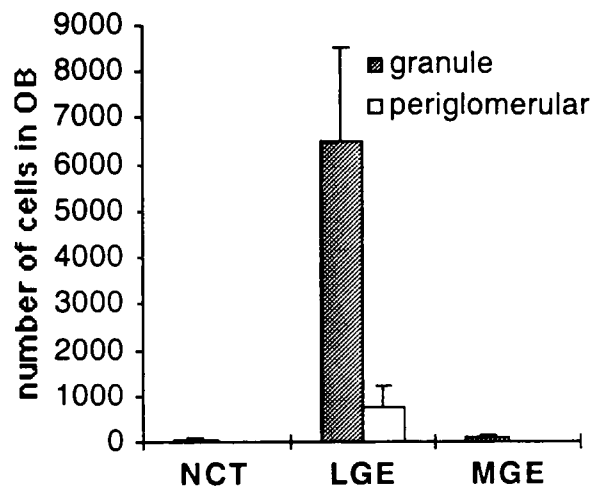
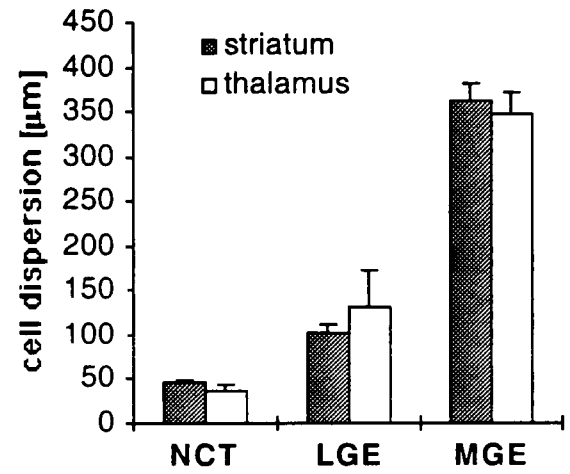
LGE cells migrate from SVZ to the olfactory bulb

As a control tissue I transplanted neuronal precursors from the neocortex, which normally migrate along radial glia and do not migrate in my in vitro assay. Cortical cells grafted into the SVZ differentiated into a large mass of neurons at the site of transplantation, unable to follow the tangential migratory pathway to the olfactory bulb (Fig. 16A) (Zigova et al., 1996). In contrast, large numbers of LGE neuronal precursors grafted into the adult SVZ migrated to the olfactory bulb, where they differentiated into granule and periglomerular neurons (Fig. 16A,20). The behavior and potential of LGE precursors was similar to that of postnatal (Luskin, 1993) or adult SVZ cells (Lois and Alvarez-Buylla, 1994) suggesting that these two populations are closely related. This finding strongly indicates that the postnatal SVZ is the direct descendant of the

Figure 16: Quantification of neuronal dispersion and migration from the SVZ to the olfactory bulb.

A, Numbers of neurons which migrated into the olfactory bulb (OB) from grafts placed into the SVZ (n=4). Filled bars represent neurons in the granule cell layer, open bars represent periglomerular neurons. Error bars correspond to S.E.M. Significantly larger number of LGE neurons migrated into the olfactory bulb compared to MGE or neocortical grafts (t-test, $p < 0.01$).

B, Histogram of average distances which neurons penetrated into the host brain. Filled bars represent transplants into striatum (n=5), open bars represent transplants into thalamus (n=4). Error bars correspond to S.E.M. The distance migrated by MGE cells is significantly greater than the distance migrated by neocortical or LGE cells (t-test, $p < 0.01$).

A**B**

embryonic LGE. Interestingly, the embryonic LGE is considered to give rise mainly to striatal neurons. For this reason it has been extensively studied as a source of neuronal precursors for brain repair in striatal neurodegenerative diseases (Freeman et al., 1995; Nakao et al., 1996; Isacson and Deacon, 1997). When early LGE cells (~E12) are grafted into the adult striatum lesioned with excitotoxic acids they give rise to a large mass of neurons with organization and neurotransmitter characteristics similar to those of the host striatum (Deacon et al., 1994; Grasbon-Frodl et al., 1996; Watts et al., 1997) (Olsson et al., 1995). It is possible that the early LGE contains mostly precursors of striatal neurons. Later in development this region might also accommodate a population of olfactory bulb precursors, which then predominate in the adult brain, when the LGE transforms into the postnatal SVZ.

MGE cells disperse through the adult brain

Surprisingly, very few MGE precursors grafted into the adult SVZ followed the rostral migratory stream to the olfactory bulb (Fig. 16A). Instead, neurons from MGE transplants dispersed through the adult brain tissue, differentiating into neurons in striatum, cortex, and septum. This result was truly unexpected. No one before has described dispersion of primary neuronal precursors through the adult brain parenchyma. To the contrary, it is generally believed that the adult brain contains molecules inhibitory for cell migration and axonal outgrowth (Cadelli and Schwab, 1991; Fawcett and Asher, 1999; GrandPre et al., 2000; Chen et al., 2000). To test whether MGE cells are able to migrate through other parts of the adult brain I transplanted embryonic tissue into striatum, neocortex and thalamus. Neurons derived from neocortical and LGE grafts placed into these regions remained close to the site of transplantation (Fig. 17A,B,D,E;

16B). In contrast, neurons from MGE grafts (on average 1630 ± 140 graft-derived neurons per brain) dispersed extensively through the host striatum, thalamus (Fig. 17C,F; 16B), and neocortex. These cells migrated up to 1.3 mm away from transplantation sites. Some neurons from striatal grafts crossed into septum and cortex (Fig. 17C). MGE cells dispersed in all directions with very few neurons differentiating at the sites of transplantation (star, Fig. 17F). This suggests that most grafted cells are migratory, or that non-migratory MGE neuronal precursors either failed to differentiate into neurons or died when grafted into the adult brain. My study of MGE cell migration in the Matrigel indicated that migratory behavior of these cells is comparable to the LGE and the postnatal SVZ cells (Table 2). What seemed to be different was the ability of MGE cells to penetrate the extracellular matrix. Now I have shown that MGE cells have extraordinary capacity to disperse through adult brain parenchyma. It is therefore possible that the increased capacity of MGE cells to invade Matrigel in vitro reflects the ability of these cells to migrate through adult brain tissue.

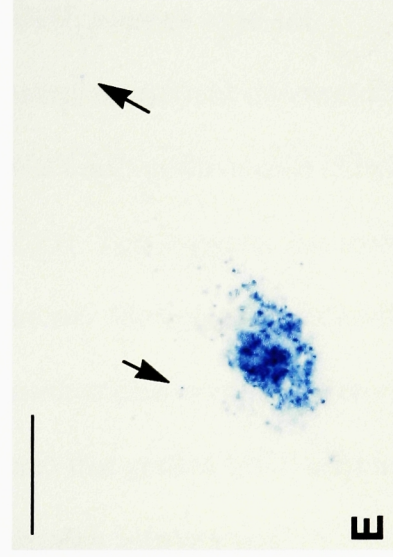
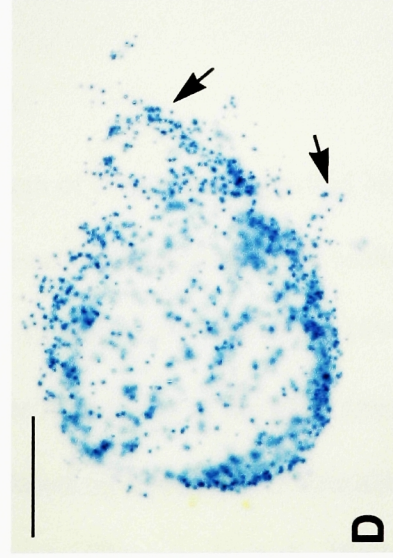
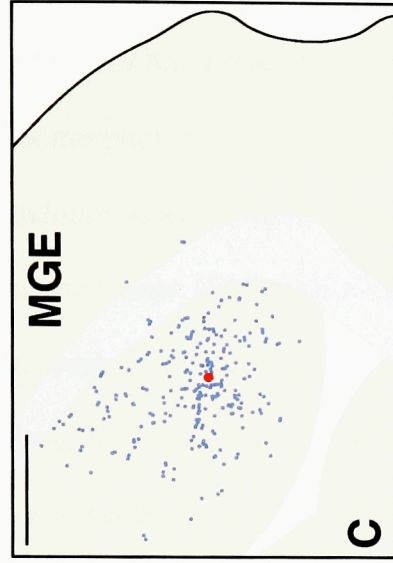
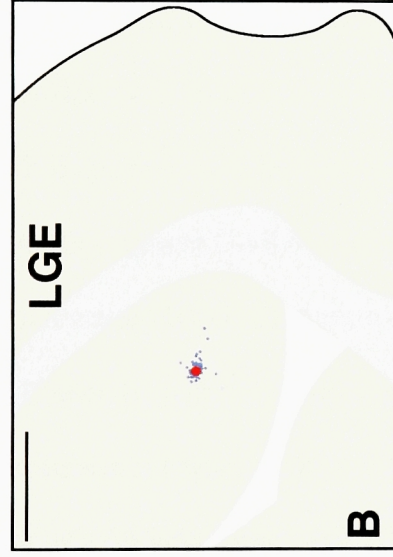
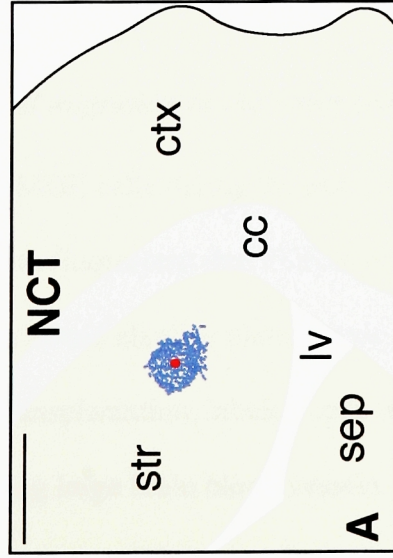
Dispersed LacZ-positive MGE cells are neurons.

Cells dispersed from MGE grafts expressed β -galactosidase under control of the NSE promoter suggesting that they had differentiated into neurons. However, despite the name, NSE is also expressed in some glial cells (Herrera et al., ; Lin and Matesic, 1994). It is known that glial cells have the ability to migrate in the adult brain environment (Emmett et al., 1991; Hatton et al., 1992). Therefore it was critical for my study to show that MGE derived cells are neurons. I confirmed the neuronal phenotype of MGE-derived, LacZ-expressing cells by immunocytochemistry and electron microscopy. Graft-derived cells stained by X-Gal also expressed neuronal markers NeuN (Latov et al.,

Figure 17: Transplants of neocortex, LGE and MGE into the adult striatum.

A-C, Maps of horizontal sections through host brains 1 month after transplantation with blue dots representing LacZ-expressing grafted neurons. Red dot is the site of transplantation. Anterior is to the right, lateral to the top. cc - corpus callosum; ctx - cortex; lv - lateral ventricle; sep - septum; str - striatum. Scale bar = 500 μ m

D-F, Photographs of corresponding graft sites. neocortical grafts contained a large mass of neurons (blue X-Gal staining) with clearly demarcated boundaries. Clusters of cells protruded short distances into the host parenchyma (arrows, D). Sites of LGE grafts contained smaller number of cells. Few neurons penetrated a short distance into the host tissue (arrows, E). Most of MGE cells have dispersed in the host striatum (arrows, F). Very few neurons remained at sites of MGE grafts (star, F). Scale bar = 100 μ m



1979) (Fig. 18A) and Hu (Marusich et al., 1994), but not GFAP or vimentin (not shown). MGE cells in semithin sections had a large round or oval nuclei with one or two central nucleoli (Fig. 18B); this morphology was very similar to that of neighboring striatal neurons. Sixty-two randomly selected MGE cells expressing LacZ were resectioned for analysis at the electron microscope (EM). All 62 cells studied at the EM were neurons. They had Nissl bodies, rough ER, Golgi apparatus, mitochondria and subsurface cisternae typical of neurons (Peters, E., Palay, S. L., and Webster de F., H.1991). Most of the neurons also had deep nuclear invaginations, and in some of the sections axo-somatic synapses were observed (Fig. 18E,F). Transplanted MGE neurons were not immunoreactive for tyrosine hydroxylase or choline acetyl transferase (markers for dopaminergic and cholinergic neurons), but one-third of LacZ-positive cells ($32\pm 7\%$) were stained with antibodies against GABA (Fig. 18C,D). This suggests that some of the grafted MGE cells differentiated into GABAergic neurons. More experiments will be required to determine if neurons with other neurotransmitter phenotypes are derived from the migrating MGE precursors. These results confirmed that grafted MGE cells migrate a long distance, integrate into the host tissue, and differentiate into neurons.

Dynamics of MGE cell migration in the adult brain.

To follow the MGE cells during the early stages of dispersion, I grafted MGE cells either labeled with fluorescent dye PKH26 or dissected from transgenic animals expressing membrane bound alkaline phosphatase in all cells (Deprimo et al., 1996). Two hours after the transplantation, labeled cells were found only at the graft site (Fig. 19A) or deposited along large brain blood vessels. Based on a similar perivascular location of grafted astrocytes, Schwann cells and neurons (Kawamura et al., 1988;

Figure 18: Characterization of transplanted MGE cells.

Horizontal sections of host brains stained with X-Gal.

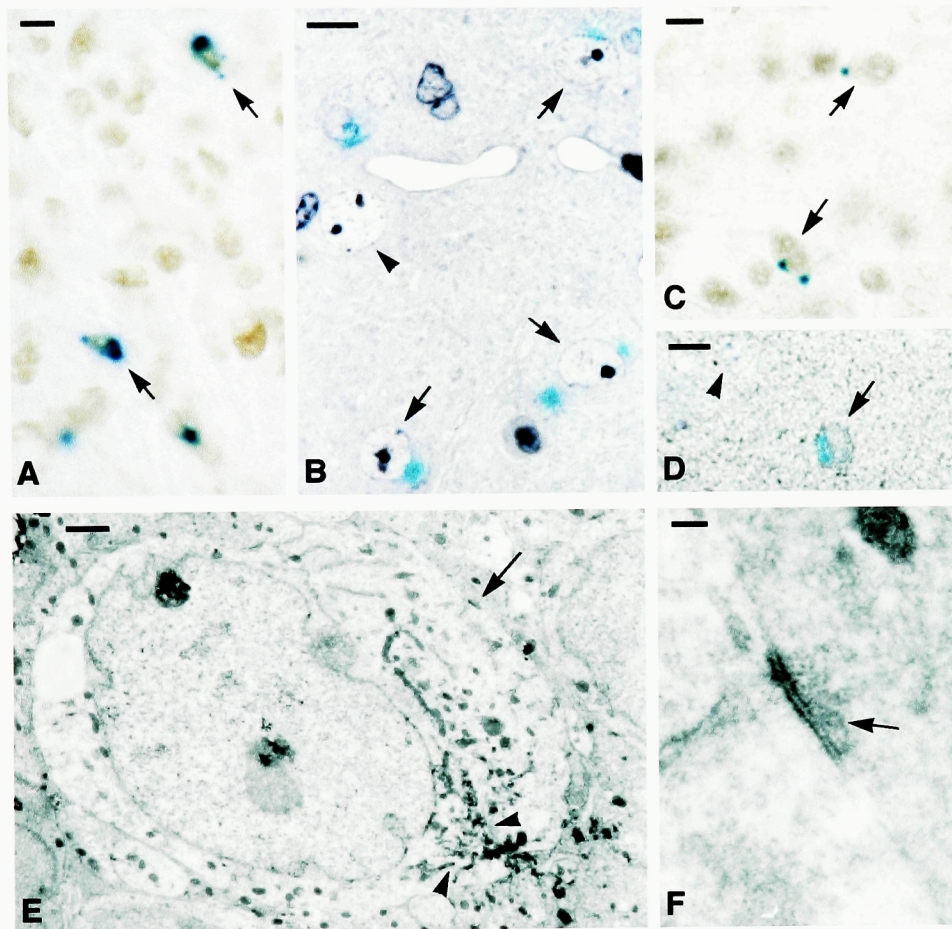
A, Immunostaining with NeuN antibody (brown color) revealed that many of the transplanted cells expressing LacZ are also immunoreactive for this neuron specific marker (arrows). Scale bar = 10 μ m

B, Semithin section counterstained with toluidine blue. Transplanted neurons (arrows) resemble morphologically the resident striatal neurons (arrowheads). Scale bar = 10 μ m

C, D, Transplanted LacZ-positive cells are double labeled with antibodies against GABA (brown color). Colocalization of LacZ and GABA staining in thick sections was confirmed in semithin sections (d). Because the blue X-Gal precipitate is lost during immunostaining of semithin sections, two images before and after staining for GABA were superimposed. Arrows point to double labeled cells, arrowhead points to a GABA negative resident striatal neuron. Scale bar = 10 μ m

E, Electron micrograph of a transplanted MGE cell. The X-Gal precipitate is visible close to the nucleus (arrowheads). Arrow points to an axo-somatic synapse. Scale bar = 1 μ m

F, Higher magnification of the synapse from (E) reveals synaptic vesicles at the axon terminal (arrow). Scale bar = 100 nm



Raisman et al., 1993; Andersson et al., 1993), it has been suggested that blood vessels may provide a pathway for cell migration in the adult brain. Although I do not exclude this possibility, our observations indicate that many of the graft-derived cells found close to brain vasculature, were passively deposited into perivascular spaces during the transplantation. It is possible that during intraparenchymal injection of cells, local increase in hydrostatic pressure causes momentary collapse of large blood vessels, allowing grafted cells to fill the perivascular space.

At 24 hours after transplantation many cell processes tipped with growth cones were observed extending from the site of transplantation. Few cells escaped the graft at this survival time and started to penetrate into the host brain parenchyma.

By 48 hours many grafted MGE cells extended processes and migrated individually through the brain tissue (Fig. 19B). MGE cells moved as far as 580 μm from the site of transplantation, suggesting that between day 1 and 2 after transplantation MGE cells actively invade the host brain. Migrating MGE cells were frequently found associated with large axonal tracts suggesting that some MGE cells disperse using axons as guides (Fig. 19B) (Rakic, 1990; O'Rourke et al., 1995). However, some MGE cells were oriented perpendicularly to the main axonal bundles, suggesting that they can use other substrates as well. It will be interesting to determine the cellular and molecular mechanism used by MGE neuronal precursors to migrate in the adult brain. This information may allow the modification of other cell types enabling them to disperse through the adult brain, which could have major clinical implications. The morphology of these cells was very similar to other tangentially migrating neuronal precursors (Luskin, 1993; Lois and Alvarez-Buylla, 1994; O'Rourke et al., 1995) (Fig. 19B,E).

Figure 19: Timecourse of MGE cell dispersion in the adult brain. Horizontal sections through the adult brain with transplanted cells expressing alkaline phosphatase.

A) Two hours after transplantation MGE cells are concentrated at the site of transplantation. There are no migratory cells or neuronal processes extending into the host brain tissue.

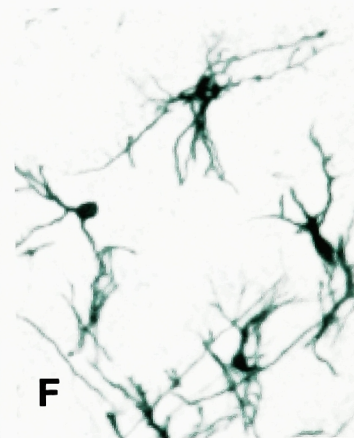
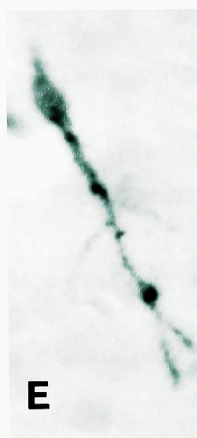
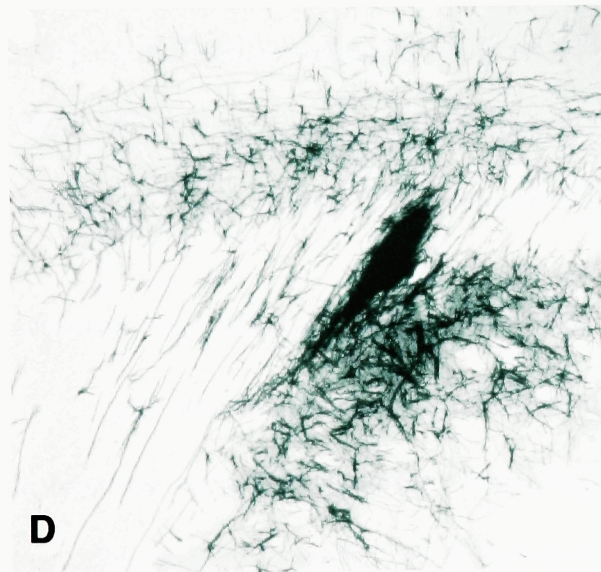
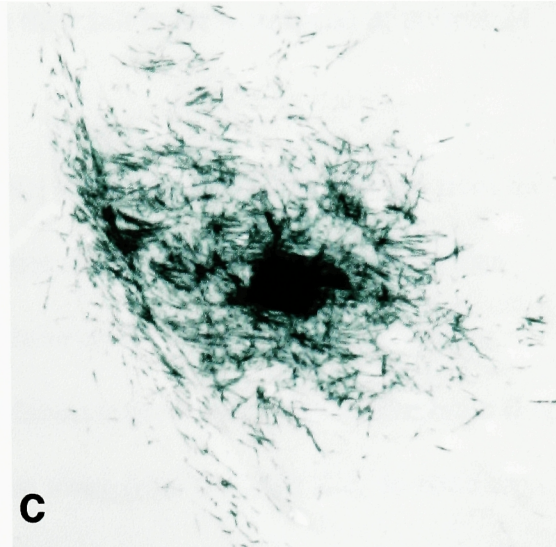
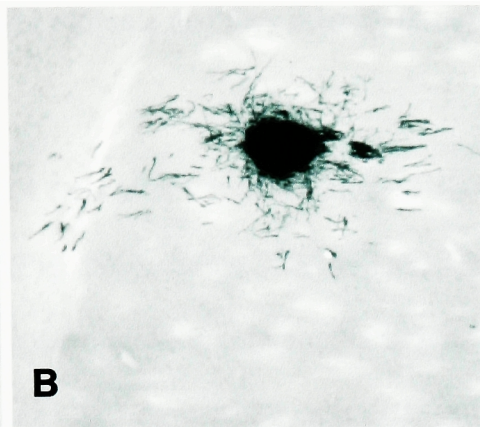
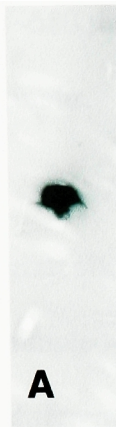
B) At two days there are many MGE cells migrating through the adult brain tissue. Notice that elongated migratory cells are often aligned with white matter tracts in the striatum (in the anteroposterior direction or left-right in this figure). The most anterior cells (at the left) reached corpus callosum and aligned themselves with callosal fibers (lateromedial orientation or top-bottom in this figure)

C) Hundreds of graft-derived migrating cells are penetrating into the host brain at four days after transplantation.

D) At seven days after transplantation many MGE derived cells started to differentiate. Notice long neurites extended from many cells. Almost no bipolar migratory cells are observed at this time point.

E) Example of a typical bipolar migratory cell observed at 2 days after transplantation.

F) Shape of differentiating MGE cells in the adult brain 7 days after transplantation.



They were bipolar with a long leading process (sometimes bifurcated) tipped with a growth cone and occasionally showing a fine trailing process (Fig. 19E). Based on the difference in the position of MGE cells at 24 hours and 48 hours after transplantation (~580 μm) I estimate that they migrate through the adult brain with speed of at least 24 $\mu\text{m/hr}$.

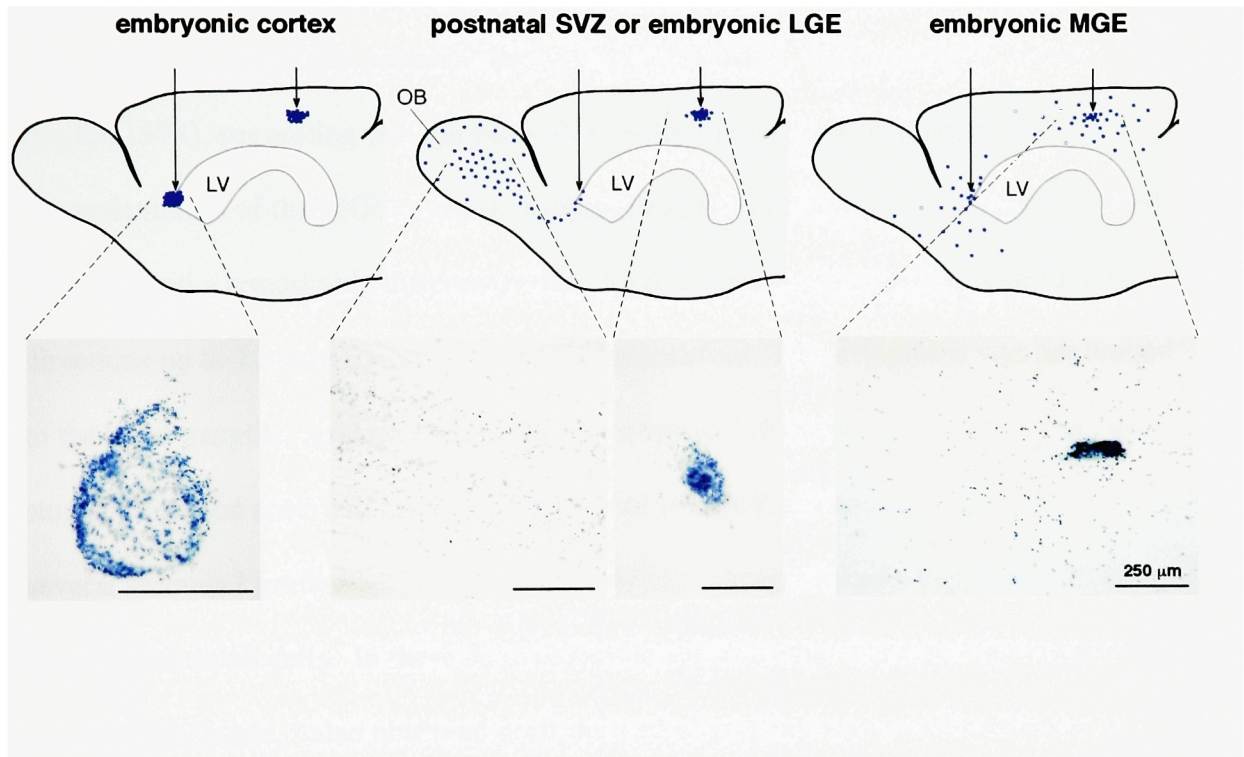
At 4 days after transplantation, MGE cells were found as far as 900 μm from the graft site (Fig. 19C). Some MGE cells had longer and more elaborate processes than simple bipolar migrating cells suggesting that these cells settled down and started to differentiate. At 7 days, MGE cells extended elaborate processes into the host brain (Fig. 19D,F). MGE cells were found as far as 1.1 mm away from the graft site. In contrast to MGE, labeled neocortical or LGE cells grafted into striatum extended neurites into the host tissue, but no migration of neuronal precursors was observed.

Migratory behavior as a unique characteristics of neuronal precursors

The developing embryonic brain is divided into compartments defined by differences in anatomy, patterns of gene expression, cellular phenotypes, and regional fates (Redies and Takeichi, 1996; Levitt et al., 1997; Rubenstein et al., 1998). Here I studied cells from three distinct telencephalic regions in vitro and after transplantation into the adult brain. I show that in addition to the above-mentioned differences, neuroblasts derived from each of these regions exhibit unique migratory behaviors (Fig. 20).

Neocortical neuronal precursors, when grafted into adult brain, differentiate into a large mass of neurons at transplantation sites. The limited migration of grafted cortical

Figure 20: Summary of embryonic cell transplantation into the adult brain. Neuronal precursors from embryonic cortex remain at the site of transplantation unable to migrate in the adult brain. LGE cells are unable to migrate when grafted into the brain parenchyma, but migrate into the olfactory bulb when grafted into the adult SVZ. MGE cells disperse in all directions from the site of transplantation, but do not follow the migratory route from the SVZ to the olfactory bulb.



precursors is likely due to the absence of radial glial guides in the adult brain (Rakic, 1990). In contrast, LGE cells migrated selectively and on a large scale along the restricted migratory pathway from the SVZ to the olfactory bulb, where they differentiated into granule and periglomerular neurons. LGE cells, however, were unable to migrate long distances through the striatum or thalamus. This behavior and potential is very similar to that of the postnatal and adult SVZ (Luskin, 1993; Lois and Alvarez-Buylla, 1994), suggesting that the adult SVZ is derived from the embryonic LGE. Transplantation of the MGE, a region neighboring the ventromedial LGE, into the adult brain yielded a remarkably different result. MGE neuronal precursors dispersed in all directions up to 1.3 mm from the site of transplantation. This dispersion was not limited to the basal ganglia—grafted MGE cells also dispersed in thalamus and neocortex. In my studies I focused specifically on the behavior of neuronal precursors. However, in several animals I grafted MGE cells dissected from transgenic mice expressing alkaline phosphatase in all cells. In these animals besides neurons that dispersed far from the transplantation site, I also observed graft-derived oligodendrocytes differentiating in the white matter in the corpus callosum and striatal white matter. In contrast to neurons, oligodendrocytes did not disperse long distances in the adult brain ($<500\text{ }\mu\text{m}$). Of the neurons derived from MGE grafts, about one third was stained with an antibody to GABA, suggesting that many of the graft-derived cells differentiated into inhibitory neurons.

Identification of two compartments in the ventral forebrain which contain cells capable of chain migration through Matrigel suggested that this new form of neuronal translocation (first identified in postnatal and adult SVZ) is used widely during forebrain

development. I found that differences in the extent of Matrigel penetration translate into differences in migratory behavior in the adult brain. The unprecedented capacity of MGE cells to disperse long distances after transplantation into the adult brain has obvious therapeutic application which will be discussed in Chapter 5 of my dissertation.

So far, I detected increased migratory capacity of MGE cells in vitro and after transplantation into the adult brain. The unique migratory behavior of MGE cells and specifically their capacity to penetrate the adult brain parenchyma suggest novel mechanisms for neuronal migration. In the next chapter, I will explore the possibility that the extensive dispersion of MGE cells in the adult brain may reflect their normal behavior in the embryo.

CHAPTER 4: MEDIAL GANGLIONIC EMINENCE CELLS: THE BRAIN INVADERS

In the previous chapter, I described a population of neuronal precursors in the ventral forebrain (MGE) which have an extraordinary capacity to migrate through the adult brain. Is it possible that MGE cells behave similarly in the embryo? It is believed that during the brain development medial ganglionic eminence (MGE) gives rise to pallidum (ventral basal ganglia). This conclusion is based on anatomical observations (Smart and Sturrock, 1979; Fentress et al., 1981) and patterns of gene expression (Shimamura et al., 1995; Pera and Kessel, 1998), both of which might be misleading. Anatomical studies follow development of morphologically defined structures and can easily miss cells migrating from one brain region to another. Genes which seem to be regionally expressed labeling one cell population might be downregulated when cells move from their original place into a new environment.

Since MGE cells could migrate easily in relatively impermeable environment of the adult brain, it is possible that similar migration happens also in their normal environment in the embryonic brain. Recently it has been proposed that some neocortical neurons might originate in an extracortical region in the ventral forebrain (DeDiego et al., 1994). This hypothesis was supported by in vitro observations of labeled cells migrating from ganglionic eminences to neocortex (De Carlos et al., 1996; Tamamaki et al., 1997) and by analysis of *Dlx1/2* double knockout mice (Anderson et al., 1997a). However, it remains unclear how extensive this migration is and exactly in which region these cells originate. Another migratory pathway, from ganglionic eminences to the thalamus, was discovered in human and primate brain (Rakic and Sidman, 1969; Letinic and Kostovic, 1997). With the reference to these discoveries, it would be interesting to determine

whether the MGE might be the source of widely dispersing neurons populating the neocortex and thalamus in the embryonic brain.

Organotypic slices

Tracing of DiI labeled cells

Initially I analyzed MGE cell migration using organotypic cultures of embryonic brain slices. This system allows precise labeling of specific regions of the developing brain. In order to compare the migratory potential of precursors in different embryonic brain regions, I used localized injections of DiI in E14 brain slices cultured on floating polycarbonate filters. Twenty-four hours after DiI injections into the MGE, I observed large numbers of labeled MGE cells (247 ± 82 , $n=13$) migrating dorsolaterally into the developing striatum and piriform cortex, with some cells detected as far as neocortex (Fig. 21). A neuronal migratory pathway from ganglionic eminences to thalamus has been identified in human embryonic brain (Rakic and Sidman, 1969; Letinic and Kostovic, 1997). To test if similar migration might happen in rodent brain I prepared a set of brain slices cut in varying angles to include ganglionic eminences and hypothalamus or thalamus. Although in all preparations I observed MGE cell migration dorsally towards the neocortex, I never observed a single MGE cell migrating ventrally into diencephalon (Fig. 21A,B). This experiment suggests that migratory cues present in brain slices guide MGE cells dorsally or that ventral regions are inhibitory for MGE cell movement. Cells migrated individually; their shape was bipolar similar to that observed when MGE cells were grafted in the adult brain (Fig. 21C). The majority of labeled MGE cells moved dorsally in a wide area within the developing striatum. A smaller

number of labeled MGE cells migrated within the ventricular zone of the LGE, and only rarely were MGE-derived cells observed within the SVZ of the LGE (Fig. 21A,B).

Dorsal migration of neuronal precursors born in rodent ganglionic eminences has been proposed in several recent publications (DeDiego et al., 1994; De Carlos et al., 1996; Tamamaki et al., 1997; Anderson et al., 1997a). However, it remains controversial whether these cells originate in LGE, MGE or both. Initial studies describing the tangential cell migration into neocortex concluded that the LGE is the source of these neuronal precursors (De Carlos et al., 1996; Tamamaki et al., 1997; Anderson et al., 1997a). The LGE origin of some cortical neurons was questioned by more recent studies (Lavdas et al., 1999) (Sussel et al., 1999). Analysis of *Lhx6* expression, a gene expressed in MGE but not LGE, revealed that this homeobox gene is expressed in the intermediate and marginal zones of neocortex, suggesting that *Lhx6* positive cells of neocortex might originate in the MGE (Lavdas et al., 1999). Additional evidence for MGE origin of some cortical neurons came from the analysis of *Nkx2.1*, another gene expressed in MGE but not in LGE. It has been suggested that MGE is phenotypically transformed into the LGE in *Nkx2.1* null mice (Sussel et al., 1999). Although LGE develops normally in these mice, there is a reduction in the number of cortical GABAergic cells suggesting that the missing neurons might be produced normally in the MGE (Sussel et al., 1999).

My experiments support the hypothesis that the MGE is the origin of many if not all neurons invading the neocortex. I observed large numbers (247 ± 82 , $n=13$) of DiI-labeled MGE cells migrating dorsolaterally towards the neocortex in cultured embryonic brain slices (Fig. 21D). In contrast, when DiI injections were placed into LGE, ten times fewer labeled cells (22 ± 11 , $n=7$) were observed to migrate away from the injection site.

Figure 21: Migration of cells in the embryonic brain.

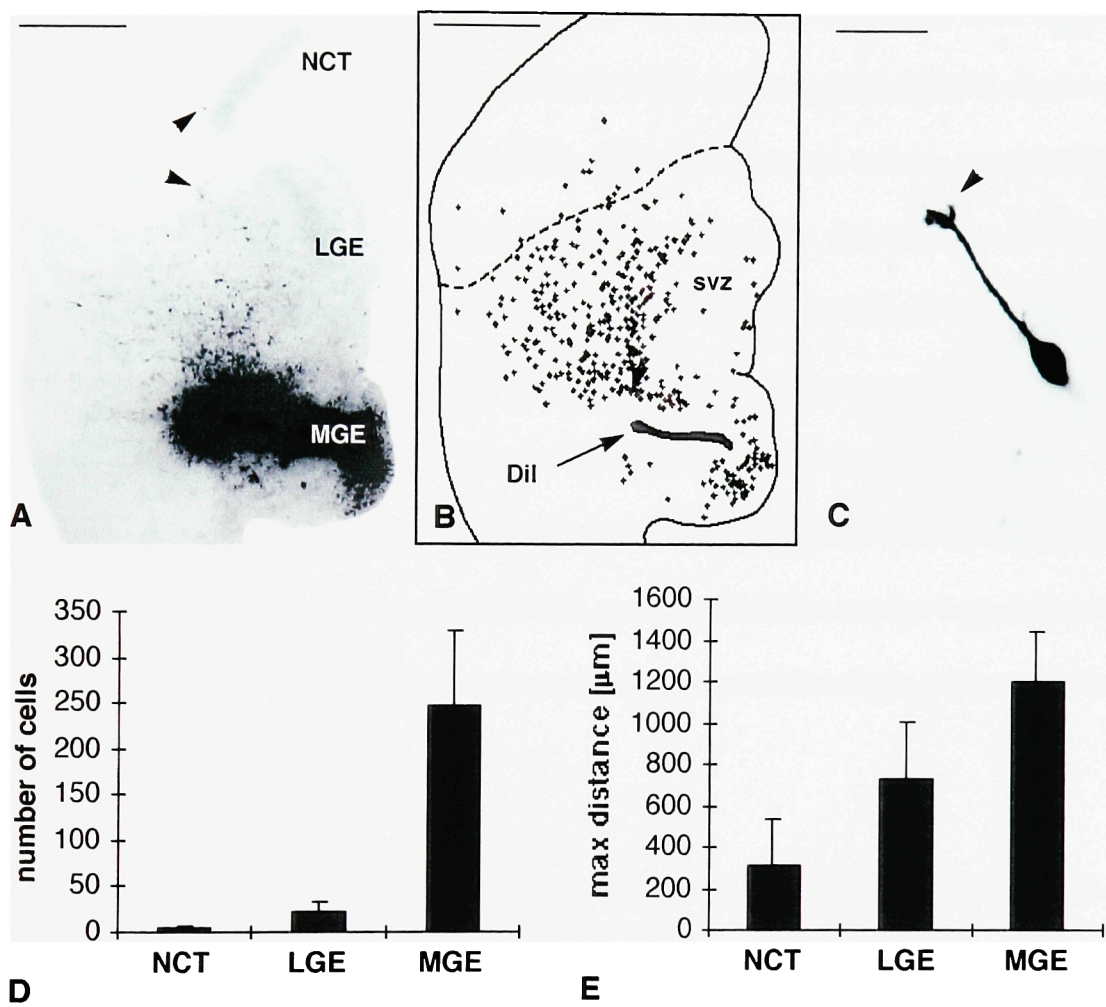
A, Section of embryonic brain (E14) with Dil injection in the MGE. Dorsal is to the top, lateral ventricle is to the right. Cohorts of Dil-labeled cells (black) are migrating dorsolaterally 24 hours after injection. Some cells already crossed into the neocortex (arrowheads). Scale bar = 500 μ m

B, Map of a section from (A). Notice that cells are migrating dorsolaterally—no cells are found migrating ventrally into diencephalon. Although cells are migrating in a distributed manner, they seem to avoid SVZ of LGE. Dashed line represents a boundary between LGE and neocortex. Scale bar = 500 μ m

C, Example of MGE-derived migrating cell in the neocortex. Embryonic MGE cells have a typical migratory morphology—similar to that of MGE cells grafted into the adult brain or migrating in vitro. Arrowhead points to a growth cone at the tip of leading process. Scale bar = 25 μ m

D, Histogram of number of cells migrating out of injection sites in MGE, LGE or neocortex. Error bars correspond to S.D. Significantly more cells migrated out of labeled MGE than from other regions (t-test, $p < 0.01$).

E, Histogram of maximum distances which neurons migrated in embryonic brain slices. Maximum distance was calculated as an average distance of five neurons which migrated farthest away in each brain slice. Error bars correspond to S.D. The distance migrated by MGE cells is significantly greater than the distance migrated by neocortical or LGE cells (t-test, $p < 0.01$).



Based on current experiments I was unable to distinguish whether labeled LGE cells represent a separate source of migrating embryonic neuronal precursors destined for neocortex or whether they correspond to MGE cells labeled when passing through the LGE. Even fewer cells (4 ± 2 , $n=9$) were observed to disperse tangentially from injection site into the neocortex (Fig. 21D). The distance MGE cells migrated in brain slices was also longer than that of LGE cells (Fig. 21E). Within 24 hours in culture, some MGE cells had moved as far as 1.2 ± 0.25 mm from the injection site. During the same time interval, LGE or neocortical cells migrated a significantly shorter distance (Fig. 21E). From the above I infer that some MGE cells migrate through the organotypic slice with a speed of at least 50 $\mu\text{m}/\text{h}$. These results indicate that MGE cells possess a unique capacity to penetrate and migrate not only in the adult brain but also through embryonic brain tissue. MGE cells can cross MGE/LGE as well as LGE/neocortex boundaries and may differentiate into neurons scattered across large areas of developing telencephalon.

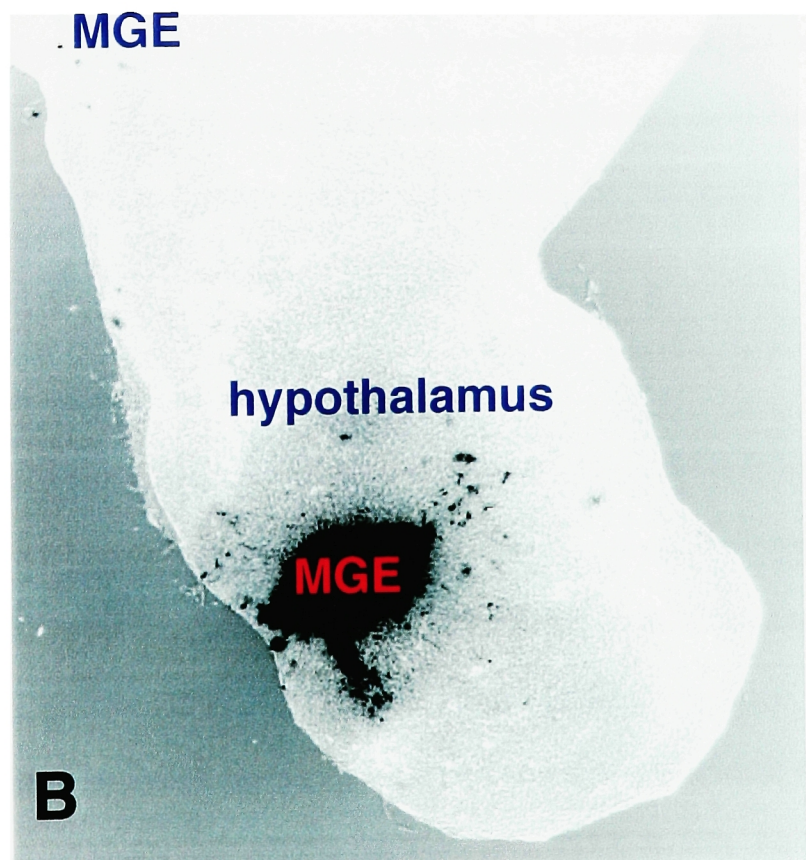
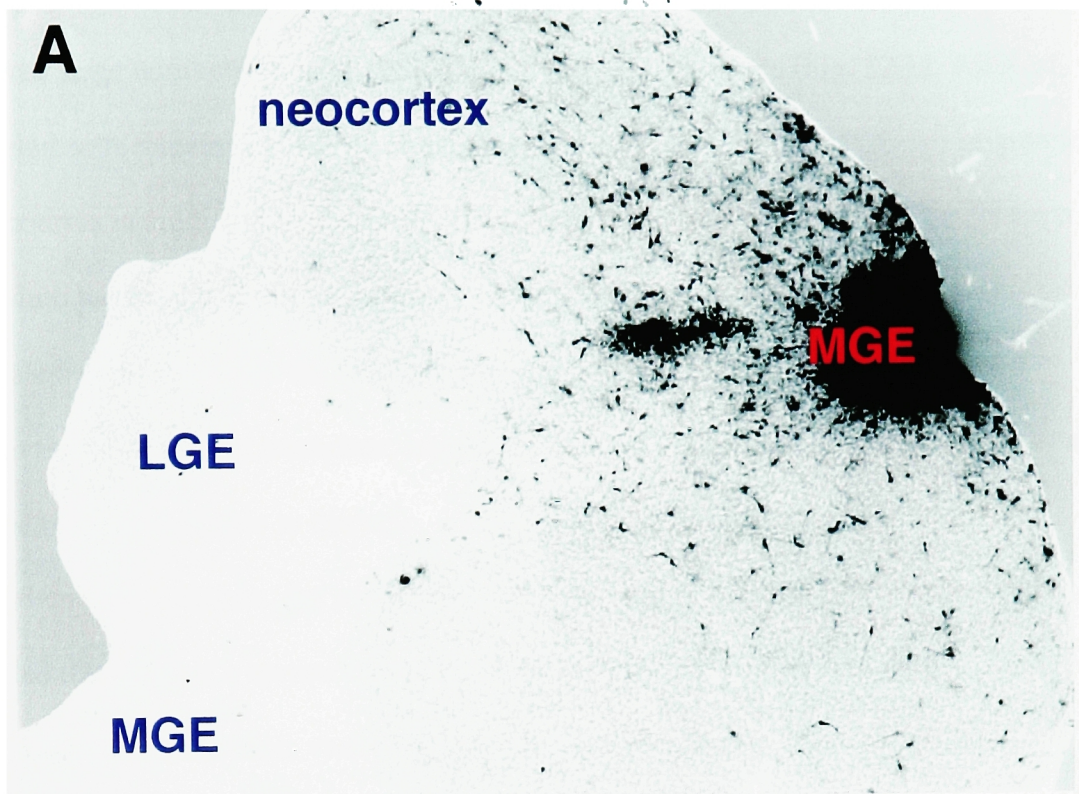
Microtransplantations of MGE cells

The observation that MGE cells do not migrate to a neighboring diencephalon, while they readily migrate to more distant neocortex triggered my interest. How is this directional migration achieved? One possibility is that MGE cell migration is directed by diffusible or short range guidance cues as has been suggested for growing axons (Tessier-Lavigne, 1992; Tessier-Lavigne, 1994). Alternatively, the diencephalon might be a restricted area non-permissive for MGE cells dispersion. There might be a barrier at the boundary between MGE and diencephalon or the entire diencephalic parenchyma might be a non-permissive environment for MGE cell migration. To test this possibility I transplanted labeled MGE cells heterotopically into hypothalamus, thalamus and

Figure 22: Microtransplantation of labeled MGE cells in embryonic brain slices.

A) When MGE cells are grafted ectopically into the embryonic cortex, they disperse throughout the cortical area (black cells). Only very few MGE cells migrate ventrally into the LGE suggesting that cell migration from the MGE to neocortex is unidirectional.

B) Only very few MGE cells grafted into hypothalamus migrated away from the site of implantation.



neocortex in embryonic brain slices. Two days after MGE transplantation into neocortex I observed large numbers of MGE cells dispersing in all directions (Fig. 22A). Most of the labeled cells remained confined in cortical region, indicating that MGE cell migration into neocortex is unidirectional. Similar dispersion was observed when MGE cells were grafted into the LGE and piriform cortex. In contrast, MGE cells transplanted into hypothalamus did not leave the site of transplantation (Fig. 22B). I observed labeled fibers extending from the transplant, suggesting that MGE cells grafted into diencephalon survived but were not able to disperse. I tested multiple different areas within hypothalamus or thalamus, but never observed MGE cell migration. These experiments suggested that it is not a narrow non-permissive boundary between MGE and diencephalon which restricts ventral migration of MGE cells, but rather the entire diencephalic parenchyma is non-permissive for MGE cells movement. This provides an explanation as to why I never observed MGE cells dispersing into diencephalon in organotypic cultures. I propose that diencephalon is a non-permissive environment for the migration of MGE cells and serves as a barrier preventing the movement of MGE cells ventrally.

Dot assay: MGE co-cultures with other brain cells

Microtransplantation experiments suggested that different embryonic brain regions have different capacity to support MGE cell migration. However, it was difficult to analyze MGE cell migration quantitatively after microtransplantation, because different tested regions have different shapes and uneven cellular composition. To assess the capacity of various embryonic brain regions to support migration of MGE cells I developed a simple and highly reproducible dot assay (Fig. 23A). Briefly, the substrate

Figure 23: Dot assay of MGE cell migration in different brain regions.

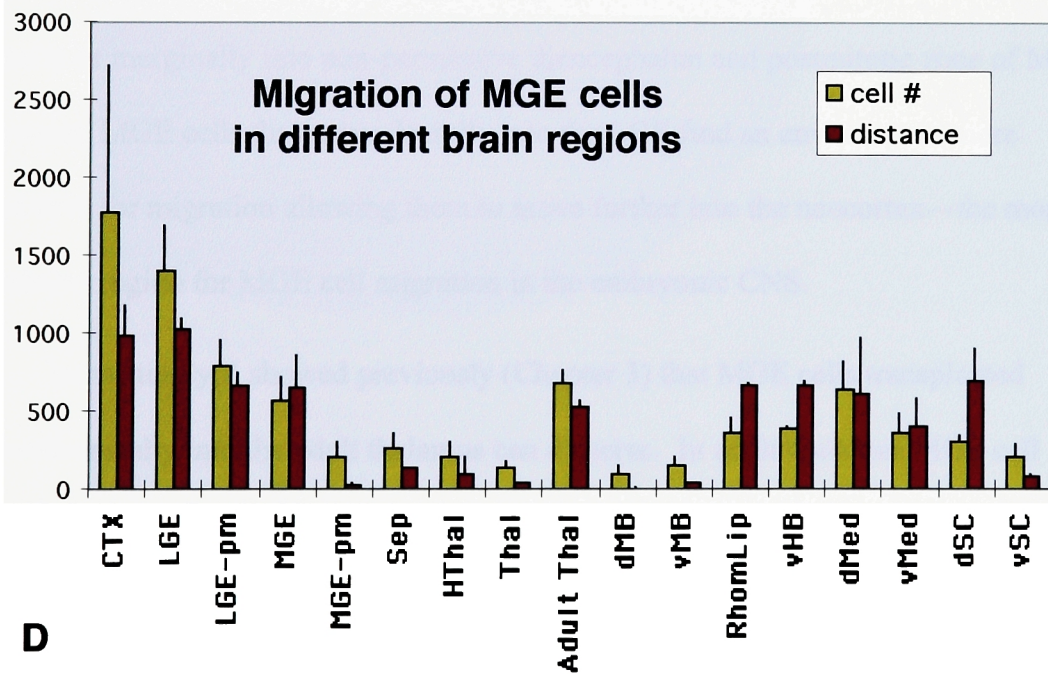
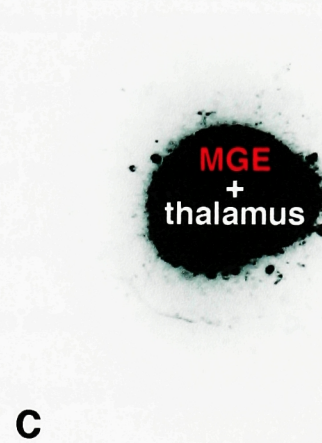
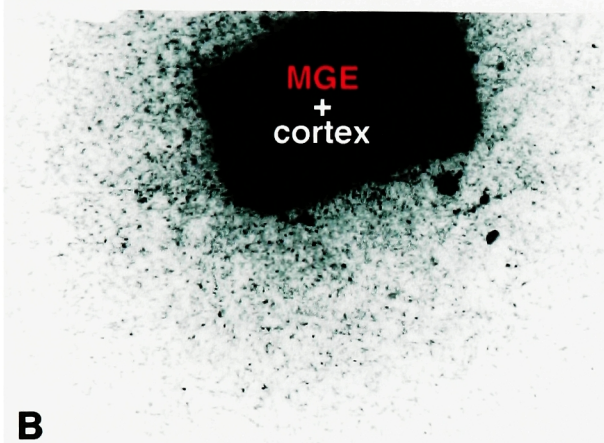
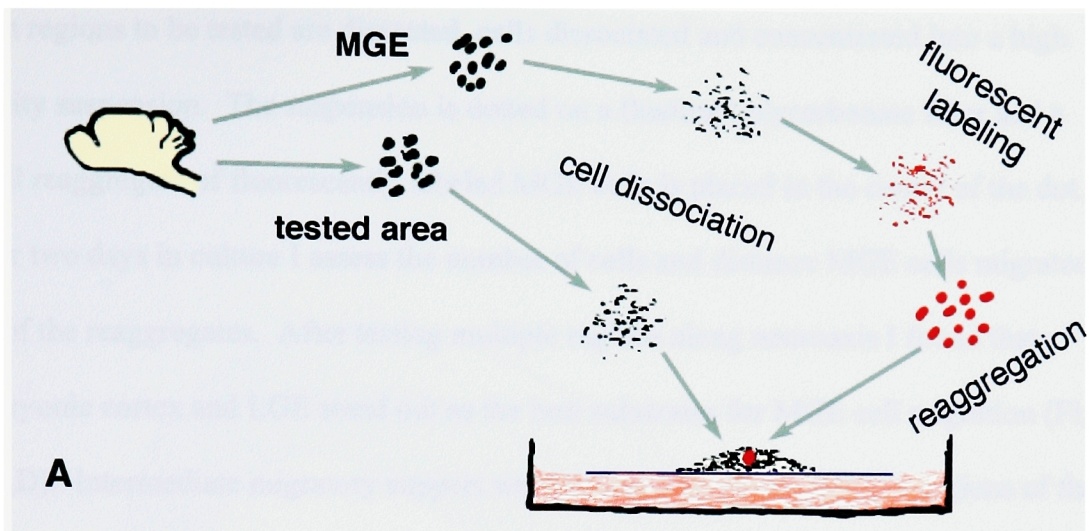
A) Diagram of dot assay cultures. MGE as well as a tested brain region are dissected and cells are dissociated. MGE cells are fluorescently labeled and reaggregated. Dissociated cells from the tested region are dotted on a floating filter inside a culture dish.

Reaggregate of labeled MGE is placed in the center of the dot.

B) After 48 hours in vitro, MGE cells (black) disperse through a dot made of cortical cells.

C) In contrast, MGE cells are not able to migrate into the dot made of thalamic cells

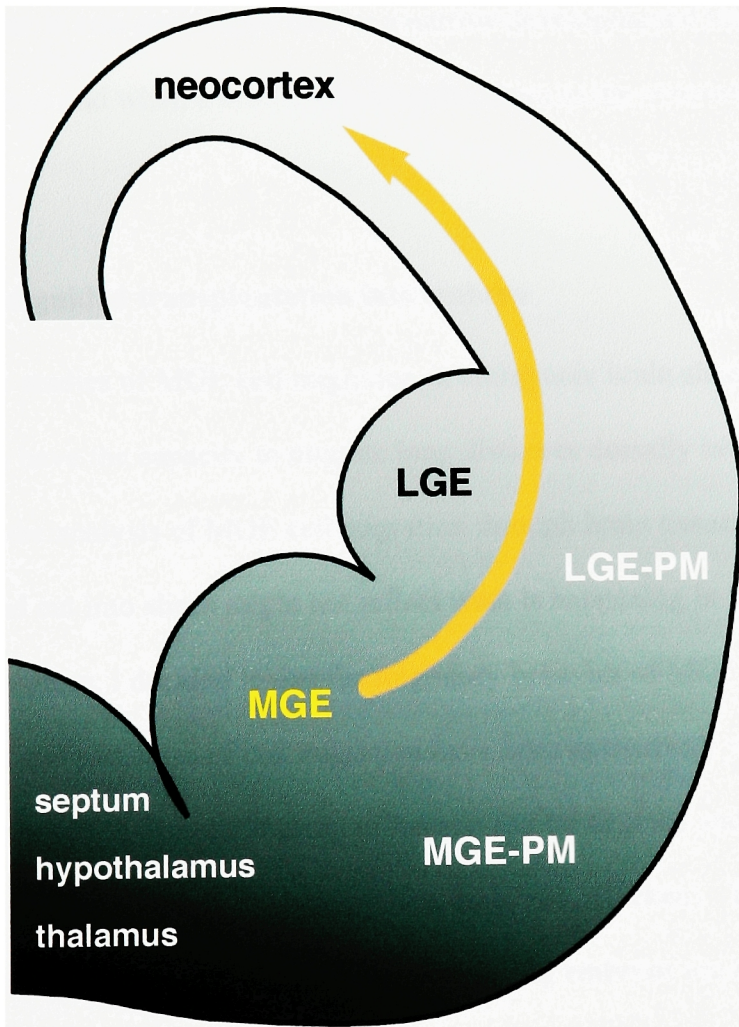
d) Extent of cell migration in dots made of different embryonic and adult tissue was analyzed. Number of cells as well as the distance MGE cells migrated from the edge of reaggregate was plotted. Notice that embryonic thalamus is non permissive while adult thalamus allows MGE cell dispersion.



brain regions to be tested are dissected, cells dissociated and concentrated into a high density suspension. The suspension is dotted on a floating polycarbonate filter and a small reaggregate of fluorescently labeled MGE cells is placed in the center of the dot. After two days in culture I assess the number of cells and distance MGE cells migrated out of the reaggregates. After testing multiple regions along neuroaxis I found that embryonic cortex and LGE stand out as the best substrates for MGE cell migration (Fig. 23B,D). Intermediate migratory support was provided by the postmitotic regions of the LGE (LGE-PM, which consists of the developing striatum and the piriform cortex) and the MGE itself. The postmitotic regions of the MGE (MGE-PM), the entire hypothalamus, thalamus and midbrain did not support migration of MGE cells (Fig. 23C,D). These results indicate that differential permissiveness of forebrain tissues for MGE cell migration might be the primary mechanism directing MGE cell movement dorsally to the neocortex (Fig. 24). In this model MGE cells are produced in a region with medium permissiveness for migration, they start to disperse, but cannot migrate ventrally or marginally into non-permissive diencephalon and postmitotic zone of MGE. Dispersing MGE cells that move dorsally into the LGE find an environment more permissive for migration allowing them to move further into the neocortex—the most permissive region for MGE cell migration in the embryonic CNS.

Interestingly, I showed previously (Chapter 3) that MGE cells transplanted heterochronically into the adult thalamus can disperse. In adult thalamus MGE cell migration was comparable to migration in the adult striatum or neocortex (Fig. 16B). In contrast, the embryonic diencephalon was non-permissive for MGE cell migration. I used the dot assay to compare the ability of thalamic cells isolated from the embryonic

Figure 24: Model of differential permissiveness of the embryonic forebrain for MGE cell migration. I propose that MGE cells are directed towards the neocortex by differential permissiveness of different forebrain tissues. MGE cells cannot migrate ventrally, medially or superficially, because hypothalamus, thalamus and postmitotic MGE regions are non permissive for MGE cell migration. Thus, MGE cells can move only dorsally through the LGE and into the most permissive region in the embryonic brain, the neocortex.



and adult brain to support MGE cells movement. Results were consistent with grafting experiments: the adult thalamus was permissive in the dot assay (Fig. 23D). The blocking mechanism, which restricts MGE cell migration in the embryonic diencephalon, is eliminated at some point during fetal or postnatal development. It is possible that diencephalon is non-permissive only at a narrow developmental time when MGE cells are being born and when a mechanism steering them to the neocortex needs to be in place.

Ultrasound guided transplantation into embryo

My studies of MGE cell migration in embryonic brain slices strongly indicate that MGE cells have the capacity to migrate long distances dorsally into the neocortex. However, the analysis of MGE cell migration through brain tissue dissected out from the embryo and cut into slices might not reflect what is happening in the intact embryonic brain. Therefore, I decided to test the migratory behavior of MGE cells directly in vivo. Fate-maps and pathways of cell migration have been extensively studied in embryonic organisms in which discreet regions of interest can be targeted and labeled by microinjection or microtransplantation (e.g. frog, fish, chicken). This direct approach is much more difficult in mammals, where developing embryos are hidden inside the uterus and protected by several membranes. Therefore our understanding of mammalian brain development stems historically from anatomical studies, which were recently supplemented by the analysis of patterns of gene expression. This molecular mapping led to a proposal that brain neuroepithelium is subdivided early in the development into regions (neuromeres) characterized by a unique pattern of gene expression (Puelles and Rubenstein, 1993; Krumlauf et al., 1993; Shimamura et al., 1997). It has been proposed

that individual neuromeres are separated by non-permissive boundaries preventing intermixing of most neuronal cells born in neighboring regions (Fraser et al., 1990). One such inhibitory region limiting tangential dispersal was identified in the developing forebrain between the neocortex and LGE (Fishell et al., 1993; Neyt et al., 1997). However, single cell labeling studies in chicken hindbrain revealed that besides cells which are restricted to the place of their origin, some cells can consistently cross neuromeric boundaries (Birgbauer and Fraser, 1994). In the following section, I studied the behavior of MGE cells in the embryo, in an attempt to find out whether MGE cell migration is restricted or whether they violate the neuromeric organization of the forebrain.

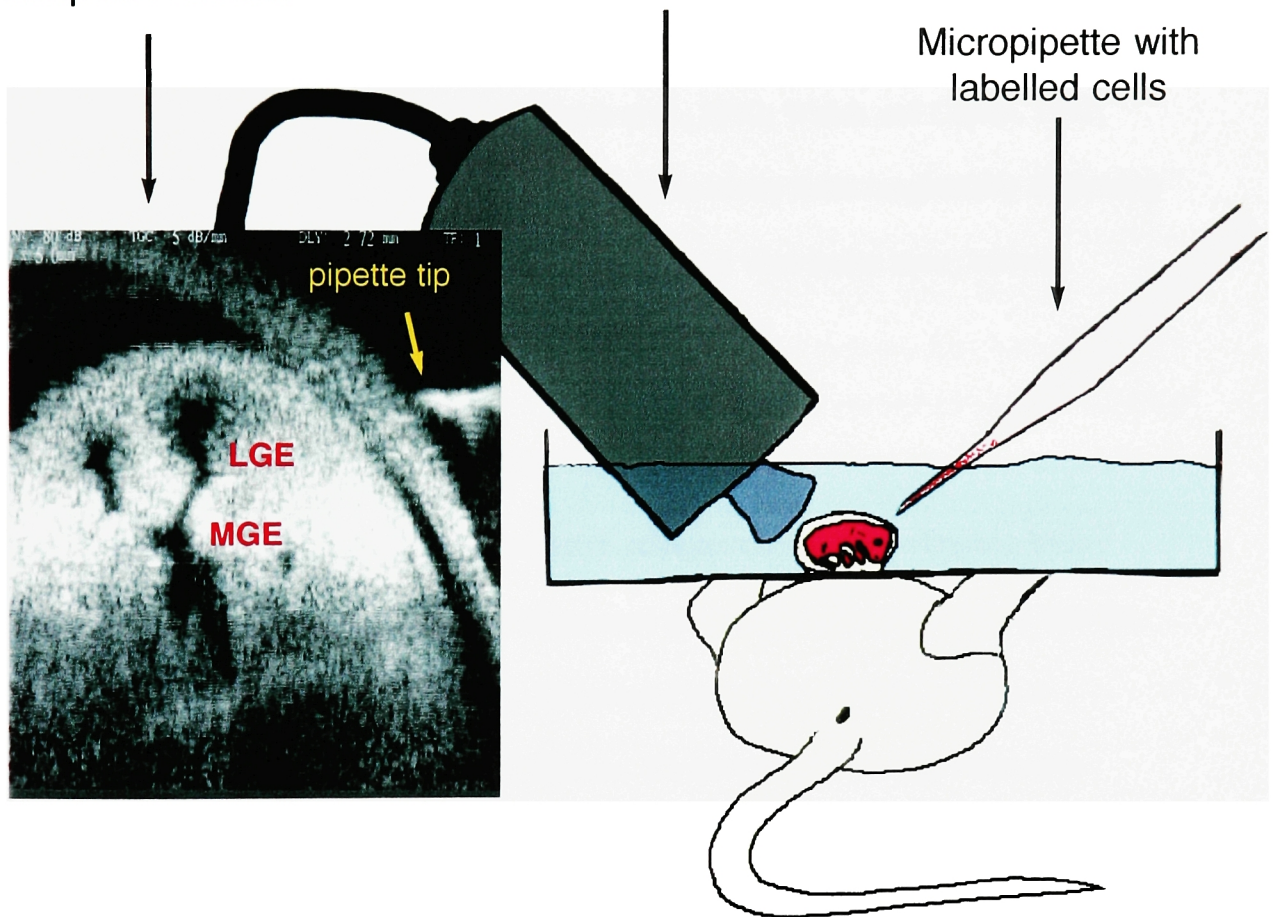
Fate of neural precursors in the mammalian brain was addressed more directly when a technique of microinjection into embryonic brain ventricles was developed. Labeled neural precursors injected into the brain ventricle adhere to the ventricular zone at multiple sites and integrate into the developing brain. The developmental potential of neuronal precursors found in different brain regions was studied using this method (Campbell et al., 1995; Brüstle et al., 1995; Lim et al., 1997). Since the sites of cell integration after intraventricular injections are not known, the extent of migration cannot be inferred from these studies. The intraventricular injection of retroviruses carrying a genetic marker, which permanently labels infected cells and all of their progeny, made possible a study of neural lineages in developing brain (Price and Thurlow, 1988; Walsh and Cepko, 1988). This approach posed two problems: 1) there is no control over which cells are infected and no record where these cells resided at the time of infection, and 2) the potential multiplicity of infection sites in one brain makes the determination which

Figure 25: Schematic representation of the ultrasound guided transplantation. Exposed uterus of an anesthetized mouse with an embryo is passed through a hole in the bottom of a Petri dish. The dish is filled with PBS, into which is immersed the ultrasound transducer. The embryo is positioned under the transducer so the LGE, and MGE can be easily identified. The tip of a pipette with labeled cells is inserted into the target region (MGE or LGE) and a small volume (~20nl) of cells is microinjected.

Embryonic brain
displayed on
computer monitor

Ultrasound
transducer

Micropipette with
labelled cells



labeled cells belong to the same clone difficult, if not impossible. The latter problem was resolved by the construction of a large retroviral library containing viruses carrying many different genetic tags. Using this approach it was found that clonally related cells in the mammalian cortex can be spread over large distances, suggesting that extensive tangential migration of young neurons contributes significantly to the histogenesis of the telencephalon (Walsh and Cepko, 1990; Walsh et al., 1992; Walsh and Cepko, 1992; Walsh and Cepko, 1993). What remains a mystery is where these tangentially dispersing cells originate. Do their precursors reside in the cortical ventricular zone, cortical subventricular zone or is their origin extracortical?

MGE cells populate the embryonic neocortex

In order to study the migration of MGE cells directly in the embryonic brain I used a recently developed technique of ultrasound guided microtransplantation into the embryonic brain (Olsson et al., 1997b). Ultrasound imaging is widely used as a noninvasive method to visualize human fetuses. Development of a high resolution ultrasound backscatter microscope made possible targeted transplantation into much smaller mouse embryos (Fig. 25) (Turnbull, 1999). MGE cells dissected from donor mice were dissociated and fluorescently labeled with PKH26 dye. Using the ultrasound microscope, labeled MGE cells were grafted back to the MGE of age-matched embryos. MGE cells migrate most actively within the first three days in vitro or after transplantation into the adult brain (Chapter 3). Therefore, I decided to analyze the extent of MGE cell dispersion in the embryo at four days after transplantation. At this timepoint I found many labeled MGE cells dispersing throughout the dorsal forebrain, with most of them located in the neocortex (Fig. 26A, 28). Many MGE cells were concentrated

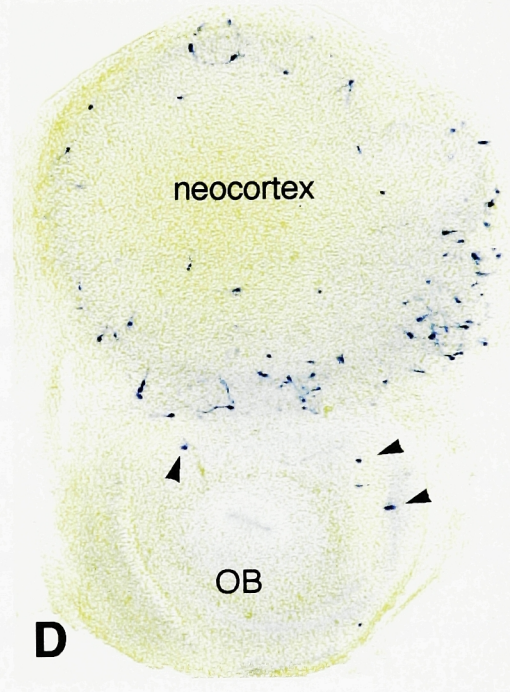
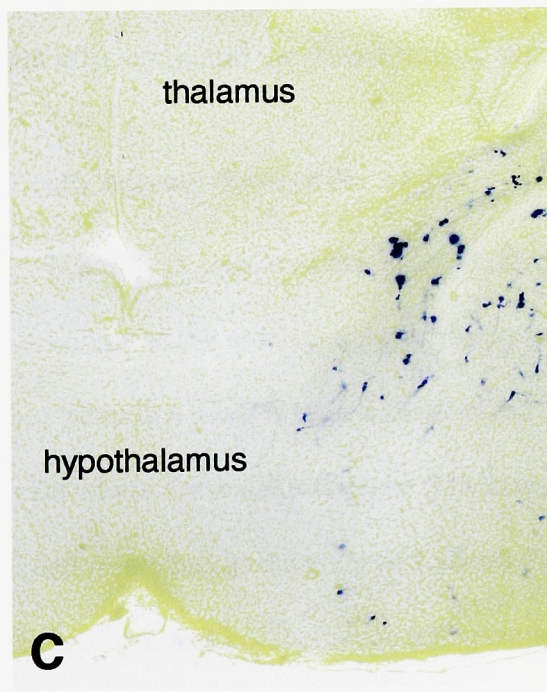
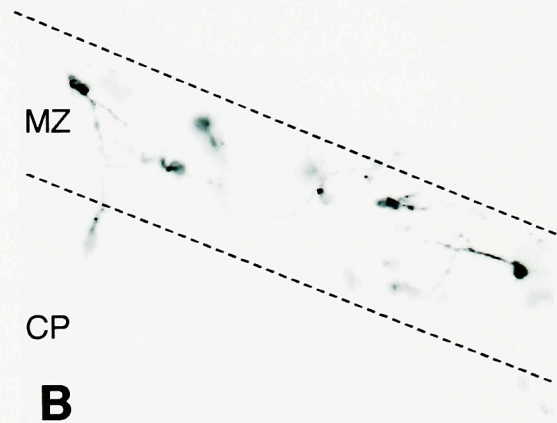
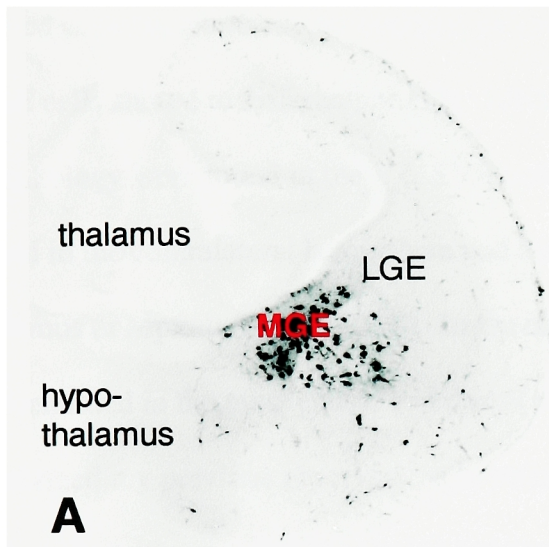
Figure 26: MGE cell migration 4 days after the homotopic transplantation. Coronal sections of E17.5 embryonic brains which were injected on embryonic day 13.5.

A) Low magnification view of the injection site in the MGE. MGE cells (black) are dispersed within the neocortex, but no labeled cells are found in the hypothalamus or thalamus. In the neocortex MGE derived cells are concentrated in the marginal zone.

B) Higher magnification of labeled cells in the marginal zone. Many of MGE-derived cells extended several processes, which is an indication that they started to differentiate.

C) Higher magnification of the boundary between diencephalon and telencephalon reveals that although MGE cells (blue) disperse even in ventral parts of the telencephalon they do not cross into the hypothalamus or thalamus.

D) Section through the rostral forebrain reveals striking difference between the large number of cells in the marginal zone of the neocortex and very few cells found in the olfactory bulb (arrowheads).



superficially in the marginal layer of neocortex (Fig. 26B, 28). In addition, MGE cells also dispersed within developing striatum (LGE). Four days after transplantation some grafted cells started to extend multiple processes from cell bodies, suggesting that some MGE cells started to differentiate (Fig. 26B). Some cells with more immature morphology were found in the VZ, SVZ and in the cortical plate. No labeled cells were found in the contralateral hemisphere and MGE cells did not migrate into hypothalamus, thalamus or septum (Fig. 26C, 28). In the olfactory bulb I found only few cells concentrated in the most caudal and dorsal regions (Fig. 26D). These *in vivo* findings confirmed my previous observations in embryonic slices and using the dot assay. MGE cells migrate through the developing striatum and disperse in the neocortex, but do not migrate efficiently into the diencephalon, septum or olfactory bulb.

MGE cells migrate through the SVZ

It has been proposed that tangentially migrating neurons in the cortical marginal zone originate in the anterior subventricular zone. These SVZ cells are thought to migrate through a narrow region close to the olfactory peduncle to reach the surface of the brain where these cells disperse (Gadisseux et al., 1992). To see if this is the route of MGE cell migration in the embryo I allowed some embryos to survive one or two days after homotopic and homochronic transplantation. At one day, MGE cells were confined at the site of transplantation with only few cells migrating within the LGE (Fig. 27A,C). There were no labeled cells found in the cortex at this time. In contrast at two days after transplantation hundreds of MGE cells were found migrating through the cortical SVZ and intermediate zone (Fig. 27B,D-F, 28). Many cells were already at this time found in the most distant regions of the developing neocortex over 2 millimeters away from the

Figure 27: MGE cell migration 1 and 2 days after the homotopic transplantation. Coronal sections of E14.5 (A,C) and E15.5 (B,D,E,F) embryonic brains which were injected on embryonic day 13.5.

A) Low magnification of the injection site one day after transplantation. Notice the needle track and the targeted deposition of labeled MGE cells in the MGE.

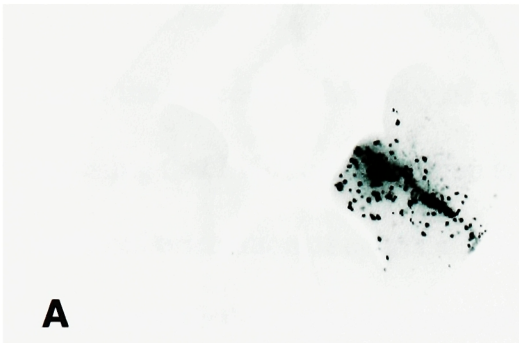
B) Low magnification of the injection site two days after transplantation. Notice that labeled MGE cells reached the most distant areas of the neocortex at this time.

C) Higher magnification of the injection site at 1 day after transplantation. There are very few cells which started to migrate into the host tissue (arrowhead) at this timepoint.

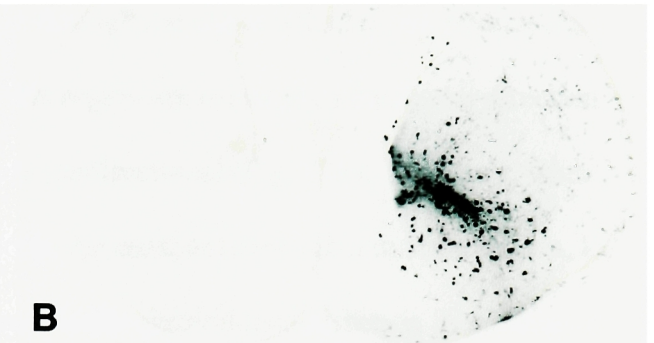
D) Large scale migration of MGE cells through the cortical SVZ and intermediate zone. Very few MGE cells reached marginal zone at this point as compared to 4 day survival (Fig. 26 A,B,D).

E) Anterior section reveals many migrating cells in the intermediate/SVZ zone cut in a tangential plane (center of the neocortex). Again there are almost no cells found in the olfactory bulb.

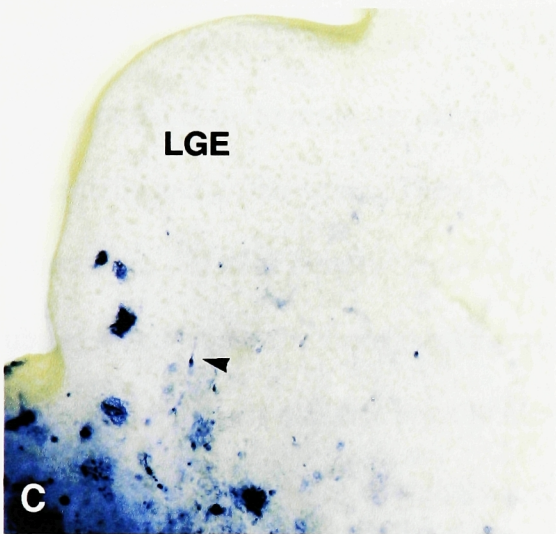
F) Larger magnification of migrating cells in the subventricular zone of the embryonic cortex. Most cells have typical migratory shape and their leading process is oriented dorsally suggesting they are moving unidirectionally to the neocortex.



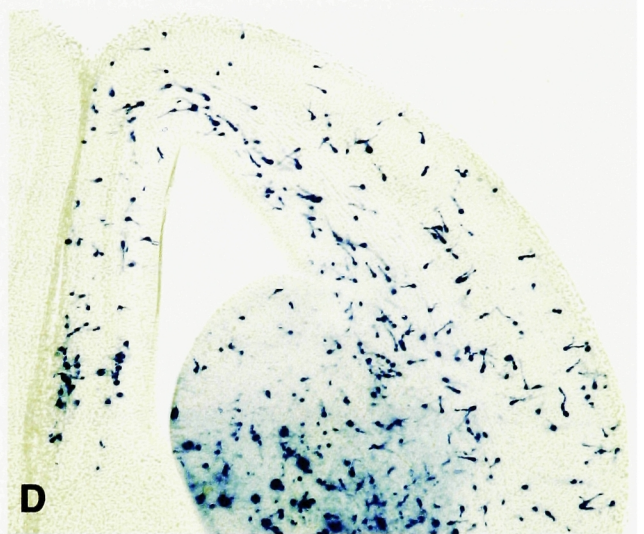
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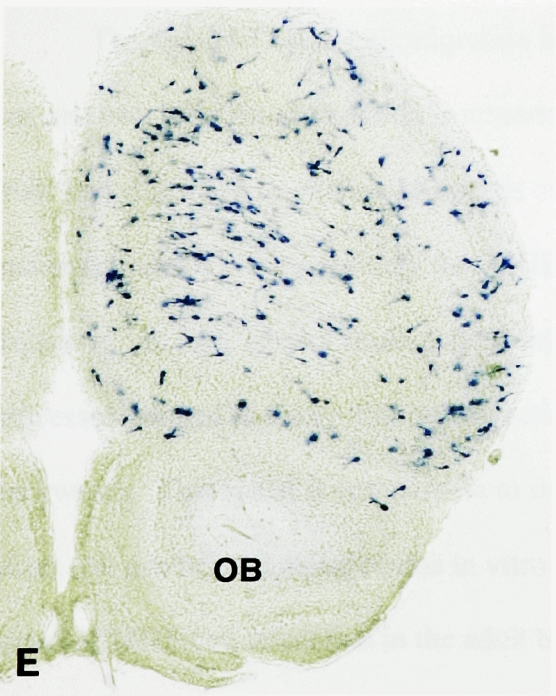
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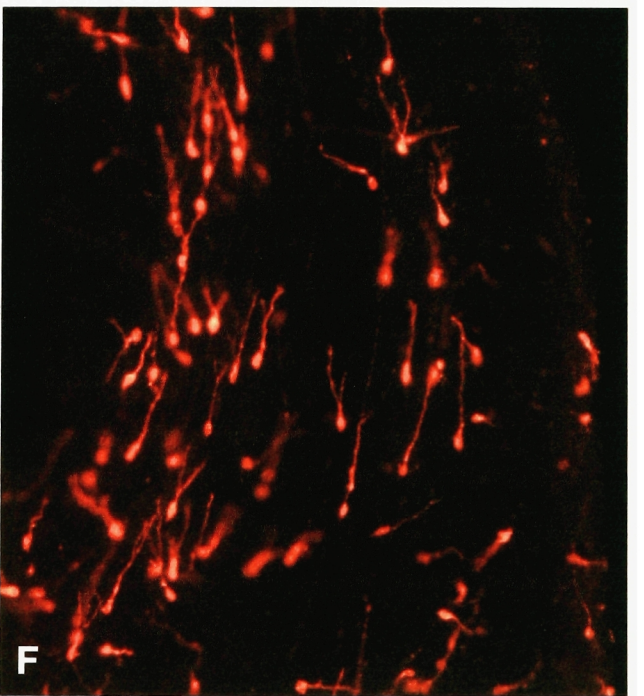
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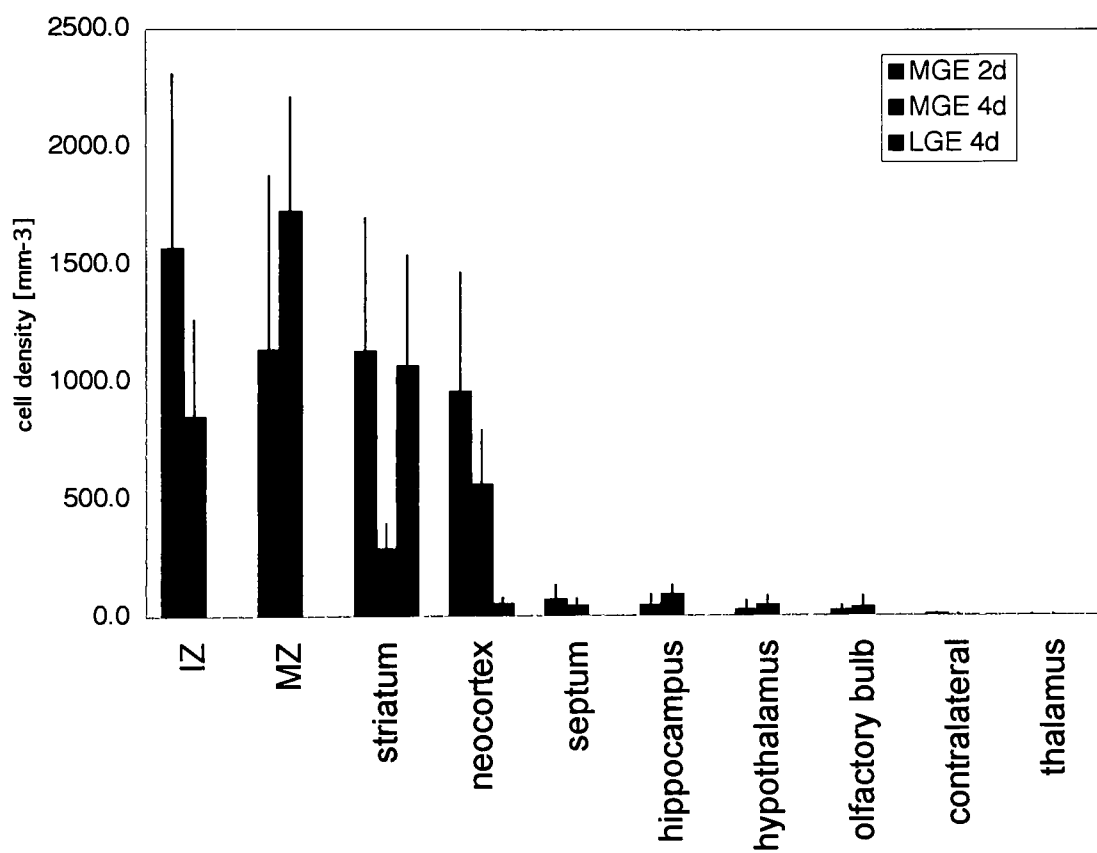
F

injection site (Fig. 27B,D). Two days after transplantation only very few cells were found in the cortical marginal layer (Fig. 27D, 28). These results indicate that between day one and two, MGE cells rapidly disperse through the subventricular and intermediate zone of the neocortex. Majority of cells in this region are oriented in the dorsal direction, suggesting that MGE cell migration is largely unidirectional (Fig. 27F). Similarly, when I analyzed orientation of neuronal precursors in the most anterior subventricular region, I could conclude that migration is unidirectional in the mediodorsal direction in this region (Fig. 27E). In days 2-4 MGE cells move from the SVZ and intermediate zone to the surface of the brain to reach the marginal layer, where they may further disperse tangentially and differentiate. These results indicate that the pathway of MGE cells dispersion is different from the pathway described for subpial granule cells. While subpial granule cells are thought to migrate through a narrow region in the anterior forebrain, MGE cells disperse broadly throughout the subventricular and intermediate zones before they reach the marginal zone.

The speed of MGE cell migration inferred from the position of labeled cells at day one and two after transplantation is remarkable. The leading cells found furthest away from transplant had to move with speeds of at least $\sim 80 \mu\text{m/hr}$. This speed is likely an underestimate: it is very unlikely that MGE cells migrated along the shortest possible trajectory. In fact, when I studied the morphology of migrating cells their leading processes pointed in many different directions and some of them were oriented backwards. This speed is comparable to the speed of MGE cell migration in organotypic slices ($50 \mu\text{m/hr}$) and along chains in vitro ($107 \mu\text{m/hr}$), but it is faster than the estimated speed of MGE cell migration in the adult brain ($24 \mu\text{m/hr}$).

Figure 28: Density of MGE and LGE cells in different embryonic brain regions after 2 and 4 day survival. Notice that at 2 days there are more MGE cells in the intermediate zone (IZ) than in the marginal zone (MZ) of the neocortex. This ratio is reversed at 4 days after transplantation.

Density of grafted MGE and LGE cells in different brain regions 2 or 4 days after transplantation



When different cells from the embryonic brain were tested in the dot assay for their capacity to support MGE cell migration in vitro I found that the best substrate was provided by cortical and LGE cells. The preferential migration of MGE cells in the intermediate zone of the neocortex in vivo suggests that the corticofugal axons running through this region and invading ganglionic eminences might provide the permissive substrate used by MGE cells (O'Rourke et al., 1992; O'Rourke et al., 1995; Metin and Godement, 1996; Metin et al., 2000). Similar substrate (commissural axons) for tangential migration of cholinergic interneurons has been identified in the spinal cord (Phelps and Vaughn, 1995). Between two and four days MGE cells migrate through cortical plate and marginal zone suggesting that besides corticofugal axons other substrates might be used by migrating MGE cells. It is interesting, that in all of my experiments (in vitro migration assay and transplantation into the adult and embryonic brain) most of cell migration happens in the first four days after explantation or transplantation. It is possible that MGE cells are preprogrammed to migrate only for a limited period of time before they differentiate. This would explain why there is a limit to the extent of MGE cell dispersion through the adult brain. If we could prolong this migratory period MGE cells would likely distribute over larger area in the adult brain, which could be therapeutically advantageous (see Chapter 5).

LGE and neocortical cells remain in the developing ventral forebrain

To study whether the long distance migration to the neocortex is a specific feature of MGE cells or whether also LGE cells can invade the neocortex, I grafted labeled LGE cells back into the embryonic LGE. LGE cells dispersed effectively through the developing striatum, but very few cells migrated into the neocortex (Fig. 29A). There

Figure 29: LGE and neocortical grafts 4 days after transplantation. Coronal sections of E17.5 embryonic brains which were injected on embryonic day 13.5 with LGE cells (A-D) or embryonic neocortical cells (E,F).

A) Low magnification of the LGE graft placed in the MGE. Notice that many labeled cells are dispersed within striatum, but only few scattered cells are found in the neocortex.

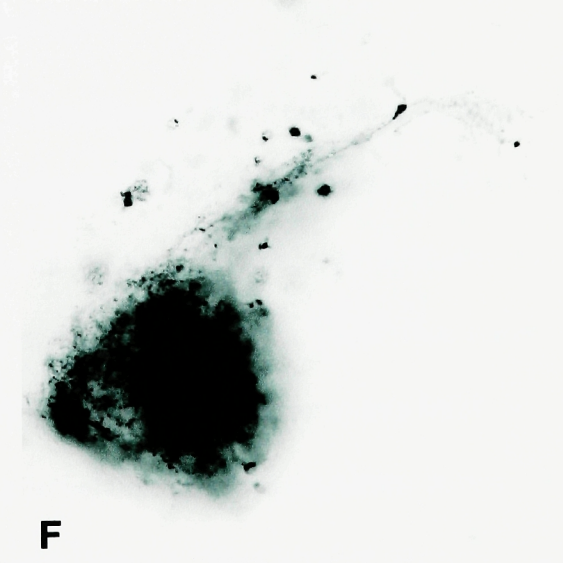
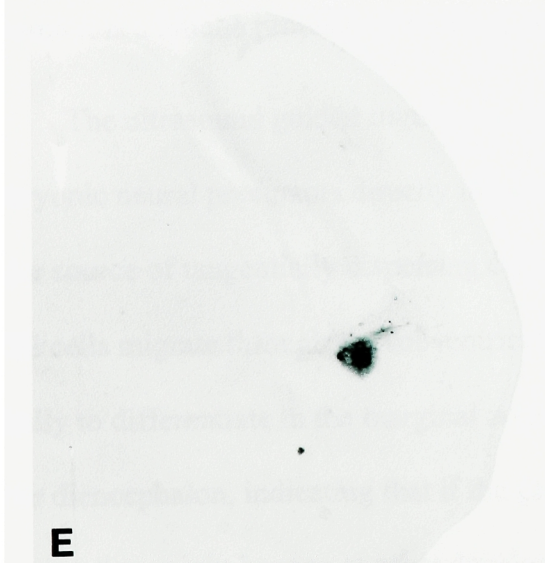
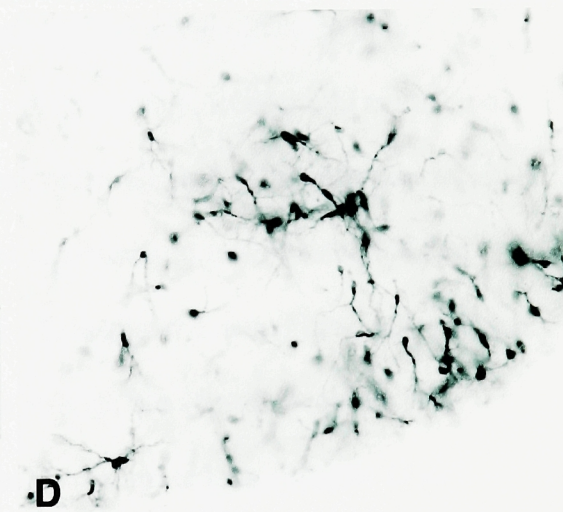
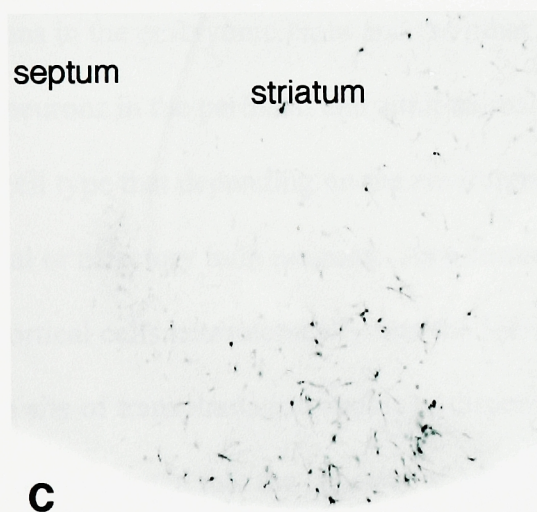
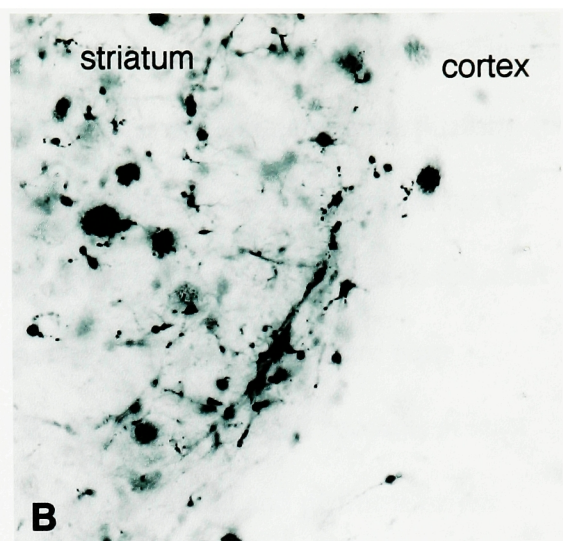
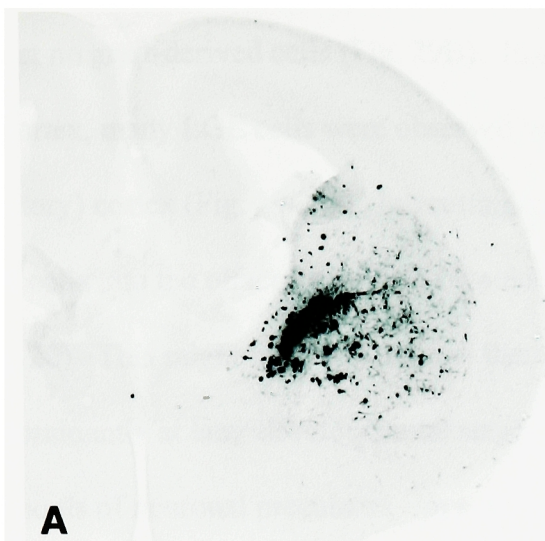
B) Higher magnification of the boundary between developing striatum (postmitotic LGE) and neocortex. There is a clear boundary which is rarely crossed by labeled LGE cells.

C) More anterior section depicts LGE cells that migrated ventrally into the piriform cortex (ventral from the striatum). This region is far from the injection site suggesting that LGE cells migrated effectively through the rhinencephalon.

D) Higher magnification of LGE cells differentiating in the piriform cortex.

E) Low magnification of the neocortical graft. The graft is confined to a small area with very few cells migrating out of the graft site.

F) Higher magnification of the cortical graft reveals neurites extending from the graft site. Suggesting that grafted cells survived, but did not migrate through the embryonic ventral forebrain.

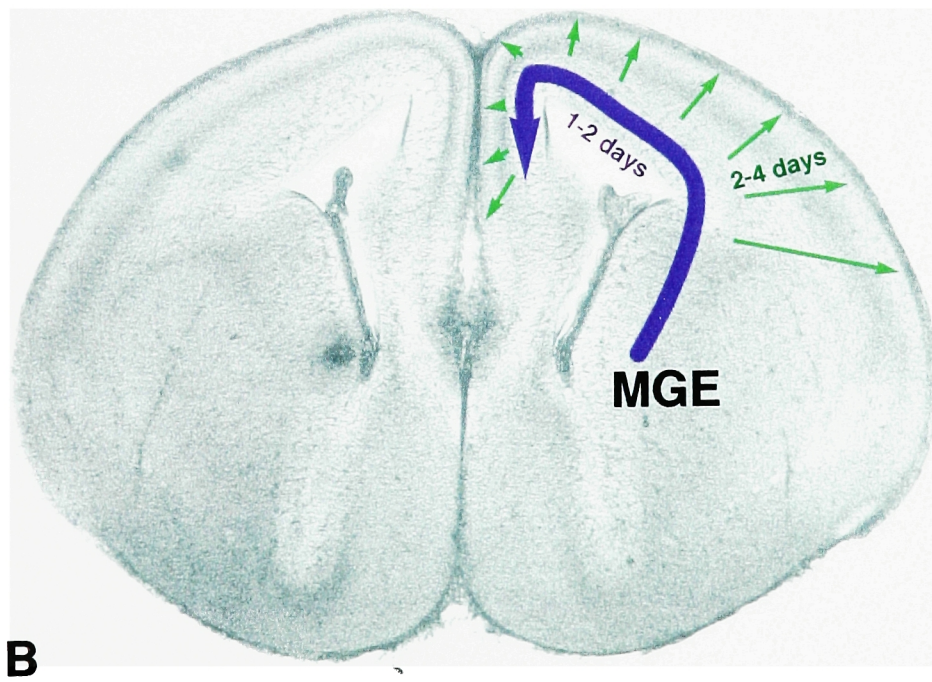
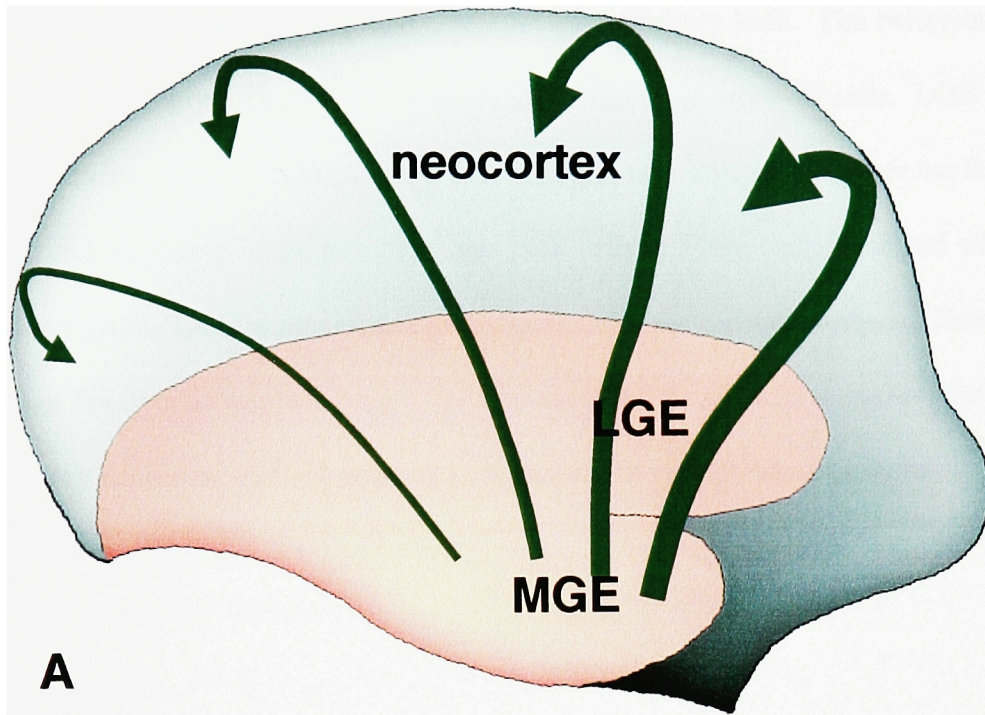


was a striking difference between the striatum, filled with grafted cells, and cortex with almost no graft-derived cells (Fig. 29B). In contrast to the absence of labeled cells in the neocortex, many LGE cells were observed to disperse and differentiate in the piriform (or olfactory) cortex (Fig. 29C,D). Interestingly, I did not observe extensive migration of LGE cells into the olfactory bulb as I would have predicted from my studies in the adult (Fig. 20). This might be due to the fact that olfactory bulb interneurons are born predominantly at later developmental stages. This indicates that LGE contains at least two pools of neuronal precursors—one giving rise to the striatal and piriform cortex neurons in the embryonic brain and the other giving rise to the olfactory bulb interneurons in the perinatal and adult animal. Alternatively, LGE might contain only one cell type that depending on the environment or developmental time produces either striatal or olfactory bulb neurons. As a control experiment, I transplanted labeled neocortical cells heterotopically into the MGE. Transplanted neocortical cells remained at the site of transplantation unable to disperse through the ventral forebrain (Fig. 29E,F). This demonstrated that the capacity of MGE cells to migrate long distance to the neocortex is a unique property of this cell population.

The ultrasound guided transplantation allowed me to study migratory pathways of embryonic neural precursors directly in the developing brain. I confirmed that MGE is a major source of tangentially dispersing cells in the mammalian forebrain. I showed that MGE cells migrate through the subventricular zone to the neocortex, where they move radially to differentiate in the marginal zone (Fig. 30). I did not observe any MGE cells in the diencephalon, indicating that if the gangliothalamic pathway is present in rodents, the migration might happen at other developmental time or might involve other cells than

Figure 30: Diagram of MGE cell migration in the embryonic forebrain.

Neuronal precursors born in the embryonic MGE migrate tangentially in dorsal direction and disperse in all regions of the developing neocortex (A). MGE cells move initially (between day 1 and 2 after transplantation) within the subventricular and intermediate zones of the neocortex (B, purple arrow). Later (between day 2 and 4) they move superficially to settle in the marginal zone of neocortex).



those in MGE. I confirmed that MGE cell migration to the neocortex is directional, they do not invade neighboring diencephalon, septum and olfactory bulb. The behavior of MGE cells is strikingly different from behavior of LGE or neocortical cells. LGE cells remain constrained to the developing striatum and piriform cortex. Whether the few LGE cells observed migrating in the neocortex are LGE cells or MGE cells dissected when passing through the LGE is impossible to distinguish by our current analysis. However, the fact that fewer cells migrate from LGE to cortex than from MGE suggests that MGE is the primary source of cortical neurons born outside the palial neuroepithelium.

CHAPTER 5: IMPLICATIONS FOR BRAIN REPAIR

In previous chapters, I have identified a population of embryonic neuronal precursors, which have a unique migratory potential. I have shown that MGE cells migrate dorsally from their site of birth and disperse throughout the developing neocortex. MGE cell migration is not limited to the embryonic environment. When MGE cells were grafted into the adult brain they dispersed several millimeters through the host tissue and differentiated into neurons. No other primary neuronal precursors has been before shown to migrate in this manner in the adult mammalian brain (outside the SVZ, rostral migratory stream and olfactory bulb) (Graybiel et al., 1989; Pakzaban et al., 1993; Olsson et al., 1995; Olsson et al., 1997a). The absence of the radial glial guides (Schmechel and Rakic, 1979; Pixley and De Vellis, 1984) and accumulation of molecules inhibiting growth cone movement in the mature brain (GrandPre et al., 2000; Chen et al., 2000) are believed to inhibit the migration of neuronal precursors in the adult brain. My findings that MGE cells can disperse and differentiate into neurons in the adult brain suggest that the adult brain environment is not as restrictive as was originally thought, which could have important clinical implications.

Transplantation of neuronal precursors into the adult brain could be therapeutically used to replace neurons lost by injury or disease (Bjorklund, 1991; Lindvall, 1991; Isacson and Deacon, 1997). Ideally, one would like to place young healthy brain cells into a patient's brain, which would then disperse throughout the damaged region and integrate into brain circuitry, specifically at sites where new nerve cells are needed. I have shown that MGE cells can migrate in the intact adult brain. However, during and after injury the brain undergoes physiological, biochemical and

structural changes which could interfere with the migration of MGE cells. Reactive gliosis and presence of microglial scavengers accompany some neurodegenerative diseases (Berry et al., 1983). Wounds in the adult brain are enclosed by glial scar tissue characterized by the deposits of molecules inhibitory for axonal outgrowth and cell migration (e.g. neurocan, phosphacan, brevican) (Fawcett and Asher, 1999). In addition, mesodermal cells (meningeal cells and macrophages) become part of the brain parenchyma in the core of the lesion (Berry et al., 1983). The growth of axons and dendrites through the lesion is ultimately obliterated, limiting the potential for brain repair (Berry et al., 1983; Fawcett and Asher, 1999). Therefore, it was possible that migration of MGE-derived neuronal precursors could be restricted in the injured adult brain (Pakzaban et al., 1993; Olsson et al., 1995).

Here I studied the potential of MGE cells to migrate through excitotoxin-lesioned adult striatum. Excitotoxic acids (kainic, ibotenic or quinolinic acid) cause a necrotic lesion at the injection site (core) and neuronal loss, accompanied by glial activation and axonal sparing in the adjacent areas (Coyle and Schwarcz, 1976; Beal et al., 1986). This pathology is very similar to that of several neurological disorders accompanied by a surge of excitatory aminoacids (glutamate, aspartate) such as stroke, head trauma or neurodegenerative diseases (for review see (Choi, 1988)). In this study I compared the survival and migration potential of MGE cells when grafted into the necrotic core or into the adjacent lesioned zone where neuronal loss and vimentin-positive astrocytes were evident. I found that MGE cells grafted into the lesioned zone could not only disperse within this zone, but they could also differentiate into mature neurons and integrate anatomically as shown by the presence of synaptic contacts. Furthermore, the expression

of c-fos in grafted cells upon haloperidol administration suggested functional integration within the lesioned striatum. The behavior of MGE cells and specifically their capacity to penetrate the injured adult brain demonstrates a unique migratory potential that could be exploited for the treatment of neurological disorders.

Migration of MGE cells in lesioned brain

Histology of the lesioned striatum.

The injection of kainic acid into the adult mouse striatum resulted in two clearly distinct zones, as previously described (Coyle and Schwarcz, 1976; Beal et al., 1986). Close to the injection site there was a region (necrotic core) of approximately 0.5 mm in diameter that was characterized by the presence of pycnotic cells, macrophages and a dense grouping of unidentified cells. Surrounding the necrotic core, and clearly separated from it, there was an area of total neuronal loss (lesioned zone) (Fig. 31A). While the lesioned zone did not contain any neurons, there were scattered pycnotic cells, glial cells and macrophages with lipidic inclusions and phagocitized debris. Axons, dendrites and synaptic contacts were spared in the lesioned striatum, however, in contrast to the typical striatal myelinated axons grouped in bundles these were more disperse, and less uniform in diameter. The lesioned core was characterized by a marked increase in vascularization. Astrocytes did not encapsulate the lesion but showed abundant intermediate filaments consistent with reactivity. Astrocytes in the lesion site were strongly vimentin and GFAP positive 5 weeks after lesioning (Fig. 31B,C).

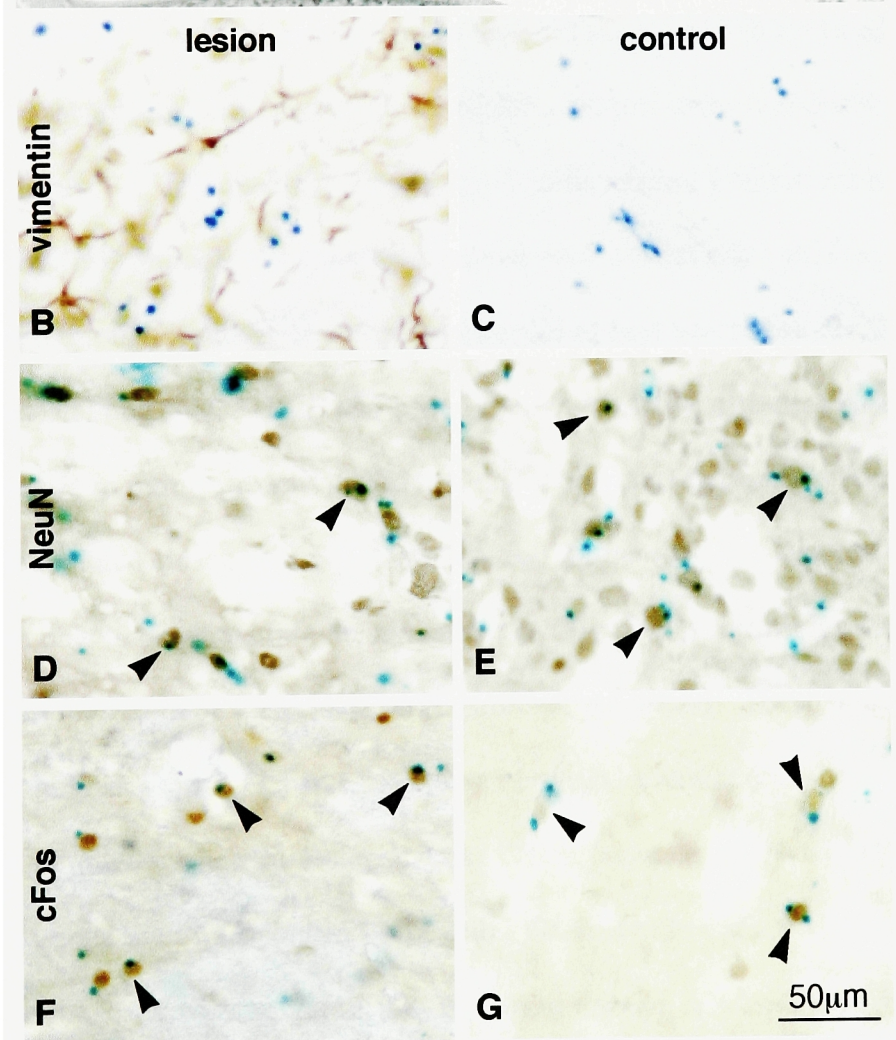
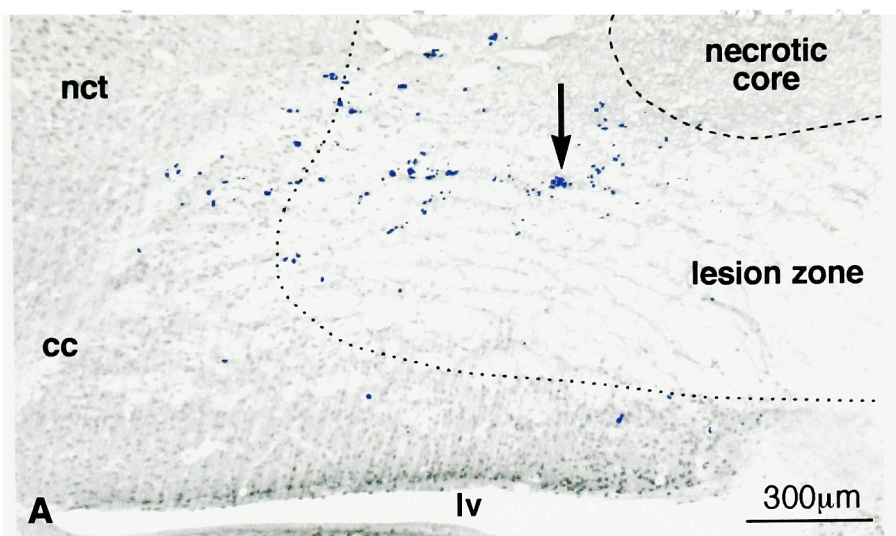
Figure 31: Transplantation of MGE cells into the adult brain lesioned with kainic acid (1 month after transplantation).

A: Horizontal section stained with X-Gal (blue) and NeuN antibody (black). Lesioned and necrotic zones are indicated. Site of transplantation (arrow) is outside of the necrotic area but within the lesioned zone, blue staining of MGE derived neurons was computer enhanced.

B, C: Immunostaining with anti-vimentin (brown color) in kainic acid injected striatum (B) and in a non-lesioned striatum (C). Note the intense immunoreactivity seen in the lesioned striatum as opposed to a non-lesioned grafted striatum.

D, E: Immunostaining with NeuN in kainic acid injected striatum (D) and in a non-lesioned striatum (E). Arrowheads point to immunoreactive MGE-derived cells.

F, G: Immunostaining with c-fos in kainic acid injected striatum (F) and in a non-lesioned striatum (G) 2 hours after haloperidol administration. Arrowheads point to c-fos positive MGE-derived cells. cc - corpus callosum, lv - lateral ventricle, nct – neocortex



MGE cells survive and migrate in the lesioned adult brain.

To assess the potential of MGE cells to migrate through the lesioned brain, I transplanted genetically labeled MGE cells into the necrotic and lesioned zones. Placement of MGE cells into the necrotic core resulted in poor survival and no migration of grafted neuronal precursors. Diffuse blue color, indicating the presence of transplanted tissue could be observed, but very few cells were identified. In contrast, when grafts were placed in the lesioned zone (approximately 0.5 mm from the necrotic core) MGE cells survived and dispersed up to 2 mm from the site of transplantation. Furthermore, average migration distance in the lesioned zone was $398 \pm 110 \mu\text{m}$, comparable to the average migration distance of $365 \pm 50 \mu\text{m}$ in the non-lesioned adult tissue (see Chapter 3). The maximal distance MGE cells migrated within the lesion was $1320 \pm 610 \mu\text{m}$ ($n=3$ animals; 4 sections per animal were quantified). MGE cells were able to migrate through lesion zone completely devoid of host neurons as confirmed by NeuN immunostaining (Fig. 31D,E) and EM. Extensive reactive gliosis as assessed by GFAP and vimentin immunoreactivity did not hinder neuronal dispersion. Some MGE neurons differentiated close to the boundary between the necrotic and lesioned zone but they were not able to penetrate into the necrotic core (Fig. 31A), confirming that this area cannot support migration or survival of MGE neuronal precursors. There were 160 ± 95 cells per $50 \mu\text{m}$ thick section dispersed within the lesioned striatum (only 20% (40 ± 25) of grafted cells migrated outside the lesioned area). Thus, lesioning of the adult brain did not prevent dispersion of transplanted MGE cells. Grafted cells did not accumulate specifically at the lesioned zone, rather they dispersed through lesioned brain in a manner very similar to that of MGE cells grafted into the intact adult brain.

Interestingly, previous studies of MGE transplants did not reveal the migratory capacity of MGE cells that I describe here (Graybiel et al., 1989; Pakzaban et al., 1993; Olsson et al., 1995; Olsson et al., 1997a). Since MGE cells in these studies were grafted into the same area where excitotoxins were previously injected, it is possible that necrotic tissue at the core of the lesion limited the migration of MGE cells. The discrepancy might also be due to differences in the techniques used to visualize grafts. While most previous studies utilized histochemical staining to visualize graft boundaries, I transplanted genetically marked cells allowing me to map graft-derived neurons that migrated far from transplantation sites and intermixed with the host tissue. It is also possible that in previous experiments the donor tissue contained mainly postmitotic zone of the medial ganglionic eminence, which is rich in postmitotic, postmigratory neurons (unpublished observation).

Mechanism of MGE cell migration in lesioned brain

It is known that brain tumors as well as immortalized cell lines often have the capacity to migrate within the adult brain (Snyder et al., 1997; Bayer et al., 1999). It would be interesting to find out whether MGE cells utilize similar mechanisms for their dispersion. In one specific case it has been shown that immortalized neuronal cells grafted into a novel model of brain injury migrate along radial glial processes towards the site where neurons have been lost (Leavitt et al., 1999). This model, unlike ours, is characterized by neuronal loss without glial reactivity. Since vimentin staining in our preparations failed to reveal radial glial fibers extending from the site of transplantation, it is unlikely that MGE cells utilize radial glia for their translocation. Our finding that MGE cells can effectively migrate through zone devoid of neurons indicates that

neuronal cell bodies are not required for their migration. MGE cells may employ axonal tracts, glia, blood vessels or extracellular matrix secreted by cells in the lesioned zone as a substrate for their translocation.

MGE cells differentiate into neurons in the lesioned brain.

The neuronal phenotype of MGE-derived, LacZ-expressing cells was confirmed by immunohistochemistry and electron microscopy. Graft-derived cells stained by X-gal also expressed the neuronal marker NeuN but not the astrocyte marker glial fibrillary acidic protein (GFAP) or vimentin. MGE-derived cells in semithin sections had a large round or oval nuclei with one or two central nucleoli. This morphology was similar to that of neighboring striatal neurons (in non-lesioned areas). Twenty randomly selected MGE cells expressing LacZ were resectioned for analysis by EM. All 20 cells studied by EM were neurons (Fig. 32B) had several Golgi apparatus, mitochondria, subsurface cisternae and abundant rough endoplasmic reticulum. The typical electron-dense X-gal product precipitate was easily identified in the EM within the rough endoplasmic reticulum. Most of the neurons also had deep nuclear invaginations, and in some of the sections axosomatic synaptic contacts were observed (Fig. 32C). Approximately 30% of grafted MGE cells were GABA positive as assessed by immunostaining and X-gal reaction.

Functional integration of grafted MGE cells

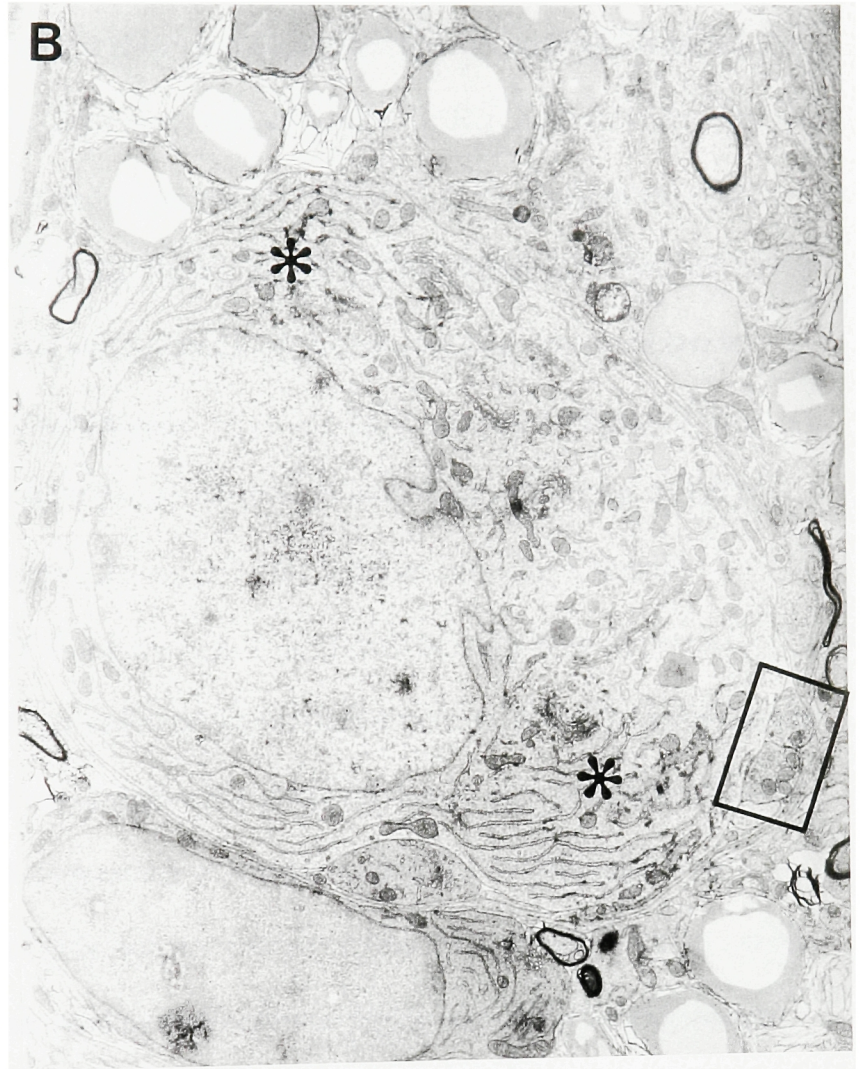
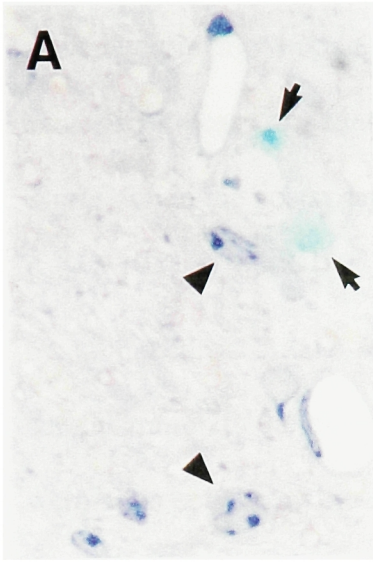
Functional integration of neurons transplanted into striatum was previously assessed by systemic administration of haloperidol, which induces c-fos expression in a subset of striatal neurons (Dragunow et al., 1990b; Schulz et al., 1994). This is thought

Figure 32: Ultrastructural analysis of grafted MGE-derived neurons in the lesioned striatum.

A. Semithin section of kainic acid-lesioned striatum. MGE-derived grafted cell with distinct x-gal deposits (arrows) is observed. No neurons from the host are seen but several glial cells are present (arrowheads). Irregularly shaped vessels and abundant lipidic drops in the neuropil are noted as well (thin arrows) , likely within macrophages.

B, Photograph at the EM level of neuron seen in A. Note the abundant Golgi apparatus and rough endoplasmic reticulum. Typical electrondense X-gal deposits are observed within the endoplasmic reticulum cisternae (asterisks). Observe abundant lipidic drops (LD) close to the grafted neuron.

C. Detail of axosomatic symmetric synaptic contacts.



to be mediated by blocking the inhibitory dopaminergic receptors located in glutamatergic presynaptic terminals (Maura et al., 1988). Thus, haloperidol is thought to induce the release of glutamate from corticostriatal axons, which in turn induces c-fos expression in the postsynaptic striatal neurons via NMDA receptors (Dragunow et al., 1990a; Herrera and Robertson, 1996). To test whether grafted MGE cells received functional corticostriatal innervation I examined expression of c-fos upon haloperidol administration to the transplanted animals. Approximately $9.84 \pm 4\%$ of grafted cells were c-fos positive in the striatum 2 hours after injection of haloperidol (Fig. 31F,G). Labeled MGE cells were distributed throughout the lesioned striatum. The same treatment resulted in $16.2 \pm 3.5\%$ of c-fos expressing striatal neurons in non-lesioned transgenic mice (not significantly different from lesioned animals, $p=0.27$). Transplanted mice injected with saline did not contain any c-fos positive graft-derived neurons in their striatum ($n=2$). These results suggest that at least some neurons that migrated long distances in the adult lesioned brain become integrated into the host brain circuitry. It is likely that these cells are innervated by corticostriatal projections. This is in agreement with the identification of axosomatic synaptic contacts on MGE cells at the EM level.

In this chapter I demonstrated that MGE cells can migrate and functionally integrate in experimentally lesioned adult brain. This finding may have important clinical implications. It directly demonstrates that the adult brain is not as restrictive for neuronal migration as was always thought. MGE cells could be directly used for treatment of neurologic conditions where GABAergic cells are needed. Alternatively, once we identify molecular mechanisms underlying MGE cell migration in the adult brain, we might be able to genetically modify other neural cells to make them capable of

similar dispersion after transplantation in the damaged host brain. This could dramatically improve the integration of grafted neuronal cells and the recovery of impaired brain functions.

CHAPTER 6: CONCLUSIONS

The twentieth century has witnessed revolutionary progress in the natural sciences culminating in the recent boom in molecular biology and genetics. Despite the progress in understanding the basic biochemical and molecular processes governing cells, there are many fundamental questions concerning the nature of life that remain largely unanswered. On a conceptual level, we understand very little how form and function arise during ontogenesis. Even worse, we have almost no clue how awareness, thoughts and feelings emerge from interactions between our brain, body and the world around us. During brain development, millions of cells must organize themselves into the complex network of brain tissue. How is this construction orchestrated? How are individual cells instructed where to go, what to do and who to contact?

In my thesis, I touched upon some of these questions. I started by analyzing a novel form of tangential neuronal migration, so called chain migration, in the postnatal subventricular zone (SVZ). I developed a simple in vitro assay which allowed me to study migratory behavior of SVZ neuronal precursors by time-lapse microscopy. Using the same assay, I identified two other regions in the embryonic brain containing cells capable of extensive migration. I focused then on neuronal precursors found in a region called medial ganglionic eminence (MGE) because, out of all tested cells, MGE neuronal precursors were the best migrants in vitro. Quite surprisingly, I found that MGE cells can also migrate through the adult brain tissue. This migratory capacity has not been previously demonstrated for any other primary neuronal precursor. Analysis of MGE cell migration in the embryonic brain revealed that long-distance migration is normal behavior for MGE cells during development. In contrast to the adult brain where MGE

cells disperse indiscriminately in all directions, in embryonic brain, MGE cells migrate specifically in the dorsal direction, passing through the LGE and invading the neocortex. I demonstrated that brain tissues neighboring MGE ventrally and medially (hypothalamus and thalamus) are non-permissive for MGE cell migration. I propose that differential permissiveness of individual forebrain regions may be the main mechanism directing MGE cells into neocortex. The finding that extracortical cells participate in the histogenesis of the neocortex clearly demonstrates that long-range neuronal migration plays an indispensable role in brain development. Long-distance neuronal migration might provide an additional level of complexity to the cortical cytoarchitecture, which is otherwise constrained by the limited space and time during development (see section on "Why are MGE cells imported to the neocortex?").

Are all tangentially migrating neurons equal?

Tangential migration is defined as a neuronal movement in a direction perpendicular to radial glia or parallel to the plane of the brain surface. It would be a mistake to assume that all tangentially migrating neurons use the same mechanisms and substrates for translocation. My experiments clearly demonstrate that individual populations of tangentially migrating neurons behave very differently. For example, postnatal SVZ, embryonic LGE and MGE cells all migrate extensively through Matrigel in vitro. In contrast, cells from rhombic lip, which migrate tangentially through the external granule layer of cerebellum, do not migrate readily in this in vitro assay. While SVZ and LGE cells migrate after transplantation from the adult SVZ to the olfactory bulb, MGE cells are unable to follow this migratory pathway. Instead they disperse in all directions through the adult brain tissue. In the embryo, LGE cells migrate within the

developing striatum and through piriform cortex. MGE cells, on the other hand, migrate a long distance to the neocortex. These are just a few examples of how different the migratory behavior can be of different tangentially moving cells in different conditions and environments. It is likely that different molecular mechanisms at the cellular level are employed to govern migration of different pools of tangentially migrating cells.

The in vitro assay I developed to study migration of postnatal SVZ cells allowed me to study two other populations of tangentially migrating neuronal precursors – the embryonic LGE and MGE. With this assay I was able to study and compare migratory behavior of these three cell populations which revealed interesting differences (see the next section). This in vitro assay will also help to elucidate the molecular mechanisms underlying tangential migration. Neuronal migration in Matrigel has been used to determine that blocking of the $\alpha 6 \beta 1$ integrin can disrupt chain migration of neural cells grown as neurospheres in vitro (Jacques et al., 1998). In addition, this assay helped to demonstrate that secreted molecule slit might act as a repulsive factor involved in directional guidance of postnatal SVZ neuronal precursors migrating in chains (Wu et al., 1999).

Mechanism of MGE cell migration

When SVZ, LGE and MGE cells are cultured in Matrigel there is a clear difference in the extent of their migration (Table 2). MGE cells can penetrate into the Matrigel much faster than LGE or SVZ cells. Interestingly, the speed of individual MGE cells migrating along established pathways is the same as in LGE or SVZ cultures. When we studied cultures of SVZ cells migrating in Matrigel by electron microscopy, I noticed

that migrating cells digest tunnels through the Matrigel (Fig. 7A). This suggests that migrating cells at the tips of growing chains produce proteases that breakdown the extracellular matrix. Matrigel cultures has been previously used to assess invasiveness of tumor cells in vitro. It is known that highly invasive tumors express proteases which allow them to digest and penetrate Matrigel (Rao et al., 1993; Tonn et al., 1999). Similarly, it is possible that MGE cells produce more potent proteolytic enzymes than SVZ or LGE cells, helping them to invade the extracellular matrix more effectively. As shown in Chapter 3, MGE cells (and not LGE or postnatal SVZ cells) can disperse effectively through the adult brain tissue. Thus, specific proteolytic enzymes may also allow MGE cell penetration into the adult brain tissue.

It is interesting that MGE cells grafted into the adult SVZ do not migrate along the rostral migratory stream. This indicates that MGE cells are not responsive to the guidance cues which direct migration of LGE or SVZ cells to the olfactory bulb. MGE cells grafted into striatum migrate preferentially along white matter tracts. Within striatum, migrating MGE cells are oriented in the antero-posterior direction along striatal white matter, but when they enter the corpus callosum they orient themselves medio-laterally along callosal fibers (Fig. 19B,C). It is possible that MGE cells align with white matter fibers because it is the least resistant route for their migration. From my studies, I cannot conclude whether white matter is a specific substrate for MGE cell migration or whether the alignment of MGE cells along white matter fibers results from mechanical constraints in the adult brain.

In the embryonic brain MGE cells invade the neocortex through the subventricular and intermediate zone. Ultrastructural and immunohistochemical study of

the embryonic intermediate zone revealed that tangentially migrating cells are in close contact with corticofugal axons running through this region towards ganglionic eminences (O'Rourke et al., 1992; O'Rourke et al., 1995; Metin and Godement, 1996; Metin et al., 2000). It is interesting that both in the embryo and in the adult, MGE cells preferentially migrate along axonal tracts. Since many axons in the adult white matter are covered with myelinating oligodendrocytes, it is unlikely that MGE cells use adult axons directly as a substrate for translocation. Indeed, ultrastructural reconstruction of MGE cells in the adult did not reveal direct contacts between migrating cells and axons. Therefore, it is possible that axonal tracts are preferentially used for MGE cell migration either for mechanical reasons, or axons release some substances which might promote or attract neuronal migration. It has been shown that tangentially migrating cells in the embryonic intermediate zone express glutamate AMPA receptors which could be stimulated by glutamate released from corticofugal axons in the intermediate zone (Metin et al., 2000). Glutamate and calcium signaling is thought to play an important role in radial migration. It has been shown that blocking glutamate transmission through NMDA channel can inhibit radial migration in vitro (Komuro and Rakic, 1993; Komuro and Rakic, 1998). It is possible that glutamate which is released from corticofugal axons in the intermediate zone, plays a similar role in tangential migration of MGE cells into the developing neocortex.

Non-permissive nature of the diencephalon

Besides the migration of cells from ventral forebrain to neocortex, it has been proposed that the ganglionic eminences might give rise to some thalamic neurons. However, this gangliothalamic migratory stream has been so far detected only in primate

and human embryonic brains, and it has been argued that this migration does not happen in other vertebrates (Rakic and Sidman, 1969; Letinic and Kostovic, 1997). Here I studied whether MGE cells have the capacity to migrate into the diencephalon in embryonic rodent brain. I failed to detect cell migration from MGE to the thalamus. Moreover, I found that developing diencephalon is non-permissive for MGE cell movement. It is interesting that this inhibition is removed later in development, which explains why MGE cells grafted into the adult thalamus were able to disperse from the site of transplantation. This suggests that the embryonic brain actively prevents MGE cells from the ventro-medial migration. It is possible that this block develops to help steer MGE cells into the developing neocortex. Alternatively, MGE cells might be detrimental to the histogenesis of the diencephalon, and this block may have evolved to prevent MGE cells from invading this region. Although I did not detect any MGE cells migrating into the thalamus, I cannot conclude that gangliothalamic migration is absent in the rodent brain. It is still possible that this migration happens at other developmental times, or that the source of cells migrating into thalamus might be other than MGE, the region I studied. Studies of the gangliothalamic pathway in the human brain revealed that migrating neuronal precursors in this region are confined to a narrow stream on the surface of the thalamus. This migration is more reminiscent of the SVZ chain migration in the adult brain than of widespread dispersion of MGE neuronal precursors in the embryonic or adult brain, as described in Chapters 3 and 4. It is possible that even in the human brain, thalamus is not permissive for widespread migration of ganglionic eminence cells. Gangliothalamic pathway may be a specialized region, similar to the adult SVZ, which is permissive for migration of cells from ganglionic eminences.

Tangential migration, radial units and neocortical development

Original models of cortical development assumed that all cortical neurons are derived from palial neuroepithelium. In this model, cells born in the neocortical ventricular zone migrate radially into the cortical plate. The route of these neurons is predominantly radial, although some cells have been observed to switch between radial and tangential migration, resulting in slight dispersion before neurons settle in their final destination. This type of migration could account for conical shape of radial clones found by retroviral lineage study and in X-LacZ chimeric mice (see Introduction). However, it became clear that another population of neuronal precursors with very different migratory behavior contributes to cortical development. These cells migrate predominantly in the tangential direction dispersing throughout the entire neocortex, ignoring the radial code of cortical development.

Analysis of neuronal phenotypes in radial and dispersed clones revealed that there is a bias in the ratio of glutamatergic and GABAergic cells. Dispersed neurons tend to be GABAergic, while radially migrating neurons tend to be glutamatergic (Tan et al., 1998). This finding provides the basis for a new hypothetical model of cortical development, which could reconcile the controversial "radial unit hypothesis" with the demonstration of widespread tangential migration during forebrain development. Regional differences in cortical neuroepithelium defined by different patterns of gene expression might provide the initial blueprint for functional specialization of different cortical areas (Donoghue and Rakic, 1999a; Donoghue and Rakic, 1999b). Since the internal structure of cortical circuits seems to be modular across the cortical plate, the most important determinant of cortical function might be the long distance connections between cortical

and subcortical regions. In other words, the proper functional differentiation of cerebral cortex is dependent on the correct innervation from the thalamus (i.e. which thalamic axons innervate which cortical area) and proper cortical projections (i.e. which cortical neurons innervate which subcortical or other cortical areas). As I mentioned above, it is possible that the genetic code in the early cortical neuroepithelium could provide the necessary instruction for the principal wiring of the developing brain. Therefore, it seems important for the projecting pyramidal neurons to maintain their regional molecular code and to avoid extensive tangential dispersion. Principal pyramidal cortical neurons are glutamatergic, which correlates with the observation that the radial columns are composed predominantly of these neurons (Tan et al., 1998). In contrast, we can speculate that modulatory non-pyramidal cortical interneurons do not need to carry any specific positional information. It is possible that they integrate and function in a similar way across the entire neocortex. Therefore, there is no reason why they should be specified locally within individual "radial units". There might be no evolutionary constraint to prevent them from tangential dispersion across many different functional areas in the cortex. It is possible that GABAergic neurons have to be in place very early in development, before glutamatergic cells start to function, to dampen the excessive release of glutamate which might result in excitotoxic cell death or produce uncontrollable seizures. For this reason it might be advantageous to let cortical GABAergic interneurons disperse evenly throughout the developing neocortex. Tangentially migrating GABAergic cells might also regulate the early steps of corticogenesis by producing signals affecting the proliferation, migration or commitment of young neurons produced in the neocortical ventricular zone.

Thus, this “dualistic” hypothesis of cortical development postulates that there are two general types of cells participating on cortical development. One type consists mainly of principal cortical neurons which carry positional information needed for proper brain wiring. Migration of these neurons is predominantly radial, restricted to a narrow brain region. A second type of neurons consists mainly of modulatory neurons which do not carry any positional information and are expected to function similarly in any cortical area where they land. Although the primary functional parcellation of the developing cortex might be genetically encoded, I believe that the fine structural differentiation of individual cortical areas into specialized cortical columns is independent of a predetermined molecular code. This later refinement likely results from selective neuronal recruitment, neuronal death and dendritic sprouting and pruning dependent on brain activity and function.

Why are MGE cells imported to neocortex?

In my thesis I show that some of the tangentially dispersing neurons found in developing neocortex do not originate in the palial neuroepithelium but are born in the opposite (ventral) part of the forebrain in the medial ganglionic eminence. I show that MGE cells engage in a long journey across most of the forebrain to invade and diffusely populate the growing neocortex. For now, I can only offer a speculative suggestion why this cumbersome migration happens and what kind of role it may play in the cortical development and brain function. There may be many different reasons for this migration. Here I will consider one possibility which seems to me quite interesting.

The brain is a complex organ with many specialized cell types that are intermixed in a highly organized tissue. Each of these cells has to be produced during development

and instructed where to go and what to do. Neural precursors in germinal zones might be modulated and shaped into specific phenotypes by their interactions with the environment. Different signals might trigger different responses from individual cells leading to a production of specific cell types. It is conceivable that only a limited number of signaling pathways can be used without interference in one place at one developmental time. Therefore, in order to produce all the different cell types needed for proper brain function it might be necessary to isolate individual fate decisions for the production of different brain cells in time or space. For example, it is known that neuronal precursors in the cortical ventricular zone do not have the same potential at different developmental times. It has been shown that while early cortical progenitors have fairly broad developmental potential, later cortical precursors are restricted to produce only later, more superficial neuronal phenotypes (McConnell, 1988; McConnell, 1995). This is an example of temporal isolation of individual fate determination events, which correlate with temporal gradient in the production of different types of cortical neurons. However, the number of different cell types generated in this way might be limited by the short time allocated to brain histogenesis. It is also possible that certain signaling molecules required for the generation of one set of neurons are incompatible with production of a different set of brain cells. Therefore, spatial isolation followed by a long distance tangential migration might be the only possible solution for the generation of a full complement of brain cells. Not only does spatial isolation result in parallel production of different types of brain cells, but it also allows different cells to be exposed to different potentially incompatible signaling molecules. It is foreseeable that ventral signaling molecules which are necessary for the specification of MGE cells (like sonic hedgehog or

FGF8) might be disruptive for the determination of neocortical phenotypes, and vice versa, molecules present in the dorsal forebrain (like different Wnt molecules or BMPs) might be detrimental to the formation of MGE specific cell types. If this is the case, MGE cells could never form in the dorsal cortical neuroepithelium, and the only way to get this neuronal phenotype in the dorsal brain would be to import cells from the ventral region whose phenotype was already determined by the ventral factors. Thus, tangential migration may be a fundamental process required for proper brain development.

In conclusion, I propose that there might be a limit to how many different signaling pathways affecting cell behavior, shape, commitment or fate can be activated in one place during one developmental period. This could possibly set limits on how complex a tissue can get. In order to produce cortical tissue capable of higher cognitive functions, evolution had to find a way to overcome this limitation. I propose that tangential migration leading to the import of neuronal cell types from neighboring germinal regions into the neocortex might be an elegant solution to this problem.

MGE cells and brain repair

In my thesis I identified a novel migratory behavior of primary neuronal precursors. I demonstrated that MGE cells can migrate through the adult brain parenchyma. This finding is very exciting, because it gives us a hope that neural transplantation could become an effective way to repair a brain damaged by disease or injury. In previous studies, cells transplanted into the adult brain remained at the site of transplantation and did not integrate very well into the host brain tissue. Grafts are often surrounded by a glial scar tissue, isolating transplanted cells from direct interactions with the host brain cells. The adult brain environment and particularly the damaged brain

tissue is believed to be inhibitory for axonal outgrowth and cell migration. This is considered a major hindrance for the successful graft integration and recovery of damaged brain function. In an ideal scenario, we would like to implant neuronal precursors in the damaged brain that could integrate specifically in sites where new neurons are needed. Thus, dispersion of transplanted neuronal precursors through damaged brain would greatly facilitate brain repair and functional recovery. In Chapter 5, I showed that MGE cells are not only able to migrate through the intact brain, but they can disperse and functionally integrate even in experimentally lesioned adult brain tissue. The fact that MGE cells can migrate throughout excitotoxin-lesioned striatum and integrate into the host brain suggests new prospects for brain repair. These results demonstrate that the injured adult brain environment may not be as limiting for cell migration and axonal outgrowth as was previously thought. Analysis of the phenotype of grafted MGE cells in the adult brain suggests that they are medium size GABAergic inhibitory neurons. The fact that I did not detect any other neuronal types among grafted MGE neurons indicates that the fate of dispersed MGE cells is predetermined. This suggests that MGE cells are not going to be a magic bullet healing all types of neural impairments. MGE cells might be used directly to treat neurologic conditions where GABAergic cells are missing (like Huntingtons disease) or where inhibitory modulatory neurons might alleviate brain dysfunction (like in some types of epilepsy). Alternatively, identification of the intrinsic mechanisms underlying MGE cell migration might allow modification of other neuronal precursors to permit their dispersion throughout the adult injured brain. MGE cells could also serve as vehicles for the delivery of neurotrophic factors, cytokines or other molecules preventing further degeneration or aiding brain

recovery. Further work on mechanisms that facilitate MGE cell migration in the adult brain and on factors that direct differentiation of MGE precursors into appropriate neuronal types is required. Only then we will be able to take full advantage of the MGE as a source of neuronal precursors for brain repair.

APPENDIX I: CD-ROM MOVIES

The attached CD contains movies of SVZ, LGE and MGE cell migration in vitro (for details see Chapter 2).

SVZ movies

Compact chain: (Comp CD.mov) shows cells closely associated to each other in a chain. Only individual nuclei are visible. Notice how cells move along the chain in both directions. Six frames from this series are presented in Fig. 10. Explant is to the lower right. Real time 1 hour. (small version)

Handshake: (Hand 60.mov) shows a chain of migrating cells in the lower part of the frame. Above this chain notice a cell on the left, which extends a leading process in search of another cell. When the growth cone detects another leading process it tightly attaches and the cell body starts to move forward.

Jumping cell: (Jump 60.mov, Jump CD.mov) proximal part of the culture where chains form a dense web. Notice cell that transfers from one chain to a neighboring chain. During this exchanges the behavior of the leading process and growth cone is clearly displayed. The leading process extends and the growth cone contacts a neighbor. At this time, the cell body that has been relatively quiescent translocates crossing from one chain to the next. See also Fig.12. Explant to the lower right. Real time 1 hour.

X chain: (X 60.mov, X CD.mov) shows the intersection formed by 2 chains and behavior of migrating cells in this intersection. Notice the cell that migrates along the upper left

arm of the X and transfers in the intersection to the upper right arm. The explant is down.

Real time 1 hour.

LGE movies

LGE 20x.mov: lower magnification view of a long chain extending from the LGE explant.

LGE 40x.mov: higher magnification view of a network of migrating cells. Notice that LGE cell movement is indistinguishable from the SVZ movement.

MGE movie

MGE 20x.mov: low magnification of an MGE explant. Movie starts when only few processes are extending into the Matrigel. Notice how rapidly and massively MGE cells invade to extracellular substrate compared to the LGE or SVZ cells.

APPENDIX II: METHODS

Cultures of subventricular zone. Brains from three to ten day old CD-1 mice were placed in ice cold Leibovitz's L-15 medium (Gibco). The subventricular zone from the lateral wall of the anterior horn of lateral ventricle was dissected and cut into pieces 50-300µm in diameter. The explants were mixed with Matrigel (Collaborative Biomedical Products) in a 1:3 ratio and allowed to congeal in a culture dish, which was prepared by cutting a 7.5 mm diameter hole in the bottom of 35 mm Petri dish (Corning) and attaching an 18 mm #1 cover slip with paraffin. The gel containing the explants was overlaid with 2 ml of serum-free Neurobasal medium (Gibco) containing B-27 supplement (Gibco), 0.5mM L-glutamine (Gibco) and penicillin-streptomycin antibiotics (Gibco). Cultures were maintained in a humidified, 5% CO₂, 37°C incubator (Heraeus).

DiI labeling. Crystals of lipophilic dye DiI (DiI, Molecular Probes) were inserted into explants of SVZ. Unincorporated crystals were washed off with excess of L-15 medium and explants were embedded in Matrigel as described above. After 24 hrs in culture individual labeled cells were visualized with fluorescent illumination and photographed on an Olympus inverted microscope IX70.

Immunocytochemistry. Immunological characterization of cells in chains was performed directly in culture dishes. Samples were fixed with 3% paraformaldehyde solution in phosphate buffered saline pH 7.3 (PBS) for 1 hr at rt. and blocked for 3 hrs in 10% horse serum, 1% BSA, 0.5% Triton X-100 in PBS. Incubation with primary monoclonal antibodies (IgM anti-MEN-B to stain polysialylated N-CAM (Rougon et al., 1986) (a gift from G. Rougon, Marseilles, France), 1:1000 dilution; TuJ1 (Lee et al.,

1990; Moody et al., 1996), 1:500 dilution (a gift from A. Frankfurter, University of Virginia, Charlottesville); anti-GFAP (Sigma), 1:200 dilution; anti-vimentin 40E-C (Alvarez-Buylla et al., 1987) undiluted; anti MAP2 (Sigma), 1:500 dilution was carried out overnight at 4°C. The samples were washed with 0.5% Triton X-100 in PBS and incubated either with goat anti-mouse IgM conjugated with RITC (to visualize anti-MEN-B antibodies and 40E-C) or with biotinylated horse anti-mouse IgG (Vector, 1:200 diluted) overnight at 4°C. After washing the cultures with 0.5% Triton X-100 in PBS, the biotinylated antibodies were visualized by incubating samples with FITC conjugated avidin (Vector, 20 µg/ml) overnight at 4°C. Samples washed in 0.5% Triton X-100 in PBS were mounted in glycerol and photographed as described above.

Complement-mediated lysis. After 6 hours in vitro, cultures were incubated with 7B11 monoclonal antibody (Szigeti and Miller, 1993) diluted 1:50 in medium for 2 hours at 37°C. Cultures were washed 3 times with medium and incubated with rabbit complement (Cappel), 1:5 dilution in medium for 2 hours at 37°C. Cultures were washed 2 times with medium. The procedure was repeated at 30 hours in vitro. The cultures were fixed after 48 hours in vitro and processed for GFAP immunoreactivity as described above. Explants incubated in anti-PSA-NCAM antibody diluted 1:1000 and complement under the same conditions as above resulted in the killing of cells in the chains.

Ultrastructural analysis. Twenty four hours after explantation, cultures were fixed in 3.5% glutaraldehyde for 30 min. at 37°C. Postfixed in 1% OsO₄ for 30 min. at rt. and stained in 5% aqueous uranyl acetate in the dark for 1 hr. at 4°C, rinsed, dehydrated in ethanol and infiltrated overnight in araldite (Durcupan, Fluka). Following polymerization, embedded cultures were detached from the coverslip, by freezing in

liquid nitrogen and thawing. Serial semithin (2 μm) and ultrathin (0.05 μm) sections were prepared in an Ultracut (Reichert), stained with lead citrate and photographed on a Jeol 100CX electron microscope. In some of the cultures the migrating cells studied by time-lapse video microscopy were subsequently identified at the electron microscope.

Time-lapse videomicrography. After 10-30 hrs in culture, selected dishes were overlaid with 2 ml of mineral oil (Sigma) and inserted into a heated culture chamber (Medical Systems Corp.). Steady flow (12.5 ml/minute) of 100% CO_2 gas over the oil surface maintained neutral pH of the culture medium. The chamber was mounted on an inverted Olympus microscope equipped with Nomarski differential interference contrast optics and a monochromatic CCD camera Hitachi Denshi KP-M1U. Composite signal from the camera was recorded onto a Toshiba time-lapse video KV-6300A, set at 1:360 time compression and simultaneously captured into Power Macintosh 8500 at a rate of 5 frames/minute (1:360 time compression) at a resolution of 640X480 pixels using Premiere 4.2 (Adobe) software. The cultures were recorded for time periods ranging from 3 to 20 hrs.

Calculation of cell flow through a chain. To calculate a bulk flow of cells through individual chains I first counted how many cells migrating away from explant pass an imaginary line drew across a selected chain (N_A) and the number of cells crossing the line migrating towards the explant (N_T) during a time period T . Rate of cell migration away from explant was calculated as $(N_A - N_T)/T$. Next I expressed thickness of the chain as the length of the chain corresponding to a single cell (L/C), where C is the number of cells contained in the chain of length L . The speed of bulk flow was then calculated by multiplying the rate of cell migration with the length of chain corresponding to a single

cell: $((N_A - N_T)/T) * (L/C)$. Positive value of the calculated speed means that the overall cell movement is in the direction away from explants.

Tissue dissection and embryonic culture conditions. LGE and MGE germinal regions were dissected from E14 embryonic CD-1 mice (Cavanagh, 1970). Only the ventricular zone and the SVZ was harvested from each region. Bordering tissue between adjacent regions was discarded to avoid contamination. Neocortical ventricular zone was dissected from cortical roof dorsal to LGE. Explants were embedded in Matrigel (Collaborative Biomedical Products) and cultured in neurobasal medium (Gibco) for 24 hours (Wichterle et al., 1997).

Transplantation. Small pieces (~200-400 μ m in diameter) of tissue dissected from NSE::LacZ transgenic E14 embryos (Forss-Petter et al., 1990) were loaded into a glass micropipette and grafted into brains of anesthetized adult CD-1 mice using a stereotactic apparatus (Lois and Alvarez-Buylla, 1994). Coordinates from bregma (anterior, lateral, deep from pia) -- striatum: 0.5 mm, 1.5 mm, 2.3 mm; thalamus: -1.5 mm, 1 mm, 3 mm; SVZ: 1 mm, 1 mm, 2.3 mm. After 1 month survival, brains were fixed and 50 μ m horizontal sections were stained with X-Gal (Lois and Alvarez-Buylla, 1994). Transplanted neurons were mapped using a computer aided mapping system (Alvarez-Buylla and Vicario, 1988). All experimental animals were treated in accordance with Laboratory Animal Research Center guidelines.

Immunostaining of embryonic cells. Two months after transplantation, mice were sacrificed and their brains were processed as described above. Floating brain sections were immunostained with antibodies against NeuN (1:500, Chemicon), Hu (12 μ g/ml) (Marusich et al., 1994), GFAP (1:1500, Sigma), vimentin (1:5) (Alvarez-Buylla et

al., 1987), tyrosine hydroxylase (1:1500, Pel Freeze), choline acetyl transferase (5 µg/ml, Boehringer), GABA (1:500, Immunotech) using biotinylated secondary antibodies, ABC kit (Vector) and DAB. Additional GABA staining was performed on semithin sections. Semithin sections (see Electron Microscopy) were exposed to 20% toluene, 30% acetone in sodium etoxide solution for 15 minutes, hydrated and washed once in 3% H₂O₂, three times in water, once in 1% sodium borohydride in PBS, and finally three times in water. Sections were then immunostained with GABA antibodies (1:1000, Immunotech), biotinylated secondary antibodies, ABC kit (Vector) and DAB.

Electron Microscopy. One month after MGE transplantation, mice were deeply anesthetized with Nembutal and transcardially perfused with 0.9% saline followed by 100 ml of 4% paraformaldehyde and 0.5% glutaraldehyde. The brains were then washed in 0.1 M PB for 2 hours. Horizontal 100 µm sections were cut on a Vibratome. The sections were stained for X-Gal, post-fixed in 2% osmium for 2 hours, rinsed, dehydrated and embedded in araldite (Durcupan, Fluka). Semithin sections (1.5 µm) were cut with a glass knife and stained lightly with 1% toluidine blue. For the identification of individual X-Gal labeled cells, ultrathin (0.05 µm) sections were cut with a diamond knife, stained with lead citrate and examined under a Jeol 100CX electron microscope.

Cell dissociation and fluorescent labeling. Dissected MGE and neocortex were incubated in 0.06 µg/ml papain (Worthington) in 120 mM NaCl, 5 mM KCl, 25 mM glucose, and 20 mM Pipes for 10 minutes at 37°C, dissociated to single cell suspension in Neurobasal medium (Gibco) with 1 mg/ml DNase (Worthington) and labeled with PKH26 fluorescent dye (Sigma) diluted 1:200 with diluent C. Cells were washed with Neurobasal medium and microinjected (0.1 µl) into striatum of adult mouse. Brains were

processed 20 and 48 hours after transplantation as described above. Sections were wet mounted in PB and photographed using epifluorescent microscope (Olympus, IX70). Each experiment was repeated five times.

Organotypic cultures. Approximately 300 μm thick transversal sections were cut from the embryonic forebrains. Sections containing diencephalon, MGE, LGE and neocortex were transferred on floating polycarbonate filters (Nucleopore, Costar). Cells in MGE, LGE or neocortex were labeled with DiI diluted in diluent C from PKH26 kit (Sigma) using glass micropipette and microinjector. Filters with sections were floated on 1 ml of Neurobasal medium with B27 supplement (Gibco) for 24 hours at 37°C in a tissue culture incubator (5% CO₂). Sections were then fixed in 3% paraformaldehyde, wet mounted in PB, photographed and the location of DiI-labeled cells mapped using a computer aided mapping system (Alvarez-Buylla and Vicario, 1988).

Excitotoxic lesions: Adult CD-1 mice (2-3 month old) were anesthetized with pentobarbital (50 mg/kg i.p.) and placed in a stereotaxic apparatus. The skin over the skull was opened and an approximately 1 mm diameter hole was drilled in the skull. A micropipette filled with kainic acid (1 mg/ml in 0.9% NaCl) was lowered into the striatum and 0.25 ml of solution was injected. Coordinates from Bregma (anterior, lateral, deep) were 0.0mm, 2.5 mm and 3.2 mm. Animals were treated in accordance with the Rockefeller University animal research facility guidelines.

Transplantation into lesioned brains: Animals received grafts seven to nine days after lesioning. Small pieces of MGE tissue dissected from NSE::LacZ transgenic E14 embryos (Forss-Petter et al., 1990) were loaded into a glass micropipette and grafted into brains of lesioned mice using a stereotaxic apparatus. The same coordinates as for

the lesion were used for transplantation into the necrotic core. For transplantation into lesioned area adjacent to the necrotic core I used the following coordinates (anterior, lateral, deep) 0.5 mm, 2.0 mm and 3.2 mm from Bregma. After 1 month survival, mice were deeply anesthetized with pentobarbital (50 mg/Kg) and intracardially perfused with 0.9% saline followed by 3% paraformaldehyde, 0.5-2% glutaraldehyde. Brains were fixed and 50-µm horizontal sections were stained with X-gal. Transplanted neurons were mapped using a computer-aided mapping system (Alvarez-Buylla and Vicario, 1988).

Immunostaining: One month after transplantation, mice were sacrificed and their brains were processed as described above. Floating brain sections were immunostained with antibodies against NeuN (1:500, Chemicon), vimentin (1:5), GABA (1:500, Immunotech), GFAP (1:500, DAKO) and c-fos (1:20,000 Chemicon) using biotinylated secondary antibodies (Vector) ABC kit (Vector) and diaminobenzidine (DAB). Drug administration: Grafted mice (n=4) received haloperidol 2mg/kg i.p., 2 hours prior to perfusion (2 mg of haloperidol were dissolved in 40 ml of acetic acid and then brought to 1 ml with 0.9% saline). In addition, two non-lesioned NSE::LacZ transgenic mice were administered 2 mg/kg of haloperidol i.p. and perfused 2 hours later as indicated above. Counting of c-fos labeled cells: c-fos immunoreactive cells were counted in 4 different sections per grafted striatum. The number of double labeled β -galactosidase positive and c-fos immunoreactive cells were counted. In addition total number of β -galactosidase positive cells were counted. Percentage of double labeled cells over total β -galactosidase positive cells was calculated and compared with the percentage observed in the non-grafted transgenic mice upon identical treatment.

Electron microscopy of lesioned brains: Perfusion, sectioning and β -galactosidase histochemical processing was as for light microscopy. Selected sections containing X-gal positive cells were post-fixed in 1 % osmium with 7% glucose for 2 hours, rinsed in maleate buffer, pH 7.4, 3 times, 15 minutes each, placed in uranyl acetate 2 % maleate buffer for 2 hours at 4 °C. Sections were then dehydrated in ethanol, placed in propylene oxide for no more than 4 minutes (if left more time there was a noticeable loss of X-gal staining) and embedded in Araldite (Durcupan, Fluka Biochemika). Serial 1.5 μ m semithin sections were cut with a diamond knife and lightly stained with 1% toluidine blue. Semithin sections were analyzed for the presence of X-gal-positive cells and photographed. These 1.5 μ m sections were glued to an Araldite block and then detached from the slide for resectioning and analysis of labeled cells at the EM. Ultrathin sections (50 nm) were cut with a diamond knife, stained with lead citrate and examined under a Jeol 100 CX electron microscope.

Dot assay. Embryonic MGE and other tested brain regions were dissected from 13 to 14 day old embryos (~5-10 embryos per experiment). Tissue was mechanically dissociated by repeated passing through the yellow pipette tip in 200 μ l of L15 medium. Dissociated cells were diluted in 1 ml of L15 medium containing 0.1 mg/ml of DNase. Larger pieces of undissociated tissue, which settled at the bottom of the tube were discarded. Cells were collected by centrifugation in benchtop centrifuge (2000 rpm for 5 minutes) and washed 2x by resuspension in 1 ml of L15 medium. MGE cells were then labeled using PKH26 kit (Sigma) and collected by centrifugation. MGE cell pellet was let to aggregate for ~15 minutes at room temperature and cut into small pieces in L15 medium. Tested cells were collected by centrifugation and resuspended in 1 volume of

L15 medium. One microliter of resuspended cells was dotted on polycarbonate filter (Whatman, 0.4 μ m) floating on the surface of 1 ml of neurobasal medium supplemented as described above. Dishes were kept for 2 days in standard tissue culture incubator. Filters were then fixed with 3% paraformaldehyde and photographed using digital SPOT camera attached to a fluorescent microscope. MGE migration was analyzed using NIH Image 1.62b7 program.

Ultrasound guided transplantation. MGE, LGE or neocortex were dissected from E13.5 donor embryos (~10-15 embryos per experiment) and cells were dissociated and labeled with PKH26 kit as described above. Labeled cells were collected by centrifugation and resuspended in 5 μ l of L15 medium containing 0.1 mg/ml of DNase. Cell suspension was loaded into beveled glass pipettes (~40 μ m diameter). The pipette with labeled cells was mounted in a microinjector (Narishige) attached to a stereotaxic apparatus aligned with the transducer of the ultrasound backscatter microscope. Recipient pregnant mouse (E13.5) was anesthetized with nembutal, uterus was exposed and an embryo was passed through a silicon membrane covering a hole in the bottom of a deep Petri dish. The dish was fixed above the anesthetized mouse and filled with PBS (containing Ca⁺⁺ and Mg⁺⁺). The assembly was placed under the transducer of ultrasound backscatter microscope. The tip of the pipette with labeled cells was inserted into the target region in the embryonic brain and 10-30 nl of cell suspension were injected.

Processing of embryonic brains. Surviving embryos were recovered 1-4 days after transplantation, intracardially perfused with 3% paraformaldehyde and 0.25% glutaraldehyde. Brains were removed and cut coronally on vibratome (50 μ m thick

sections). Brain sections were wet mounted and photographed on fluorescent microscope (using 2x objective and digital cooled CCD camera SPOT). Images were analyzed using NIH Image 1.62b7 program.

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