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The multipolar stage and disruptions in neuronal migration

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The genetic basis is now known for several disorders of neuronal migration in the developing cerebral cortex. Identification of the cellular processes mediated by the implicated genes is revealing crucial stages of neuronal migration and has the potential to reveal common cellular causes of neuronal migration disorders. We hypothesize that a newly recognized morphological stage of neuronal migration, the multipolar stage, is vulnerable and is disrupted in several disorders of neocortical development. The multipolar stage occurs as bipolar progenitor cells become radially migrating neurons. Several studies using *in utero* electroporation and RNAi have revealed that transition out of the multipolar stage depends on the function of filamin A, LIS1 and DCX. Mutations in the genes encoding these proteins in humans cause distinct neuronal migration disorders, including periventricular nodular heterotopia, subcortical band heterotopia and lissencephaly. The multipolar stage therefore seems to be a critical point of migration control and a vulnerable target for disruption of neocortical development. This review is part of the *INMED/TINS* special issue *Nature and nurture in brain development and neurological disorders*, based on presentations at the annual *INMED/TINS* symposium (<http://inmednet.com/>).

Introduction

Appropriate neuronal positioning is essential for normal neocortical function. Perhaps the best example of this to date is the connection between altered neuronal positioning and susceptibility to epilepsy [1–6]. Experimental [3,7] and genetic disruptions that alter neuronal migration and laminar patterning invariably create neocortical circuits that have aberrantly enhanced excitability [7–9]. Currently less well understood, more subtle alterations in neocortical development might contribute to other alterations in neural function, including dyslexia [10,11] and schizophrenia [12–14].

Developmental mechanisms that ensure normal neocortical patterning include laminar and areal fate determination, neuronal migration and activity-dependent refinement [15]. In this mechanistic sequence, neuronal migration must preserve the earlier fate-determined patterns or risk significant developmental

disruption that cannot be corrected easily by subsequent activity-dependent processes. The radial-unit hypothesis provides a theoretical framework whereby the fated pattern established in a population of dividing neural progenitors is maintained by neurons migrating along parallel radial glial fibers [16–18]. In addition to such parallel migration, neocortical neurons can migrate obliquely to the radial glia. Interneurons migrate into neocortex in this way [19,20], and many pyramidal neuron precursors also migrate non-radially [21,22]. The complement of cellular and molecular mechanisms that control and regulate neuronal migration in the neocortex is becoming increasingly well known and these are reviewed elsewhere [23–26]. Here, we focus on studies that identify molecular requirements for a cellular transition that occurs for most, if not all, migrating pyramidal neurons, and we propose that spatially distinct phases of the multipolar stage are particularly vulnerable to disruptions that can result in neuronal migration disorders.

A multipolar stage in pyramidal neuron development

Radial progenitors of the ventricular zone (VZ) that give rise to neocortical pyramidal neurons are uniformly bipolar in morphology: one long process extends to the pia and a shorter process extends to the ventricular surface (Figure 1). Migrating neurons generated from radial progenitors [27] also adopt a characteristic bipolar morphology, with a leading process directed towards the pia and a trailing process extending below [18]. Migrating neurons that have bipolar morphologies are prevalent throughout the intermediate zone (IZ) and cortical plate (CP) of developing neocortex through periods of migration [28,29]. Cells with non-bipolar, complex morphologies are also prevalent in the subventricular zone (SVZ) and lower IZ throughout migration. Until relatively recently, it was unclear whether these morphologically more complex cells in the SVZ and IZ were a population separate from pyramidal neuron precursors, such as migrating interneurons or glial progenitors, or whether some were multipolar intermediates of migrating pyramidal neurons.

Live-cell imaging and *in utero* electroporation experiments have now unambiguously shown that many of the multipolar cells in the SVZ and IZ do in fact originate from radial progenitors [29–31]. Progenitors, labeled either by infection using an enhanced green fluorescent protein (eGFP) retrovirus to label individual clones [31] or by *in utero* electroporation to label larger numbers of cells [29],

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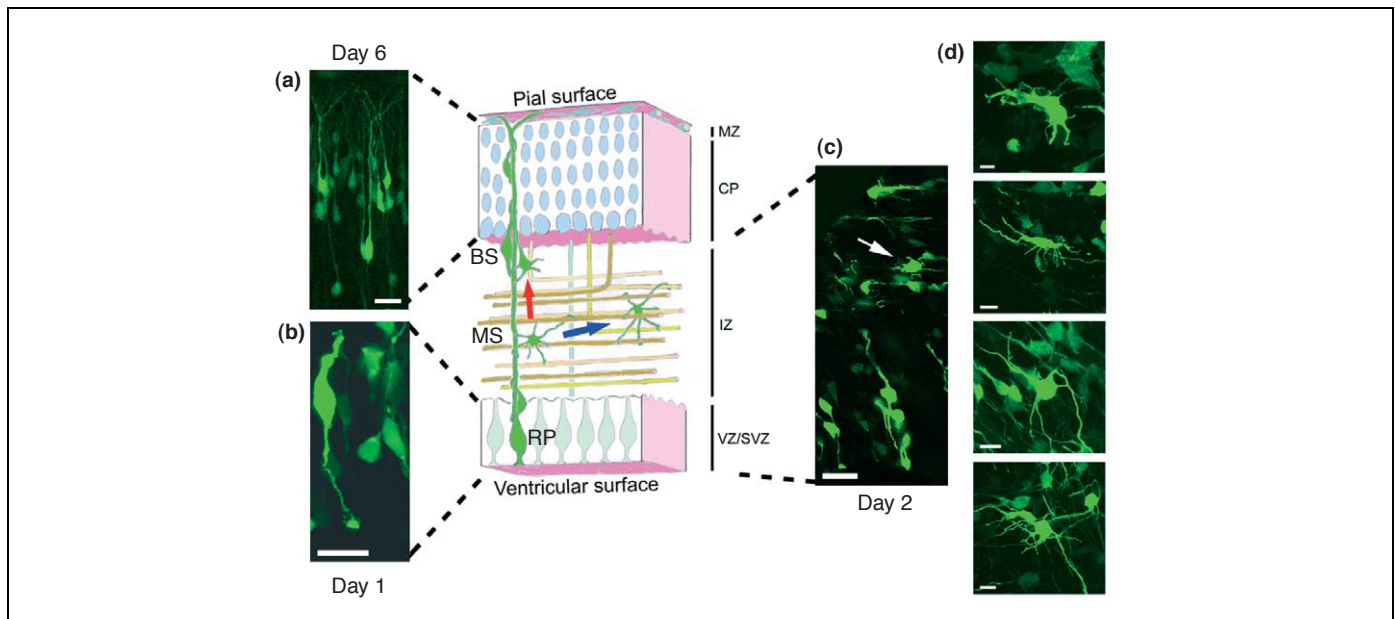


Figure 1. Morphological stages of migrating pyramidal neurons in developing neocortex. Radial progenitors (RP) in the ventricular zone (VZ) (b) give rise to simple bipolar precursor neurons and neocortical neurons (a). Cells transit into a multipolar stage (MS) in the subventricular zone (SVZ) (c,d) and continue to be at this stage when in the intermediate zone (IZ) (d). Within the IZ, VZ and SVZ, cells in the multipolar stage transit into a bipolar stage (BS) of migration and migrate through the IZ and into the cortical plate (CP). Additional abbreviation: MZ, marginal zone. Red and blue arrows indicate transitions of radially and horizontally migrating cells in the multipolar stage. Panels (a–d) show examples of eGFP-transfected cells in the different morphological stages seen one, two and six days following transfection of cells at the VZ surface by *in utero* electroporation. The white arrow in (c) indicates a multipolar cell in the IZ, and the four panels in (d) show higher-magnification examples of such labeled cells in the IZ. Scale bars, 20 μm (a–c) and 10 μm (d). Adapted from Ref. [43].

become multipolar cells in the SVZ. The multipolar stage is transient, and cells eventually adopt a morphology characteristic of bipolar migrating pyramidal neuron precursors in the IZ [32]. Results from *in utero* electroporation and retroviral labeling experiments indicate that the percentage of pyramidal neuron precursors that go through the multipolar stage *in vivo* is large, and might include every migrating pyramidal neuron. Two days following transfection, 88% of migrating cells in the SVZ and IZ are in the multipolar stage [29]. Moreover, because entry into and exit from the multipolar stage can be asynchronous across the population, this is an underestimate of the actual fraction of migrating pyramidal neurons that transit through the multipolar stage at some point during their lifetime. Tangentially migrating interneurons also transit into a multipolar stage near the neocortical SVZ [33], and multipolar stages have been described for migrating cells in brainstem auditory nuclei [34]. Thus, a multipolar stage in migration might be a common intermediate for many neurons, and for neocortical neurons a stage that occurs before directed migration along radial glia.

Migration behavior of neurons in the multipolar stage

Neurons in bipolar stages can migrate by two modes: locomotion and somal translocation [35]. In the locomotion mode, cells migrate at rates of $\sim 10 \mu\text{m h}^{-1}$ towards the pia as the leading process and soma alternately extend and follow [35]. In the somal translocation mode, the soma smoothly translocates at rates of $10\text{--}50 \mu\text{m h}^{-1}$ along a previously extended long leading process reaching towards the pial surface. [35] The migratory behavior of cells in the multipolar stage is distinct from that of cells in the two modes of radial migration. Whereas somal translocation and

locomotion are both characterized by a steady progression in the direction of a leading process, the multipolar stage is characterized by meandering and seemingly random motions [29,30,32]. For some periods, the somas of cells in this stage are immobile. Cellular processes extending from the somas, by contrast, are dynamic and highly motile. During other periods in the multipolar stage, cells migrate towards the ventricular surface, extend a process that touches the surface, and then reverse direction [30,32]. As a consequence of these behaviors, the overall migration rate in any single direction is much slower in the multipolar stage ($1\text{--}6 \mu\text{m h}^{-1}$) than for the two bipolar modes of migration, and the average radial progress made by cells in the multipolar mode is $\sim 2 \mu\text{m h}^{-1}$ [29].

The distribution of cells with bipolar or multipolar morphologies following a single *in utero* electroporation suggests that cells enter the bipolar state in different locations and at different rates. Some cells have morphologies consistent with bipolar stages 1–2 d following transfection throughout the IZ, whereas others remain in the multipolar stage for several days within the IZ [29,36]. It is not currently known whether the apparent variability in time spent by cells in the multipolar stage is determined by cell-intrinsic differences or differential exposure to signaling or cellular interactions.

Molecular requirements for transition out of the multipolar stage

In addition to revealing the detailed morphologies and dynamics of migrating pyramidal neuron precursors, *in utero* electroporation [37,38] has become a powerful tool for probing molecular mechanisms required for migration (Box 1). RNA interference (RNAi)-mediated loss-of-function, dominant-negative and gain-of-function approaches

Box 1. *In utero* RNAi and subcortical band heterotopia

In utero electroporation, initially introduced by Tabata *et al.* [38], is an effective way to transfect patches of cells at the surface of the ventricular zone (VZ) in developing neocortex. Advantages of this method include spatial and temporal control of transfection. The transfection site along the VZ surface can be spatially directed by orienting the electric field of the pulse (Figure 1a) to label dorsal cortex (Figure 1b), lateral cortex (Figure 1c) or interneurons from the ganglionic eminence [77]. Transfection can also be carried out at early or late times of neocortical neurogenesis. Early transfection [embryonic day (E)13 in rat] labels cells of deeper layers (Figure 1b,c) and later transfection (E15) labels more superficial cells (Figure 1d), indicating that the technique can be a method for spatially restricting birth dating.

Both loss-of-function and gain-of-function approaches have been adapted to *in utero* electroporation. High co-transfection rates of ~90% enable effective co-transfection of multiple plasmids to simultaneously label cells with fluorescent markers proteins, to overexpress genes of interest, and to knockdown target genes using RNAi by short hairpin RNA (shRNA) vectors. Loss-of-function using RNAi mediated by transfection of plasmids encoding shRNA has been a particularly powerful approach for studying the role of gene products in radial migration. Effective knockdowns of DCX [43], FILIP [44], LIS1 [36], doublecortin-domain-containing 2 (DCDC2) [11], doublecortin-like kinase (DCLK) [73,78] and the LIS1-interacting protein NDEL1 [79] have been shown by *in utero* electroporation to impair radial migration in developing neocortex. In addition, the approach is amenable to any mammalian species. For example, as shown by Bai *et al.* [43] and Ramos *et al.* [71], the approach has led to generation of a rat model of double-cortex syndrome (Figure 1e,f), which could not be produced using either RNAi or genetic approaches in mice [70,71,73]. The model created by RNAi against DCX displays subcortical band heterotopia and scattered dysplastic neurons [71] (Figure 1f). Future experiments are now needed to determine the degree to which this morphological model in rats can also serve as a model of the functional disruptions that occur in humans with double-cortex syndrome.

Potential problems and limitations of *in utero* RNAi

There are several limitations, precautions and controls that must be considered in any RNAi experiment. First, RNAi is not a genetic deletion, and therefore it must be assumed that residual protein is present even after full RNAi effect. Such 'knockdown' as opposed to 'knockout' might obscure the absolute function of a protein if there is lack of an RNAi phenotype. Alternatively, in some cases knockdown might reveal a function by preventing compensatory mechanisms from being engaged. Second, RNAi is known to have off-target effects [80,81]. In several studies using microarrays and protein profiling, it has been demonstrated that RNAi methods always decrease the levels of transcripts and proteins not directly targeted by the RNAi vector. Therefore, it is crucial to have a rescue control [82] in which expression

of the targeted protein is added back by an exogenous expression system or plasmid. For example, DCX expression nearly completely rescues and eliminates the formation of subcortical band heterotopia and laminar dysplasia created by *in utero* RNAi of DCX [71]. Another crucial control for RNAi is that more than one RNAi sequence targeted against the same target gene must lead to the same phenotype. For example, in LIS1 RNAi experiments [36] this control was used to show specificity for the LIS1 gene *Pafah1b1*. Finally, another significant difference between *in utero* RNAi and knockout approaches, which might be an advantage or a disadvantage, is that *in utero* RNAi targets only some cells at a particular time and place in development. This might result in absences of phenotypes in RNAi experiments; for example, if cell interactions are required then *in utero* RNAi might not reveal a phenotype or function of a gene. Alternatively, such mosaic disruptions as produced by *in utero* RNAi might reveal cell-autonomous functions of a gene that are difficult to resolve in knockout animals.

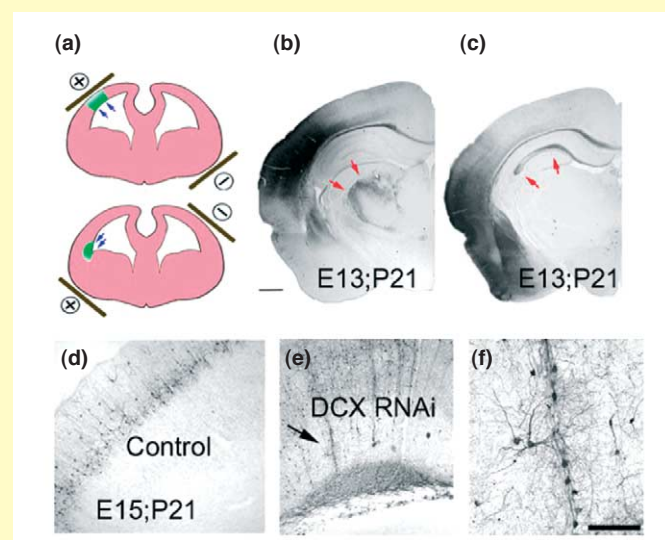


Figure 1. Electroporation and *in utero* RNAi creates an animal model of subcortical band heterotopia. Blue arrows in (a) indicate approximate locations of two patches of electroporation. Red arrows in (b,c) indicate location of axons projecting to the thalamus (b) or hippocampus (c) at postnatal day (P)21 following electroporation at E13 of medial and lateral areas of the developing cortex, respectively. Transfection at E15 labels more superficial cells (d). (e,f) Electroporation of an animal model of subcortical band heterotopia created by RNAi against DCX. The arrow in (e) indicates a minicolumn of neurons shown in a higher magnification in (f). Scale bar in (b), 500 μ m for (b,c); scale bar in (f), 100 μ m for (d,e) and 250 μ m for (f).

have all been successfully applied by co-transfecting combinations of plasmids. Some of the first gene products manipulated in this way have been those previously identified by positional cloning of mutations that disrupt neocortical laminar patterning in humans [39–42]. Manipulating expression of the rodent genes encoding filamin A (*Flna*), lissencephaly 1 (*Pafah1b1*) and doublecortin (*Dcx*) by *in utero* electroporation significantly alters the number of cells in the multipolar stage [36,43,44]. Furthermore, the location of cellular phenotypes suggests that there are at least two substages within the multipolar stage (Figure 2).

Filamin A

Mutations in the human X-linked gene encoding filamin A (*FLNA*) cause periventricular nodular heterotopia, a

malformation type that has been associated with epilepsy [45,46] and more recently with reading disorders [47]. The location of neuron aggregates at the ventricular surface in this malformation indicates that neurons either fail to migrate away from the VZ surface or inappropriately migrate backwards towards the ventricular surface. *In utero* electroporation experiments indicate a cellular role of filamin A for exit from the multipolar stage [44,48] (Figure 2). Excess expression of the rodent filamin A gene *Flna*, induced either by plasmid overexpression or by RNAi of *Filip1* (which encodes a filamin-A-binding protein that targets filamin for degradation) [48], reduces the number of multipolar cells in the SVZ and IZ [44]. Conversely, overexpression of *Filip1* and the resulting decrease in *Flna* expression causes accumulation of cells

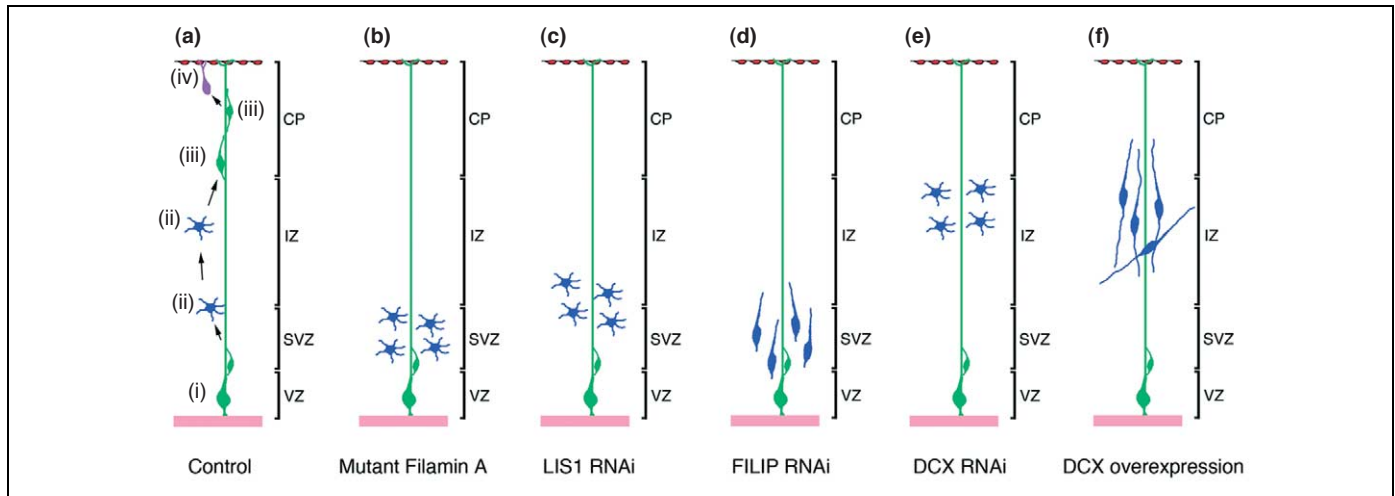


Figure 2. Summary of phenotypes created by manipulating the function of filamin A, LIS1 and DCX in migrating neurons in embryonic rodent neocortex. Normal migration is summarized in (a); numbers represent the radial progenitor (i), multipolar (ii) and bipolar (iii,iv) stages. Filamin A (b) and LIS1 (c) loss-of-function cause cells in the multipolar stage to accumulate in the VZ and SVZ [36,44], and doublecortin loss-of-function (e) causes multipolar-stage cells to accumulate in the IZ [43,71]. Conversely, increasing filamin A activity by RNAi of FILIP [44] accelerates the transition to a bipolar morphology stage in the SVZ (d), and DCX overexpression [71] increases numbers of bipolar cells in the IZ (f).

in the multipolar stage [44]. So too does expression of truncated *Flna*, which has a dominant-negative effect; the transfected cells do not migrate, despite having multipolar processes that continue to be dynamically extended and retracted. By contrast, *Filip1* overexpression reduces dynamic multipolar process extension without interfering with radial migration [44]. Filamin A is thus required for migration within the multipolar stage, and for the transition into bipolar modes of migration.

LIS1

Sporadic germline mutations in one allele of *LIS1* cause inherited, classical or type I lissencephaly, and rarer somatic mosaicism of *LIS1* mutation has been associated with subcortical band heterotopia [49–51]. The cell biology of LIS1 and its homologs in eukaryotes has been extensively studied and reviewed elsewhere [23,52–54]. Briefly, LIS1 interacts with dynein, dynactin [55–57], Ndel1 [58,59] and Nde1 to regulate nuclear movement and cell migration [60]. Mouse knockout models show that allelic variations of the LIS1-encoding gene *Pafah1b1* have effects on developmental processes including cell division, and neuronal migration [61–63] of both pyramidal neurons and interneurons [64]. The pleiotropy of the effects of *Pafah1b1* mutations in mouse neocortical development has made it difficult to distinguish between direct and secondary roles of LIS1 in developing neurons. Using LIS1 RNAi and *in utero* electroporation, Vallee and colleagues have shown that LIS1 directly affects nuclear movements necessary for cell division and migration in the neocortex [36,52]. Moreover, LIS1 RNAi arrests most cells in the multipolar stage of pyramidal neuron development. Using a powerful combination of *in utero* RNAi and live-cell imaging, Tsai *et al.* showed that LIS1 RNAi causes accumulation in the VZ and SVZ of multipolar cells that have reduced capacity for migration. Similar to disruption of filamin A function, LIS1 RNAi increased numbers of highly motile secondary branches on

multipolar cells [36,52]. Overexpression of LIS1-GFP does not have clear effects on the distribution or morphology of migrating multipolar cells [36] (and Wang and LoTurco, unpublished), indicating that increasing LIS1 expression might not facilitate the transition out of the multipolar stage. LIS1, like filamin, is therefore required for exit from the multipolar stage, but not for dynamic extension and retraction of processes.

DCX

Mutations in the X-linked gene *DCX* are the most common genetic cause of subcortical band heterotopia, or double-cortex syndrome, in females, and a major cause of lissencephaly in males [49]. *DCX* encodes doublecortin, which binds to and stabilizes microtubules when not phosphorylated; when DCX is phosphorylated, its binding to microtubules is reduced [49,65–68]. DCX has thus been hypothesized to have a role in the signaling-induced dynamics of microtubules in migrating and differentiating neurons [23,68,69]. *Dcx* knockout mice surprisingly do not show disruptions in neocortical lamination [70], whereas DCX RNAi in rats disrupts radial migration and causes formation of subcortical band heterotopia in a DCX-dependent fashion [71]. One potential reason for this difference might be revealed by double knockouts for *Dcx* and the gene encoding doublecortin-like kinase (*Dclk*), which do show neocortical migration deficits [72,73]. Mice might therefore have a compensatory *Dclk*-mediated mechanism that is missing in humans and rats. Nevertheless, DCX RNAi in rats causes cells to accumulate in the multipolar stage [43] in the IZ. Over-expression of *Dcx*, by contrast, causes cells to adopt a highly bipolar morphology [43,71] (Figure 3). Therefore, similar to *Flna*, *Dcx* loss-of-function and gain-of-function cause respective increases or decreases in the number of cells in the multipolar stage. Two differences between *Flna* and *Dcx* mutant phenotypes are the locations of extra multipolar cells in loss-of function animals and of bipolar cells

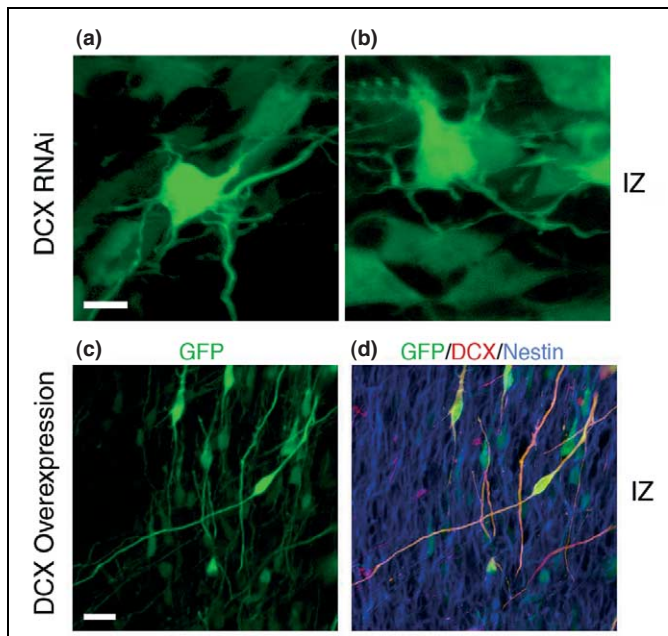


Figure 3. Cells at the multipolar and bipolar stages following disruption of DCX function. Examples of multipolar-stage cells in the IZ four days following DCX RNAi (a,b) and examples of bipolar stage cells in the IZ induced by DCX overexpression (c,d). Scale bars, 10 μm (a,b) and 20 μm (c,d). Adapted from data in Refs [43,47].

following overexpression. Filamin manipulations affect cells much closer to the VZ surface [44], within the VZ and SVZ, whereas manipulations of DCX alter the morphologies of migrating cells in the IZ [43,71]. The shift in the location of phenotypes is consistent with the spatial expression of *Flna* and *Dcx*: filamin A is first expressed while cells are in the VZ and SVZ [48,74], whereas DCX is not expressed significantly until cells exit the cell cycle and the VZ [66].

A multistage model for exit from the multipolar stage and implications for neuronal migration disorders

The cellular phenotypes observed in RNAi experiments, and the different locations of cortical malformations that result from *Flna* and *Dcx* mutations, point to a model in which the multipolar stage has at least two and perhaps more substages susceptible to interruption (Figure 4). The RNAi and cortical malformation phenotypes of periventricular nodular heterotopia and subcortical band heterotopia indicate at least two stages within early migration before the lamination of neurons in the cortical plate. Filamin A disruption by mutation or RNAi arrests migration near the VZ and SVZ, and DCX disruption arrests migration more superficially in the IZ and axon tracts of the white matter. Interruption of the second multipolar phase appears to lead to accumulation of cells in the IZ, which leads to formation of subcortical band heterotopia (Figure 4). LIS1 is likely to have a role at every level of early migration and neuronal polarization; however, because cases of subcortical band heterotopia have been associated with mosaicism in LIS1 mutations [51], it is possible that LIS1 is particularly important in the second multipolar stage. Tests of this sequential model can now be performed using combinations of *in utero* RNAi and overexpression. For example, one prediction of the

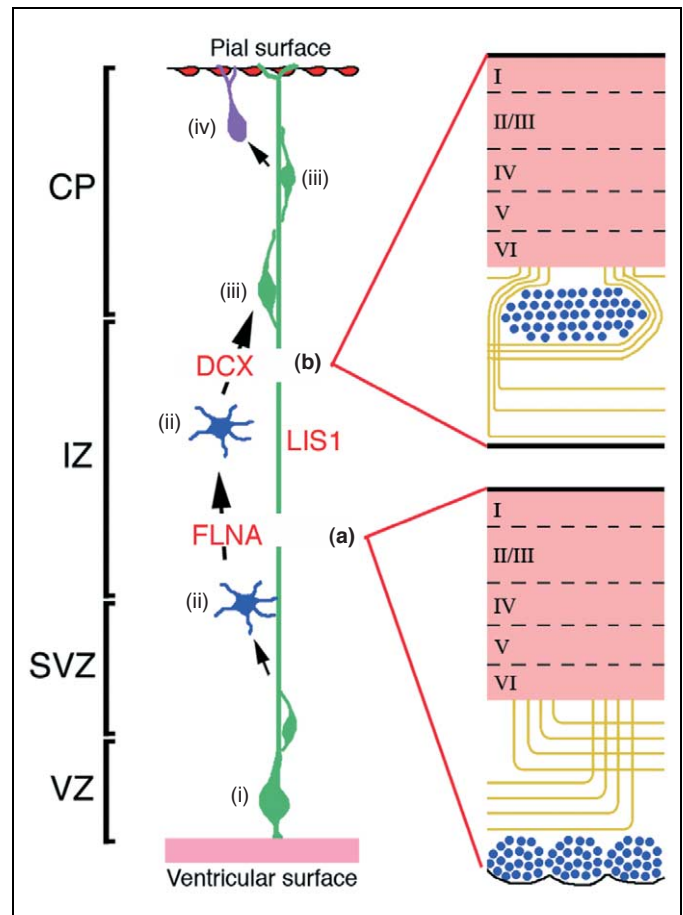


Figure 4. Relationship between phases of the multipolar stage and two neuronal migration disorders. Interruption of early phases of the multipolar stage by mosaic loss of *FLNA* function creates nodular periventricular heterotopia (blue dots) because cells are unable to polarize and make radial progress through the IZ (a). Numbers correspond to different stages of migration, as in Figure 2. Depletion of the VZ through neurogenesis results in heterotopias remaining along the VZ surface. By contrast, loss of DCX or LIS1 function interrupts transition out of a later phase of the multipolar stage by interfering with selection and extension of a primary leading process in the IZ. This causes formation of heterotopia embedded within the forming axon tracts of neocortical white matter (b).

model is that DCX RNAi would block FILIP-induced premature transition into bipolar stages in the SVZ, and dominant-negative forms of filamin A would block induction by DCX of bipolar morphologies in the IZ.

Cellular interactions in the multipolar stage

Although their morphology would suggest that cells in the multipolar stage do not interact strongly with oriented cellular elements such as radial glia or axons, there is evidence for interactions between cells in the multipolar stage. First, live-cell imaging of clonally related cells show that cells in the multipolar stage can remain in contact with their originating radial progenitor [32]. In addition, results from sequential RNAi electroporation experiments show that interruption of cells in the multipolar stage interferes with other cells not directly affected by RNAi [43]. In fact, DCX RNAi interrupts the migration both of pyramidal neurons and of non-pyramidal neurons in the IZ that are not directly affected by DCX RNAi [71].

Evidence for complex cellular interactions is also suggested by the differences between lissencephaly and subcortical band heterotopia. The lissencephalic

neocortex, although severely disrupted, has a clear pattern of four neuronal layers [75] above white matter. By contrast, in human double-cortex syndrome in females heterozygous for *DCX* mutations, and in some hemizygous males [51,76], radial migration is interrupted within the white matter and subcortical band heterotopia develops [71]. Thus, when all cells lack *DCX* function, lamination above the white matter can occur; however, when a subset of cells lacks *DCX*, such as in the rat RNAi model, in human females heterozygous for *DCX* mutant alleles, or in human males with certain rare *DCX* alleles, migrating neurons remain trapped within the white matter. This suggests that there is more disruption to migration of cells when they are developing within the context of normal cells, and also perhaps when all cells have attenuated mutant alleles. Taken together with the multipolar-arrest findings in the RNAi models, this suggests that transition from the multipolar stage of migration, similar to many developmental processes in the neocortex, has elements of competitive interaction. One possibility is that cells compete for limited radial glial scaffolding that would guide them into the cortical plate.

Additional questions

Several questions lead directly from the suggestion that the multipolar stage is a point of vulnerability to disruption. First, do the multipolar stages of different migrating neuron types have different vulnerabilities to interruption? In both *LIS1* RNAi and *DCX* RNAi experiments, a few cells attained bipolar morphologies and migrated. It is not yet clear whether these represent an insensitive population, or simply a population in which RNAi is less effective. If some cells are less sensitive to disruption, then identifying the causal differences might provide insights into prevention of migration disorders. Another question that might have particular relevance to epilepsy is whether interactions between interneurons and pyramidal neurons in the multipolar stage help to establish the appropriate balance of inhibitory and excitatory cells in neocortical circuits. A final, more general question is what function, beyond being a target for disruption, might the multipolar stage have in neuronal migration? One hypothesis is that the multipolar stage is a plastic and dynamic stage during which migration trajectories can be changed by cell-to-cell interactions. As such, the multipolar stage might mark a point of cellular plasticity that can normally enable flexibility in neocortical development.

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