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Advanced Microscopic Imaging Methods to Investigate Cortical Development and the Etiology of Mental Retardation

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Abstract

Studies on human patients and animal models of disease have shown that disruptions in prenatal and early postnatal brain development are a root cause of mental retardation. Since proper brain development is achieved by a strict spatiotemporal control of neurogenesis, cell migration, and patterning of synapses, abnormalities in one or more of these events during prenatal development can lead to cognitive dysfunction after birth. Many of underlying causes of mental retardation must therefore be studied in developing brains. To aid in this research, live imaging using laser scanning microscopy (LSM) has recently allowed neuroscientists to delve deeply into the complex three-dimensional environment of the living brain to record dynamic cellular events over time. This review will highlight recent examples of how LSM is being applied to elucidate both normal and abnormal cortical development.

Keywords

live imaging; multi-photon; autism; fragile X syndrome; lissencephaly; Down syndrome

It is widely accepted that proper cognitive development in humans requires appropriate interactions with one's environment through sensorial exploration, didactic training, and social experience. However, evidence has shown that cognitive ability is also specified by a genetic component. The most striking example of this is seen in the increasing number of reports linking genetic abnormalities to various forms of mental retardation. Studies in the past decade have identified some of the genetic components and cellular processes necessary for proper brain development. The subject of this review is how recent technological advances in imaging have allowed closer examination of how these genes influence critical events during brain development.

Many of the underlying causes of congenital forms of mental retardation arise early in development, either already by birth or soon thereafter. While much progress has been made in describing embryonic vertebrate development through the use of in vitro and static in vivo experiments, studies have illustrated that cellular processes can occur differently within the intact living brain due to the increased complexity of the environment. Initially, the relative inaccessibility of the gestating embryo slowed progress in determining how both rapid and slow events proceed in the three-dimensional context of the brain. This barrier has been

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overcome by new imaging methods that have opened the door to the multitude of dynamic events that occur during brain development, for the first time allowing researchers to study cells in real time while they interact within their native environment.

SPATIOTEMPORAL REQUIREMENTS OF CORTICAL DEVELOPMENT

Development of all brain structures proceeds in a precisely controlled progression of cell division, migration, and differentiation, which is conserved across mammalian species. For the purposes of this review, I have compartmentalized this progression into five separate stages (Fig. 1). It is important to emphasize that these events are functionally linked to one another. For example, abnormal neurogenesis can lead to downstream abnormalities in migration or synapse formation. In addition, it is also important to note that several cellular processes can occur simultaneously within the same stage. For instance, programmed cell death, a main component of the refinement, stabilization, and segregation of pathways and circuits (here represented in Stage 5) also plays a large role in specifying brain size during the first two stages of development by impacting the number of proliferating neuronal stem cells [Blaschke et al., 1996; Haydar et al., 1999b; Kuida et al., 1998]. Taken together, work on embryonic development in wild-type and mutant animals suggests that disruptions, or even short-lived delays, in any one of the stages of brain development can lead to lasting defects in subsequent stages. I will focus on the development of the cerebral cortex, which, as the site of our executive brain function, truly defines our species and controls how we interact with the world. Many congenital disorders with embryonic origins directly impact cerebral cortical function by interfering with one or more stages of its growth.

EXPANSION AND COMMITMENT OF NEURAL PRECURSORS

The first stage of neocortical growth is the exponential expansion in the number of neural stem cells in the ventricular zone (VZ), a pseudostratified neuroepithelium that surrounds the lateral ventricles. This process begins at neural plate stage and lasts until the onset of neurogenesis, which occurs on the 11th day of gestation (E11) in mice [Takahashi et al., 1996] and by the 7th gestational week (7GW) in humans [His, 1904; Molliver et al., 1973]. The duration of Stage 1 is critical for brain development and is a major factor in the resulting differences in cortical size and function between species [Caviness et al., 1995; Rakic, 1995].

Stage 2 begins as the first neurons are generated from the VZ, which has rapidly expanded to include multipotential stem cells as well as committed neuronal and glial progenitors [Alvarez-Buylla et al., 2001; McCarthy et al., 2001; Temple, 2001]. Neurogenesis lasts from E11 until E17 in mice [Takahashi et al., 1996] and from 7GW to 20GW in humans. The longer duration of Stage 2 in primates is thought to contribute to their increased cortical volume and cognitive function. The production of specific classes of committed progenitors from stem cells and the duration of their cell cycle are also critical factors influencing later cognitive ability [Caviness et al., 1995, 2000; Rakic, 1995].

Many infants with congenital abnormalities are born with reductions in head circumference and brain size (microcephaly) due to perturbations during Stages 1 and 2. Defective cell divisions caused by mutations in the *ASPM* (abnormal spindle) gene, linked to autosomal recessive primary microcephaly [Bond et al., 2002], may reduce the founder cell population in these patients. In addition, mice deficient for the *Ndel* and *LIS1* genes exhibit primary mitotic defects [Faulkner et al., 2000; Liu et al., 2000; Gambello et al., 2003; Feng and Walsh, 2004], which may be causally related to the microcephaly seen in human lissencephaly. Similarly, the reduced brain size found in Down syndrome (DS) [Colon, 1972; Schmidt-Sidor et al., 1990; Wisniewski, 1990; Wisniewski et al., 1993] and microcephaly vera [Mochida and Walsh, 2001] may be due to altered cell divisions that occur during Stages 1 and 2. These changes can be as subtle as a slight increase in cell cycle duration, such as that found in DS [Schneider and

Epstein, 1972; Bernert et al., 1996] and DS mouse models [Haydar et al., 2000a], which leads to the production of fewer VZ founder cells and a delay in the radial expansion of the cerebral wall during neurogenesis.

Deployment of Nascent Neurons

Neuronal migration (Stage 3) begins as the first neurons are generated from the VZ and migrate by at least two modes into the superficial neocortical wall to establish the cortical plate (CP). Neuronal migration can occur radially, from the dorsal telencephalic VZ outward into the expanding CP [Angevine and Sidman, 1961; Marin-Padilla, 1971; Rakic, 1972, 1974; Tan et al., 1995, 1998], or tangentially, from the ventral telencephalic VZ into the dorsal telencephalon [de Carlos et al., 1996; Anderson et al., 1997; Lavdas et al., 1999; Wichterle et al., 2001; Jimenez et al., 2002]. In addition, studies using clonal analysis of retrovirally infected neural precursors, which demonstrate that lineally related neurons can disperse throughout the neocortex [Walsh and Cepko, 1992, 1993; Reid et al., 1995, 1997; Ware et al. 1999], suggest the possibility that additional modes of migration may yet be found for particular groups of neurons in the dorsal or ventral telencephalon.

Radial neuronal migration has been described both in rodents [Nadarajah et al., 2001, 2003; Nadarajah and Parnavelas, 2002; Noctor et al., 2001, 2004] and in nonhuman primates [Rakic, 1972 1978, 1990]. Neurons migrate along the fibers of bipolar radial glial cells (RG), which extend processes from the surface of the lateral ventricle to the pial surface just above the CP (Fig. 1). As new neurons arrive, the CP expands into the mature six-layered cortex in an inside-out manner: the first-born neurons occupy the deepest layers and each new round of young neurons migrates past older neurons to occupy progressively more superficial layers [Angevine and Sidman, 1961; Rakic, 1974]. Thus, the laminar destination of cortical neurons is related to their day of genesis. This fact is critically important since the dorsally derived radially migrating neurons, which are primarily excitatory projection neurons [Chan et al., 2001; Gorski et al., 2002], are joined by ventrally derived inhibitory interneurons that have migrated tangentially into the neocortex (Fig. 1). Birth-dating experiments using tritiated thymidine in nonhuman primates have shown that cortical neurons born on the same day eventually occupy the same cortical lamina [Rakic, 1974]. Therefore, there must be a finely tuned coordination between the dorsally and ventrally derived neurons to result in the comingling at their final destination. Taken together, these results suggest that the mechanisms controlling the tempo and fidelity of radial and tangential migration are critical for the arrival of excitatory and inhibitory neurons to the proper cortical space.

Data from human brain malformations and mouse models of disease illustrate how disruptions in cell migration lead to cognitive impairment [Rakic, 2000]. The abnormal placement, or heterotopia, of dorsally derived neurons is found in a wide variety of clinical syndromes [Ross and Walsh, 2001]. The disturbance of interneuron migration and integration may also be involved in a variety of developmental disorders involving deficits or misplacement of GABAergic cells, such as in cortical dysplasia causing epilepsy [Roper et al., 1999; Powell et al., 2003], Tourette's syndrome [Leckman and Riddle, 2000], and autism [Bauman and Kemper, 1994; Casanova et al., 2002a, 2002b].

Several studies have now identified some of the gene mutations that underlie these abnormalities. Deficiencies in the extracellular matrix glycoprotein Reelin, encoded by the *RELN* gene, have been found in children with autism [Persico et al., 2001] and lissencephaly [Hong et al., 2000; Fatemi, 2001]. Mutations in the *Ndel1*, *LIS1*, and *DCX* genes contribute to the heterotopias found in several lissencephalies including Miller–Dieker syndrome (Lo Nigro et al., 1997; Ross et al., 1997; des Portes et al., 1998; Gleeson et al., 1998; Pilz et al., 1998; Ross and Walsh, 2001). Transgenic mouse imaging studies have also begun to investigate the role of genes in neuronal migration, most recently including ataxic Scrambler mice, which are

deficient in Disabled-1 (mDab1). The mDab1 protein is an intracellular docking protein for cAbl kinase, which is phosphorylated when Reelin binds to its receptor [Hiesberger et al., 1999]. It was hypothesized that Scrambler mice exhibit defects in the inside-out formation of the neocortical plate because later-born neurons are unable to establish superficial positions and instead “pile up” underneath earlier-derived neurons [Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997]. Indeed, time-lapse imaging revealed that the migration defect in these animals was due to defective detachment of neurons from their radial glial guides, causing a “traffic jam” that prevented the superficial placement of new neurons [Sanada et al., 2004]. Recently, work on Close Homologue of L1 (CHL1)-deficient mice, a gene whose human orthologue is mutated in human 3p syndrome [Angeloni et al., 1999; Frints et al., 2003], has shown that defective radial migration leads to ectopically positioned projection neurons, which consequently develop misoriented dendrites [Demyanenko et al., 2004]. Thus, significant disruptions in radial migration can precede, and perhaps cause, deficits in differentiation and synaptic development.

Cell Differentiation, Synaptogenesis, and Programmed Cell Death

Once in their final resting place, cortical neurons differentiate into a specific phenotype by synthesizing appropriate neurotransmitters and receptors, by extending dendrites and dendritic spines to receive connections, and by sending axons out to synapse with their appropriate targets. As such, this phase of development (here termed Stage 4) is a balancing act, where cells must first be competent to receive afferent connections prior to the ingrowth of contacting fibers and, vice versa, targets of efferent pathways must be ready to respond by developing postsynaptic specializations when contacted. In addition, the numbers of synapses on each cell relates to cell class and function. Therefore, any disturbance delaying the onset of synaptogenesis or preventing the formation of the appropriate numbers of synapses can adversely affect later cognitive function.

Several congenital disorders exhibit abnormalities in Stage 4 [Kaufmann and Moser, 2000]. For example, loss of the Fragile X mental retardation protein (FMRP) is postulated to interfere with proper translation of mRNA localized at synapses [Jin and Warren, 2003; Bassell and Kelic, 2004]. This could lead to the markedly abnormal dendritic spines and deficits in synaptic maturation found in Fragile X syndrome [Hinton et al., 1991; Irwin et al., 2000; Greenough et al., 2001]. In addition, there is paucity of dendritic spines in DS [Marin-Padilla, 1976; Suetsugu and Mehraein, 1980; Ferrer and Gullotta, 1990] and Rett syndrome [Belichenko et al., 1994; Armstrong et al., 1995]. The specific causes of the spine abnormalities in the latter two syndromes have yet to be identified. However, relating back to the axiom presented above, defects in early stages of neocortical development can precede abnormalities in synapse formation. For example, a delay in neocortical expansion in the trisomy 16 (Ts16) mouse, a model for DS, results from both a decrease in the number of founder cells during Stage 1 and from delayed neurogenesis in Stage 2 [Haydar et al., 2000a]. Despite the fact that the Ts16 VZ compensates for this delay by eventually producing a neocortical wall of normal thickness [Haydar et al., 1996, 2000a], delays in the formation of early neocortical neurons causes reduced innervation of the neocortex by thalamic afferents just before birth [Cheng et al., 2004b]. Thus, lasting deficits observed in Stage 4 can be established by abnormalities in the early expansion of neural stem cells and by delays in neurogenesis.

After neurogenesis, migration, and synaptogenesis, Stage 5 ensues as a critical period of development during which neuronal survival is supported by synaptic activity and the maintenance of connections. In addition, many cells serve transient roles and are removed during early postnatal life via programmed cell death (PCD) [Spreafico et al., 1995; Valverde et al., 1995]. Thus, genetic anomalies that interfere with the onset or rate of synaptic development or that increase PCD can reduce the numbers of cortical neurons and lead to

cognitive disability. For instance, high rates of programmed cell death have been found in postnatal DS neocortex [Busciglio and Yankner, 1995; Wolvetang et al., 2003]. Underscoring the interdependence of developmental stages, the etiology of DS is multifactorial and may include causally related abnormalities in neural stem cell proliferation, synapse formation and maintenance as well as PCD. In summary, proper development of the cerebral cortex requires a strict sequence of events, each of which can be adversely affected by abnormalities in preceding stages of growth.

BENEATH THE SURFACE WITH LASER SCANNING MICROSCOPY

Uncovering the Dynamics of Development

Several factors that have inhibited the study of these developmental stages (e.g., neural precursor proliferation, migration, synaptogenesis) are 1) the inaccessibility of the in utero embryo, 2) the confounding mix of extremely rapid biochemical events, which can occur within milliseconds and within the context of comparatively slow morphogenic processes, which can happen over hours, days, and months, and 3) the difficulty of following cells in their three-dimensional environment, where kinetic processes occur in the context of intercellular interactions, extracellular matrix, and diffusible morphogenic factors. Cell culture techniques have been instrumental in describing many intracellular mechanisms. However, while fundamental processes can, and in many cases must be, studied in dissociated cell preparations, drawbacks to in vitro examination of cells include the cellular trauma of the dissociation process and the lack of short- and long-range environmental influences. In addition, since developmental milestones are dependent on the success of preceding stages, there is a need to follow cells over protracted periods within their native environment.

The invention of laser scanning microscopy (LSM), the development of fluorescent reagents, and the availability of low-cost high-powered computers have revolutionized scientific imaging and have made time-lapse investigation of cellular processes a viable experimental paradigm in the laboratory. LSM has become a powerful tool to categorize the dynamic processes that occur in the intact, living, and developing embryonic brain. In the past 20 years, several different methods with LSM technology at their core have been developed to study rapid events, such as calcium sparks, as well as the relatively slow processes of cell proliferation, migration, and synaptic maturation. A detailed description of LSM design and theory can be found in a number of other publications [Pawley, 1995; Potter, 1996b; Paddock, 1999; So et al., 2000]. In this review, a general description of LSM technology will introduce a discussion of current applications. The extent to which this technology can be used to evaluate brain development in disease models will be highlighted.

The Core LSM Technology

Two methods now in widespread use are confocal (CLSM) and multi-photon laser scanning microscopy (MPLSM), both of which are designed to collect the photons emitted from a discrete optical space at the focal plane of the microscope objective. This property is exceedingly important when imaging thick tissue as it results in high clarity images by eliminating out-of-focus light from elements above and below the focal plane. Thus, a series of thin optical sections through the depth (the z-dimension) of the sample, termed a z-stack, can be used to reconstruct the sample in three dimensions (3D), and z-stacks can be acquired over time to perform four-dimensional (4D) experiments.

While both methods usually excite the fluorophores in the specimen with lasers, they differ in the excitation wavelengths used as well as the amount of irradiation and penetration of the sample. In CLSM, continuous wave lasers emitting visible wavelengths irradiate the entire sample (Fig. 2A), resulting in emission from fluorescent molecules throughout its depth. A

pinhole aperture, placed in front of the detector, is then used to reject the out-of-focus light and pass through only the emission from the focal plane. The drawbacks of this excitation paradigm include the large amount of excitation during sample illumination, the corresponding bleaching and phototoxicity throughout the sample, and the loss of much of the emitted light at the pinhole [Sandison et al., 1995]. In addition, the diffraction and absorption of visible light by cellular membranes and organelles typically limits the depth of CLSM imaging, preventing analysis much deeper than 50–100 μm below the sample surface. These drawbacks can simply be considered annoying byproducts when scanning fixed tissue and can be avoided by thin sectioning of the tissue, for example. However, the phototoxicity in CLSM can confound studies in living preparations [Squirrell et al., 1999], and the limited penetration confines CLSM studies to the surface of living samples, which are oftentimes damaged during the preparative process.

In contrast, multi-photon excitation avoids some of these pitfalls and can therefore be more effective for imaging of living specimens. MPLSM uses ultrafast infrared (IR) pulse lasers where each individual pulse is extremely short ($\sim 10^{-13}$ s) and has very high instantaneous power. However, because the repetition rate of these pulses are spaced relatively far apart ($\sim 10^{-8}$ s), the amount of power felt at the sample is reduced to the milliwatt range [Denk et al., 1995; Potter 1996a]. In addition, because the pulse repetition rate is longer than the fluorescence decay time, which is typically 10^{-9} s for commonly used fluorophores, only one pulse at a time can excite each fluorescence molecule, thereby preventing saturation. Most importantly for multi-photon excitation, the energy of individual photons within each pulse is small, and the absorption of two or more photons is therefore needed to excite a fluorescent molecule. This multiple absorption event is only favored at a diffraction-limited spot in the focal plane of the objective (Fig. 2B), removing the requirement of a pinhole since fluorophores above and below the focal plane are not excited. In addition, the increased transmission of the longer IR wavelength through living tissue allows for deeper imaging [Centonze and White, 1998]. For these reasons, MPLSM has been exploited for time-lapse imaging in living tissue [Potter, 1996b; Squirrell et al., 1999; Megason and Fraser, 2003]. Several drawbacks of MPLSM imaging include the significant increase in cost due to the expense of IR pulse laser systems ($\sim \$150\text{K}$) and the increased maintenance needs of the IR lasers, as they require frequent cleaning and alignment. Newly developed turn-key “IR systems in a box” promise to reduce the amount of this maintenance. In part driven by the advances in live tissue imaging afforded by CLSM and the capability of deep live imaging with MPLSM, several methods for maintaining living preparations have recently been optimized to enable 4D imaging.

Experimental Systems

One method for investigating cellular behavior in intact tissue is the organotypic slice, where a 300 – 400 μm brain slice can be cultured acutely for several days [Stoppini et al., 1991; Haydar et al., 1999a] or for weeks to months [Gahwiler et al., 1997]. Acute slices are cut from freshly isolated brain and then cultured either in an incubator between imaging bouts [Noctor et al., 2001, 2002, 2004] or on the stage of an LSM microscope for up to several days [Nadarajah et al., 2001, 2002; Ang et al., 2003; Haydar et al., 2003]. Even when cultured in minimal defined media lacking serum or growth factors, neocortical organotypic slices recapitulate the neurogenesis, neuronal migration, and very low levels of PCD found in vivo [Haydar et al., 1999a]. Thus, it is now possible to study many of the dynamic cellular properties and stages of development in organotypic slices using 4D LSM. A modification of this technique, called the slice overlay assay, can be used to investigate intrinsic and extrinsic cues in neural development [Polleux and Ghosh, 2002]. This involves plating dissociated cells onto a slice, allowing integration of the exogenous cells for “mix and match” experiments. For example, studying the migration or proliferation of cells derived from mutant animal models after plating them onto wild-type slices can determine whether the abnormality seen in the mutant cells is

intrinsic or is specified by the extracellular environment. Advantages of slice imaging include the ability to stabilize the specimen to avoid large movements in the imaging field, the relative ease of slice preparation from different developmental ages, and the possibility to study time-lapse events in an array of species—including in resected human tissue.

There are, however, several potential disadvantages to slice culture that must be continuously evaluated since they may alter cellular behavior. These include the trauma of sectioning, the degeneration of cells and processes at the cut surfaces of the slice, and the composition of the media. Because the sectioning unavoidably causes cell death at the edges of the slice, deep imaging is required to observe cells not affected by this trauma, therefore, MPLSM is preferred for slice imaging. Regarding media formulation, often it is preferable to use a minimal defined media that contains only the identified components necessary for slice health. Several studies have taken the approach of observing and cataloging the basal rates and modes of cellular behavior in slices cultured in minimal media [Komuro and Rakic, 1992, 1993; Haydar et al., 1999b, 2000b, 2003; Ang et al., 2003] so that the role of exogenous factors in neurogenesis, migration, and synapse formation may be investigated in a quantitative manner by adding them back to the defined media. For example, although precursors in embryonic and fetal brain slices do not require serum or growth factors, a recent study on migration of postnatal mouse SVZ precursors determined that 5% fetal calf serum was necessary to maintain survival [Gadea, Aguirre, Haydar, and Gallo; unpublished observations]. Most importantly, regardless of the steps taken to duplicate the in vivo macroenvironment with culture conditions, the slice preparation represents a substantial manipulation of the tissue. A validation of results obtained from live-imaging slice experiments is therefore often necessary. Increasingly, groups are confirming the conclusions from their slice data by performing time-lapse imaging in vivo.

Two methods have thus far been used to perform MPLSM in vivo. One procedure involves restraint of an anesthetized animal on the microscope stage. This has been used to achieve MPLSM time-lapse recordings of calcium dynamics in axons, dendrites, and movement of cortical dendritic spines [Svoboda et al., 1997; Chen et al., 2000; Cox et al., 2000; Lendvai et al., 2000] as well as the migratory characteristics of GABAergic interneurons in the embryonic brain [Ang et al., 2003]. Another technique makes use of a newly developed miniature MPLSM that is mounted onto the head of an adult animal and is tethered by fiber optic cable to the IR pulse laser [Helmchen et al., 2001; Brecht et al., 2004]. This allows time-lapse imaging of superficial cortical neurons in freely moving, awake and behaving subjects. Although the mini-MPLSM technique is still being refined, it clearly represents the cutting edge of live in vivo LSM imaging. One of the confounding technical problems with in vivo MPLSM includes the gross movements of the specimen by heartbeat and breathing. These rhythmic movements can be avoided by fast scanning, for example, line scanning, at intervals in between heartbeats and breaths [Svoboda et al., 1997; Lendvai et al., 2000; Brecht et al., 2004]. In addition to the choice between an in vitro or in vivo preparation, a major component of a successful imaging experiment is the choice of label.

Labeling Methodologies

There are a plethora of strategies for fluorescently labeling cells and organelles for LSM imaging of living tissue, ranging from expressing fluorescent proteins in all cells of the body via transgenic technology to the analysis of cell compartments or individual organelles with fluorescent dyes. While a comprehensive description of all of these methods is prohibitive, I will discuss several techniques used for either for widespread labeling or for labeling of restricted classes of cells that may be of particular interest to those investigating the underlying causes of mental retardation and developmental disabilities.

Apart from transgenic technology, several useful tools for labeling large numbers of cells and intracellular compartments have been developed. For example, the lipophilic carbocyanine dye

DiI, which incorporates and migrates within the membrane bilayer, has been injected into the lateral ventricle of embryonic and perinatal mouse brains to label migrating neurons [O'Rourke et al., 1992]. In addition, pial placement of DiI was used to label cells in contact with the pia, including the long bipolar RG, which span the full thickness of the cerebral wall [Miyata et al., 2001]. In the Miyata et al. [2001] study, DiI-labeled RG in slices were followed over time with CLSM to demonstrate that RG maintain their elongated morphology throughout all phases of the cell cycle. In addition, vital dyes can be used to label all cells in a slice simply by immersion in dye-containing cell culture medium. For example, CellTracker Green CMFDA (Molecular Probes, Inc.) was used as a cytoplasmic label of neocortical cells and enabled MPLSM migration studies of migrating neurons in prenatal neocortical slices [Ang et al., 2003]. In addition, Ang et al. [2003] developed an in utero surgical technique to expose and label superficial neocortical neurons with Cell-Tracker Green while keeping the embryos viable and in the uterine environment during MPLSM imaging. Similar studies using CLSM have labeled migrating neurons in slices by bath application in Oregon Green BAPTA [Nadarajah et al., 2001, 2002, 2003]. Bath application of various other dyes, such as SYTO dyes to label nucleic acid [Chenn and McConnell, 1995; Haydar et al., 2003], potentiometric dyes to monitor membrane potential, and endoplasmic reticulum- and mitochondrial-specific dyes (ER- and Mito-Tracker, Molecular Probes, Inc.) can be used to label intracellular organelles.

Recently, several methods have been developed for labeling embryonic brain cells in utero. One of these, termed in utero electroporation, makes use of the inherent negative charge of DNA molecules. This technique requires injection of mammalian expression plasmids through the uterine musculature and amniotic sac and into the cerebral ventricles, followed by brief voltage pulses between two electrodes placed outside the uterus to create pores and to drive the DNA into cells lining the ventricle [Akamatsu et al., 1999; Miyasaka et al., 1999; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001; Hatanaka and Murakami, 2002]. This technique has been used successfully to transfect VZ and SVZ cells with fluorescent reporter genes, to assay the roles of diffusible morphogens [Fukuchi-Shimogori and Grove, 2001], and has also been used to deliver siRNA plasmids to knock down genes of interest [Bai et al., 2003]. One advantage of in utero electroporation is the high transfection efficiency: this procedure typically transfects a 500 to 1,000- μ m-wide swath of cells situated beneath the anode, and transfection rates within this swath can reach up to 100%. In addition, any group of cells bordering the ventricle can be selectively transfected merely by changing the position of the electrodes so that the anode is above the region of interest. One major limitation of in utero electroporation is that mouse embryos much younger than E12 cannot be reliably transfected, due to their size and fragility.

Another in utero injection technique makes use of high resolution ultrasound backscatter microscopy (UBM) to gain access to very early stages of embryonic development [Olsson et al., 1997; Liu et al., 1998], for example, as early as E8.5 mouse embryos [Gaiano et al., 1999]. UBM-assisted studies have used retroviruses for gain-of-function analysis of Sonic Hedgehog in telencephalic precursors, and UBM-assisted focal homotopic injections of ventral telencephalic cells have delineated the roles of each portion of the striatal anlage in the allocation of interneurons throughout the telencephalon [Wichterle et al., 2001; Nery et al., 2002].

These labeling techniques and reagents, especially when used in combination with the organotypic slice and in vivo imaging methods, present a very powerful capability to rapidly catalog the molecular elements that control cellular behaviors in the complex environment of thick living tissue. Using these techniques, several studies have recently presented fundamental properties of proliferating, migrating, and differentiating cells that can be used to evaluate the development in animal models of congenital abnormalities.

DYNAMIC CELLULAR BEHAVIORS DURING CORTICAL DEVELOPMENT

Mitotic Spindle Rotation and Mode of Cell Division

A series of LSM imaging studies have described the mechanics of neural precursor proliferation and established a dataset from normal animals to which results from mutant animal models can be compared [Chenn and McConnell, 1995; Adams, 1996; Haydar et al., 2003]. The cellular strategy controlling the growth of the founder cell population and the rate of neurogenesis has been termed the mode of division. There are two main modes of division: symmetrical (S-type) cleavages yield two similar daughter cells with the same proliferative fate while asymmetrical (A-type) divisions generate different daughters; for example, one daughter that remains proliferative and another that differentiates into a neuron (Fig. 3). Developmental regulation of S-type and A-type cleavages is thought to control the size and eventual complexity of the neocortex [Rakic, 1988, 1995; Caviness et al., 1995; Takahashi et al., 1996]. There are multiple types of S- and A-type divisions. For example, S-type_{in} divisions generate daughter cells that remain proliferative and stay in the cell cycle whereas both daughter cells from S-type_{out} cleavages become postmitotic and exit the cell cycle. S-type_{in} divisions occur early in neurogenesis, yield more progenitors, and lead to an exponential expansion of the VZ population. S-type_{out} divisions, which mainly occur later during neurogenesis, increase the rate of neuronal production, and exhaust the VZ of precursor cells as the last cortical projection neurons are born. Although less well described in mammals, A-type divisions have been shown to generate different classes of neocortical neurons [Qian et al., 2000]. Thus, the changing proportions of each mode of division over the course of cortical neurogenesis play a large role in the eventual size, composition, and function of the neocortex. However, despite the critical role that proliferative events play in directing cortical growth, the mechanisms controlling the transition between modes of division have been unexplored until the advent of LSM imaging techniques.

The orientation of the mitotic plate just before cleavage is one of the mechanisms for specifying the mode of division during brain development. Time-lapse and static imaging studies of progenitor cells in vivo have correlated changing cleavage plane angles with alternate cell fates in both invertebrates [Horvitz and Herskowitz, 1992; Skop and White, 1998; Theesfeld et al., 1999; Doe and Bowerman, 2001] and vertebrates [Chenn and McConnell, 1995; Kaltschmidt et al., 2000; Haydar et al., 2003]. These studies suggest that vertical cleavage planes perpendicular to the ventricular surface identify S-type divisions. In contrast, horizontal cleavage planes parallel to the ventricle signify A-type divisions (Fig. 3).

In addition to acquisition of the cleavage plane, a recent time-lapse MPLSM study also confirmed and extended the original observations [Adams, 1996] of a startling feature of the developing neocortical wall: the mitotic spindle of dividing VZ cells undergoes significant rotation and oscillation throughout metaphase before acquisition of the final cleavage plane [Haydar et al., 2003] (see Movie 1). Moreover, the final cleavage orientation relates directly to the amount of preceding mitotic spindle rotation such that S-type divisions occur quickly with proportionately less rotation than in the longer A-type cleavages. Importantly, the average cleavage angle in mitotic VZ cells correlates with the graded neuronal commitment over the course of neurogenesis and therefore may be causally related to the changing VZ output. Thus, for the first time, MPLSM can be used to analyze mitotic spindle dynamics to measure the rapid intracellular movements that control founder cell expansion and the onset, rate, and duration of neocortical neurogenesis in animal models of disease.

Radial and Tangential Neocortical Migration

The application of LSM to the study of neuronal migration has led to several recent advances in determining the intracellular and extracellular components that control cell migration

[Komuro and Rakic, 1998b]. The CLSM work of Komuro and Rakic, which showed in acute slices that cerebellar granule cell migration is regulated by spontaneous intracellular calcium fluctuations controlled by the action of N-type calcium channels [Komuro and Rakic, 1992] and NMDA receptors [Komuro and Rakic, 1993], could only have been accomplished by combining LSM with live imaging. In addition, several different modes of radial and tangential migration in the neocortex have been characterized both in vivo and in slices, making it increasingly clear that the mode of neuronal migration may change with respect to developmental time point or may be specific for certain cell classes [Nadarajah and Parnavelas, 2002]. Thus, the characteristics of cells displaying a particular migratory phenotype may be used to assess the health and proper development of the system.

Radial migration from the neocortical VZ occurs as new neurons are generated during the course of neurogenesis. Thus far, two modes of radial neuronal migration have been established using LSM on both fixed and living tissue. In glial-guided migration, the ascending processes of radial glial cells are used as a migrational substrate [Rakic, 1972, 2003; Sidman and Rakic, 1973; Hatten and Mason, 1990; Nadarajah et al., 2001, 2003; Noctor et al., 2001,]. In contrast, some neuronal precursors that span the neocortical wall can migrate by somal translocation after becoming postmitotic. This occurs when the ventricular foot process of these bipolar cells is retracted, followed by migration of the cell soma into the CP within the pial-attached process [Morest, 1970; Miyata, et al., 2001; Nadarajah et al., 2001; Rakic 2003]. In addition to recording the morphological changes associated with glial-guided and somal translocation, CLSM experiments have also measured the rate of glial-guided migration in the neocortex to be between 14 $\mu\text{m/h}$ [O'Rourke et al., 1992], 27 $\mu\text{m/h}$ [Gongidi et al., 2004], and 35 $\mu\text{m/h}$ [Nadarajah et al., 2001], while averaging only 9 $\mu\text{m/h}$ in the cerebellum [Komuro and Rakic, 1998a]. The different migration rates reported may be due to differences in tissue, cell type, or media composition. In contrast to glial-guided migration, somal translocating cells appear to migrate faster at 60 $\mu\text{m/h}$ [Nadarajah et al., 2001], presumably because they do not need to maintain contact with a migrational substrate. It is unclear whether specific neuronal classes require either radial glial migration or somal translocation, and the preponderance of each type of radial migration has not yet been quantified with respect to developmental age. Once fully characterized and assigned to specific classes of cells, these two modes of radial migration can be used in humans and in animal models to determine Stage 3-specific abnormalities.

Although many groups have described the tangential migration of ventrally derived interneurons into the dorsal neocortical wall [de Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999], the cellular mechanisms providing for this long-distance movement through densely packed parenchyma were unknown until recently. Static and time-lapse LSM imaging is now beginning to describe the behavior of tangentially migrating neurons in detail and uncover how these cells may find their proper targets [Polleux and Ghosh, 2002; Ang et al., 2003]. Interneurons from the ventral telencephalon migrate tangentially through all layers of the neocortical wall but are most highly concentrated in the intermediate and marginal zones. Migrating interneurons are bipolar with a long growth cone-tipped leading process and a short trailing process (Fig. 4). The leading process is highly motile and is clearly used to explore the environment before somal translocation moves the cell forward [Polleux and Ghosh, 2002; Ang et al., 2003]. In addition, when traversing cortical zones and prior to changing directions, interneurons often stop migration and initiate multiple processes before proceeding [O'Rourke et al., 1992; Polleux and Ghosh, 2002]. In a study examining the mode of interneuron migration through the superficial neocortical wall, it was found that there are three separate streams of interneurons that emerge from the ventral telencephalon on different embryonic days beginning on E11.5 [Ang et al., 2003]. Each stream appears to follow a specific vector across the telencephalic surface into the dorsal neocortex. Moreover, interneurons migrate in two specific stages: a rapid unidirectional long-range migration of 60–70 $\mu\text{m/h}$ [Polleux and Ghosh, 2002; Ang et al., 2003] is followed by a slower

phase (3–4 $\mu\text{m}/\text{h}$) during which cells undergo multiple process extensions and retractions prior to integrating into the proper cortical area [Ang et al., 2003]. The temporal and spatial resolution of the time-lapse studies clearly suggest that interneuron growth cones are used to sense the cues available in the local environment during this second phase (see Movie 2) and that these as yet unidentified cues are used for proper integration. Nevertheless, the time of onset, direction, and rate of migration in the migratory streams can now be used to assess abnormalities of interneuron migration in animal models of disease.

Synaptic Development and Maintenance

In addition to characterizing the first several stages of embryonic cortical growth, LSM is now being used to investigate the conditions necessary for establishment and maintenance of synapses and is often uncovering phenomena that are not detectable using more traditional methods. Fast-scanning LSM systems have been used with membrane potential dyes and calcium indicators to study neuronal physiology both at the whole-cell level and with higher resolution at the level of dendritic spines. For example, Jin et al. [2003] demonstrated, via 3D reconstruction of biolistically transfected interneurons, that the growth and branching of interneuron dendrites is dependent upon depolarization, which is itself influenced by brain-derived neurotrophic factor. Another recent study investigating the regulatory mechanisms of GABA release illustrated that live imaging can uncover differences between spines that are undetectable using electrophysiological methods [Brager et al., 2003]. Using time-lapse CLSM, this group was able to elucidate the variance in exocytosis parameters from different GABA boutons by showing that the regulation of GABA exocytosis is in part specified by the composition of voltage-gated calcium channels (VGCC) present on the presynaptic membrane. Furthermore, the marked differences in the types of VGCC present on different GABAergic boutons correspondingly results in variations in exocytosis parameters.

Several groups have also made advances in describing the physiology of synapse development and maintenance using MPLSM. Of particular note, studies by the Denk, Tank, and Svoboda laboratories have described time-dependent changes in neuronal physiology *in vivo*. In a seminal study, Svoboda et al. [1997] combined *in vivo* MPLSM imaging of superficial pyramidal neurons in adult rat somatosensory cortex with sharp electrode recording to determine how cell depolarization and whisker stimulation influenced calcium fluctuations. Prior to this study, *in vitro* work had suggested that sodium and calcium action potentials can propagate into the dendritic tree and thereby affect synaptic plasticity. However, the authors showed *in vivo* that calcium action potentials remained more localized to the cell soma and did not invade the distal dendrites. The authors posited that several variables introduced during *in vitro* culturing of neurons, such as changes in resting membrane potential and the ionic composition of the media, may have contributed to the different physiology found *in vivo*.

In another series of elegant experiments, *in vivo* live MPLSM imaging showed that spine morphology changes rapidly in response to the level of target innervation [Chen et al., 2000; Lendvai et al., 2000]. It was previously hypothesized from *in vitro* studies that dendritic filopodia and spines are motile during synaptogenesis as new contacts are explored and existing synapses are strengthened [Ziv and Smith, 1996]. Indeed, *in vivo* MPLSM imaging showed that the length of dendritic spines and filopodia on layer 2/3 neurons of the rat barrel cortex changed quite dramatically, often appearing *de novo* or disappearing within 10–30 min. To test whether the amount of sensory input could affect these spine dynamics, the authors removed the whiskers to deprive the cortical neurons of their sensory input and found that spine movements significantly decreased. The results also indicated that spiny protrusions were most affected when the sensory deprivation occurred during a discrete time window between P11 and P13. This experience-dependent morphogenesis suggests that synaptic formation and maintenance is specified during a narrow developmental time window and, therefore, that early

abnormalities in the production and differentiation of cortical neurons may have severe developmental consequences. Together, these studies illustrate that LSM imaging techniques can be used to track the progression of developmental stages and to assess synaptic development and physiology in the intact brain of mouse models of disease.

LIVE IMAGING IN ANIMAL MODELS OF CONGENITAL DISORDERS

Animal models are a powerful tool for exploring the underlying cellular and molecular causes of mental retardation. Many new models are appearing for a variety of cognitive defects, and the use of live LSM imaging to analyze the dynamic properties of brain development in these models is now possible. Importantly, although symptoms of congenital disorders may not appear for months or even years after birth, recent studies in autistic brains as well as in animal models of lissencephaly, fragile X syndrome, and Down syndrome suggest that a broader examination of all stages of embryonic development may provide new perspectives on the etiology of these disorders.

The several mutant mice generated as models of human lissencephaly and Miller–Dieker syndrome (including the *Nde1*, *Ndel1*, *Lis1*, *Ywhae*, and *Mnt*-deficient animals) all point to specific defects in migration that result in altered cortical formation [Hirotsume et al., 1998; Toyo-oka et al., 2003, 2004; Feng and Walsh, 2004]. The reasons for the migrational defects reside in the interaction of these proteins with the motor functions of cytoplasmic dynein and its subsequent interaction with microtubules. However, consistent with the role dynein plays in cell division [Pfarr et al., 1990; Steuer et al., 1990; Echeverri et al., 1996], there is also considerable evidence suggesting that defective neurogenesis may precede the migrational abnormalities in these mutant mice [Faulkner et al., 2000; Liu et al., 2000; Feng and Walsh 2004]. For example, neurogenesis is delayed in *Nde1*-deficient mice, and the neural precursor cells in the neocortical VZ have abnormal cleavage orientations [Feng and Walsh, 2004]. Therefore, given the association between mitotic spindle rotation and cleavage plane orientation, it is now important to measure spindle rotation in these models and the extent to which mitotic abnormalities 1) contribute to the overall microcephaly found in lissencephaly and 2) are causally linked to the migration defects, perhaps by affecting the development of different neural precursor groups. This is important since any future attempt to correct prenatal migration in lissencephaly patients might fail if the neurogenesis defects are not concurrently addressed.

Autism is another disorder in which an LSM-based study of neurogenesis may provide new insights. Autistic infants undergo two separate stages of altered brain development prior to the onset of behavioral symptoms: decreased brain size at birth is followed by a second phase during which the brain grows larger than normal. This second phase begins in the first several months of life and lasts for 2–4 years [Courchesne et al., 2003; Courchesne, 2004]. Despite the increases in both cortical grey and white matter during the second overgrowth phase, subsequent brain maturation does not occur normally in autism, leading to mental retardation and often to seizures. A basic structural unit of the cerebral cortex is the minicolumn, a radial array of cells and synapses ranging from the deep cortical layers to the pial surface. Each cortical area contains a different number of minicolumns, and the size and cell density of each column is suggested to reflect the maturation and functional ability of the cortex [Casanova et al., 2003]. In young autistic brains, while there is an increase in the number of minicolumns, each column is smaller than normal and has reduced cell density [Casanova et al., 2002b]. Thus, microcephaly at birth precedes defective brain maturation and synaptic development in autism. One parsimonious explanation for the defects in autism is therefore that defective neural production during the embryonic period can lead to disrupted synapse formation and strengthening during the critical period. Although no single model for autism has yet been established, live LSM imaging can be used to measure parameters of neural precursor

expansion, neurogenesis, and spine motility (differentiation/synaptogenesis) in the many animals that model specific autistic traits [Belmonte et al., 2004; Murcia et al., 2004].

A number of symptoms found in DS, such as the microcephaly and spine and synapse abnormalities, may be investigated by applying LSM to the two different animal models of DS [Galdzicki et al., 2001]. The trisomy 16 (Ts16) mouse has a full triplication of mouse chromosome 16 (MMU16) [Reeves et al., 1986], which is partially syntenic with human chromosome 21 [Holtzman and Epstein, 1992; Reeves and Miller, 1992]. Ts16 embryos exhibit delayed neurogenesis and deficits in corticothalamic connections [Haydar et al., 1996; Haydar et al., 2000a; Cheng et al., 2004a], but die before birth. Therefore, it has not been possible to determine the extent to which delayed neurogenesis may alter later stages of growth, such as neuronal differentiation and synaptogenesis. In contrast, the partial trisomy (Ts65Dn) mouse, which has a triplication of just a segment of MMU16 genes thought to be critical for the cognitive deficits in DS patients [Epstein, 1993; Gardiner et al., 2003], has been extensively characterized postnatally. Ts65Dn mice exhibit motor and sensory deficits as well as spatial learning abnormalities [Escorihuela et al., 1995, 1998; Reeves et al., 1995; Demas et al., 1996, 1998; Holtzman et al., 1996; Costa et al., 1999; Hyde et al., 2001]. Similar to DS patients [Marin-Padilla, 1976; Suetsugu and Mehraein, 1980], recent studies have shown that adult Ts65Dn hippocampal neurons have enlarged spines and boutons [Belichenko et al., 2004] and altered dendritic morphology [Dierssen et al., 2003], suggesting that synaptogenesis and/or synaptic maintenance are altered in this model. However, since Ts65Dn breeding colonies are hard to establish due to the cost of the animals and the sterility of the males, embryonic studies on Ts65Dn animals have not yet been performed. Obvious future steps to uncover the etiology of the cognitive deficits in DS are to perform embryonic neurogenesis and tract tracing studies on Ts65Dn embryos and to supplement those studies with MPLSM imaging of dividing neural precursors, migrating neurons, and the movement of spines during and after the early postnatal critical period for synaptogenesis.

CONCLUSION

New imaging methods have been recently used to increase our understanding of the cellular events that occur during brain development. Many of these processes, in particular mitosis, neuronal migration, and synapse formation, are highly dynamic and are controlled by signals from the extracellular space and from cell–cell contacts. The locations, movements, and interactions between cells in the three-dimensional environment of the living brain are therefore critical components of proper cortical growth. Live LSM imaging has opened the door to these remarkable events and has stimulated new avenues of research. LSM has been critical for the recent studies illustrating the behavior of the growth cone and leading process during interneuron migration (Movie 2), which have focused attention on the factors the growth cone is sensing and the intracellular signaling pathways mediating proper integration of these cells. In addition, LSM imaging of mitotic spindle orientation will be important for identifying the molecular determinants of cell division and fate specification.

More than just elaborating processes of normal brain development, LSM imaging methods will be instrumental in identifying possible treatment strategies when applied to animal models of congenital disorders. For example, understanding how tangentially migrating interneurons seek and integrate into the proper domain of the dorsal telencephalon is important for a variety of epileptic disorders, including autism, lissencephaly, cortical dysplasia, and schizophrenia. However, as noted above, the dependence of synapse formation and resulting cognitive function on preceding stages of brain growth suggests that many diseases presenting with mental retardation may have their origins in utero. While surgical and medical treatments for embryos are by no means common, one goal of the field of developmental neuroscience is to gain the level of knowledge required to make this critical period of growth clinically

approachable. The above studies, and the particular use of LSM to uncover the molecular and cellular bases for abnormal brain development, have begun an exciting phase of exploration that will elucidate the elemental causes of mental retardation and provide an important diagnostic tool for efforts at its amelioration and prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Neocortical development proceeds in a series of stages. During Stage 1, the neural precursor cell population expands exponentially via symmetrical cell divisions. Each mitosis generates two daughter cells that remain in the cell cycle and continue to divide. Stage 2 begins as neurons are generated from the ventricular zone (VZ). The birthday of each neuron specifies its position within the expanding cortical plate (CP). Early born neurons take up deep CP positions and are passed by later generated neurons that migrate to the superficial CP. During Stage 3, excitatory neurons generated from the dorsal VZ (cells with black nuclei) migrate radially across the intermediate zone (IZ) and into the CP along radial glial cells, whose fibers span the width of the neocortical wall from the ventricular (V) to the pial (P) surfaces. Simultaneously, inhibitory interneurons (cells with grey nuclei) generated in the ventral telencephalon migrate tangentially to integrate into the dorsal CP plate. After settling into the appropriate area and layer, neurons begin to differentiate in preparation to send and receive connections during Stage 4. Once synapse formation has begun, connections are either strengthened and refined or eliminated. During Stage 5, supernumerary cells and those not receiving the proper number and variety of synapses are removed by programmed cell death.

Fig. 2.

Two methods for fluorescent imaging in thick samples are (A) confocal (CLSM) and (B) multi-photon laser scanning microscopy (MPLSM). CLSM makes use of visible lasers that contain high energy as they pass through the depth (z dimension) of a sample. Elements in the path of the beam are irradiated and fluorescent molecules above and below the focal plane are excited to emit photons. The visible wavelengths of CLSM lasers can be absorbed by organelles and cause phototoxic damage to living cells. In contrast, the ultrafast infrared pulse lasers used in MPLSM can penetrate deeper into living tissue. In MPLSM, the increased likelihood of absorption of two or more photons results in selective fluorophore excitation at the focal plane. This provides for fluorescence emission in a diffraction-limited area (white spot) while fluorophores above and below the focal plane are not excited.

Fig. 3.

The mode of cell division specifies growth of the neocortex during Stages 1 and 2 of development. During early development, proliferating neural precursors divide symmetrically and both daughter cells remain *in* the cell cycle (CC). These S-type_{in} divisions result in an exponential expansion of the VZ precursor population. Neurogenesis starts as VZ cells initiate A-type divisions, yielding one proliferative and one postmitotic daughter cell. Finally, during the later stages of cortical neurogenesis, S-type_{out} divisions generate two postmitotic neuron daughter cells that migrate away from the VZ and deplete the neural precursor population. The angle of the cleavage plane, compared to the surface of the ventricle, is specific for each mode of cell division. Mitoses with perpendicular cleavage planes typify symmetrical divisions, while horizontal cleavage planes occur during asymmetrical divisions. See Movie 1 in supplementary material for an example of neural precursor cell divisions in an acute organotypic slice.

Fig. 4.

Migrating cortical interneurons negotiate a complex environment with their leading process before locating the proper address for their integration. In these panels, an interneuron moves tangentially through the cortical plate in a MPLSM experiment performed on a slice obtained from an E14.5 embryonic mouse brain. The leading process and growth cone (highlighted by arrowheads) extend through the parenchyma before rapid translocation of the cell body. The slice was stained with CellTracker Green (Molecular Probes) and imaged on a Zeiss LSM 510 NLO system using an excitation wavelength of 800 nm (see Ang et al., 2003 for a full description of methods). The frames in A–C are separated by 6 min of elapsed time and were

extracted from the full movie (Movie 2 in supplemental material) contributed by Ang E Jr, Haydar TF, and Rakic P.