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FEATURE ARTICLE
Strategies for Analyzing Neuronal Progenitor Development and Neuronal Migration in the Developing Cerebral Cortex

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The emergence of functional neuronal connectivity in the developing cerebral cortex depends on 1) neural progenitor differentiation, which leads to the generation of appropriate number and types of neurons, and 2) neuronal migration, which enables the appropriate positioning of neurons so that the correct patterns of functional synaptic connectivity between neurons can emerge. In this review, we discuss 1) currently available methods to study neural progenitor development and differentiation in the developing cerebral cortex and emerging technologies in this regard, 2) assays to study the migration of descendents of progenitors (i.e., neurons) in vitro and in vivo, and 3) the use of these assays to probe the molecular control of these events in the developing brain and evaluation of gene functions disrupted in human neurodevelopmental disorders.

Keywords: cortical development, cortical progenitors, neuronal migration,

Introduction

The emergence of functional neuronal organization and connectivity in the developing cerebral cortex depends on 2 crucial early developmental events: 1) the differentiation and proliferation of neural progenitors, leading to the timely generation of appropriate numbers of cortical neuronal subtypes, and 2) migration of neurons to specific locations in the cortex where they establish functional synaptic connections with other neurons (Marin and Rubenstein 2003). Disruptions in these developmental events can have severe consequences, including mental retardation, lissencephaly, tumorigenesis, and neuropsychiatric disorders like autism and schizophrenia (Ayala et al. 2007; Kerjan and Gleeson 2007; Jackson and Alvarez-Buylla 2008). Furthermore, analogous events occur in the postnatal brain, where new neurons are born in neurogenic niches and targeted to where they are needed as part of either normal maintenance of the neural circuitry or in response to disease or injury (Falk and Frisen 2005; Ghashghaei et al. 2007; Zhao et al. 2008; Kriegstein and Alvarez-Buylla 2009). Thus, our ability to study the complexities of neural progenitor differentiation and neuronal migration is essential to understanding normal cerebral cortical formation, the prevention and treatment of neurodevelopmental disorders, and the regeneration of functional connectivity in damaged or diseased sites in the adult brain.

Since neural progenitor differentiation and neuronal migration depend on complex intercellular and intracellular interactions, the most effective methods to study these processes should combine an ability to genetically target and visualize relevant cells in their native environment with an analysis of global patterns of progenitor formation and neuronal movement. Traditional techniques for studying progenitor differentiation and neuronal migration have relied on either analysis of fixed tissue or in vitro culture of individual cells. But the recent advances in live imaging techniques and genetic labeling of distinct cell types in the developing brain have accelerated our ability to analyze the complexities of the fundamental processes of cerebral cortical formation. This review will describe the currently available and emerging methods for studying neural progenitor differentiation and neuronal migration, evaluating their uses and limitations, and will propose how these methodologies can be adapted and improved to resolve fundamental issues in cerebral cortical development and disease.

The first part of the review will focus on assays to study the differentiation of different types of neural progenitors (neuroepithelial cells, radial progenitors, and intermediate precursors) in the developing cortex. In the second part, we will survey neuronal migration assays, addressing 2 significant aspects of migration: 1) movement of neurons from their places of birth to positions where they form functional synaptic connections and 2) migratory pathway selection and migration mode differences among neuronal subtypes. Finally, we will describe how these methods can be adopted to test the functions of genes that are rapidly being identified as candidate susceptibility genes for human brain disorders in which progenitor development or neuronal migration is affected.

Progenitor Differentiation

During cerebral cortical development, after closure of the anterior neural tube, neuroepithelial cells proliferate within the walls of the ventricle, populating the ventricular zone (VZ) with a pseudostratified layer of bipolar cells (Fig. 1). At the onset of neurogenesis, neuroepithelial cells transform into radial glial progenitors (RGPs), which are characterized by a highly polarized morphology and a glial-like molecular identity (reviewed in Götz and Huttner 2005). Initially, RGPs expand their population by dividing symmetrically to produce 2 daughter RGPs. Then, as neurogenesis proceeds, an increasing number of RGPs begin to divide asymmetrically, giving rise to a daughter neuron or intermediate progenitor (IP) and a self-renewing daughter RGP (Malatesta et al. 2000; Miyata et al. 2001; Anthony et al. 2004; Noctor et al. 2004, 2008). IPs move to the subventricular zone (SVZ), where they form an

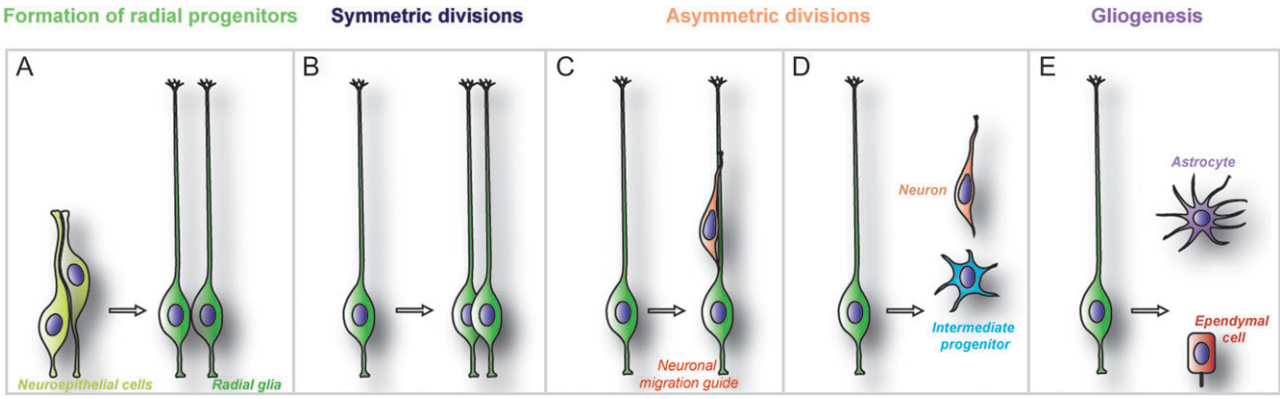

Figure 1. Progression of radial progenitor development in embryonic cerebral cortex**.** Initially, RGPs are established from an undifferentiated sheet of neuroepithelial cells (*A*). Radial glia then divide symmetrically and generate more radial glia (*B*). Subsequent asymmetric division of radial glial cells gives rise to daughter neurons and other radial glial cells (*C* and *D*). Neurons either somally translocate or attach to radial glial guides (*C*) as they migrate to the cortical plate. Asymmetric divisions of a radial glial cell can also yield a daughter neuron and an IP (*D*). IPs divide and generate neurons. During late stages of corticogenesis, as neurogenesis and migration dwindle, radial progenitors give rise to astrocytes or become ependymal cells (*E*). These distinct phases of progenitor development can be studied using clonal analysis of isolated progenitors, neurosphere assays, real-time tracking of labeled progenitors in embryonic cortex, or in utero imaging. An important point to note is that significant molecular and morphological heterogeneity of radial progenitors exists within the VZ of the developing cerebral cortex and is not outlined in this figure.

additional neurogenic niche that supplies the majority of projection neurons to all layers of the cerebral cortex (Haubensak et al. 2004; Kowalczyk et al. 2009). As neurogenesis wanes, asymmetric neurogenic RGP divisions decrease in frequency due to the terminal differentiation of RGPs into glia and ependymal cells (Noctor et al. 2008) (Fig. 1). During neurogenesis, significant molecular and morphological heterogeneity among neural progenitors in the VZ has been characterized and this heterogeneity may be essential to create neuronal diversity in cerebral cortex (Gal et al. 2006; Mizutani et al. 2007; Anthony and Heintz 2008; Howard et al. 2008; Kawaguchi et al. 2008; Hansen et al. 2010; Stancik et al. 2010).

In addition to their role as cortical progenitors, RGPs perform another important function as guides for neuron migration. Specifically, the pial-directed radial process of RGPs provides a permissive and instructive scaffolding for the oriented migration and placement of newly generated neurons (Ayala et al. 2007). Thus, as neuronal progenitors and migratory guides, RGPs perform multiple evolving functions as cortical development unfolds (Fig. 1), making it necessary to develop tools to analyze each of these functions.

Time-lapse Analysis of Adherent Neural Progenitor Clones

A fundamental question in radial progenitor development during corticogenesis is what governs the timing and mode of RGP division and the gradual restriction of daughter cell fate as development proceeds. Is the sequential production of RGPs, IPs and neurons, and then glia regulated by cell-intrinsic programs, extrinsic cues, or a combination of both? Similarly, what signals impinging on progenitors determine the orderly generation of layer-specific projection neurons?

In vitro assays using isolated, single cortical progenitors from different embryonic ages can be used to selectively study and manipulate RGP proliferation, differentiation, and cell fate in a defined environment. In this method, dissociated cortical progenitors are plated on an adherent substrate at clonal density, so that cell–cell contacts are minimized, and the only extracellular cues present are produced from the clones themselves or exogenously added to the defined culture medium (Shen et al. 2006). Over several days in vitro, single progenitors generate other progenitors, distinct neuronal subtypes, and glia, with a timing and order that parallels the timing and order observed in vivo (Shen et al. 2006). The identity of daughter cells generated from isolated progenitors can be determined using cell type–specific markers, thus defining the mode of progenitor division. For instance, if an isolated progenitor gives rise to only RGPs, it underwent symmetric self-renewing divisions (Fig. 1*A*). If the daughter cells are of different cell types (i.e., neurons, intermediate precursor, or astroglia), they are likely the result of asymmetric divisions (Fig. 1*C*,*D*). While this assay is ideal to test the cell-intrinsic capacities of cortical progenitors from different developmental stages (Fig. 1), extrinsic cues critical for distinct patterns of progenitor division can also be presented to the isolated clones and tested for their influence on the mode of progenitor division.

Time-lapse analysis of neural progenitor clones is a further extension of this technique where long-term live-cell imaging of isolated progenitors and their descendents (Fig. 2), followed by correlative staining for progenitor, neuronal, and glial subtypes, can be used to establish a comprehensive lineage tree (Al-Kofahi et al. 2006; Shen et al. 2006). The effects of extrinsic factors or cell–cell contacts on progenitor division can be examined by adding diffusible cues to the medium or culturing progenitors at higher density, respectively (Ravin et al. 2008; Shen et al. 2006). Additional refinements to the system might include methods to specify the identity of the initial progenitors. For example, one can use fluorescence activated cell sorting to purify progenitors from transgenic mice expressing XFP under the control of a promoter active in a specific progenitor subpopulation (e.g., BLBP-GFP to isolate RGPs, Tα1-GFP to isolate short neural precursors, or Tbr2-GFP to isolate IPs; Table 1).

The advantage of this technique over neurosphere assays, which are also used to evaluate progenitor proliferation, is that it permits complete registering of individual cell divisions and

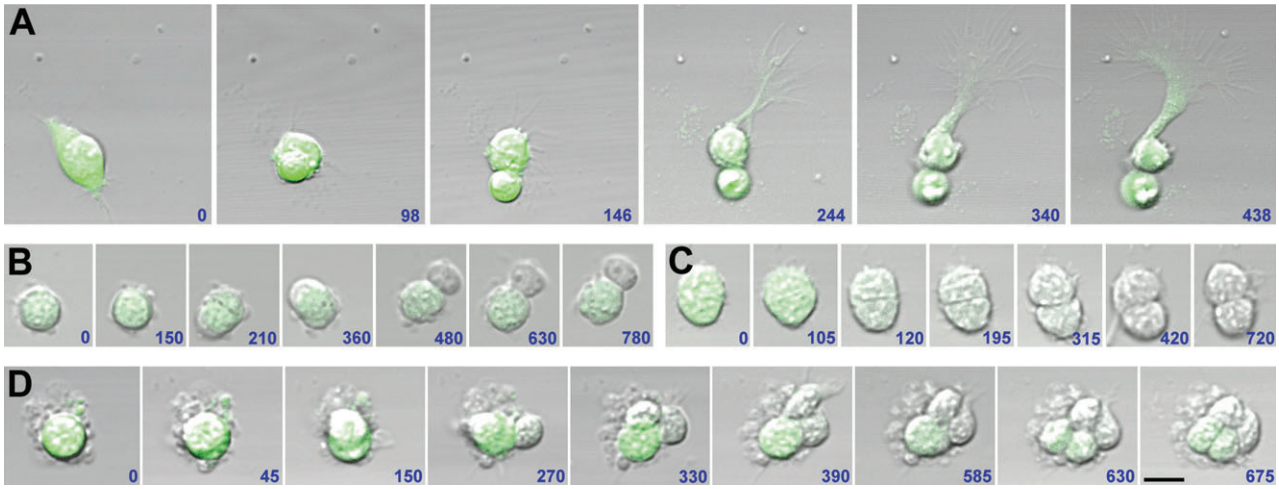

Figure 2. Analysis of isolated cortical progenitor development. Radial progenitors micro dissected from the VZ of E14.5 BLBP-GFP embryonic cortices were plated at clonal density and isolated, and genetically defined radial progenitors (GFP^+) were time-lapse imaged. (*A*) Division of GFP^+ progenitor resulting in 2 other GFP^+ cells, 1 of which extends a basal process. (*B*) Division of GFP^+ progenitor resulting in 1 GFP^+ cell and 1 GFP^- cell. (*C*) Division of GFP^+ progenitor resulting in 2 GFP^- cells. (*D*) Generation of a mixed clone containing GFP^+ and GFP^- cells. The combined use of time-lapse analysis of genetically defined cortical progenitors with correlative immunolabeling of daughter cells at the end of imaging can be used to study patterns of normal and altered progenitor development. Time elapsed between observations are indicated in minutes. Simultaneous GFP and phase light images were obtained using a Zeiss inverted confocal microscope equipped with live-cell incubation chamber. Scale bar = 25 μm.

Table 1
Progenitor-specific transgenic mouse lines

Line	Time of initial expression	Labeled progenitor population	Reference
Rat α1-tubulin-YFP	E9.5	Neuroepithelium IPs	Sawamoto et al. (2001)[a]
Sox2-GFP	E10.5	Neuroepithelium RGPs Postnatal dentate gyrus Postnatal SVZ	Ellis et al. (2004)
Rat Nestin-GFP	E11.5	Neuroepithelium RGPs Postnatal dentate gyrus Postnatal SVZ and RMS	Yamaguchi et al. (2000)
GLAST-dsRed	E12.5	RGPs Postnatal dentate gyrus	Glowatzki et al. (2006)
Eomes/Tbr2-GFP	E12.5	IPs	Arnold et al. (2009), Kowalczyk et al. (2009)
Human GFAP-GFP	E13.5	RGPs Astrocytes	Malatesta et al. (2000), Zhuo et al. (1997)
BLBP-GFP, YFP, dsRed	E14.5	RGPs Astrocytes Postnatal dentate gyrus	Schmid et al. (2006)
ALDH1L1-GFP	E16.5	RGPs	Anthony and Heintz (2007, 2008), Barres (2008)
Hes5-GFP	N.D.	RGPs	Akazawa et al. (1992), Gong et al. (2003)
Fezf2-GFP	E12.5	Progenitors of deep layer projection neurons	Kwan et al. (2008), Gong et al. (2003)
Tis21-GFP	E10.5	IPs and some RGPs	Haubensak et al. (2004), Attardo et al. (2008)

Note: N.D., not determined.
[a]Also expressed in early born neurons (Coksaygan et al. 2006).

behavior and a clear analysis of intrinsic mechanisms at work in choosing cell fate. Neurospheres, which are essentially a collection of cells arising from a progenitor proliferating in suspension, do not permit such analysis. However, the neurosphere culture is a useful assay to expand progenitors (Fig. 1*B*) using extrinsic factors. Neurospheres thus generated can then be differentiated on an adhesive substratum to generate neurons and glia.

An obvious drawback of this method of clonal analysis of isolated cortical progenitors is that dissociated progenitors neither encounter the permissive microenvironments (i.e., progenitor niches) found in vivo nor do they maintain critical features such as polarity and orientation they would have in the intact brain. This limitation might have major consequences considering that coordinated patterns of symmetric and asymmetric progenitor divisions in vivo rely on appropriate cell–cell contacts (Lu et al. 2001) that are absent in isolated cultures. Cortical slice assays help overcome these limitations of isolated progenitor cultures.

Cerebral Cortical Slice Assays for Real-Time Analysis of Progenitor Development

Improvements in tissue culture methods and live-cell imaging in tissue slices have made possible the real-time tracking of individual progenitors and their progeny within tissue explants. Currently, this involves targeted expression of a fluorescent protein in progenitors followed by time-lapse confocal imaging of slice cultures. Depending on the objective of the experiment, single or multiple progenitors can be targeted in specific proliferative regions of both embryonic and postnatal cortices. The most common modes of fluorescent gene expression rely on electroporation (in utero and ex utero), viral transduction, and germ line genetic manipulation, each having unique advantages. For example, while viral transduction enables tracking of individual cells in isolation, electroporation and genetic manipulation allow targeting of cohorts of specific progenitor subpopulations.

Likely the most rapid method of progenitor transfection, DNA electroporation results in 1 or more regions of the cortex expressing a fluorescent transgene in multiple progenitors and their neuronal and glial progeny. In this method, concentrated (2–5 mg/mL) purified plasmid DNA containing the gene of interest is injected with a micropipette into the lateral

ventricle of embryos, either removed from the uterus (ex vivo) (Gongidi et al. 2004) or left intact in the embryonic sac (in utero) (Tabata and Nakajima 2001). A low-voltage current is then passed through the cortex, and the DNA is incorporated into progenitors lining the lateral ventricles. The current's path can be oriented differently so that plasmid DNA will be taken up preferentially by progenitors that fall within the current's path. In this way, progenitors in the dorsal (Tabata and Nakajima 2001), ventral, and hippocampal (Navarro-Quiroga et al. 2007) anlages can be exclusively targeted. After ex utero electroporation, the brains are removed, sliced, and maintained in vitro. After sufficient time for transgene expression (24–48 h), long-term live-cell imaging can be performed on labeled progenitors in slices to analyze their patterns of differentiation (Xie et al. 2007; Yoon et al. 2008; Yokota et al. 2009) using a confocal microscope equipped with live-cell incubation system. With in utero electroporation, the embryos are returned to the mother, where they develop for the desired number of additional days. The advantage of the ex utero approach is that it avoids the potential for embryonic death that, at times, occurs from the insults associated with in utero electroporation and allows for rapid analysis of large numbers of cortical progenitors. In contrast, in utero electroporation allows the cortex to develop intact until the effect of transgene expression in progenitors is ready to be analyzed.

In its simplest form, electroporation followed by live-cell imaging is a powerful method for observing the dynamics of progenitors as they differentiate, divide, and generate neurons. With the development of brighter, stable fluorescent markers, critical features of progenitors such as interkinetic nuclear movement, interprogenitor interactions, and pial end-feet dynamics can be followed in RGPs in both normal cortex and models of neurodevelopmental disorders (Xie et al. 2007; Yokota et al. 2009). Moreover, combining fluorescent labeling with genetic manipulation of progenitors, by coelectroporating mutated genes or short hairpin RNA (shRNA) constructs, can be an extremely efficient way to analyze the molecular mechanisms that regulate progenitor morphology, proliferation, and neurogenesis (LoTurco et al. 2009; Loulier et al. 2009). For example, the efficiency and reproducibility of electroporation make this technique amenable to rapid screening with shRNA libraries or shRNA screens targeting specific susceptibility genes for neurodevelopmental disorders. Further, by varying the timing of electroporation and using progenitor-type specific promoters, one can target and study radial progenitors and their progeny at distinct stages of development (Mizutani et al. 2007; Anthony and Heintz 2008). Postnatally, electroporation can be used to label and study progenitors and new neurons in the neurogenic niches (e.g., SVZ) of the neonatal or mature brain (Barnabe-Heider et al. 2008) This approach can significantly facilitate our ability to study adult neurogenesis and the response of adult neural progenitors to injury and disease.

Viral transduction offers an alternative to electroporation. In this technique, high-titer retroviral particles expressing a gene of interest and a fluorescent marker are injected into the lateral ventricle 1–2 days prior to slice culture and imaging. The viral particles diffuse evenly through the ventricular space and infect single isolated progenitors, which can then be visualized and tracked as they divide and generate daughter neurons (Noctor et al. 2001, 2004, 2008). Because viral vectors stably integrate into the genome, both progenitors and their progeny will express the transgene at relatively consistent levels. This is in contrast to electroporated cells where the plasmid DNA does not always integrate, and expression levels can vary widely among cells. However, when analysis of large cohorts of progenitors and rapid gene manipulation are required, electroporation-based methods are optimal.

In contrast to electroporation or viral transduction, germ INS > line genetic manipulation provides a non-invasive technique for labeling progenitors. Recently, a number of transgenic mouse lines have been produced that express fluorescent protein tags under the control of different progenitor-specific promoters (Table 1). These lines label neuroepithelial precursors (Ellis et al. 2004), RGPs (Zhuo et al. 1997; Malatesta et al. 2000; Gong et al. 2003; Haubensak et al. 2004; Schmid et al. 2006; Regan et al. 2007; Attardo et al. 2008; Kwan et al. 2008), and IPs (Sawamoto et al. 2001; Gong et al. 2003; Haubensak et al. 2004; Attardo et al. 2008; Kwan et al. 2008; Arnold et al. 2009) and offer uniform labeling of a majority of cells in a specific progenitor population. These lines are powerful tools to investigate progenitor dysfunctions in neurodevelopmental disorders because when crossed with mutant models of neurodevelopmental diseases, they can reveal defects in large cohorts of progenitors in distinct cortical areas that might not be seen with more focal labeling of progenitors achieved with electroporation or retroviral vectors. A recent study demonstrating how defects in neurogenesis may lead to the increased inhibitory drive in the brains of Down's syndrome mouse model illustrates the insights that could be gained by combining neurodevelopment disease models with other relevant genetic tools (Chakrabarti et al. 2010).

Advanced Techniques to Study Progenitor Development in Intact Brains

In spite of many advantages, the isolated progenitor cell culture methods and the slice-based assays do not entirely preserve the neural progenitor cell niche. Neural stem cell niche in the developing brain, consisting of the extracellular matrix, inter-progenitor adhesive contacts, and the vasculature within the proliferative zone, can dynamically influence the appropriate unfolding of neural progenitor division and differentiation (Chenn and Walsh 2003; Klezovitch et al. 2004; Shen et al. 2004; Rasin et al. 2007; Loulier et al. 2009; Stubbs et al. 2009; Weimer et al. 2009). As such, it will be vital to develop methods to observe and study neural progenitor development in live embryonic brains in utero. Multiphoton microscopy-based methods can be employed to evaluate neural progenitor development in the intact developing cerebral cortex in utero (see Supplementary Figure 1 and Supplementary Movie 1). Embryonic mice expressing XFP in neural progenitors can be used to image specific types of neural progenitors at different stages of cortical development in vivo. Although technical issues such as mechanical stability of embryos necessary for repeated long-term imaging of an identical area of the proliferative zone and the increasing thickness of cerebral wall during development can present obstacles for this type of imaging, the ability to follow neural progenitor differentiation and behavior in utero holds great promise for our understanding of how patterns of

progenitor differentiation influence the formation of cerebral cortex.

Neuronal Migration

Once generated from proliferating progenitors, postmitotic neurons migrate from the proliferative VZ and SVZ to their final sites of differentiation. During development, the waves of neuron migration that commence in distinct regions of the brain differ in their timing, mode of movement, cytoskeletal dynamics, and response to guidance cues. Developmental cascades triggered by migratory mode and pathway choice are thought to lead to the emergence of distinct neuronal subtypes. How these complex patterns of neuronal movement and identity development are orchestrated so that the right types and numbers of neurons are deposited into appropriate areas and layers of the cerebral cortex remains unclear.

In general, neurons utilize 2 modes of migration, radial and tangential, depending on their origin and destination. In the dorsal telencephalon, early generated neurons of the preplate and deeper layers migrate radially using somal translocation, whereas later generated neurons utilize elongated radial glial guides to attain their more distant locations at the interface between cortical plate and marginal zone (Rakic 1972, 2003; Sidman and Rakic 1973; Takahashi et al. 1990; Miyata et al. 2001; Nadarajah et al. 2001; Hatten 2002; Fig. 1). Meanwhile, in the ventral telencephalon, GABAergic cortical interneurons are generated in the ganglionic eminences, and they migrate tangentially into the cerebral wall (de Carlos et al. 1996; Anderson et al. 1997; Ang et al. 2003; Marin and Rubenstein 2003). Postnatally, radial migration continues in the dentate gyrus of the hippocampus as newly generated granule neurons migrate from the SVZ into the granular layer (Nowakowski and Rakic 1979). Tangential migration also occurs throughout life, exemplified by olfactory bulb neuroblasts that migrate from the anterior SVZ to the olfactory bulb, through the rostral migratory stream (Lois and Alvarez-Buylla 1994; Marin and Rubenstein 2003).

Several developmental brain disorders have been linked to defects in radial migration in humans, including periventricular heterotopia and lissencephaly (Kerjan and Gleeson 2007; Sarkisian et al. 2008). Also, aberrant migration, placement, and connectivity of the inhibitory GABAergic interneurons have been implicated in disorders such as schizophrenia, autism, and Tourette's syndrome (Di Cristo 2007). Thus, understanding the global orchestration of neuronal migration and the molecular mechanisms of cell movement will contribute toward delineating the biological basis of a plethora of neurodevelopmental disorders. Further, strategies to promote functional recovery in the injured adult brain depend on the generation of new neurons and the appropriate guidance of these neurons to where they are needed. Thus, an understanding of the process of neuronal migration could be of use in neural disease settings where there is a demand for neurogenesis and targeted guidance of neurons to repair and maintain functional neural circuitry.

In Vitro Migration Assay Using Dissociated Neuronal Cells

Migration assays using dissociated neurons and glial cells are highly suitable for the analysis of intracellular signaling activities (e.g., calcium transients or cytoskeletal dynamics), cell–cell and cell–substratum interactions during neuronal migration. In these assays, dissociated neurons and astroglia from embryonic cortical tissue can be recombined in vitro to promote neuronal attachment and migration on astroglial processes (Hatten and Liem 1981; Gregory et al. 1988; Elias et al. 2007). Tissue imprints of embryonic cortex made by peeling off adhered cortical slices can also yield neurons attached to radial glial processes (Barres et al. 1990; Anton et al. 1996). Alternately, dissociated neurons or micro explants from embryonic cortices can also be plated on specific adhesive substratum to analyze neuronal response to surface bound cues (Fishman and Hatten 1993; Lois et al. 1996; Wichterle et al. 1997; Tsai et al. 2007). Neuronal motility in all these different assays can be easily tracked using live-cell imaging. The advantage of these methods lies in the ability to simultaneously visualize individual isolated neurons and their intracellular cytoskeleton or signaling components (e.g., calcium, endosomes), making possible the study of cytoskeletal or signaling dynamics during neuronal migration. For example, using dissociated, migrating cerebellar granule neurons, it has been shown that the physical coupling of the nucleus and centrosome is a key feature of migrating neurons (Solecki et al. 2004; Tanaka et al. 2004; Bellion et al. 2005; Schaar and McConnell 2005) and that defects in this coupling are seen in neurons taken from mutant models of migration disorders (Tanaka et al. 2004; Koizumi et al. 2006; Tsai et al. 2007). These assays were instrumental in examining the role of neuron–glial contact and adhesion in neuronal migration, as they can be used to selectively perturb neuronal or glial components involved (Anton et al. 1997; Edmondson et al. 1988). Further, these techniques are also commonly used to study the role of guidance cues in directing migration, where neurons migrating from an explant are confronted with a choice of permissive/nonpermissive substratum or with a gradient of a soluble chemoattractant or repellant (Hu and Rutishauser 1996; Wu et al. 1999; Ward et al. 2003; Ward et al. 2005; Higginbotham et al. 2006).

Real-time Neuronal Migration in Embryonic Brain Slice Assay

A major deficit of migration assays using dissociated neurons is that they disrupt many of the endogenous signals critical for patterned migration within the developing cerebral cortex. Evaluating neuronal migration in living embryonic slices helps overcome this deficit. This method of tracking neurons as they migrate through their native environment has helped expand our understanding of aspects of migration that had only been postulated using fixed tissue, such as distinct phases and modes of projection or interneuron migration (Noctor et al. 2004), neuronal–RGP interactions (Gongidi et al. 2004), and the mechanisms of maintaining neuronal polarity during migration (Xie et al. 2003; Guerrier et al. 2009). Further, analysis of human neuronal migration disorders indicates that disruptions in selective stages of migration can have vastly different effects on cerebral cortical formation and function (e.g., disrupted initiation of migration linked to periventricular heterotopia, aberrant maintenance of migration associated with lissencephaly and cortical band heterotopia, and deregulated terminal phase of migration leading to over migration of neurons in Walker–Warburg syndrome (Gleeson and Walsh 2000). The cortical slice assay in which all different stages of

neuronal migration can be visualized is highly amenable to the study of mechanisms underlying selective stages of neuronal migration. Migrating neurons in embryonic slices can be labeled with either fluorescent dyes or XFP transgenes. Lipophilic or vital dyes, such as DiI, DiO, CMTMR, and Oregon Green, can be introduced into the ventricles or on top of cortical slices to label large cohorts of migrating neurons in the embryonic cortex and postnatal RMS (Honig and Hume 1989; de Carlos et al. 1996; Doetsch and Alvarez-Buylla 1996; O'Rourke et al. 1997; Nadarajah et al. 2001; Anton et al. 2004; Miyata et al. 2004). Dye- or transgene-coated gold particles can also be directly introduced into migrating neurons using a biolistics delivery approach (Gan et al. 2000; O'Brien and Lummis 2006). In utero or ex vivo electroporation, as described above, will achieve similar results, without the risk of the lipophilic dyes diffusing from cell to cell (Tabata and Nakajima 2001).

A more reliable and exact method to label migrating neurons is to use transgenic mice, where fluorescent markers are expressed exclusively in distinct migrating neuronal populations in both embryonic brain and adult brain. Table 2 describes some of these transgenic lines and the neuronal populations where fluorescence is expressed. A useful feature of some of these lines is the *cis*-expression of a fluorescent protein and Cre under the control of a neuronal-specific promoter. When crossed to mice with a floxed allele, one can simultaneously delete expression of the floxed gene and visualize the mutant neurons, allowing imaging of the altered

Table 2
Neuron-specific transgenic mouse indicator lines for migration assays

Line	Time of initial expression	Labeled neuronal population	Reference
Dcx-GFP, dsRed	E11.5	Projection neurons and interneurons Postnatal neuroblasts in SVZ and RMS	Gong et al. (2003), Koizumi et al. (2006), Wang et al. (2007)
Cdk5-GFP	E11.5	Postmitotic projection neurons	Gong et al. (2003), Tsai et al. (1993)
NeuroD6-GFP	E11.5	IPs and postmitotic projection neurons	Gong et al. (2003), Shimizu et al. (1995)
p35-GFP	E12.5	IPs and postmitotic projection neurons	Delalle et al. (1997), Gong et al. (2003)
Cx43-GFP	E12.5	Postmitotic projection neurons	Gong et al. (2003)
β-3-tubulin-GFP	E12.5	Postmitotic projection neurons	Gong et al. (2003)
Fezf2-GFP	E12.5	Postmitotic deep layer projection neurons	Kwan et al. (2008)
Nkx2.1-GFP	E10.5	Interneurons from MGE	Gong et al. (2003)
Lhx6-GFP	E11.5	Interneurons from MGE	Cobos et al. (2006), Gong et al. (2003)
Dlx5/6-Cre-IRES-GFP	E12.5	Interneurons from LGE and MGE	Stenman et al. (2003), Yokota, Gashghaei et al. (2007)
Dlx1-GFP	E12.5	Interneurons from ventral telencephalon	Cobos et al. (2005), Gong et al. (2003)
CXCR4-GFP	E12.5	Interneurons from ventral telencephalon	Gong et al. (2003), Tissir et al. (2004)
GAD65-GFP	E12.5	Interneurons from ventral telencephalon Postnatal neuroblasts in SVZ and RMS	De Marchis et al. (2004), Lopez-Bendito et al. (2004), Tanaka et al. (2006)

Note: LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence.

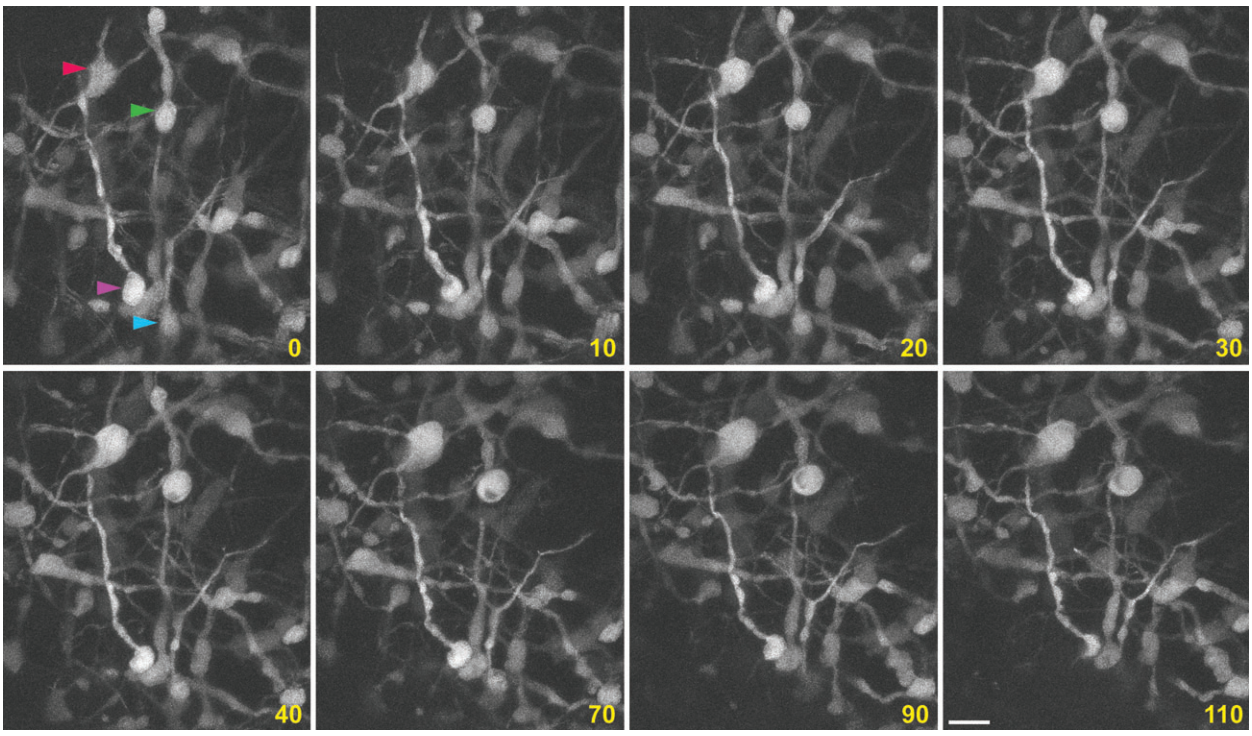

Figure 3. In utero imaging of interneuronal dynamics. GFP^+ interneurons in the developing cerebral cortex (parietal cortical area) of E16.5 Dlx5/6-CIE embryos were repeatedly imaged using 2-photon microscopy. Time-lapse imaging of these neurons in living embryos illustrates dynamic changes in their position, process growth, or neighbor relationships. Arrowheads indicate sample neurons undergoing such changes. Time elapsed is indicated in minutes. Scale bar = 30 μm.

patterns of neuronal migration resulting from gene deletion (Stenman et al. 2003; Stanco et al. 2009). Electroporation of postmitotic neuron-specific promoters driving XFP (e.g., NeuroD-GFP) can also be used to selectively target and label migrating neurons in embryonic cortex (Yokota, Ring, et al. 2007; Heng et al. 2008; Guerrier et al. 2009). This approach, when used in transgenic mice expressing a different fluorescent protein under the control of a progenitor-specific promoter, can provide differential labeling of migrating neurons and radial progenitors and thus enable the investigation of complex interactions between radial glia and migrating neurons (Yokota, Gashghaei, et al. 2007). Similarly, generation of bitransgenic mice in which projection neurons and interneurons are labeled with different fluorescent tags (Table 2) will be highly useful in evaluating how interneurons and projections neurons coordinate their patterns of migration to generate cortical laminar organization.

4-D Neuronal Migration Analysis and Examination of Neuronal Migration in Living Embryos

Although the migration assays using embryonic cortical slices have yielded extensive insights into the modalities and molecular control of neuronal migration, other fundamental questions related to neuronal migration, such as how subtypes of interneurons and projection neurons coordinate their migration and placement in specific layers and areas of the developing cerebral cortex, remain unanswered. Simultaneous live imaging of genetically defined populations of interneurons, projection neurons, and radial glia in the entire rostral–caudal extent of developing cerebral cortex from different embryonic ages is essential to understand how specific subtypes of neurons achieve their laminar and areal positions within the developing cortex. Slices from the entire extent of embryonic cortex can be repeatedly scanned in real time, using a confocal microscope equipped with *x*–*y*–*z*–motorized stage and live-cell incubation chamber. The images collected can be compiled and analyzed to get a 4D view of neuronal movement within the cortex at a particular stage of embryonic development. Further, multiphoton microscopy provides a powerful tool to map neuronal movement and dynamics within the emerging neocortex of intact living embryos (Fig. 3; Ang et al. 2003). For example, specific analysis of interneuronal movement in living embryos using multiphoton imaging indicates that multidirectional migration within local areas of the developing cortex may facilitate the appropriate placement of interneurons within distinct cortical regions (Yokota, Gashghaei, et al. 2007). This type of combined large-scale neuronal imaging in the developing brain of normal and genetic models of neurodevelopmental disorders can yield unexpected and significant insights in to how different types of neurons get to where they have to go in the developing brain and how disruption of this process can cause a specific developmental disorder.

Future Directions

The methods to study progenitor development or migration of their neuronal progeny are driven by the need to understand the molecular and cellular basis of these phenomena during normal brain development and in neurodevelopmental disorders. An emerging goal of these efforts is to map and study the behavior of large cohorts of developing progenitors and their progeny with high temporal, spatial, and molecular resolution across development. For example, the evolving functional status of critical polarity regulators during progenitor differentiation can be probed with functional state-specific fluorophores, such as bioprobes for CDC42 or Rac activity (Nalbant et al. 2004; Wu et al. 2009). Similarly, cell state–specific probes (e.g., Fucci to define different stages of cell cycle) can be developed and used to map changing differentiation states. Further, tagging critical signaling components with fluorescent timers (Subach et al. 2009), photoactivatable modifiers (Wu et al. 2009), and photoconvertible fluorophores such as Kaeda can be used to track the dynamics of critical molecular regulators (e.g., Wave1) (Yokota, Ring, et al. 2007) or organelles (Wang et al. 2009) during neuronal development. This approach of using biosensors (Miyawaki 2005; Miyawaki and Schnitzer 2007; Matsuda et al. 2008; Wu et al. 2009) specific to a particular functional state of key molecular determinants or particular cell state to query the dynamics of normal or altered development in mouse genetic models of neurodevelopmental disorders will be essential to fully understand how cerebral cortex forms and to define the neurodevelopmental pathways whose disruption is likely to lead to brain developmental disorders.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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