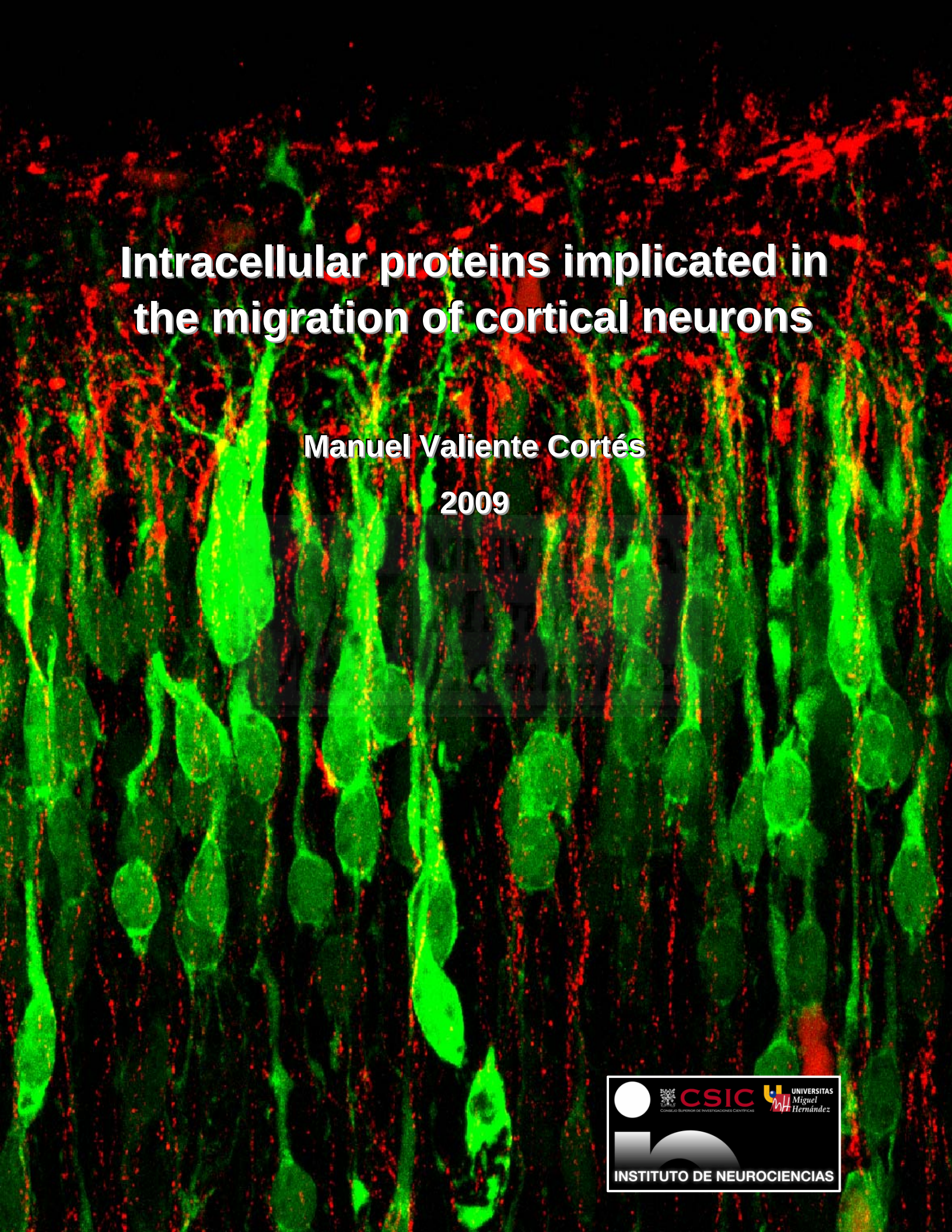

Intracellular proteins implicated in the migration of cortical neurons

Manuel Valiente Cortés

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CSIC
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INSTITUTO DE NEUROCIENCIAS

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Manuel Valiente Cortés

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Universidad Miguel Hernández

Instituto de Neurociencias

Prof. Juan Lerma, Director del Instituto de Neurociencias de Alicante, centro mixto de la Universidad Miguel Hernández y el Consejo Superior de Investigaciones Científicas,

DA SU CONFORMIDAD a la presentación de la Tesis Doctoral presentada por D. Manuel Valiente Cortés titulada "INTRACELLULAR PROTEINS IMPLICATED IN THE MIGRATION OF CORTICAL NEURONS", que ha sido realizada bajo la dirección del Prof. Oscar Marín Parra.

Para que así conste a los efectos oportunos, firma el presente certificado.

En Sant Joan d'Alacant, a 17 de Octubre de 2009.

Fdo: Prof. Juan Lerma

Director

Instituto de Neurociencias (CSIC-UMH), Alicante

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Consejo Superior de Investigaciones Científicas

Universidad Miguel Hernández

Instituto de Neurociencias

D. Oscar Marín Parra, Profesor de Investigación del Consejo Superior de Investigaciones Cientificas,

AUTORIZA la presentación de la Tesis Doctoral titulada “INTRACELLULAR PROTEINS IMPLICATED IN THE MIGRATION OF CORTICAL NEURONS” realizada por D. Manuel Valiente Cortés, bajo su dirección y supervisión en el Instituto de Neurociencias (UMH-CSIC), y que presenta para la obtención del grado de Doctor por la Universidad Miguel Hernandez.

Para que así conste a los efectos oportunos, firma el presente certificado.

En Sant Joan d’Alacant, a 17 de Octubre de 2009.

Fdo: Prof. Oscar Marín Parra

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Introduction

General organization of the neocortex

The cerebral cortex is the most complex structure of the organism due to the extreme diversity and connectivity among its cellular components (Molyneaux et al., 2007; Thomson and Lamy, 2007). This complexity sustains the high order functions of the brain, like speech, though and emotion, and also governs motor functions and interpret sensory information. The cerebral cortex comprises several classes of cortices, which differ in the number of their constituent layers: the six-layered neocortex, also known as isocortex; two different types of tri-laminar cortices, the piriform cortex and the hippocampus, which collective constitute the allocortex; and the four-layered mesocortex, a transition cortex that bridge isocortical and allocortical areas.

Functional organization of neocortical areas

The neocortex is divided into different areas according to their cytoarchitecture, chemoarchitecture, connectivity, differential gene expression and function. In the mouse, the main primary cortical areas are the motor cortex (M1 and M2), located in the rostral aspect of the neocortex, the somatosensory (S1 and S2) and auditory (A1) cortices, found at intermediate regions, and the visual cortex (V1 and V2), placed in the caudal aspect of the neocortex. Each of these areas receives inputs from specific nuclei in the thalamus and, after processing the information, sends projections to other cortical or subcortical areas.

The functional specification of cortical areas begins early during development, around embryonic day (E) 9.5 in the mouse, with the expression of several patterning genes in restricted areas of the cortex. One of these genes is *Fgf8*, which is induced in the neuroepithelium at the anterior aspect of the cortex. Consequently, FGF8 distributes in a high rostral to low caudal gradient throughout the neocortex (Figure 1). FGF signaling functions as an organizing center that induces the activation or repression of several transcription factors in progenitor cells of the cortex (Fukuchi-Shimogori and Grove, 2001), which acquire distinct identities through the expression of a certain set of transcription factors depending on their distance to the source of FGF. For example, progenitor cells located close to the source of FGF8 express high levels of *Pax6* and *Sp8*, while cells placed at caudal positions primarily express *Emx2* and *COUP-TF1* (O'Leary and Sahara, 2008). This genetic parcellation of the cortex progresses during development giving rise to the delineation of distinct cortical areas.

Although the genetic specification of the cortex is the very first determinant of cortical arealization, additional mechanisms are required for its refinement and maintenance. Thalamocortical axons (TCAs) play an important role in these later processes. Axons from different thalamic nuclei enter the cortex around E14.5, wait for a few days in the subplate until neurons at this location reach their final position and then invade layer IV. TCAs appear to influence several aspects of cortical arealization, such as the size of different areas and their final identity (López-Bendito and Molnar, 2003).

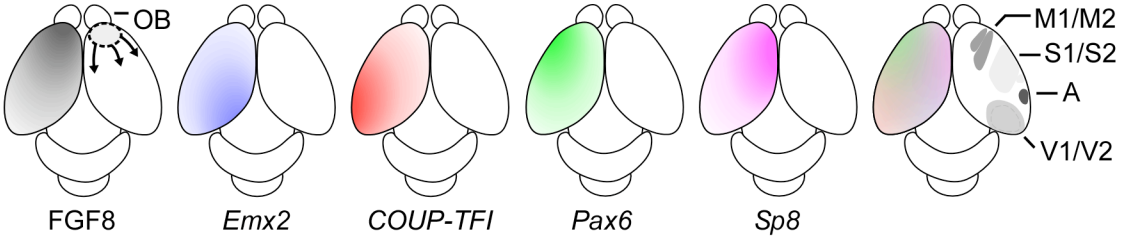

Figure 1. Morphogens, transcription factors and functional areas in the cerebral cortex. Different functional areas in the cerebral cortex are formed as a result of a complex combination of transcription factors differentially expressed (left hemispheres of *Emx2*, *COUP-TFI*, *Pax6* and *Sp8*), induced by diffusible proteins (morphogens) that creates gradients (left hemisphere in FGF8) in the cortical surface. The most important morphogen and the transcription factors induced in the arealization of the cerebral cortex are schematically represented in this figure. Their combination gives as a result the formation of motor (M1/M2), somatosensory (S1/S2), auditory (A) and visual (V1/V2) areas. (OB) olfactory bulb.

Functional organization of neocortical columns

The neocortex is also patterned in the vertical dimension. In the mouse, the neocortex consists of six layers. Each layer contains distinct population of neurons, with specific connectivity (afferent and efferent connections), molecular profiles, morphology and function. Neocortical neurons are organized in modules known as cortical columns, which group neurons throughout the depth of the cortex that share a given receptive field and encode for similar features. The information that arrives to each neocortical column is processed following specific patterns of connections through which different layers communicate with each other generating intracortical circuits (Thomson and Lamy, 2007). Schematically, the main cortical circuit begins with the arrival of sensory information from the thalamus to layer IV. Layer IV neurons send their output to supragranular layers II-III, which after processing send the signal to infragranular layers V and VI. These layers represent the output station of the cortex, sending their projections to subcortical and subcerebral targets. Many feedback loops exist in this circuitry. For example, layer VI neurons also send information back to layers III and V.

Each column of the neocortex contains two main classes of projections neurons (Molyneaux et al., 2007), which on the basis of their connectivity are known as commissural and subcortical:

- **Commissural** neurons localized to layers II-III, V and VI. These cells project to cortical areas in the contralateral cortex by sending their axons through the corpus callosum and, to a minor extent, the anterior commissure.

- **Subcortical** neurons are mainly located in layers V and VI and send their axons out of the cortex (basal ganglia, thalamus, brainstem and spinal cord). The neurons with the largest soma in the neocortex, found in deep layer V, are included in this group. Layer V neurons can be classified in different groups according to their specific subcerebral projection: corticostriatal (motor, associative and limbic neurons targeting the basal ganglia), corticotectal (visual neurons projecting to superior colliculus and collateral rostral pons), corticopontine (motor neurons mainly targeting the rostral aspect of the brainstem, the pons), and corticospinal (motor neurons projecting to the spinal cord, with collateral axons to the striatum, red nucleus, caudal pons and the caudal aspect of the brainstem, known as the medulla oblongata).

Inhibitory interneurons are found in every layer of the cortex and therefore also contribute to the organization of functional circuitry in the neocortex. They primarily modulate excitatory flow in the layer in which they are positioned (Thomson and Lamy, 2007), although they may also contribute to regulate the output of projection neurons or other interneurons located in different layers.

Excitatory and inhibitory neurons employ different neurotransmitters to exert their function. In contrast to projection neurons (also known as pyramidal cells) which produce glutamate as their main neurotransmitter, interneurons use γ-aminobutyric acid, GABA. The function of these neurotransmitters depends on many different factors, most importantly the functional state of the receiving neurons. In general, however, glutamate depolarizes neurons and therefore is considered an excitatory neurotransmitter. GABA, on the other hand, tends to hyperpolarize neurons in the adult cortex, and so is referred to as an inhibitory neurotransmitter. The balance between excitation and inhibition is crucial for proper cortical functioning, and disturbances of this dynamic balance are thought to cause severe neurological syndromes. A defective cortical developmental program, among other causes, may cause such unbalance in cortical function.

A brief account of the development of the cerebral cortex

Regionalization of the neural tube

During neurulation, the neural plate gives rise to the neural tube. The neural tube then differentiates into the spinal cord and the brain, which grows from a dilatation within the rostral aspect of the tube. This primitive dilatation is further subdivided into three vesicles: the prosencephalon or forebrain, the mesencephalon or midbrain, and the rombencephalon or hindbrain (Figure 2). Further differentiation of these vesicles gives rise to the telencephalon and diencephalon from the forebrain, while the hindbrain originates the metencephalon and the myelencephalon. These five subdivisions undergo additional morphogenetic processes to give rise to each of the individual regions that can be distinguished in the adult brain. So, the telencephalon generates the cerebral hemispheres and the basal ganglia, the diencephalon give rise to the thalamus, hypothalamus and optic vesicles, the metencephalon produces the pons and the cerebellum, and the myelencephalon differentiates into the medulla oblongata.

Acquisition of pallial and subpallial identities

Patterning of the telencephalon begins with a primary division into pallial and subpallial domains, which largely delineates the extent of glutamatergic and GABAergic phenotypes, respectively, in the mature telencephalon (Hebert and Fishell, 2008). Pallial and subpallial identities result from the differential expression of specific sets of genes at different locations within the telencephalon. These transcription factors stabilize their expression through mutually repressive interactions. For example, the zinc-finger transcription factor *Gli3* is broadly expressed in the telencephalic anlagen at E8.5. Following neural tube closure, *Gli3* expression becomes progressively restricted to the pallium. In contrast, the gene encoding for the morphogen Shh has the opposite expression pattern, being mostly confined to the ventral midline. *Shh* represses *Gli3* expression and thus blocks the acquisition of a pallial fate in subpallial territories. Other genes are responsible for the specification of subpallial identity. For instance, analysis of *Foxg1*

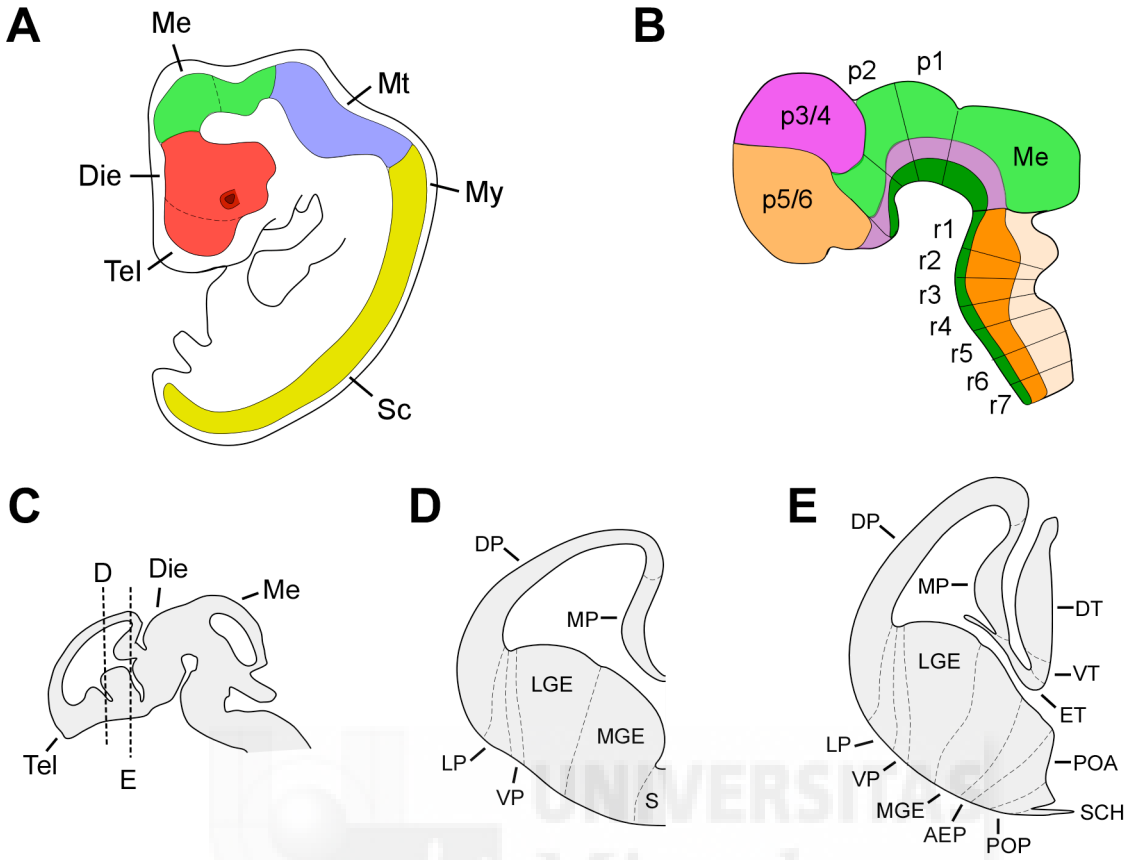

Figure 2. Development of the telencephalon. (A) The brain is subdivided in three large regions: forebrain (red), midbrain (green) and hindbrain (blue and part of yellow). The growth and development of flexures (cephalic, pontine and cervical) create further subdivisions to originate the telencephalon and diencephalon, in the case of the forebrain, and metencephalon and myelencephalon in the case of the hindbrain and mesencephalon from the midbrain. (B) The prosomeric model parcelate the forebrain in different longitudinal and transversal domains designated as p1 to p6. (C) During next stages of development new structures appear. (D, E) Coronal sections at levels depicted in (C). (Tel) telencephalon, (Die) diecenphalon, (Me) mesencephalon, (Mt) metencephalon, (My) myencephalon, (Sc) spinal cord, (p1-6) prosomeres 1-6, (r1-r7) rombomeres 1-7, (DP) dorsal pallium, (MP) medial pallium, (LP) lateral pallium, (VP) ventral pallium, (MGE) medial ganglionic eminence, (LGE) lateral ganglionic eminence, (S) septum, (AEP) enterior entopeduncular area, (POA) anterior preoptic area, (POP) posterior preoptic area, (SCH) suprachiasmatic nucleus, (ET)eminentia thalami, (VT) ventral thalamus, (DT) dorsal thalamus.

mutants suggests that subpallial patterning requires this gene. *Foxg1*;*Gli3* double mutants also have an important phenotype, which suggests that *Foxg1* is directly necessary for subpallial identity, and not just to inhibit *Gli3* from being expressed in this region (Hanashima et al., 2007; Martynoga et al., 2005; Rash and Grove, 2007). *Foxg1* expression is induced by *Fgf8*, which is also thereby implicated in patterning the subpallium. Genetic evidence strongly suggests that FGF8 is responsible for the specification of genes with restricted expression in the subpallium, such as *Lhx6*, *Lhx7*, *Nkx2.1*, and *Gsh2*, which are missing in the absence of the FGF8 receptors *Fgfr1* and *Fgfr2* or in *Fgf8* hypomorphic mouse mutants (Gutin et al., 2006; Storm et al., 2006). FGF signaling is also linked to *Shh* and *Gli3*. *Fgf8* is not expressed in *Shh* mutants, suggesting that it is downstream of this gene (Rash and Grove, 2007). However, *Fgf8* expression is present in *Shh*;*Gli3* double mutants, which indicates that *Fgf8* lies downstream of *Shh* but is also sensitive to repression of *Gli3* (Gutin et al., 2006). Consequently, *Fgf8* effect is fundamental for both rostro-caudal specification and dorso-ventral patterning. Additional morphogenetic proteins control the

patterning of the pallium. For example, Wnts and BMPs are required for the specification of dorsal midline structures, such as the choroid plexus and the hem (Stenman et al., 2003).

The action of the different morphogenetic proteins present in the telencephalon instructs pallial and subpallial progenitors to express different sets of transcription factors. The pallium express, for example, *Pax6*, while the ventral aspect of the subpallium induces *Nkx2-1*. *Gsh2* occupies the territory between *Pax6* and *Nkx2-1* expression, which effectively constitute the boundary between the pallium and the subpallium (Corbin et al., 2003; Inoue et al., 2000). Additional transcription factors are required for the specification of pallial identities, among which Lhx2, Ngn1 and Ngn2 are the best studied so far (Stenman et al., 2003).

As a result of this complex, interconnected gene networks, the pallial and subpallial components of the telencephalon give rise to different structures. In the pallium, different types of cortical regions are specified, including the piriform cortex, the neocortex and the hippocampus. In addition, non-cortical, pallial structures are found in the amygdala. In the subpallium, on the other hand, patterning mechanisms generate several transient structures: the lateral, medial and caudal ganglionic eminences (LGE, MGE and CGE, respectively), as well as the preoptic area (POA) in the telencephalic stalk domain. These structures will ultimately generate different nuclei in the base of the telencephalon, including the basal ganglia (striatum and globus pallidus) and parts of the amygdala (Figure 2).

Two different migratory behaviors are required to build up the cortex

Migration is a fundamental process in the developing nervous system

The growth of the nervous system increases the distance between proliferative areas and the final residence of newborn neurons. This growth thus requires mechanisms that guarantee the ability of neuroblasts to populate positions that are usually far away from their origin (Hatten, 1999). As in other organs, the nervous system have acquired the ability to grow both in size and complexity by using migration as a strategy to position cell types from different origins in the same final destination. So, migration of neuronal precursors is required for the development of both the central nervous system (CNS) and the peripheral nervous system. Neuronal migration is, along with axon guidance, one of the mechanisms that settle the basis to establish neural circuits in the adult brain.

There are two main strategies to migrate in the cerebral cortex attending to how cells move in relation to proliferative zones. When cells migrate orthogonal to proliferative cells, migration is designed radial migration. In contrast, the movement of cells parallel to progenitors areas is known as tangential migration (Marín and Rubenstein, 2003) (Figure 3). One of the main differences between these two types of migratory behaviors is the type of structures they generate. Radial migration is typically used to expand neuronal clones at a certain distance from their origin but maintaining a relative topological relationship with the proliferative area. For instance, projection neurons born in the somatosensory cortex will end up positioned within this specific area. By contrast, tangential migration promotes cell diversity in different brain structures, because this migratory behavior does not impose restrictions to the relative dispersion of newborn neurons from their place of origin. Consequently, tangential migration allows for the integration of cells from very different origins in the same brain region.

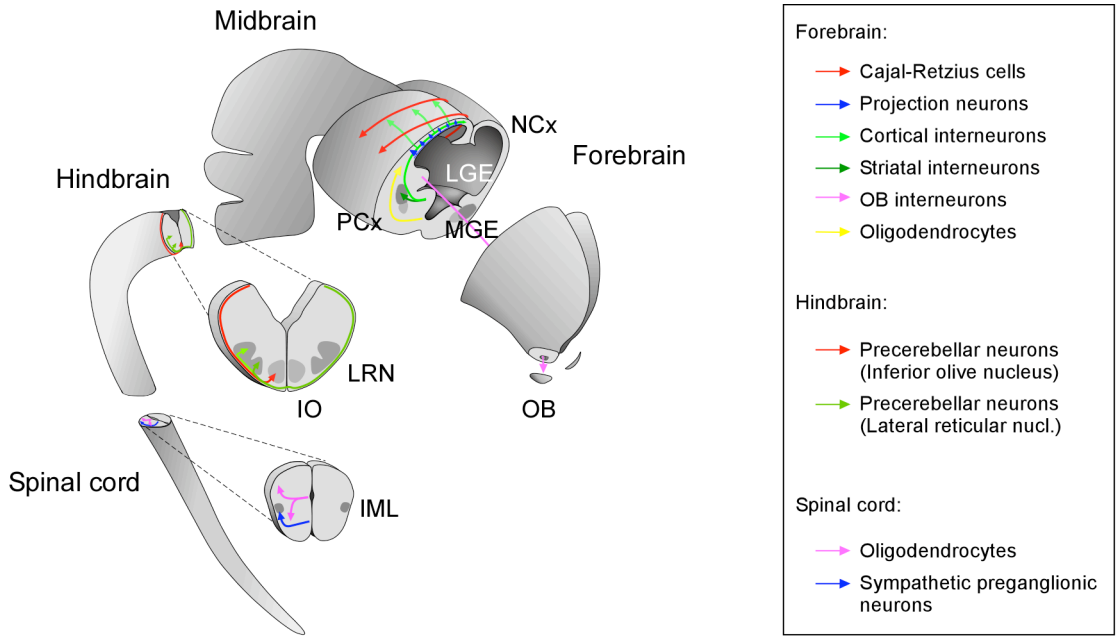

Figure 3. Representative migrations in the developing CNS. Several migrations coexist during embryonic development at different areas of the CNS. This schema summarizes some of the migrations that occurs during the second week of the embryonic period in the mouse. Tangential and radial migrations are the main strategies used by neurons to reach final destinations. Thay are not exclusive, since different neuronal precursors are able to switch from one pattern to another during different phases of migration (e.g. cortical interneurons in the forebrain and precerebellar neurons in the hindbrain). (OB) olfactory bulb, (MGE) medial ganglionic eminence, (LGE) lateral ganglionic eminence, (NCx) neocortex, (LRN) lateral reticular nucleus, (IO) inferior olive nucleus, (IML) intermediolateral region of the spinal cord.

Neuronal migration is not restricted to the embryonic brain. It is also used in restricted regions of the postnatal brain to allow the positioning of newborn neurons generated in active places of neurogenesis, such as the dentate gyrus and the telencephalic subventricular zone which gives rise to olfactory bulb interneurons (Ghashghaei et al., 2007; Lois and Alvarez-Buylla, 1994).

Overview of cell migration in the developing cortex

The assembly of neural circuits in the cerebral cortex involves the migration of many different cell types, most importantly projection neurons and interneurons, but also supporting cells such as oligodendrocytes and transient populations that are crucial for the development of the cortex such as Cajal-Retzius cells. Projection neurons use radial migration to reach their proper layer in the cortex, while most interneurons and many oligodendrocytes that were born in the subpallium migrate tangentially to their final destination. Cajal-Retzius cells arise from a few sites within the border of the pallium, and also use tangential migration to disperse throughout the entire cortical surface. Since all these migrations take place simultaneously, the different origins and guidance of the cell populations that populate the cerebral cortex create a relatively complex scene in which different classes of cells move at the same time and even share very similar routes of migration (e.g. Cajal-Retzius cells and cortical interneurons migrate in opposite directions through the surface of the cortex; see also Figures 3 and 4).

Although we have described radial and tangential migrations as two different modes of migration related to specific neuronal subtypes, this is not the case for some neuronal populations. Many neurons in the cerebral cortex and in other brain regions may switch from one mode of migration to the other at specific

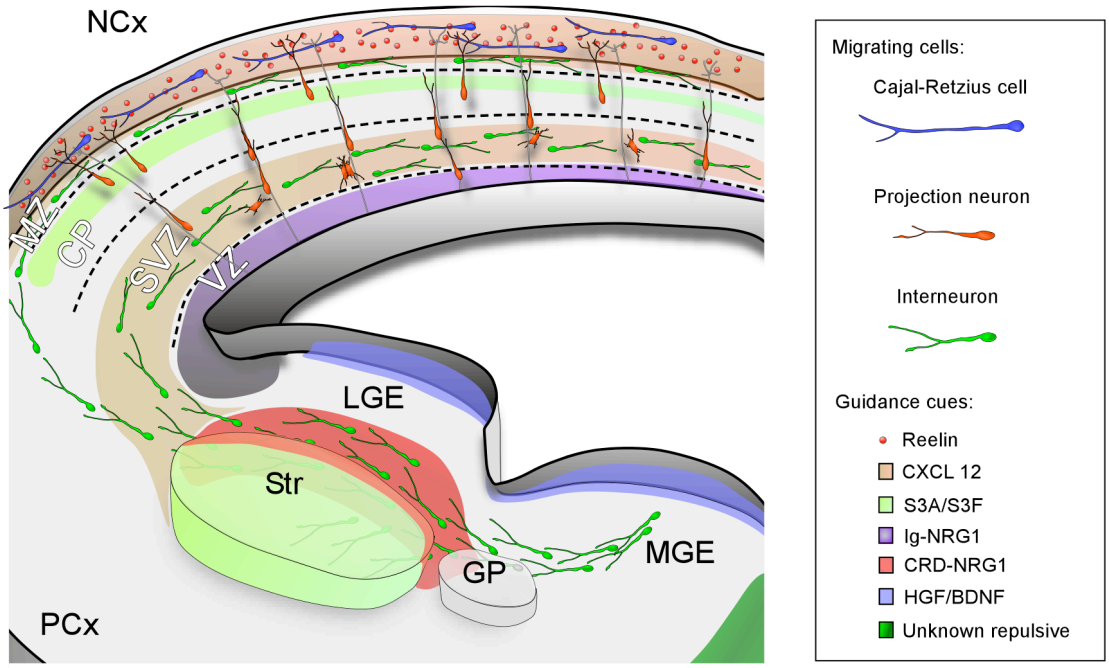

Figure 4. Guidance cues and neuronal migration in the telencephalon. The schema shows a coronal slice of the telencephalon with the main cortical migrations and their guidance cues. Migrating interneurons originated at the MGE migrate tangentially through the subpallium. Some of these interneurons enter the striatum (striatal interneurons), while others continue toward the cortex (cortical interneurons) sorting out through S3A/S3F, NRP1/2 signaling after passing through a corridor that expresses CRD-NRG1. Interneurons arrive to the cortex guided by motogenic factors (HGF/BDNF) and attractive cues (Ig-NRG1). Once in the cortex, migration occurs in two main streams (MZ and SVZ). Interneurons disperse through the cortex guided by CXCL12 gradient at those locations. Cajal-Retzius cells also use this cue through the MZ but migrate ventro-laterally in opposite direction to interneurons. These cells synthesized Reelin, which together with S3A/S3F guide the migration of projection neurons. The axon of migrating projection neurons is not included in the schema for simplicity. (MGE) medial ganglionic eminence, (LGE) lateral ganglionic eminence, (PCx) piriform cortex, (NCx) neocortex, (Str) striatum, (GP) globus pallidum, (MZ) marginal zone, (CP) cortical plate, (IZ) intermediate zone, (SVZ/VZ) subventricular/ ventricular zone.

steps within their migratory pathway. This is the case, for example, of cortical interneurons, which reach the cortex through a long tangential migration but incorporate into specific cortical layers by radial migration (Ayala et al., 2007; Nadarajah and Parnavelas, 2002).

The following sections review in detail the migratory behaviors of the two main neuronal components of the cerebral cortex, projection neurons and interneurons. Although both cell types end up sharing the same final destination, their developmental programs are quite distinct because they differ in their origin, migratory pathways, molecular regulation of migration and morphological attributes.

Migration of projection neurons

Radial glia cells are the progenitors of projection neurons

Projection neurons are generated throughout the ventricular zone (VZ) of the pallium and migrate radially to reach their specific position in cortical layers. The earliest cortical neurons form a transient structure known as the preplate, around E10 in the mouse. This primordial layer consists of Cajal-Retzius cells and

the first cohort of pyramidal neurons, which will eventually populate the subplate (SP). Cajal-Retzius cells, which play important roles during neuronal migration, arise from discrete pallial sources and colonize the entire surface of the cortex through tangential migration (Bielle et al., 2005; Takiguchi-Hayashi et al., 2004; Yoshida et al., 2006). The next cohort of pyramidal cells forms the cortical plate (CP) by intercalating in the preplate and splitting this primitive structure in a superficial layer, the marginal zone (MZ or layer I), and a deep layer, the SP. The development of the neocortex progresses with new waves of neurons that migrate towards the CP (Gupta et al., 2002). Birth dating studies have shown that neurons within the CP (future layers II-VI) are generated in an "inside-out" sequence. So, neurons generated earlier reside in deeper layers, whereas later-born neurons migrate past existing layers to form superficial layers (Angevine and Sidman, 1961; Rakic, 1974).

The progenitors of cortical projection neurons are known as radial glial cells (RGCs). This highly specialized cell is fundamental in cerebral cortex development, where it carries out two different functions: neurogenesis and guidance of newborn neurons, in particular at mid- and late stages of corticogenesis (Noctor et al., 2001; Tamamaki et al., 2001). Once neurogenesis is completed, most RGCs differentiate into astrocytes and perform additional functions in the adult brain (Kriegstein and Alvarez-Buylla, 2009).

The proliferation of the RGCs follows different patterns during development. Early in development, each RGC undergoes symmetric cell divisions to generate additional RGCs, thereby expanding the primary progenitor pool (Kriegstein and Alvarez-Buylla, 2009; Li et al., 2002). When neurogenesis begins, RGCs start generating neurons through asymmetric cell divisions (Kriegstein and Alvarez-Buylla, 2009; Noctor et al., 2004). In both scenarios, the morphology of RGCs remains relatively constant, with the soma near the ventricular lining, a short apical process contacting the ventricle, and a long basal process that expands through the width of the entire cerebral cortex until the pial basement membrane, where it typically branch profusely and generates several protrusions that constitute the endfeet of RGCs. As in other pseudostratified neuroepithelia, the soma of RGCs undergo interkinetic movements before dividing close to the ventricular surface (Miyata, 2008).

Glial-independent and glial-guided migration of projection neurons

Although the interaction between migrating neurons and the basal process of RGCs has been typically considered an axiom of radial migration, recent studies suggest that not all radially migrating neurons use the basal process of the RGCs to migrate. Specifically, it has been described that during early phases of corticogenesis (E10-E13), newborn projection neurons migrate to their final position without interacting with RGC fibers. As the cortex thickness increases (E12-E16), the migration of projection neurons becomes dependent on the radial glia scaffold (Kriegstein and Noctor, 2004; Nadarajah et al., 2003; Nadarajah et al., 2001; Nadarajah and Parnavelas, 2002).

The mechanisms supporting the migration of early born cortical projection neurons are different from those used during glial-guided migration (Figure 5). In the first case, daughter cells seem to inherit the basal process from the parental RGC. Thus, newborn neurons are already attached to the pial basal membrane, and therefore do not need to elaborate further leading processes to complete their migration (Miyata et al., 2001). Detachment of the apical anchorage is simply followed by somal translocation towards the surface of the cortex. Interestingly, the forces involved in the upward movement of the soma

seem to originate from the specific spring-like conformation of the basal process, which moves the nucleus by twisting after detachment from the VZ (Miyata and Ogawa, 2007).

As the cortex grows, the distance between the VZ and the surface of the brain increases, and the mechanism used by early born cortical projection neurons to reach the surface of the cortex is no longer useful to promote the movement of newborn neurons. At this point, projection neurons extend their leading process guided by their interaction with the basal process of RGCs, and translocate their nucleus in a saltatory pattern that is repeated for each migratory cycle (Nadarajah et al., 2001; Noctor et al., 2004). This mechanism is known as locomotion. Once cells reach their final position in the CP, they detach from the radial fiber scaffold and begin their differentiation program. The different behavior of cells undergoing somal translocation and locomotion suggests that specific molecular programs are involved in each case. Indeed, it has been found that some molecules are particularly important for glial-guided migration but in turn are largely dispensable during somal translocation (Hammond et al., 2004; Hatanaka et al., 2004).

The migration of projection neurons during mid-stages of corticogenesis occurs through a series of consecutive phases (Kriegstein and Noctor, 2004) (Figure 5). Newborn neurons adopt a bipolar morphology and migrate up to the border between the subventricular zone (SVZ) and intermediate zone (IZ), where they stop. At this specific location, where growing TCAs and corticofugal axons are also positioned, projection neurons change their morphology and become multipolar, with several processes mostly oriented tangentially (Tabata and Nakajima, 2003). This phase, known as the multipolar state, may last up to 24 hours. During this time some multipolar cells divide symmetrically to generate two daughter cells (Kowalczyk et al., 2009; Noctor et al., 2004; Tabata et al., 2009). Eventually cells repolarize, generate a primordial axon directed toward the VZ and a leading process oriented toward the pia (Noctor et al., 2004). These bipolar cells restart migration in close apposition to basal process of a RGC, and continue moving along radial glial fibers until they reach the CP. The transition of cells from the multipolar stage to the bipolar, glial-guided phase of migration, seems to be under complex molecular regulation, because defects in many genes involved in radial migration precisely impair this process (LoTurco and Bai, 2006).

Mechanisms controlling glial-guided migration of projection neurons

The movement of projection neurons during glial-guided migration depends on three primary factors: (i) the integrity of the RGC scaffold, (ii) the interaction of migration neurons with radial glial fibers, and (iii) other molecules responsible for the migration of projection neurons.

Integrity of radial glia scaffold

Glial-guided migration depends on the integrity of the fiber scaffold organized by RGCs. The basal process of RGCs is attached to the basal membrane organized below the pial surface. Extracellular matrix (ECM) molecules including type IV collagen and nidogen (Halfter et al., 2002), members of the laminin family proteins (Haubst et al., 2006; Radakovits et al., 2009) and heparan sulphate proteoglycans, such as perlecan (Costell et al., 1999), are the main components of the basal membrane. Maintenance of this structure depends on both normal production of the ECM components (Beggs et al., 2003) and the presence of certain receptors in the basal endfeet of the radial glia processes (Graus-Porta et al., 2001; Li et al., 2008b; Moers et al., 2008). Loss of function for many of these proteins leads to disruption of the

basal membrane and, subsequently RGC detachment, which finally disrupts radial migration (Kerjan and Gleeson, 2007). Some RGC intracellular proteins are also important for the formation and/or maintenance of RGC basal processes. For instance, the adenomatosis polyposis coli gene, *APC*, has been shown to be essential to determine the conformation of RGCs. Loss of APC produces defects of proliferation, process extension, and failure to respond to extracellular cues important for the maturation of RGCs (Yokota et al., 2009). One of these cues is Neuregulin 1 (Nrg1), which contributes to the maintenance of the basal process through activation of ErbB2 receptor (Schmid et al., 2003). Reelin is another cortical protein necessary for process extension in at least a subpopulation of RGCs (Hartfuss et al., 2003).

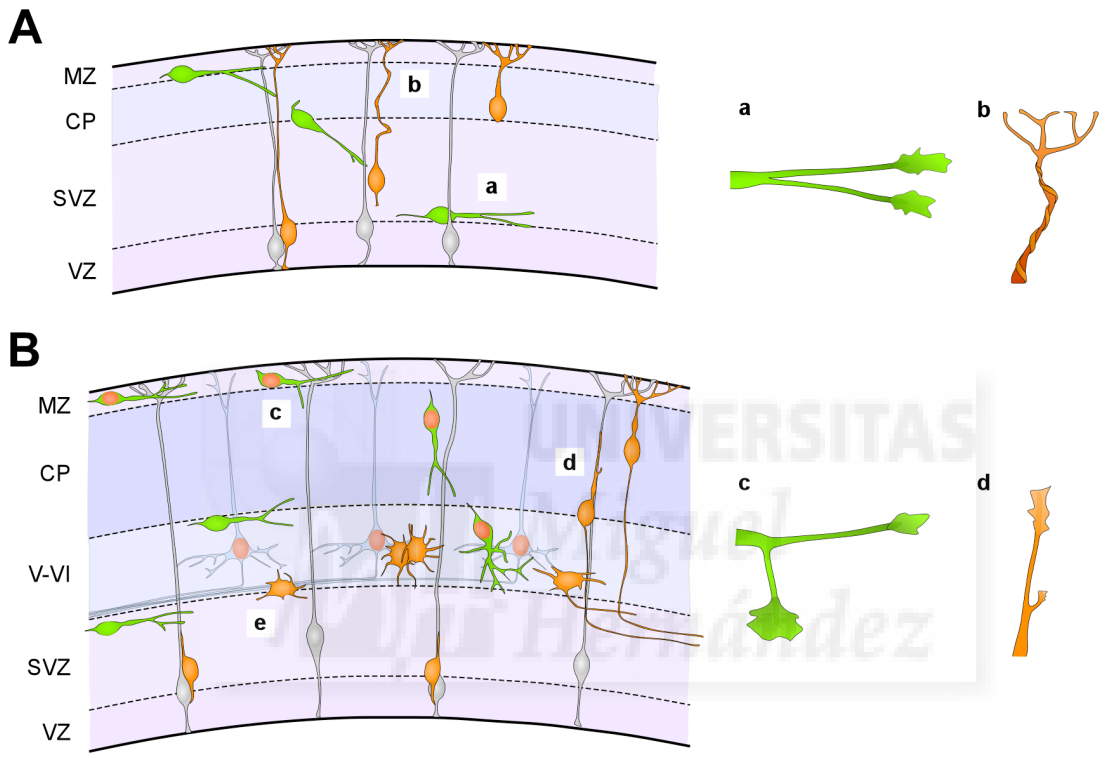

Figure 5. Leading process dynamics in cortical migrating neurons. (A) Early generated projection neurons (in orange) migrate independent of the radial glia apical process by translocating the soma upwards using a spring-like mechanism (b). When the cerebral cortex grows, the distance from the ventricular zone (VZ) to the pia also increases, and projection neurons (in orange) use locomotion to find the cortical plate (CP). (B) Cells that undergo locomotion are intimately attached to the radial glia apical process (d). Projection neurons switch to a multipolar state at the middle of their pathway developing several processes all round the cell (e). At this specific stage, the axon is also being generated toward the VZ. Cortical interneurons (in green) migrate tangentially through the cortex in defined streams (a) without invading the cortical plate. The leading process of this migratory cell develops several branches used to modify their trajectory. At a certain point, these cells generate a process oriented to the CP and radially invade a specific layer to start to differentiate (c). Both projection neurons and interneurons born at the same time end their migration at the same layers (red colored nuclei represent cells born at the same time). (MZ) marginal zone, (SVZ) subventricular zone, (V-VI) layers V-VI.

Adhesion between radial glia and migrating projection neurons

The interaction between migrating neurons and RGCs is mediated by the adhesive properties conferred by certain proteins that allow cells to recognize each other and remain in close proximity while migrating. Additional mechanisms are required to regulate the detachment of neurons from the basal process of RGCs at the end of their migration in the CP. There are two main families of proteins that have been

directly implicated in mediating the adhesive properties of migrating neurons, integrin receptors and connexins.

Several integrins have been reported to mediate the interaction between projection neurons and RGCs. For example, α3β1 integrin receptors are expressed in migrating neurons, and blocking antibodies against these receptors decrease the migratory speed of projection neurons in vitro. Moreover, α3 mutants show disorganization in the layering of the cerebral cortex (Anton et al., 1999; Schmid et al., 2004). However, since integrin receptors are also involved in the development of the basal membrane that anchors the endfeet of RGCs, it has been very difficult to dissociate the cell autonomous role of integrins in migrating neurons from a secondary defect caused by impaired attachment of RGCs to the pial surface. Indeed, recent studies argue against a direct implication of integrins in the migration of pyramidal cells. Analysis of neuron-specific conditional mutants for the β1 subunit of the integrin receptor, which dimerizes with multiple α subunits, revealed minor defects in cortical layer formation (Belvindrah et al., 2007; Radakovits et al., 2009). By contrast, genetic deletion of the ECM protein laminin-γ1 specifically from neurons or from the basal lamina impairs neuronal migration in different ways. Absence of laminin-γ1 from the basal lamina causes excessive migration and abnormal invasion of the MZ (Halfter et al., 2002), while laminin removal exclusively from migrating projection neurons leads to the accumulation of migrating neurons in lower layers, presumably caused by blocking of integrin downstream pathways (Chen et al., 2009). In sum, although the role of integrins in cortical development is multifaceted and complex, it is likely that they mediate to some extent the interaction between migrating pyramidal cells and the basal processes of RGCs.

Connexins are the main component of Gap junctions, which are structures that mediate a specific type of cell-cell communication (Wei et al., 2004). Typically, Gap junctions form channels when connexins from two adjacent cells interact. In other circumstances, connexins form hemichannels that are used to release small molecules to the extracellular space. In addition, recent studies have shown that the adhesive properties of Gap junction may contribute to associate different cell types. In particular, Connexin 43 (Cx43) and Connexin 26 (Cx26) seem to participate in mediating neuron-glia interactions. Short hairpin-mediated knockdown of *Cx43* or *Cx26* severely disrupts radial migration in a cell-autonomous manner (Elias et al., 2007). In the absence of Cx43 or Cx26 function radial glia is not morphologically affected, but projection neurons accumulate in lower layers of the developing cortex and fail to reach CP. These cells also display important morphological modifications, such as increased numbers of leading process branches. Interestingly, the role of connexins in radial migration does not depend on their ability to form channels between cells. Regulation of adhesion by connexins seems to be largely dependent of motifs found in their C-terminus (Cina et al., 2009; Elias et al., 2007; Stout et al., 2004).

Additional signaling pathways have been implicated in mediating the adhesion of migrating neurons to RGCs. For example, projection neurons deficient in p35, one of the regulatory units of Cdk5, abnormally detach from radial glial fibers during their migration towards the CP and also display increased leading process branching (Gupta et al., 2003). Finally, molecules expressed by RGCs also seem to be implicated in this process. For instance, RGCs express SPARC (secreted protein acidic and rich in cysteine)-like 1 specifically in the portion of their basal process that transit through the upper part of the CP, where neurons terminate their migration, and several lines of evidence suggest that this protein is an anti-adhesive molecule that is involved in this process (Gongidi et al., 2004).

Guidance molecules for projection neurons

Besides the proteins described above that primarily regulate the adhesive properties of migrating neurons, very few molecules have been reported to guide the radial migration of projection neurons in vivo. The best characterized is Reelin, whose function in the brain is still not completely understood despite being identified almost fifteen years ago. Reelin is a large glycoprotein secreted by Cajal-Retzius cells that binds to two lipoprotein receptors expressed by migrating projection neurons, VLDLR and ApoER2 (Trommsdorff et al., 1999) (Figure 4). Reelin binding to its receptors recruits the intracellular adaptor protein Dab1, which then becomes phosphorylated by the Src family kinases (SFKs) Src and Fyn (Arnaud et al., 2003; Howell et al., 1997; Howell et al., 2000) . Genetic evidence demonstrates that this signaling pathway is important for the maintenance of the "inside-out" pattern of migration, because identical cell positioning defects have been described in mouse deficient in Reelin (*reeler* mice), Dab1 (*Dab1*$^{-/-}$, *scramble* or *yotari* mice), or both VLDLR and ApoER2 receptors (*Vldlr*$^{-/-}$;*ApoER2*$^{-/-}$ mice). In all these mice there is a rough inversion of cortical layers, with early-generated neurons located in superficial positions and late-born neurons occupying deep layers. Moreover, one of the early events in cortical migration, the preplate splitting, fails in all these mutants. In the absence of Reelin, Dab1 or both VLDLR and ApoER2 the first cohort of pyramidal cells does not reach the surface of the cortex, and instead accumulate under subplate neurons.

Increasing experimental evidence indicates that Reelin functions near the MZ as a signal that contributes to the detachment of projection neurons from RGCs. Expression of a mutant form of *Dab1* in which specific tyrosines (Y220 or Y232) have been mutated prevents the normal migration of pyramidal cells (Sanada et al., 2004). It has been suggested that these sites in Dab1 could mediate the detachment of pyramidal cells from RGCs by repressing the expression of α3 integrin receptors, which are though to be important for neuron-glia interactions (Anton et al., 1999).

Semaphorin 3A (Sema3A) has long been implicated in the orientation of dendrites and axons of newborn pyramidal cells (Polleux et al., 1998; Polleux et al., 2000). More recently, the role of Sema3A in the migration of projection neurons has been addressed. Sema3A seems to function as a chemoattractant for these neurons through the activation of the Neuropilin-1 receptor and the co-receptors Plexin-A2, Plexin-A4 and Plexin-D1 (Chen et al., 2008) (Figure 4). Knockdown of these receptors leads to the accumulation of neurons in deep layers of the cortex. These neurons develop leading processes that are abnormally oriented respect to the pia. Similar defects are observed when the gradient of Sema3A is disrupted.

Axon development during radial migration

Migrating projection neurons develop their axons as they migrate towards the CP, a feature that distinguishes them from other migrating neurons (e.g. interneurons). In other words, migration temporally coexists with axon guidance in the same cells. Axon specification occurs at a specific moment during the migratory cycle. Time-lapse experiments have revealed that cells at the multipolar state start to generate a process that grows toward the VZ. After switching from the multipolar state to the bipolar state, cells keep growing the axon at the rear of the cell, in the opposite direction to the migration (Noctor et al., 2004). The directed growth of the axon is instructed by Sema3A, which acts as a chemorepulsive cue. Sema3A is highly expressed in the CP, while its Neuropilin-1 receptor is enriched in cells at the multipolar state (Hatanaka et al., 2009).

Axon initiation requires the polarization of neurons. This process is likely regulated in vivo by the LKB1-SAD-A/B pathway. Mouse mutants for both LKB1 and SAD-A/B have important defects in cortical axon development (Barnes et al., 2007; Kishi et al., 2005). LKB1^{S431}, which is necessary for activation of SAD-A/B kinases, is a substrate of PKA and p90RSK. These two molecules are typically activated by extracellular cues (Sapkota et al., 2001; Simon et al., 2004), which suggest that the graded distribution of extracellular cues through the cortex induce polarized intracellular events that leads to the establishment of a single axon directed in the opposite direction of the migration (Barnes and Polleux, 2009). One of the possible cues regulating this process is Sema3A, a chemorepulsive cue highly expressed in the CP (Hatanaka et al., 2009).

Migration of interneurons

Origins of cortical interneurons

In the mouse, different areas in the subpallium generate GABAergic interneurons that colonize the cerebral cortex during development. Most cortical interneurons derive from the medial and caudal ganglionic eminences (MGE and CGE, respectively), although other regions such as the lateral ganglionic eminence (LGE) and the preoptica area (POA) also seem to contribute with small populations (Anderson et al., 1997; Anderson et al., 2001; Butt et al., 2005; De Carlos et al., 1996; Gelman et al., 2009; Lavdas et al., 1999; Nery et al., 2002; Sussel et al., 1999; Yozu et al., 2005).

Cortical interneuron diversity is very large. More than twenty different types of interneurons have been described based on morphological, electrical or neurochemical criteria (Markram et al., 2004; PING, 2008). The origin of this diversity is still unclear, but the current model suggests that it is a consequence of the diversity of progenitor cells present in the subpallium. Experiments from different laboratories indicate that interneurons containing Parvalbumin (PV) or Somatostatin (SST) are generated in the MGE, whereas those expressing Calretinin (CR) or Vasointestinal peptide (VIP) come from the CGE (Flames et al., 2007; Xu et al., 2004). A small population of NPY-expressing cells seems to derive from the CGE and from the POA. MGE-derived interneurons express Calbindin (CB) and the transcription factor Lhx6 (Fogarty et al., 2007). Further analysis of the expression of several dozens of transcription factors indicated that multiple progenitor domains exist within the MGE (Flames et al., 2007), which led to the discovery of a preferential origin of SST- and PV-expressing interneurons in the dorsal and ventral aspects of the MGE, respectively (Flames et al., 2007; Wonders et al., 2008). These results suggest that a combination of transcription factors differentially specify progenitor cells within the MGE to generate PV or SST interneurons. For example, Martinotti interneurons, which express both CR and SST, specifically derive from progenitors that express the transcription factor Nkx6-2 in the dorsal MGE (Fogarty et al., 2007).

Subpallial tangential migration

In their way to the cortex, interneurons use different routes to migrate tangentially through the subpallium at different stages of development. Early generated interneurons (E12.5) migrate preferentially superficial to the developing striatum, whereas late generated interneurons (E13.5-E15.5) migrate deep to the striatal

mantle, close to the LGE SVZ (Marín and Rubenstein, 2001). The guidance of interneurons through the subpallium does not seem to directly depend on the cortex (Marín et al., 2003), suggesting the existence of a gradient of attractive or permissive cues in the route these cells follow through the subpallium and/or repulsive cues located ventral to the MGE (Marín et al., 2003; Wichterle et al., 2003).

The POA contains signals that are chemorepulsive for MGE-derived cells, but the identity of these cues remains unidentified (Marín et al., 2003; Wichterle et al., 2003). *Slit1* and *Slit2* are expressed in this region at the time when MGE-derived interneurons migrate to the cortex. Although tangentially cortical interneurons avoid cell aggregates expressing both Slit1 and Slit2 (Wichterle et al., 2003), these molecules cannot be exclusively responsible for the non-permissive nature of the ventromedial forebrain for MGE-derived interneurons because this activity is still maintained in *Slit1/2* double mutants (Marín et al., 2003).

The cellular corridor that interneurons transverse in the LGE in their way to the cortex is highy permissive for their migration. Cells in the LGE corridor express a membrane bound isoform of Nrg1 (CRD-Nrg1), which delineates the route of migrating interneurons through the subpallium. In vitro, MGE-derived cells preferentially adhere to cells expressing CRD-Nrg1, which suggests that this signal is highly permissive for interneurons. The transduction of these signals occurs via the ErbB4 receptor, which is expressed by MGE-derived cells. Overexpression of a dominant negative form of the receptor or genetic removal of *ErbB4* from migrating interneurons severely disrupts tangential migration and reduces the number of cells that reach the cortex (Flames et al., 2004). Altogether, these experiments suggest that CRD-Nrg1/ErbB4 signaling is important for the tangential migration of interneurons through subpallium.

Cortical interneurons avoid entering the striatum during their journey through the subpallium. The ability of cortical interneurons to be repelled by striatal cells relies on the expression of class III semaphorin receptors Neuropilin1 (Nrp1) and Neuropilin2 (Nrp2), which are not expressed by migrating striatal interneurons. Neuropilin receptors bind to Semaphorin-3A (Sema3A) and Semaphorin-3F (Sema3F), which are present at high concentrations in the striatal mantle, creating a non permissive/repulsive territory for cortical interneurons (Marín et al., 2001). Expression of Neuropilin receptors in MGE-derived cells depends of the expression of several transcription factors. For example, Nrp2 is repressed by direct binding of Dlx1/2, which thereby prevents the premature expression of this receptor in MGE progenitors (Le et al., 2007). In addition, postmitotic expression of Nkx2-1 represses neuropilins in striatal interneurons. Cortical interneurons turn off Nkx2-1, which allows the expression of neuropilins in these cells (Nóbrega-Pereira et al., 2008).

Several extracellular proteins that act mainly as motogenic factors enhance the movement of MGE-derived cells. These factors are Hepatocyte growth factor (HGF) (Powell et al., 2001), Brain-derived neurotrophic factor (BDNF) (Polleux et al., 2002), Neurotrophin-4 (NT4) (Polleux et al., 2002)and Glial-derived neurotrophic factor (GDNF) (Pozas and Ibáñez, 2005). All of them enhance the migratory capabilities of MGE-derived cells without providing directional guidance (perhaps GDNF), thereby promoting the ability of migrating interneurons to leave their proliferative regions.

Cortical dispersion and invasion

Once interneurons have reached the pallium, they disperse through the entire cortex using different stereotyped streams. There are three migratory streams used by migrating interneurons: Marginal zone (MZ), subplate (SP) and subventricular zone (SVZ) (Figure 5). Interneurons migrate forming a rather dense group that follows a lateromedial trajectory towards the hippocampus. Although it is not known how interneurons choose which stream they would follow as they disperse through the cortex, some extracellular guidance cues has been shown to be expressed in the position where these streams are located (Marín and Rubenstein, 2001; Métin et al., 2006; Nakajima). The complexity of cortical dispersion has emerged when high-resolution time-lapse videomicroscopy combined with sophisticated organotypic cultures and in vivo assays have been applied. Thus, migration of cortical interneurons through the cerebral cortex does not only occur in ventral to dorsal and lateral to medial trajectories, but also in other directions. It seems that different populations of interneurons, presumably originated at different times and places within the subpallium, simultaneously move in opposite directions, which suggest that different extracellular cues may influence the migratory behavior of each subpopulation of interneurons (Ang et al., 2003; Tanaka et al., 2006).

One of the factors that may contribute to the dispersion of interneurons through the cortex is the secretable isoform of Nrg1, Ig-Nrg1, which is highly expressed in the VZ of the cortex (Flames et al., 2004). Ig-Nrg1 acts as a chemoattractant for interneurons and stimulates their arrival at the cerebral cortex, although it is not clear that it influences the migratory streams through which interneurons migrate. In this regard, another important cue for the cortical dispersion of interneurons is CXCL12, also known as SDF-1. This chemokine is expressed in the SVZ and MZ of the developing cortex by projection neurons and meningeal cells, respectively (Stumm et al., 2003; Tiveron et al., 2006). The receptor for this ligand, CXCR4, is expressed by cortical interneurons (Stumm et al., 2003; Tiveron et al., 2006). Recently it has been shown that this signaling pathway is important to maintain the tangential dispersion of interneurons throughout the cerebral cortex, preventing their premature invasion of the CP (López-Bendito et al., 2008). Interestingly CXCL12 is also crucial for Cajal-Retzius cell migration (Borrell and Marín, 2006). Since Cajal-Retzius cells migrate in medial to lateral direction, it seems unlikely that CXCL12 provides directional information to both types of cells. Consistently, CXCL12 seems to function by restricting interneuron migration through the MZ and SVZ (López-Bendito et al., 2008), although some studies have suggested that CXCL12 is indeed a chemoattractant for cortical interneurons (Li et al., 2008a).

After spending a period of time to disperse through the entire cortical surface, interneurons start the invasion of the CP (López-Bendito et al., 2008). Layering acquisition by MGE-derived interneurons also follows a pattern in which early-generated interneurons populate deep layers and late-generated interneurons occupy superficial layers (Valcanis and Tan, 2003). Consequently, projection neurons and interneurons generated at the same time end up roughly located in the same cortical layers, suggesting a high degree of coordination of both migratory programs. Since projection neurons reach their final destination prior to the invasion of the CP by interneurons (López-Bendito et al., 2008; Pla et al., 2006), it is likely that layer acquisition by interneurons is dependent on the correct position of their projection neurons counterparts. Consistently, cortical ectopias formed after deleting genes required for the migration of projection neurons also cause the accumulation of interneurons in the ectopic clusters (Beggs et al., 2003; Ramos et al., 2006). Moreover, experimental evidence suggests that interneurons are not

affected by loss of Dab1 function, which indicates that interneuron layering does not involve Reelin signaling. So, the current model suggests that signals provided by projection neurons, maybe as a consequence of their maturation, instruct interneurons to reach the appropriate layer within the CP.

Although interneurons switch their orientation from tangential to radial migration during CP invasion, it is not clear whether they use the basal processes of RGCs to support their radial migration, as projection neurons do (Nadarajah and Parnavelas, 2002; Yokota et al., 2007). The mechanisms mediating the radial migration of interneurons and their invasion of the CP are just beginning to be identified. Although no specific chemoattractants have been identified to control the CP invasion by interneurons, it seems that the process requires both CP attraction and loss of sensibility to the instructive cues present outside the CP that regulate the tangential dispersion of interneurons. Additional instructive signals might be required to halt interneuron migration in the correct place within the CP (Bortone and Polleux, 2009). Blocking signals that contribute to the intracortical tangential dispersion of interneurons, such as CXCL12, Netrin-1 or GDNF alters the distribution of subsets of interneurons in the cerebral cortex, in some cases by facilitating a premature switch from tangential to radial migration (López-Bendito et al., 2008; Stanco et al., 2009).

Cell biology of neuronal migration

Most migrating cells are polarized in the direction of movement (Iden and Collard, 2008). Polarity proteins activate signaling pathways that organize cytoskeleton components to adapt the specific morphological requirements of cell migration (Hatanaka and Murakami, 2002; Li and Gundersen, 2008; Solecki et al., 2006). Migrating neurons are prominently polarized due to the development of relatively long leading processes in the direction of movement (Ward et al., 2005). The leading process is tipped with a growth cone-like structure, which is responsible for sensing the surrounding microenvironment in analogy to axonal growth cones (Bellion et al., 2005; Polleux et al., 2002).

The migratory cycle of neurons (including projection neurons and interneurons, in the case of the cortex) comprises a series of events that repeat continuously. This includes the extension and/or retraction of the leading process, which is followed by translocation of the cell soma. This later process takes places in two phases: (i) generation of a cytoplasmic perinuclear dilatation in the proximal side of the leading process, toward which subcellular organelles such as the Golgi apparatus and the centrosome move, and (ii) movement of the nucleus (Figures 6 and 7). While our knowledge of nucleokinesis has rapidly expanded over the past few years, the mechanisms underlying the dynamic remodeling of the leading process remain mostly unexplored. This is particularly true for cortical interneurons, for which even the morphology of the leading process is still a matter of dispute (Marín et al., 2006).

Guidance cues are thought to influence neuronal migration by influencing the motility of leading processes, modifying the dynamic behavior of cellular adhesions, and restructuring the cytoskeleton by instructing multiple actions (e.g.: actomyosin contraction, microtubule catastrophe and polymerization). Understanding the intracellular machinery that participates in the adaptation of migrating neurons to surrounding extracellular cues is starting to shed light on the basic principles that govern the process of migration. This knowledge is also proving useful to understanding some genetic diseases affecting the developmental of the nervous system.

Mechanisms regulating leading process dynamics

Microtubules are essential for maintaining migration, since blocking microtubule dynamics alters neuronal morphology and migration (Baudoin et al., 2008). It is not completely understood how microtubules are regulated during the migratory cycle. Different classes of microtubules exist, which correspond to different post-translational modifications: Tyrosinated microtubules are associated with dynamic parts of the microtubule network, while acetylated microtubules are linked to relatively stable microtubules. The different classes of microtubules are enriched at specific domains of migrating neurons, although their specific role in the dynamic behavior of the leading process is unknown (Collin et al., 2008; Umeshima et al., 2007) (Figure 6). Another very important aspect of microtubules is their polarization within the cell. Microtubules grow in the direction of their plus ends, which are typically oriented towards the membrane. In contrast, the microtubules minus ends are normally located in close proximity to a Microtubule Organizing Center (MTOC), where nucleation occurs by addition of α and β monomers of tubulin. In migrating neurons the main MTOC is the centrosome, which is enriched in γ-tubulin (Li and Gundersen, 2008).

Several proteins are known to be important for their role in regulating the dynamic behavior of the leading process, mostly by influencing the microtubule cytoskeleton. For example, the microtubule-associated protein (MAP) Doublecortin (DCX) was initially identified as a gene mutation associated with a specific type of lissencephaly in humans (Gleeson et al., 1998; Portes et al., 1998). Experiments in rodents showed that Dcx is implicated in neuronal migration, both in projection neurons (Bai et al., 2003; Deuel et al., 2006; Koizumi et al., 2006a) and interneurons (Kappeler et al., 2006), through its role in microtubule stabilization acting as an anti-catastrophe and nucleator factor (Moores et al., 2006). Another MAP, LIS1 (Sapir et al., 1997; Tamar et al., 1999), is also implicated in the migration of both types of cortical neurons (Bi et al., 2009; Cahana et al., 2001; Hirotsune et al., 1998), and mutations in the human *PAFAF1B1* gene leads to type I lissencephaly (Lo Nigro et al., 1997; Reiner et al., 1993). Defects in neuronal migration associated with both DCX and LIS1 proteins are at least in part due to the elaboration of an excessive number of leading processes during migration, probably as a consequence of the reduced stability of the microtubule cytoskeleton (Bai et al., 2003; Tsai et al., 2005). The balance between stable and dynamic microtubules seems to be regulated by MARK2/Par-1, a polarity protein that phosphorylates several MAPs. For example, MARK2/Par-1 phosphorylates DCX and induces its dissociation from microtubules, thereby promoting unstable microtubules (Drewes et al., 1997; Schaar et al., 2004). Somehow surprisingly, MARK 2/Par-1 knockdown also promotes the accumulation of cells in the multipolar state of the migration of projection neurons, suggesting that the generation of multiple processes could also be enhanced by promoting microtubule stability (Sapir et al., 2008a). Double knockdown of *Dcx* and *MARK2/Par-1* partially rescue the phenotype (Sapir et al., 2008b), which indicates that although the phenotype resulting from promoting stable versus unstable microtubules is similar, the underlying mechanisms are likely to be different. Finally, mutations in the human tubulin genes *TUBA3* (α-tubulin) and *TUBB2B* (β-tubulin) are linked to important neurological disorders. Mouse mutants for these genes have severe neuronal migratory defects, although at this point it is unclear what are the primary defects found in tubulin-deficient neurons (Jaglin et al., 2009; Keays et al., 2007).

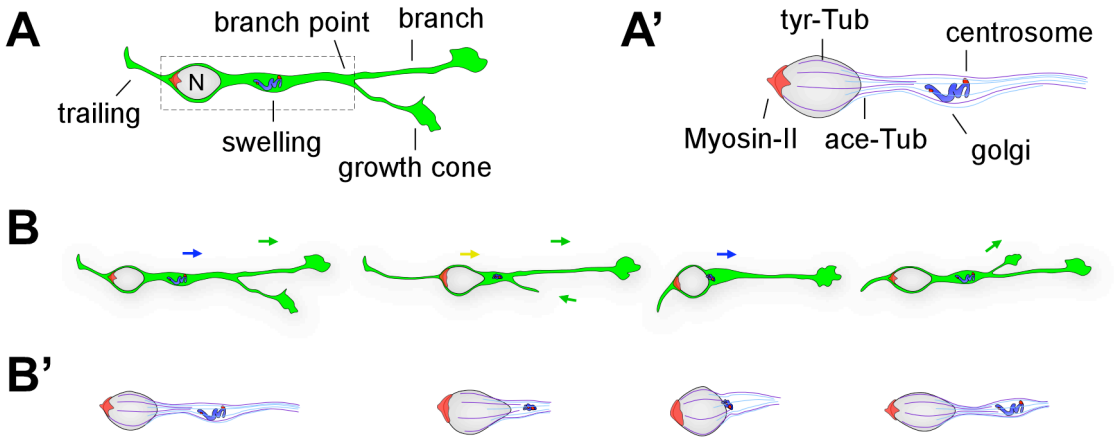

Figure 6. Migratory cycle of cortical interneurons. (A) Schema with representative structures of a migrating interneuron. (A') High magnification of the nucleus and cytoskeleton with the microtubules surrounding the nucleus and other subcellular organelles. Note that Myosin-II is enriched at the rear part of the nucleus. The schema shows only the area marked by the dashed line. (B) The migratory cycle starts with the extension of one of the processes and forward movement of the dilatation or swelling. Extension and retraction coexist in different branches of the leading process until one of them is selected. At this moment the nucleus translocates until reaching the swelling at the branch point. Non selected processes are reabsorbed by the cell whereas a new process starts to grow, and the cycle is repeated. (B') High magnification of the nucleus and cytoskeleton preceding the branch point in each of the steps of the migratory cycle. (tyr-Tub: Tyrosinated tubulin, ace-tub: Acetylated tubulin)

The actomyosin network is the other major component of the cytoskeleton. Although its implication in migrations has been extensively studied in other cell types (Le Clainche and Carlier, 2008; Okeyo et al., 2009; Petrie et al., 2009), its role in neuronal migration has remained mostly unexplored. Until recently, the role of the actomyosin network has been primarily studied in relation to the process of nucleokinesis (see below), but recent work suggests that actin and the motor protein Myosin-II are also present in the leading process. A role of the actin cytoskeleton in leading process dynamics is supported by studies on the implication of the actin-binding protein Filamin A (FLNA) (Nagano et al., 2004; Nagano et al., 2002) and the small GTP-binding protein Rnd2 (Heng et al., 2008) in the migration of projection neurons. Mutations in the human *FLNA* gene generate periventricular heterotopias, a developmental brain malformation that causes epilepsy (Feng and Walsh, 2004). FLN family proteins have been implicated in cross-linking cortical actin filaments into a dynamic three-dimensional structure (Feng and Walsh, 2004). Loss of function of FLNA in projection neurons blocks their migration to the CP, and cells remain accumulated in the SVZ/IZ where multipolar cells are normally found. Moreover, cells become rounded and are unable to transit to the bipolar state (Nagano et al., 2004). Rnd2 has also been implicated in the control of the actin cytoskeleton in different cell types (Chardin, 2006), including neurons, in which the Rnd2 target Raplostin has been shown to interact with WASP, a well-known actin nucleator protein (Kakimoto et al., 2004). Knockdown of *Rnd2* in the cerebral cortex perturbs the morphology of migrating cells, which develop multiple branches and get stalled in the multipolar state (Heng et al., 2008).

Several lines of evidence also implicate Cdk5 in the regulation of leading process dynamics in migrating neurons. Conditional deletion of Cdk5 or expression of a Cdk5 dominant-negative (Cdk5-DN) severely impairs neuronal migration, preventing the multipolar to bipolar transition (Ohshima et al., 2007). Low concentrations of Cdk5-DN have a weaker effect that complete ablation of Cdk5, but also impairs the morphology of the leading process in neurons that escape the multipolar stage and migrate into the CP (Ohshima et al., 2007). A similar phenotype was found in mouse mutant for the Cdk5 regulatory protein

p35, in which pyramidal cells develop an abnormal leading process with several branches. In this later case, the interaction between neurons and radial fibers is also disrupted (Gupta et al., 2003). Another line of evidence that support a role for Cdk5 in leading process dynamics is that it phosphorylates several proteins that are directly implicated in this process, such as DCX, FAK, NUDEL, and p27 (Kawauchi et al., 2006; Niethammer et al., 2000; Sasaki et al., 2000; Tanaka et al., 2004b; Xie et al., 2003). Interestingly, Cdk5 does not seem to be required for neuronal migration in some circumstances. For example, early generated projection neurons are not affected by the loss of Cdk5 (Gilmore and Herrup, 2001; Hatanaka et al., 2004), suggesting that this protein might be involved in the migration of cells that interact with RGCs (Miyata and Ogawa, 2007). This idea is consistent with reports indicating that the migration of cortical interneurons is not affected by the absence of Cdk5 (Gilmore and Herrup, 2001; Hammond et al., 2004), although a recent study suggests that minor abnormalities also exists in these cells (Rakic et al., 2009).

Somal translocation

The movement of the soma in migrating neurons involves two steps, the forward displacement of the perinuclear dilatation in the proximal aspect of the leading process, and the subsequent translocation of the nucleus (Bellion et al., 2005; Schaar and McConnell, 2005) (Figure 7). The perinuclear dilatation, also named as cytoplasmic swelling, is a single and discrete enlargement of the leading process in the region immediately anterior to the nucleus that develops as part of the migratory cycle of migrating neurons (Bellion et al., 2005; Hao et al., 2009; Renaud et al., 2008; Schaar and McConnell, 2005). During somal translocation, the movement of the perinuclear dilatation precedes that of the nucleus, which remains stationary during this phase. Some subcellular organelles, such as the Golgi apparatus, the centrosome and the endoplasmic reticulum relocate into the perinuclear dilatation as this structure moves in the leading process. Since the centrosome is the main MTOC of migrating neurons, the dilatation is currently perceived as a structure implicated in the organization of the microtubule network and the subsequent movement of the nucleus (Solecki et al., 2006). In addition to this function, previous studies have suggested that the perinuclear dilatation, towards which the nucleus moves during nucleokinesis, is the key structure for the adhesion of migrating neurons to their substrate (Schaar and McConnell, 2005). Remarkably, recent work has described that some connexins, mainly Cx26, are enriched in this structure, and that their adhesive properties are required for glial-guided migration (Elias et al., 2007).

The forward movement of the cytoplasmic dilatation and the subsequent displacement of the nucleus involve microtubules and actomyosin elements of the cytoskeleton. In migrating cells, microtubules are organized in a perinuclear cage surrounding the nucleus, with their plus end attached to this structure and the minus ends directed towards the centrosome (Solecki et al., 2004; Solecki et al., 2009; Xie et al., 2003). This array of microtubules has been proposed to support the pulling forces that promote nuclear translocation. The first demonstration of the functional role of this microtubule cage in nucleokinesis derives from studies on the cell polarity protein Par6α. Par6α is involved in the localization of γ-tubulin close to the centrosome, which is fundamental to polarize microtubules in the MTOC. Functional experiments showed that overexpression of Par6α delocalized both proteins away from the centrosome, disrupting the microtubule cage and, consequently, blocking neuronal migration (Solecki et al., 2004). Other molecules that interact with microtubules also play a role in this process. For instance, loss of LIS1 function leads to a defect in the movement of both the centrosome and the nucleus, likely due to the interaction of this protein with the motor protein dynein (Tanaka et al., 2004a; Tsai et al., 2007). NUDEL is

also associated to this complex, but a clear implication of this protein in nucleokinesis is missing (Niethammer et al., 2000). DCX has been also implicated in the movement of the perinuclear swelling and the nucleus in different types of tangentially migrating neurons (Kappeler et al., 2006; Koizumi et al., 2006a), and this is also the case for Focal adhesion kinase (FAK), an intracellular kinase that is enriched at the perinuclear microtubule cage. A non-phosphorylatable mutant of FAK in serine 732 seems to impair nucleokinesis. Interestingly, FAK phosphorylation at S732 is mediated by Cdk5, which suggests that a p35/Cdk5/FAK pathway could modulate nuclear movement through the microtubule cytoskeleton (Xie et al., 2003).

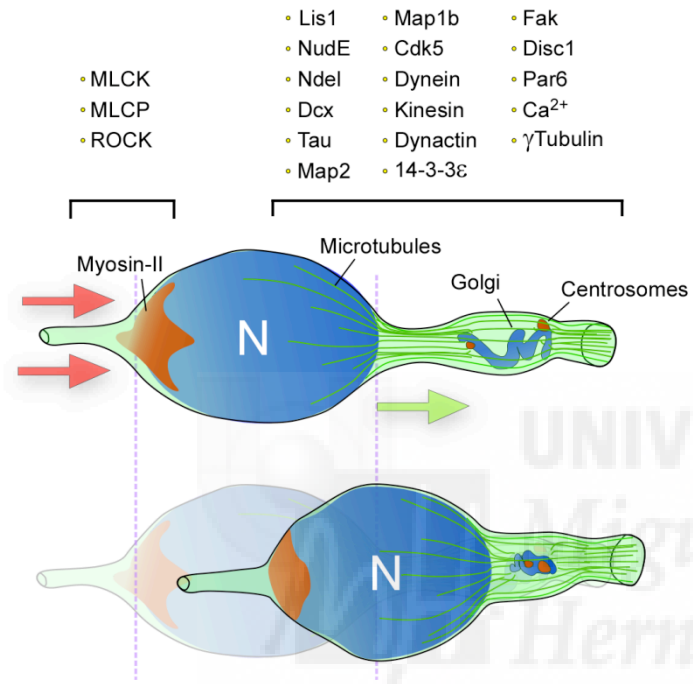

Figure 7. Nucleokinesis in migrating neurons. Nucleokinesis in migrating neurons involves both perinuclear swelling and nucleus translocations. First, the perinuclear dilatation moves forward along with the centrosome and the Golgi. The perinuclear microtubule cage (in green) pulls the nucleus forward until reaching the swelling. Forward pulling forces (green arrow) are complemented by Myosin-II at the rear, which generates pushing forces (red arrows), creating the saltatory pattern of nucleokinesis. In addition to the motor proteins dynein and myosin, several other proteins related to microtubule or acto-myosin cytoskeletons are implicated in this process. (N) nucleus.

Until recently it has been assumed that the forces responsible for the movement of the nucleus were microtubule-based, and involve pulling of the nucleus through the action of motor proteins on the perinuclear microtubule network (Figures 6 and 7). Recent studies have questioned this idea. First, microtubules surrounding the nucleus are not necessarily linked to the centrosome (Umeshima et al., 2007), which suggests that this subcellular organelle is unlikely to be the source of the forces pulling the nucleus forward and that additional MTOC structures, beyond the centrosomes, could be positioned at different locations within the cell (Luders and Stearns, 2007). Second, forward movement of the centrosome precedes nuclear translocation and can be dissociated from it. During this step the nucleus remains stationary, thereby increasing its distance to the centrosome (Bellion et al., 2005). Third, time lapse assays of migrating projection neurons have clearly shown that intracellular proteins independently regulate centrosome movement and nuclear translocation (Tsai et al., 2007). Altogether, these findings open new directions in our understanding of the relationship between microtubules, the centrosome and the nucleus.

In contrast to the pulling forces that have been proposed to displace the microtubule cage and the nucleus during nucleokinesis, recent work suggest that pushing forces originated at the rear of the cell may also influence this process (Figures 6 and 7). Pushing forces appear to require the motor protein Myosin-II,

which is localized at the rear of the nucleus in migrating neurons (Bellion et al., 2005; Schaar and McConnell, 2005). Pharmacological blocking or RNAi knockdown of Myosin-II severely impairs nucleokinesis in both interneurons (Bellion et al., 2005; Schaar and McConnell, 2005) and projection neurons (Tsai et al., 2007). In contrast, the movement of the centrosome does not seem to be affected in the absence of Myosin-II function, which reinforces the view that the movement of this structure and the nucleus are differentially regulated (Tsai et al., 2007). In summary, the current model of somal translocation suggests that this process involves both microtubule dependent dynamics, regulated in a dynein-dependent manner, and pushing forces from the back of the cell, which are dependent on myosin motor proteins (Vallee et al., 2009).

Neuron-substrate interactions during migration

The microenvironment surrounding migrating cortical neurons varies enormously during their journey. Projection neurons interact very closely with the basal processes of their progenitors during certain developmental stages, while interneurons move perpendicular to the glial scaffold during their tangential migration. In addition, each neuron migrates in close proximity to many other cells, some of which are also migrating and some others that remain stationary. Simultaneusly, cortical and thalamic axons invade the cortex during the time cortical neurons migrate to their final position, which increases even more the number of possible partners with whom migrating neurons may interact. With this in mind, it seems evident that understanding the nature of the interactions of the different classes of migrating neurons is necessary to precisely define the molecular and cellular mechanisms regulating neuronal migration in the cerebral cortex.

Neuron-glia interactions

Neuron-glia interactions have been best characterized during glial-guided migration in developing cortical structures. Integrins are probably the best characterized molecules in this process. Integrins are transmembrane receptors composed of α and β subunits that form heterodimers. Early in vitro studies emphasized a role for neuronal α3β1 integrin receptors in mediating the interaction of migrating pyramidal neurons with radial glial cells (Anton et al., 1999). Subsequent analyses of mouse mutants have in turn focused the attention to radial glial cells, as these cells and their endfeet anchorage to the meningeal basement membrane are dramatically affected in the absence of β1 integrin signaling (Belvindrah et al., 2007; Graus-Porta et al., 2001; Halfter et al., 2002). According to this later view, integrin function is only indirectly required for migrating neurons, which would otherwise migrate normally in the absence of integrin-mediated adhesion. As migration on two-dimensional substrates naturally overemphasizes the role of adhesion, it is very likely that in vivo studies may offer a much more precise idea of the contribution of integrin signaling during neuronal migration. Nevertheless, mouse genetic analyses may have overlooked subtle defects in the motility of integrin mutant neurons (Schmid et al., 2004), which may only cause a minor delay in migration and not a major disruption of cortical layering. Consistent with this notion, two recent studies suggest that integrin signaling cell-autonomously influence neuronal migration. Conditional deletion of neuronal laminin γ1, a component of the ECM deposited around migrating neurons and radial glial fibers that binds integrin receptors, disrupts the migration of pyramidal neurons (Chen et al., 2009). In addition, specific removal of α3 integrins from tangentially migrating interneurons perturbs

their normal distribution as they invade the embryonic cortex, suggesting that these receptors are required to recognize specific guidance cues present in the migratory route of interneurons (Stanco et al., 2009).

Recent studies have shown that the interaction between radial fibers and migrating neurons also relies on the adhesive properties of Gap junctions (Cina et al., 2009; Elias et al., 2007). Several connexins, the component of Gap junctions, are expressed in both radial glial cells and migrating neurons, and their association in *trans* is required for glial-guided migration. Interestingly, the channel capabilities of Gap junctions are not required for this process. The mechanisms regulating the dynamic assembly and disassembly of these transient contacts between radial glial fibers and migrating neurons are currently unknown.

When migrating projection neurons arrive at their final destination they need to detach from RGCs to start their differentiation. This process is mediated at least in part by Reelin signaling, through the intracellular adaptor protein Dab1. Dab1 associates with the cytoplasmic region of β1 integrins (Schmid et al., 2005), and it has been hypothesized that the interaction between α3β1 integrin with the Reelin signaling pathway may trigger the internalization of integrins, thus leading to the detachment of migrating neurons from radial glia processes (Dulabon et al., 2000; Schmid et al., 2005). In addition, previous work has shown that phosphorylation of specific tyrosines in Dab1 is necessary to terminate glial-guided migration, because neurons expressing non-phosphorylatable forms of these residues (Y220F or Y232F mutants) fail to downregulate the expression of α3 integrin in the cortical plate, and consequently remain attached to the radial glia (Sanada et al., 2004).

The interaction of cortical interneurons with radial fibers has only been recently explored. Contacts between interneurons and the endfeet of RGCs have been observed after interneurons have reached the cortex, although the functional meaning of these contacts is not clear (Yokota et al., 2007).

Neuron-axon interactions

Thalamocortical and corticofugal axonal projections extend through the telencephalon at the same developmental stages than cortical neuronal migration takes place. Consequently, it is conceivable that migrating interneurons and thalamic or cortical axons may interact (Lavdas et al., 1999; Métin and Godement, 1996). The first functional evidence for such interactions derives from work with a neuronal adhesion molecule member of the immunoglobulin superfamily expressed in corticofugal fibers, TAG-1. Blocking antibodies against TAG-1 impairs the migration of cortical interneurons towards the cortex in slice cultures (Denaxa et al., 2001). Somehow surprisingly, pharmacological blocking with an enzyme that cleaves GPI-anchors does not disrupt tangential migration in the cerebral cortex (Tanaka et al., 2003). Moreover, analysis of mouse mutants for *Tag1* did not show any major defect in the migration and distribution of interneuron subpopulation in the cerebral cortex (Denaxa et al., 2005).

Interactions with blood vessels

Nervous and vascular systems follow similar distributions in the cell body and in particular in the CNS (Carmeliet and Tessier-Lavigne, 2005). During development, molecules whose function was first described in axon guidance have been shown to play an important role in the development of the brain vasculature. In the adult brain, for example, it has been suggested that interactions exist between migratory neuroblasts in the rostral migratory stream (RMS) and blood vessels. In addition to act as a

physical substrate, endothelial cells seem to provide migrating neurons with a source of BDNF, which promotes their migration (Snapyan et al., 2009).

Homophylic interactions

Neurons may also develop inhibitory homotypic interactions. This mode of migration, known as contact inhibition, was first described to define the behavior of fibroblasts confronting each other in vitro, where they retract their protrusions and change direction on contact. This strategy allows neurons to achieve directional migration in the absence of chemotactic gradients, because it favors the movement of cells towards areas with less cell density. Recent in vitro experiments suggests that Cajal-Retzius cells derived from the cortical hem may use this mechanism to disperse throughout the surface of the cerebral cortex during early corticogenesis (Borrell and Marín, 2006), although the molecular mechanisms underlying this process are currently unknown. It should be noted, however, that members of the planar cell polarity (PCP) pathway have been shown to mediate contact inhibition of locomotion in neural crest cells (Carmona-Fontaine et al., 2008), and PCP proteins are likely involved in the migration of telencephalic neurons (Ying et al., 2009).

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Objectives

The general goal of my Thesis project was to elucidate new intracellular mechanisms regulating the migration of cortical neurons. To this end, we studied the migration of both interneurons and projection neurons, with two specific aims:

1. To characterize the cellular mechanisms underlying the tangential migration of cortical interneurons, with a special focus on the dynamics of the leading process.

2. To identify new molecular mechanisms involved in the regulation of adhesion dynamics during the glial-guided migration of cortical projection neurons, with emphasis on the role of Focal adhesion kinase (FAK) in this process.

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Results

Part 1. Biased selection of leading process branches mediates chemotaxis during tangential neuronal migration

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Biased selection of leading process branches mediates chemotaxis during tangential neuronal migration

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Current models of chemotaxis during neuronal migration and axon guidance propose that directional sensing relies on growth cone dynamics. According to this view, migrating neurons and growing axons are guided to their correct targets by steering the growth cone in response to attractive and repulsive cues. Here, we have performed a detailed analysis of the dynamic behavior of individual neurons migrating tangentially in telencephalic slices using high-resolution time-lapse videomicroscopy. We found that cortical interneurons consistently display branched leading processes as part of their migratory cycle, a feature that seems to be common to many other populations of GABAergic neurons in the brain and spinal cord. Analysis of the migratory behavior of individual cells suggests that interneurons respond to chemoattractant signals by generating new leading process branches that are better aligned with the source of the gradient, and not by reorienting previously existing branches. Moreover, experimental evidence revealed that guidance cues influence the angle at which new branches emerge. This model is further supported by pharmacological experiments in which inhibition of branching blocked chemotaxis, suggesting that this process is an essential component of the mechanism controlling directional guidance. These results reveal a novel guidance mechanism during neuronal migration that might be extensively used in brain development.

KEY WORDS: Cellular dynamics, Chemotaxis, Neuronal migration, Mouse

INTRODUCTION

Neuronal migration and axon guidance play central roles in the assembly of neuronal circuits. During axon guidance, directional movement appears to rely on growth cone dynamics (Dickson, 2002; Lin and Holt, 2007; Round and Stein, 2007). According to this view, growing axons are guided to their correct targets by steering the growth cone in response to attractive and repulsive cues (Kalil and Dent, 2005). Given the typical bipolar morphology of many migrating neurons, with a leading process extending in the way of their migration and a trailing process in the opposite direction, this model of directional guidance have also been extensively used to explain the chemotaxis of migrating neurons (Miyata and Ogawa, 2007; Noctor et al., 2001; Rakic, 1990; Yee et al., 1999). However, not all migrating neurons have the same morphology. In the developing cerebral cortex, for example, some migrating interneurons have been described to exhibit a bipolar morphology with a single leading process (Jiménez et al., 2002; Polleux et al., 2002), whereas many others have been reported to display branched leading processes (Anderson et al., 1997; Bellion et al., 2005; Friocourt et al., 2007; Kappeler et al., 2006; Lavdas et al., 1999; Marín and Rubenstein, 2001; Nasrallah et al., 2006; Polleux et al., 2002). The existence of branched leading processes is by no means specific to cortical interneurons, as other types of tangentially migrating neurons also adopt this morphology (López-Bendito et al., 2006; Marín and Rubenstein, 2001; Ward et al., 2005).

The issue that arises in relation to the branched morphology of some migrating neurons is how they navigate using two leading processes to explore a wide prospective territory. In the case of cortical interneurons, work over the past few years has revealed that they originate in the subpallium and migrate over long distances using multiple tangential pathways (Corbin et al., 2001; Marín and Rubenstein, 2001). Moreover, we now know that interneuron migration is controlled by a complex combination of long-range attractive and repulsive signals, short-range instructive molecules, cell-adhesion complexes and motogenic factors (Marín and Rubenstein, 2003; Métin et al., 2006). With the exception of nucleokinesis, for which the underlying principles are just beginning to be elucidated (Bellion et al., 2005; Kappeler et al., 2006; Nasrallah et al., 2006), very little is known about the cellular mechanisms that control the directional movement of cortical interneurons in response to all these multiple guidance cues. Our results suggest that dynamic regulation of leading process branching is an essential mechanism for directional guidance in tangentially migrating interneurons.

MATERIALS AND METHODS

Mice

We used wild-type (CD1 and C57/b6), *Gfp* (CD1) and *Gad65-Gfp* (C57/b6) transgenic mice (Hadjantonakis et al., 2002; López-Bendito et al., 2004). The day of vaginal plug was considered to be embryonic day (E) 0.5. Animals were maintained under Spanish and EU regulation.

Slice and explant culture experiments

Organotypic slice cultures of the embryonic mouse telencephalon were prepared as previously described (Anderson et al., 1997). In some experiments, vehicle solution (H_2O) or Y27632 (30 □m) was added to the medium 12 hours after culture and replaced after 12 hours. Slices were then cultured for another 12/24 hours. For immunohistochemistry, slices were re-sectioned to 60 □m and incubated with rabbit-anti GFP (1:2000, Invitrogen) overnight at 4°C followed by 488 Alexa donkey anti-rabbit (1:500, Invitrogen) for 2 hours at room temperature. Slices were mounted using Mowiol-Dabco with Bisbenzamide (1:1000, Sigma).

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Medial ganglionic eminence (MGE) explants were dissected out from E13.5 slices and confronted with COS cell aggregates (expressing *dsRed* alone, or *dsRed* and *Ig-Nrg1*) in Matrigel matrices. Explants were incubated for 12 hours at 37°C in Neurobasal (Invitrogen, San Diego, CA) before the addition of vehicle or Y-27632 (30 μm). Vehicle and inhibitor were replaced after 12 hours and explants incubated for a total of 48 hours. In other experiments, small pieces of the MGE from E13.5 *Gfp* transgenic mice were transplanted into wild-type host slices, as described elsewhere (Marín et al., 2003).

For acute treatment of individual neurons, micropipettes (tip diameter ~1 μm) were placed above (~10 μm) and at a distance of ~20 μm from superficial dsRed-expressing neurons. Pressure pulses (0.1-0.4 bar; duration, 20 ms) were applied at a frequency of 2 Hz for 1 hour. Pipette solution contained Y27632 (3 mM) or the recombinant EGF domain of Nrg1 (NRG1-β1, Peprotech; 13 nM), and 0.4 mM Alexa Fluor 488 sodium salt (Invitrogen) dissolved in PBS or H_2O.

In vitro focal electroporation

pCAGGS-based *Gfp* and *dsRed* expression vectors were pressure injected focally into the MGE of coronal slice cultures and focally electroporated as described before (Flames et al., 2004).

Time-lapse videomicroscopy

Slices were transferred to the stage of an upright Leica DMLFSA or inverted Leica DMIRE2 microscope coupled to a confocal spectral scanning head (Leica TCS SL) and viewed through 10–60× water immersion or 20× oil objectives. Slices were continuously superfused with warmed (32°C) artificial cerebrospinal fluid at a rate of 1 ml/minute or maintained in supplemented neurobasal medium.

Quantification

Canvas (ACD Systems) and ImageJ (NIH, http://rsb.info.nih.gov/ij/) software were used for image analyses. For single pair-wise comparisons, a two-tailed *t*-test was used. Chi-square analysis was used to compare frequency distributions.

In co-culture experiments (Fig. 4), the migration angle was measured between the virtual vertical lines positioned at 0 or 90° (for quadrants 1 or 4, respectively) and the leading process closer to the COS aggregate.

For the analysis of the orientation of migrating cells at the pallial/subpallial boundary in slice cultures (Fig. 5), a virtual box (366 μm^2×225 μm^2) was defined at the place in which interneurons arrive to the cut in each slice. Cells within the box and in the adjacent 150 μm on both sides were classified into one of three groups depending on the orientation of their leading process: type 1 (leading process parallel to the cut), type 2 (process away from the cut) and type 3 (process towards the cut) cells. In cells displaying more than one leading process branch, the one with the swelling was selected. In few cases in which this was not evident, we consistently selected the widest and/or longest of the branches. To maximize the resolution of the cell morphology, cells were identified in 15-25 μm *z*-stacks.

For the analysis of the orientation of migrating cells in the cortex of slice cultures (Fig. 6), we identified Gad65-Gfp/BrdU double-positive cells in each slice and their main leading process was drawn using Canvas software. The main process was defined as that containing the organelle swelling in front of the nucleus; in a few cases in which this was not evident, we consistently selected the widest and/or longest of the branches. For each slice, we draw a grid of virtual radial lines (lines perpendicular to the ventricular zone and the pia) and orientated each cell in relation to the most adjacent 'radial line'. Cells that deviated less than 25° from radial lines were considered as radially oriented; those that deviate more than 25° were designated as tangentially oriented (Fig. 6F). We systematically exclude from this analysis those cells located in the more lateral or medial regions of the cortex, so that the curvature of the slice in those regions would not interfere with our analysis.

RESULTS

Leading process branching characterizes many tangentially migrating neurons in the CNS

Detailed analysis of *Gad65-Gfp* embryos, in which most γ-aminobutyric acid-containing (GABAergic) neurons express *Gfp* (López-Bendito et al., 2004), revealed that a branched leading process is a common feature of many tangentially oriented neurons in different regions of the brain and spinal cord (see Fig. S1 in the supplementary material) (Ward et al., 2005). To study the dynamic behavior of leading process branching in migrating interneurons, we used confocal time-lapse videomicroscopy in organotypic slice cultures. In this preparation, migrating interneurons display morphologies that closely resemble those described in vivo (Fig. 1A,B) (Ang et al., 2003). In a first series of experiments, we carried out focal electroporation of a plasmid encoding for the red fluorescence protein dsRed in the MGE of E13.5 telencephalic slices to label putative GABAergic interneurons and performed time-lapse videomicroscopy after 12-36 hours in culture (Fig. 1C,D). Using this approach, we labeled a reduced number of relatively scattered interneurons per slice, which allowed maximal resolution of the individual processes in each migrating cell. Every tangentially migrating interneuron observed displayed stereotyped dynamics (n>200 cells from at least 30 independent experiments), including nuclear movements in register with constant remodeling of the leading process. A representative case is presented in the sequence of photographs shown in Fig. 1E. At some point during their migratory cycle, all interneurons display a branched leading process, with the soma and the nucleus located at a specific distance behind the bifurcation (Fig. 1E) (t=0:00 h). Forward movement of the nucleus occurs in a two-step sequence, as previously described (Bellion et al., 2005; Schaar and McConnell, 2005) (see Fig. S2 in the supplementary material). First, a dilatation of the cell soma located in front of the nucleus moves forward towards the bifurcation (Fig. 1E) (t=0:10 h). This movement is subsequently followed by the forward displacement of the nucleus until it reaches the bifurcation (Fig. 1E) (t=0:30 h). After reaching the branching point, which is maintained stationary during nucleokinesis, the nucleus continues moving forward along one of the branches (Fig. 1E) (t=0:52 h). Furthermore, when the nucleus enters one of the primary branches, the other branch is already retracting and eventually becomes integrated in the trailing process (Fig. 1E) (t=0:52 h).

Observation of migrating neurons for extended periods of time revealed that the sequence of steps described above represents a migratory cycle that is consistently repeated by tangentially migrating interneurons. A typical example is presented in the sequence of photographs shown in Fig. 1F, which illustrate the dynamic behavior of a tangentially migrating interneuron (see also Fig. 1F′; see Movie 1 in the supplementary material). The movement of this cell can be described by successive translocations of the nucleus along one of the branches of the leading process from one bifurcation point to the next one, in a sequence that is continuously repeated. Thus, with the exception of small and transitory branches (Fig. 1F, small arrows), the bifurcation point of the leading process consistently marks the future position of the nucleus at the end of each nucleokinesis (Fig. 1F, broken lines). In many cases, the non-selected branch retracts before the nucleus reaches the branching point (Fig. 1F) (t=6:45 h; n=47 out of 83 retracting branches, from 38 different cells). In the remaining cases, the branch that is not selected by the nucleus becomes the trailing process (Fig. 1F) (t=2:45 h; n=36 out of 83 retracting branches, from 38 different cells), and eventually disappears. Branch selection correlated with rapid changes in the morphology of the growth cones tipping the leading process branches: selected branches displayed elaborate growth cones, whereas the growth cone of non-selected branches showed a collapsed morphology prior to retraction (see Fig. S3 in the supplementary material). We also performed time-lapse videomicroscopy in acute E15.5 telencephalic slices obtained

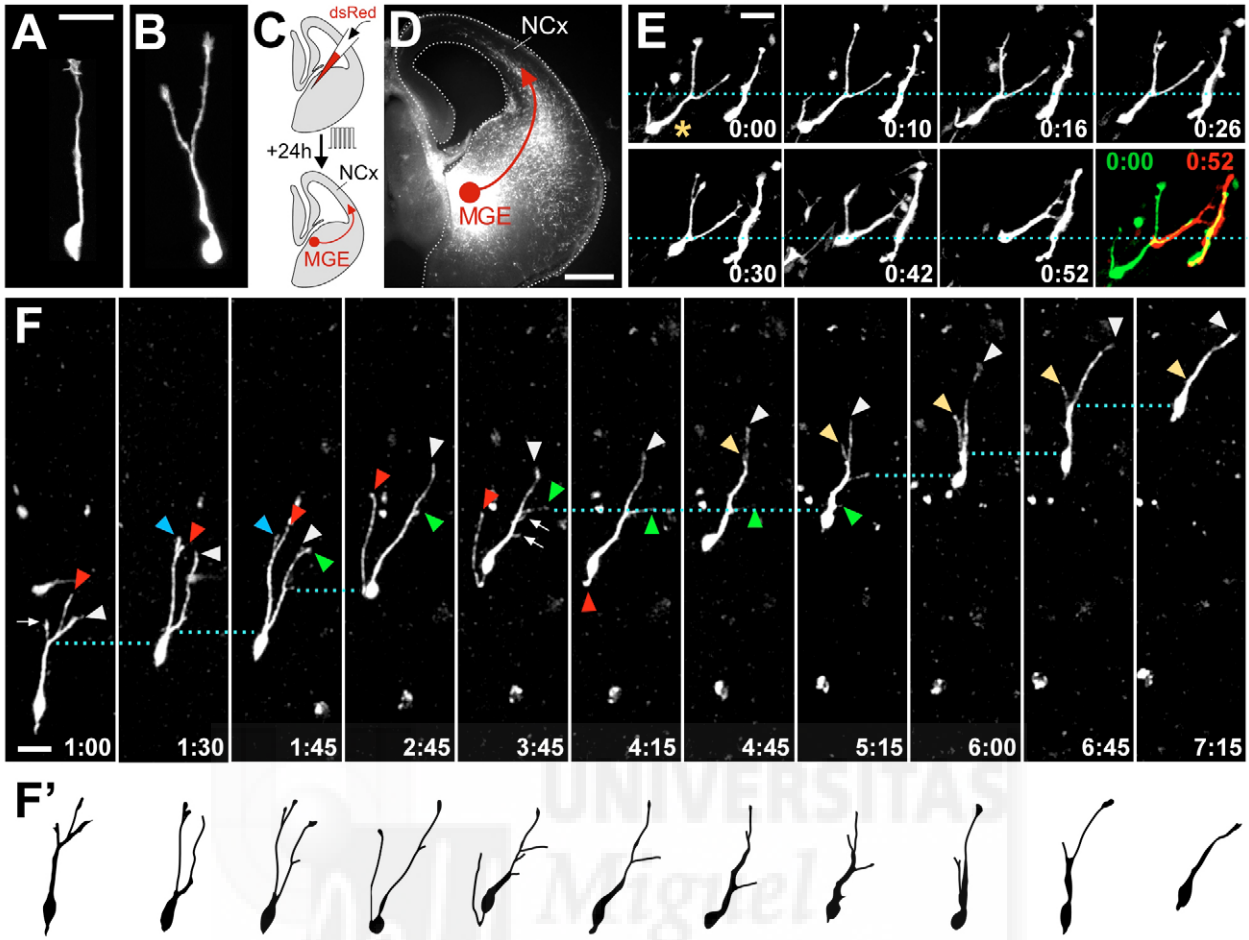

Fig. 1. Stereotyped dynamic behavior of tangentially migrating interneurons. (**A**,**B**) Different morphologies of interneurons derived from the medial ganglionic eminence (MGE). (**C**) Experimental paradigm. NCx, neocortex. (**D**) Migration of MGE-derived cells in E13.5 *dsRed*-electroporated slice after 36 hours in culture. (**E**) Time-lapse sequence of a *dsRed*-electroporated interneuron (asterisk) migrating from the subpallium to the cortex in a slice culture. Time is depicted in hours: minutes. The bifurcation point of the leading process is marked by a broken blue line. The last frame in the sequence shows superimposed images of the frame *t*=0:00 (green) and *t*=0:52 (red). (**F**,**F′**) Time-lapse sequence of a *dsRed*-electroporated interneuron migrating through the subpallium in a slice culture. This neuron was recorded for more than 7 hours to analyze several migratory cycles; only selected frames are displayed (see Movie 1 in the supplementary material for a complete movie version). The bifurcation points are marked by broken blue lines, and each leading process branch is coded with a colored arrowhead. Small arrows indicate small very transient processes. The drawings in F′ illustrate the morphology of this cell for each of the frames shown in F. Scale bars: 20 μm in A,B,E-F′; 300 μm in D.

from *Gad65-Gfp* transgenic mice. Simultaneous analysis of multiple GFP-expressing cells in the cortex of *Gad65-Gfp* embryos revealed that interneurons tangentially migrating through the subventricular zone (SVZ), intermediate zone (IZ) or marginal zone (MZ) navigate by alternative selection of leading process branches, independently of their speed of migration (see Movie S2 in the supplementary material; $n>200$ cells from at least 20 slices). In conclusion, bipolar morphologies such as those observed in static images (Fig. 1A) represent a phase in the migratory cycle of tangentially migrating neurons, in which the leading process remodels continuously as they move.

Directional guidance involves biased choices of leading process branches

How do migrating neurons with branched leading processes achieve directional guidance? Detailed observation of individual migrating interneurons revealed that leading process branches do not steer much as the cell moves (see Fig. S4 in the supplementary material). Instead, branches tend to grow or retract following the trajectory they initiate at the branch point, and so the geometry of the branches, and not the turning of their growth cones, appears to dictate the overall cell trajectory. In other words, the angle at which branches are formed determines the possible directions that can be followed by migrating cells. In cells that follow quasi-linear trajectories, such as those migrating in the subpallium (LGE in Fig. 2A,B) or in the cortical subventricular zone (SVZ) (Fig. 2A,C), the distribution of branch angles in the entire population follows a Gaussian distribution, with average angles relatively small (LGE: 45.32°±1.77°, n=109 cells) (Fig. 2B′); (SVZ: 35.76°±2.14°, n=64 cells) (Fig. 2C′). Dynamic observation of these cells also revealed that when interneurons navigate in a relatively constant direction the nucleus tends to transit alternatively through left and right branches in successive cycles (Fig. 2B″; see Movie 3 in the supplementary material). Analysis of the generation of new leading process branches revealed that neurons migrating in quasi-linear trajectories generate left or right branches with almost equal probability (50.9%

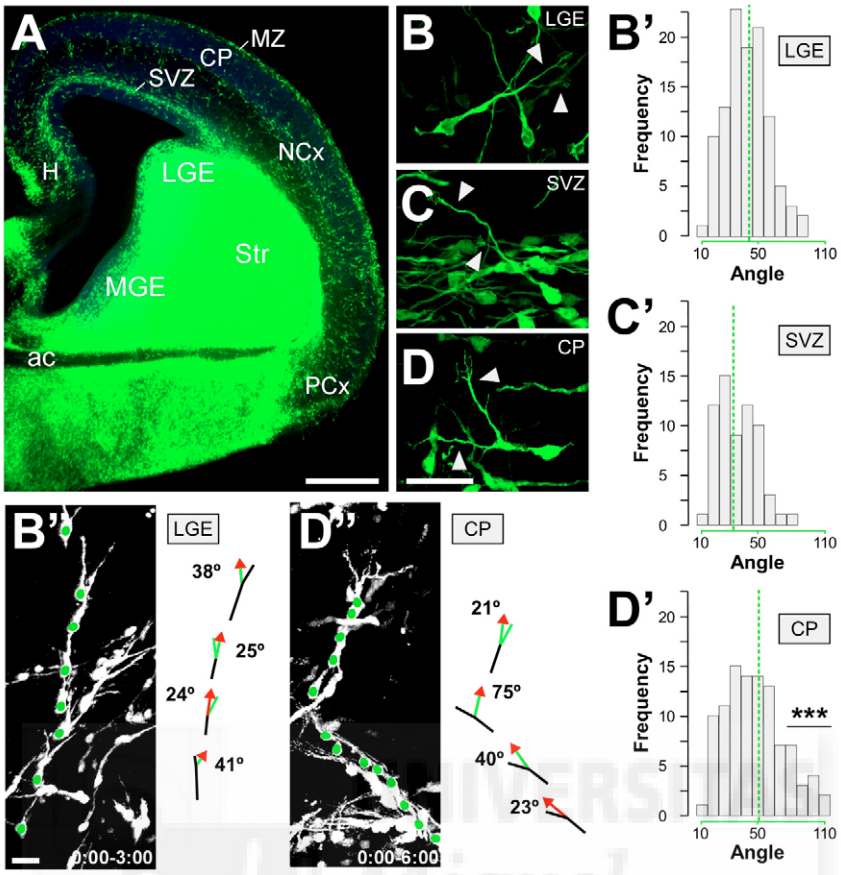

Fig. 2. Branch dynamics during tangential migration. (**A**) A telencephalic slice from an E16.5 *Gad65-Gfp* embryo. GABAergic cells are observed in green in the different regions in which migration was studied: subpallium (lateral ganglionic eminence, LGE), cortical subventricular zone (SVZ) and cortical plate (CP). Owing to the massive accumulation of migrating neurons in the subpallium, analysis of neurons migrating through the LGE was performed in slice cultures in which the MGE was electroporated with *Gfp* or *dsRed* (as in Fig. 1). (**B-D**) Representative images of the morphology of cells migrating through the LGE (B), SVZ (C) or CP (D). (**B′-D′**) Quantification of the frequency of angles form between leading process branches in cells migrating through the LGE (B′), SVZ (C′) or CP (D′). Mean angles are 45.32° (LGE), 35.76° (SVZ) and 51.83° (CP), respectively. Cells inside the highly packed SVZ generated very small branching angles with high frequency (C′), whereas neurons in the CP branched at large angles with much more frequency (D′). The frequency of angles greater than 70° is significantly higher in the CP than in the LGE or SVZ (χ^2 test, ***P<0.001). (**B″**,**D″**) Superimposed images of movie frames recreate the path followed by migrating neurons in the LGE (B″) and CP (D″). Green dots indicate the position of the nucleus in each nucleokinesis. Diagrams depict directional changes in these cells. New branches are shown in green, and the chosen branch is marked with a red arrowhead. Numbers indicate the angle formed by the branches. ac, anterior commissure; H, hippocampus; MZ, marginal zone; NCx, neocortex; PCx, piriform cortex; Str, striatum. Scale bars: 500□m in A; 25□m in B-D; 20□m in B″,D″.

left branches, 49.1% right branches; n=104 branches from 20 different cells). Moreover, the selection of a certain branch did not seem to determine the generation of the next one (left and right branches were formed in 52.6% and 47.4% of the cases, respectively, following the selection of a left branch; left and right branches were formed in 47.8% and 52.2% of the cases, respectively, following the selection of a right branch; n=84 branches from 20 different cells).

A different picture emerged from the analysis of cells migrating close to the cortical plate (Fig. 2A,D), where interneurons tend to change direction very frequently (Ang et al., 2003; Polleux et al., 2002). Thus, although the average angle between branches was only slightly larger in cells migrating close to the cortical plate than in cells migrating through the LGE or SVZ (51.74°±2.52°, n=102 cells) (Fig. 2D′), we observed a prominent increase in the frequency of cells that displayed relatively large angles (Fig. 2D′, asterisks). There were two possible interpretations of these results. One alternative is that the region of the cortical plate contains two distinct populations of migrating interneurons, which branch at different angles. Another possibility is that the same cells can make relatively small or large branch angles depending on the environment. In agreement with this second hypothesis, dynamic analysis of interneurons migrating around the cortical plate revealed that rapid changes in direction are consistently preceded by the formation of leading process branches at relatively large angles (n=7 cells) (Fig. 2D″; see Movie 4 in the supplementary material).

Consistent with prior observations in LGE-derived SVZ neuroblasts (Ward et al., 2005), our previous results suggest that tangentially migrating interneurons change direction by biased choices of leading process branches. In addition, the differences observed in the net angle formed by leading process branches between cells following quasi-linear trajectories and those rapidly changing direction suggest that the amplitude of the angle at which new branches form could be influenced by guidance cues present in the cortex. To test this hypothesis, we performed experiments in which we forced interneurons migrating in a

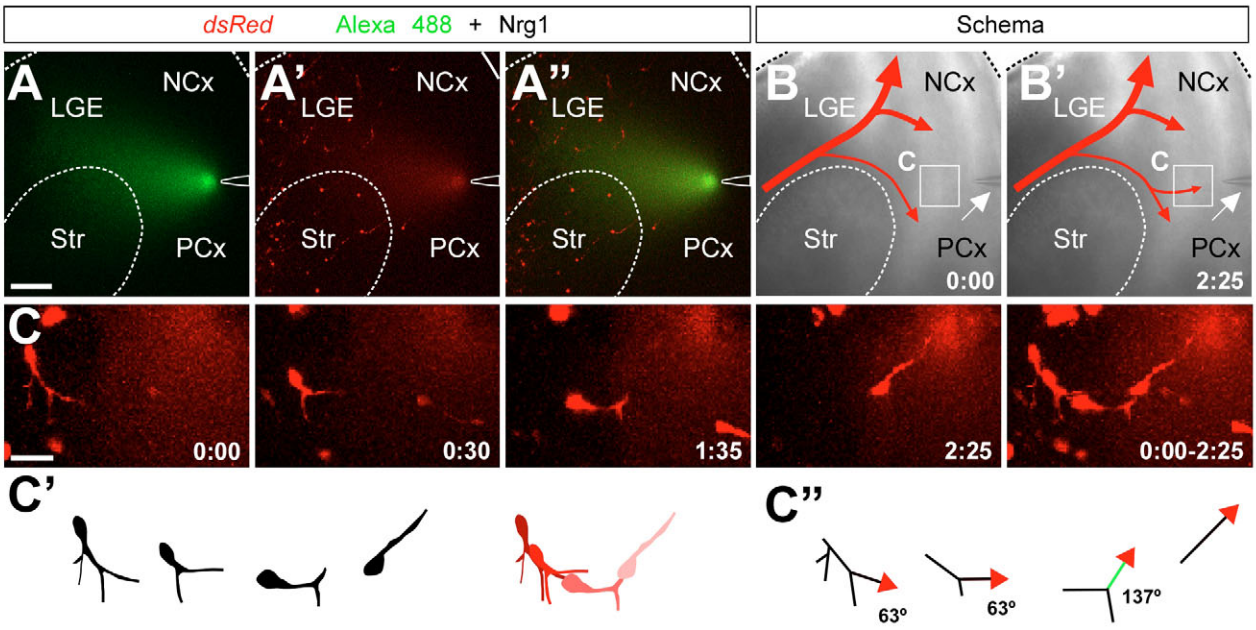

Fig. 3. Branch dynamics during Nrg1-induced chemotaxis. (**A-A″**) Images of a *dsRed*-electroporated slice perfused with a micropipette containing recombinant EGF domain from Nrg1 (13 nM) and Alexa 488 (green channel). To induce drastic changes in direction, cortical interneurons (red channel) migrating through the LGE were confronted with the micropipette at an angle that is perpendicular to their normal trajectory. (**B-B′**) Schematic representation of the trajectory change followed by the cell shown in C. White arrow indicates the micropipette. (**C**) Representative time-lapse sequences of a migrating cell that developed drastic trajectory changes in response to the chemoattractant. The cell generates a new leading process toward the pipette immediately before changing its trajectory (*t*=1:35). The angle generated before the most significant change in direction is the largest made by the neuron during this sequence (C). The cell chose the branch oriented towards the chemoattractant to continue migration. The gradient is also visualized in red owing to laser cross-contamination. (**C′-C″**) Drawings illustrate the morphology of the cell shown in C. Diagrams in C″ depict the movement of this cell. New branches are shown in green; chosen branch is tipped with a red arrowhead. The numbers indicate the angle formed by the branches. Scale bars: 100 m in A-A″,B,B′; 25 m in C,C′.

steady path to rapidly change their trajectory. For this, we focally electroporated the MGE of embryonic slices with *dsRed* and perfused the vicinity of individual migrating cells with a glass micropipette containing the cortical interneuron chemoattractant neuregulin 1 (Nrg1) (13 nM) (Flames et al., 2004). In these experiments, we consistently placed the micropipette perpendicular to the route followed by individual cells to stimulate a swift turn (Fig. 3A-A″). Observation of the migratory behavior of individual cells acutely attracted to a source of Nrg1 revealed that direction change was always preceded by the generation of a new leading process branch at a large angle (>60° in nine out of nine cells) (Fig. 3B,B′,C; see Movie 5 in the supplementary material). This new leading process branch was consistently oriented towards the source of the attractant, and was far more likely to be maintained than those extending in other directions (nine out of nine cells). This suggested that interneurons orient up intense chemoattractant gradients by branching the leading process at relatively larger angles than when they navigate in a constant direction, and by consistently choosing the new, better aligned branch, in their subsequent nuclear movement.

Chemotaxis in cortical interneurons requires leading process branching

Our previous results suggest that leading process branching is the mechanism that mediates directional sensing in cortical interneurons. To confirm this hypothesis, we performed experiments in which we pharmacologically perturbed the generation of new leading process branches in migrating interneurons. Previous studies have shown that Rho/ROCK inhibition causes the elongation of the leading process in pontine neurons through a mechanism that is likely to involve Rac activation (Causeret et al., 2004). When applied to telencephalic slices, the ROCK1/2 inhibitor Y-27632 (30 M) also decreased the migration of cortical interneurons (*n*=19 slices) (see Fig. S5 in the supplementary material). As expected (Causeret et al., 2004), decreased migration was in part due to a reduction in the frequency of nucleokinesis in tangentially migrating neurons (*n*=6 cells) (see Fig. S6 in the supplementary material). However, we also found that bath application (*n*=35 cells) (see Fig. S5 in the supplementary material) or direct pipette perfusion with Y27632 (*n*=5 cells) (see Fig. S7 and Movie 6 in the supplementary material) decreased the frequency of leading process branching in cortical interneurons.

To evaluate the chemotaxis behavior of MGE-derived interneurons when leading process branching is perturbed, we performed explant co-culture experiments with a source of Nrg1 (Flames et al., 2004). In brief, we co-cultured E13.5 MGE explants with aggregates of COS cells expressing the secretable isoform of *Nrg1* (*Ig-Nrg1*) in matrigel three-dimensional matrices (Fig. 4A). In control experiments, cells derived from an E13.5 MGE explant confronted with COS cells transfected with a mock plasmid migrated uniformly in all directions (Fig. 4B). To quantify the effect of Nrg1 on the trajectory of the neurons, we subdivided the circle enclosing the MGE explant into eight sectors of equal size and measured the maximum angle at which migrating cells deviate from the vertical (migration angle, α) (Fig. 4E). In control experiments,

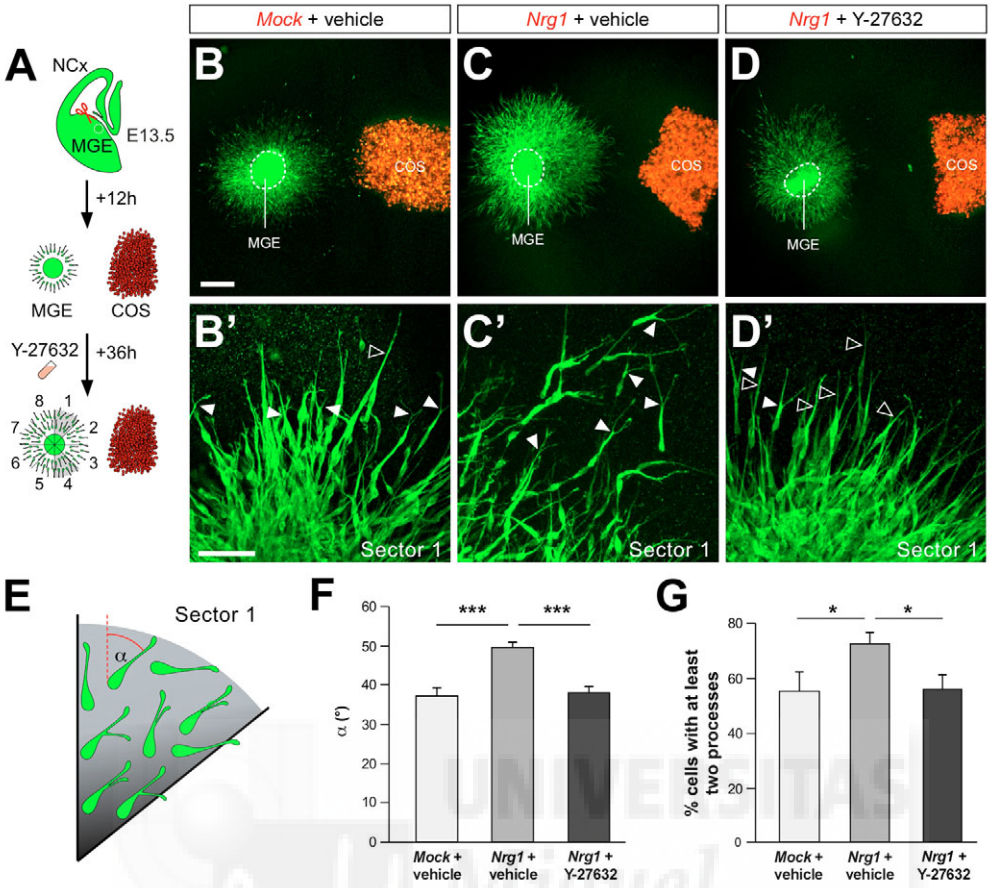

Fig. 4. Chemotaxis in MGE-derived cells requires leading process branching. (**A**) Schematic diagram of experimental design. (**B-D**) Migration of MGE-derived cells in response to mock-transfected (B) or *Nrg1*-transfected (C,D) COS cells aggregates cultured in matrigel matrices for 36 hours in the presence of vehicle solution (B,C) or the ROCK inhibitor Y27632 (30□M) (D). COS cells were also transfected with *dsRed* to aid their visualization. Broken lines indicate the limits of the explants before culture. (**B′-D′**) Confocal images of cells migrating through Sector 1, as defined in the schematic shown in A, in control (B′), Nrg1 (C′) and in Nrg1+Y27632 (30□M) (D′)-treated explants. Solid and open arrowheads indicate branched and non-branched interneurons, respectively. (**E**) Schematic view of the method used to quantify the orientation of cells (α angle). (**F**) Quantification of α angle in Sector 1. Bars show mean±s.e.m. 37.02±2.14° (control, *n*=167 cells from three independent experiments), 49.65±1.29° (Nrg1, *n*=520 cells from three independent experiments) and 38.10±1.41° (Nrg1 +Y27631, *n*=381 cells from three independent experiments). *t*-test, ***$P<0.001$. (**G**) Quantification of percentage of neurons located in Sector 1 with at least two leading process branches. Bars show mean±s.e.m. 54.85±7.41% (Ctrl, *n*=161 cells from three independent experiments), 72.21±4.49% (Nrg1, *n*=528 cells from three independent experiments) and 55.73±5.34% (Nrg1+Y27631, *n*=317 cells from three independent experiments). *t*-test, *$P<0.05$. Scale bars: 100□m in B,C,D; 40□m B′,C′,D′.

this angle was roughly 45°, with a mean value of ~35° (37.02±2.14°, *n*=167 cells from three independent experiments) (Fig. 4B′,E,F). By contrast, when MGE explants were co-cultured along COS cells expressing Nrg1, the maximum migration angle observed within sectors 1 and 4 was roughly 90°, with a mean value of ~50° (49.65±1.29°, *n*=520 cells from three independent experiments) (Fig. 4C,C′,E,F). These values reflect that many neurons derived from the MGE were attracted towards a source of Nrg1 (Flames et al., 2004), and therefore they reoriented their migratory path towards the COS cell aggregate. As in slices, addition of Y27632 to the explant culture medium modified the morphology of migrating neurons, decreasing the number of processes generated by interneurons (*n*=317 cells from three independent experiments) (Fig. 4G). In this scenario, migrating interneurons failed to reorient their migration towards Nrg1. The migration angle of MGE-derived neurons in response to Nrg1 in the presence of Y27632 was similar to that of explants confronted with control COS cells (38.10±1.41°, *n*=381 cells from three independent experiments) (Fig. 4D,D′,E,F), demonstrating that interneurons failed to reorient when leading process branching is perturbed.

The previous experiments suggest that leading process branching is required for interneurons to appropriately orient up intense chemoattractant gradients. A caveat of these experiments, however, is that blocking ROCK could directly interfere with Nrg1 signaling. To rule out this possibility, we performed another set of experiments in which we physically – rather than chemically – forced interneurons to change their migratory direction. In brief, we performed homotypic and isochronic transplants of small MGE pieces from E13.5 *Gfp*-transgenic mice into host slices from wild-type embryos and then follow the migration of MGE-derived cells (Fig. 5A). After a few hours, we cut off the pallium from organotypic slice cultures, therefore blocking the normal migratory pathway of cortical interneurons (Fig. 5A,B). In the absence of a cortex, migrating cells continue to migrate dorsally (Marín et al.,

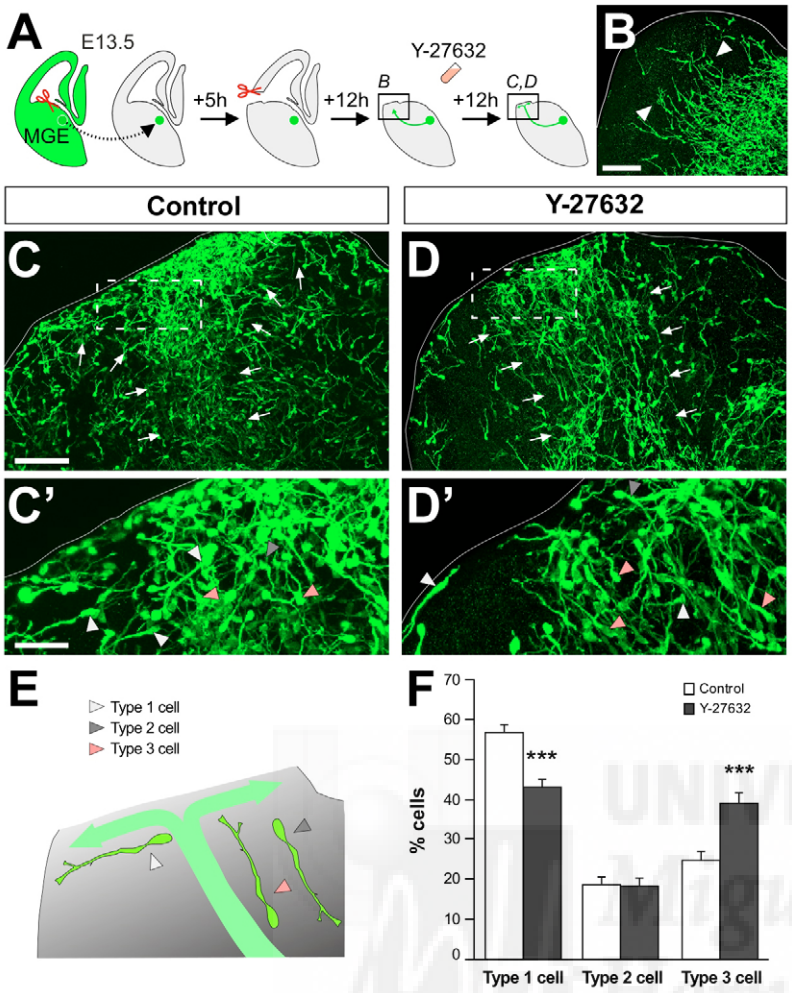

Fig. 5. Physically induced turning of interneurons requires leading process branching. (**A**) Schematic diagram of experimental design. After dissection of an E13.5 GFP MGE into small pieces, microtransplants were placed on a host E13.5 wild-type MGE and 5 hours later the pallium was removed from slices by cutting at the pallial-subpallial boundary. After 12 hours of incubation, GFP interneurons have migrated just a short distance from the cut (**B**). At this time, vehicle or Y27632 (30 M) was added to the medium and slices were incubated for another 12 hours. (**C-D′**) Images of GFP-expressing interneurons migrating in slices in the presence of vehicle (C,C′) or Y27632 (D,D′). (C′,D′) High-magnification images of the boxed areas shown in C,D, respectively. Note that interneurons reach the cut in a fairly delineated stream in both control and experimental slices (arrows in C,D). In control slices, many interneurons turn 90° to continue migrating parallel to the incision (C), while turning is diminished in the presence of Y27632 (D). (**E**) Light-gray, dark-gray and pink arrowheads indicate type 1 (leading process parallel to the cut), type 2 (leading process away from the cut) and type 3 (leading process towards the cut) cells, respectively. (**F**) Quantification of the relative proportion of type 1-3 cells in control and experimental slices. Histograms show averages ±s.e.m. Control: 56.56±1.79%, 18.68±1.86%, 24.76±2.09% for cell type 1, 2 and 3 respectively; 1151 cells from eight slices in three independent experiments. Y27632: 42.80±1.85%, 18.17±1.98%, 39.03±2.36% for cell type 1, 2 and 3 respectively; 743 cells from eight slices in three independent experiments. χ^2-test: ***P<0.001. Scale bars: 100 m in B,C,D; 25 m in C′,D′.

2003), but they are forced to turn 90° laterally when they reach the incision (Fig. 5A). Prior to the arrival of interneurons to the incision (Fig. 5A,B), we added vehicle solution or Y27632 (30 M) to the culture medium to test whether leading process branching was required for this rapid change of direction. After 12 additional hours in culture, we examined the orientation of GFP-expressing cells close to the incision (Fig. 5A). In control experiments, most migrating cells where oriented parallel to the incision (Type 1 cells) (Fig. 5C,C′,E,F), suggesting they have turned 90° after reaching the cut. In the presence Y27632, which decreases leading process branching (see Fig. S7 in the supplementary material), the percentage of migrating cells parallel to the incision was significantly reduced compared with controls (Fig. 5D,D′,E,F). Conversely, many more cells remained oriented towards the incision in Y27632-treated slices than controls (Type 3 cells) (Fig. 5D,D′,E,F). These experiments reinforced the view that leading process branching is required by interneurons to perform considerable changes in their migratory direction.

To explore whether turning also depends on branch dynamics in a physiologically relevant context, we next examined the behavior of interneurons in an environment in which they normally make profound changes in their migratory direction. For this, we analyzed the embryonic cortex at E15.5, when interneurons begin to reach their final position by rapidly deviating from their tangential routes towards the cortical plate (Ang et al., 2003; Polleux et al., 2002) (Fig. 6A). Although the molecules involved in this process are currently unknown (López-Bendito et al., 2008), we hypothesized that leading process branching would also be required for interneurons to perform this rapid change in direction (as in Fig. 2D″; see also Movie 5 in the supplementary material). To test this idea, we prepared slices from E15.5 *Gad65-Gfp* embryos that had received a BrdU pulse at E13.5 to unequivocally identify a cohort of synchronically born interneurons. At E15.5, interneurons have not yet begun to change their migration from tangential to radial, and most cells display a tangential orientation (94.56±1.61 %, n=74 cells from three slices of two independent experiments) (Fig. 6B). After 2 DIV, however, almost half of the entire population of interneurons has changed their orientation to radial (41.99±5.37% radially oriented, 58.00±6.02 % tangentially oriented; n=231 cells from four slices of two independent experiments) (Fig. 6C,E-G). By contrast, incubation of slices with Y27632 (30 M) greatly reduced the transition of migrating interneurons from tangential to radial orientation (23.21±2.42% radially oriented, 76.79±2.42% tangentially oriented, n=280 cells from four slices of two independent experiments) (Fig. 6D-F,H). Altogether, our experiments strongly suggest that leading process branching is required for the chemotaxis of cortical interneurons.

DISCUSSION

By using cortical interneurons as a model, we have found that neuronal migration do not always involve growth cone turning as the mechanism for chemotaxis. In these cells, and probably in many other tangentially migrating neurons in the brain and spinal cord, directional

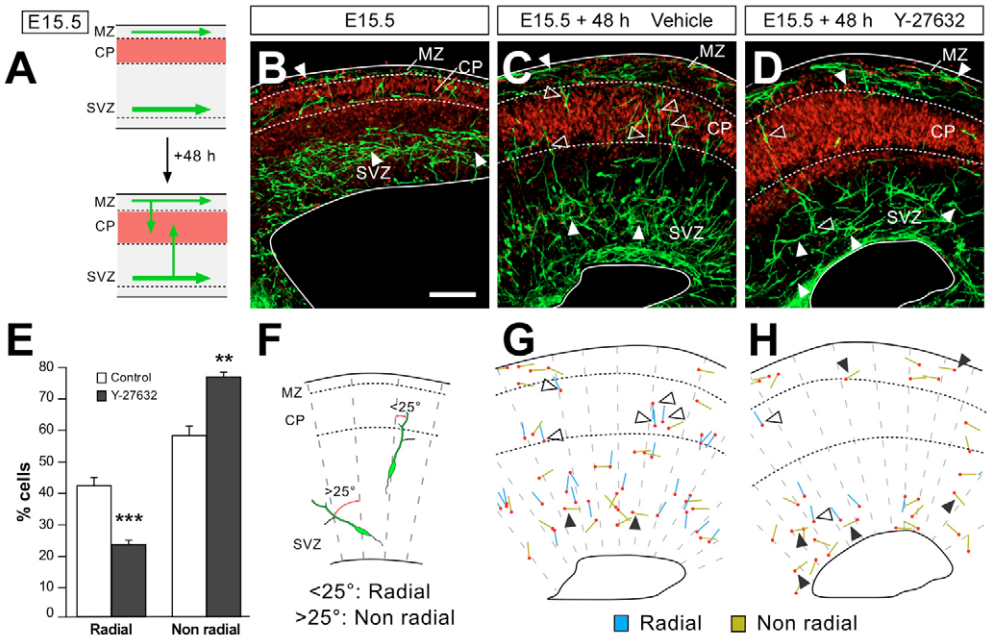

Fig. 6. Switch from tangential to radial position during CP invasion is perturbed after addition of Y27632. (**A**) Schematic showing the preferential orientation of interneurons in the cortex at E15.5 and after 48 hours in culture. Interneurons invade the CP performing a rapid change in direction (Ang et al., 2003; Polleux et al., 2002). (**B-D**) Images of cortical slices at E15.5 (B) or after 48 hours in culture (C,D) showing the distribution of GFP/BrdU+ interneurons, treated with vehicle (C) or Y27632 (D). Solid and open arrowheads indicate tangentially and radially oriented cells, respectively. (**E**) Quantification of percentage of GFP/BrdU+ radially and tangentially oriented neurons in control and Y27632-treated slices after 48 hours in culture. Bars show mean±s.e.m. Control: 41.99±5.37% (radially oriented), 58.00±6.02% (tangentially oriented), *n*=231 cells from four slices of two independent experiments; Y27632: 23.21±2.42% (radially oriented), 76.79±2.42% (tangentially oriented), *n*=280 cells from four slices of two independent experiments. χ^2-test, ***P<0.001, **P<0.01. (**F**) Schematic showing the criteria for the classification of the orientation of interneurons in cortical slices. (**G**,**H**) Schematic representation of the distribution of GFP/BrdU+ interneurons in C and D, respectively. Solid and open arrowheads indicate tangentially and radially oriented cells, respectively. Scale bar: 100□m.

sensing depends on the generation and stabilization of branches in the leading process rather than growth cone steering. Consequently, the geometry of the leading process branches (i.e. the angle at which they branch) determines the possible directions to be followed by migrating neurons. Thus, chemotaxis in neurons with leading process branches is linked to the stabilization of the most suitable branch.

Leading process branching characterizes many tangentially migrating neurons

Radially migrating neurons are frequently described as bipolar in morphology, with a single leading process oriented in the direction of movement (Nadarajah and Parnavelas, 2002). By contrast, the observation of both bifurcated and non-bifurcated leading processes in tangentially migrating interneurons have led to the suggestion that different types of cortical interneurons may use different modes of migration (Nasrallah et al., 2006; Polleux et al., 2002). Our results demonstrate that the same cells adopt these two different morphologies as part of their migratory cycle, suggesting that leading process branching is part of the mechanism used by cortical interneurons to migrate. Interestingly, many other tangentially migrating neurons appear to branch their leading process as part of their migratory cycle (López-Bendito et al., 2006; Ward et al., 2005) (this study), suggesting that this might be a general feature for very distinct neuronal types. This, however, does not imply that all neuronal populations undergoing tangential migration have branched leading processes. For example, tangentially migrating precerebellar neurons display a single leading process during their entire trajectory (Bourrat and Sotelo, 1990).

The use of branches during tangential migration has been previously interpreted as a mechanism employed by the cell to explore a wide territory, in which guidance signals encountered by the tips of both branches, attractive or repulsive, are likely to be different (Ward et al., 2005). The differential activation of guidance receptors at both branches could lead to different signaling activities, which may then translate into distinct tendencies towards growth or collapse. Once the misbalance between signals at both branches reaches a crucial level, then one of the branches retracts and the other stabilizes. These observations suggest that the dynamic behavior of the two leading processes is highly interdependent, functioning in a continuous competing mode: a one-win/one-lose dichotomy. In that sense, the behavior of cortical interneurons appears to resemble more closely that of some unicellular organisms in which chemotaxis involves the generation of new protrusions by splitting the leading edge of the cell (Andrew and Insall, 2007). Whether similar mechanisms coordinate the generation of new pseudopods in *Dictyostelium* and leading process branches in cortical interneurons remains to be elucidated.

Leading process branching mediates directional guidance

For axons, directional growth in response to chemotactic cues is thought to involve a compass-like behavior in which axons are continuously instructed to the correct direction by differential actin polymerization across the growth cone (Wen and Zheng, 2006). In the case of tangentially migrating neurons, the dynamic behavior of the leading process, which continuously branch as part of the migratory cycle, suggests that these cells do not achieve directional

migration through a mechanism that involves leading process steering. In these cells, growth cone dynamics seem to be relevant only for the initial orientation of the branch. After that, they appear to serve exclusively for the elongation or retraction of the branches, the orientation of which remains stable during their entire lifetime. Consequently, directional movement is mediated by biased choices of nuclear movements into one of the leading process branches.

During quasi-linear migration, the nucleus alternates branches to maintain a defined course. Remarkably, the probability of selecting each of the two alternative branches is almost 50% during quasi-linear migration, which suggest that interneurons are set to follow a rather constant course when migrating in relatively shallow chemotactic gradients. By contrast, our observations suggest that interneurons achieve rapid changes of direction by modifying their basic behavior of leading process branching and selection. Under the influence of intense chemoattractant gradients, interneurons retract systematically those branches that are not oriented towards the source of the chemoattractant, branch at larger angles than they normally do when migrating in quasi-linear trajectories, and preferentially select the new branches, which are better aligned with the source of the chemoattractant. The importance of leading process branching in directional guidance is illustrated by our pharmacological manipulations, which suggest that a perturbation of the frequency at which interneurons branch their leading process alters their ability to react to guidance cues (e.g. Nrg1). A caveat of these experiments, however, is that blocking ROCK could not just prevent branching, but also directly interfere with Nrg1 signaling. We believe this is unlikely because inhibition of Rho-ROCK signaling appears to enhance rather than inhibit chemoattractive guidance in spinal commissural axons (Moore et al., 2008). Moreover, ROCK inhibition does not block Nrg1-mediated cell adhesion in B lymphoblasts (Kanakry et al., 2007), suggesting that Nrg1 signaling does not involve ROCK function. In any case, inhibition of leading process branching in slice cultures also makes rapid directional changes more difficult for interneurons under other circumstances. For example, inhibition of branch formation reduce the ability of interneurons to turn under mechanical constrains or during the process of cortical plate invasion. These experiments reinforce the view that the dynamic behavior of leading process branching is required for the chemotaxis of tangentially migrating interneurons.

In conclusion, our results suggest that directional migration can be achieved not only through growth cone steering, as it happens in neurons with a bipolar shape, but also by biased choices of leading process branches in those neurons that have a leading process with a more elaborate morphology.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/1/41/DC1

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Supplemental data

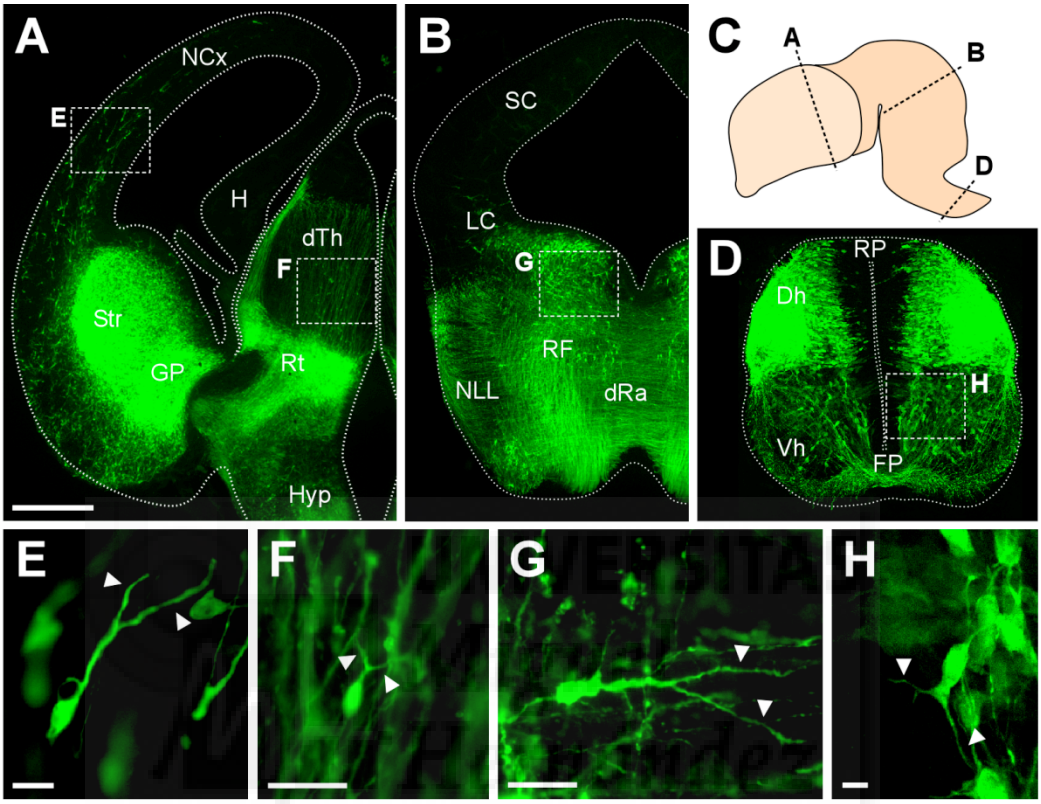

Figure S1. Leading process branching is common to many neurons in the developing brain. (A-D) Serial sections through the brain (A,B) and spinal cord (D) of Gad65-Gfp embryos at E12.5 (D) and E13.5 (A,B). In Gad65-Gfp embryos, Gfp expression is restricted to GABAergic neurons, some of which have typical morphologies of migrating cells. The schematic drawinsg in C depict the approximate localization of sections (A,B,D). (E-H) High-magnification images of cells migrating in the developing cortex (E), thalamus (F), midbrain reticular formation (G) and ventral spinal cord (H). All these cells display similar morphologies characterized by the presence of a branched leading process (arrowheads). H, hippocampus; Dh, dorsal horn of the spinal cord; dTh, dorsal thalamus; dRa, dorsal raphe nucleus; FP, floor plate; GP, globus pallidus; Hyp, hippothalamus; NCx, neocortex; NLL, nucleus of the lateral lemniscus; RF, reticular formation; RP, roof plate; Rt, reticular nucleus; SC, superior colliculus; Str, striatum; LC, locus coeruleus; Vh, ventral horn of the spinal cord. Scale bars: 300 μm in A,B,D; 10 μm in E-H.

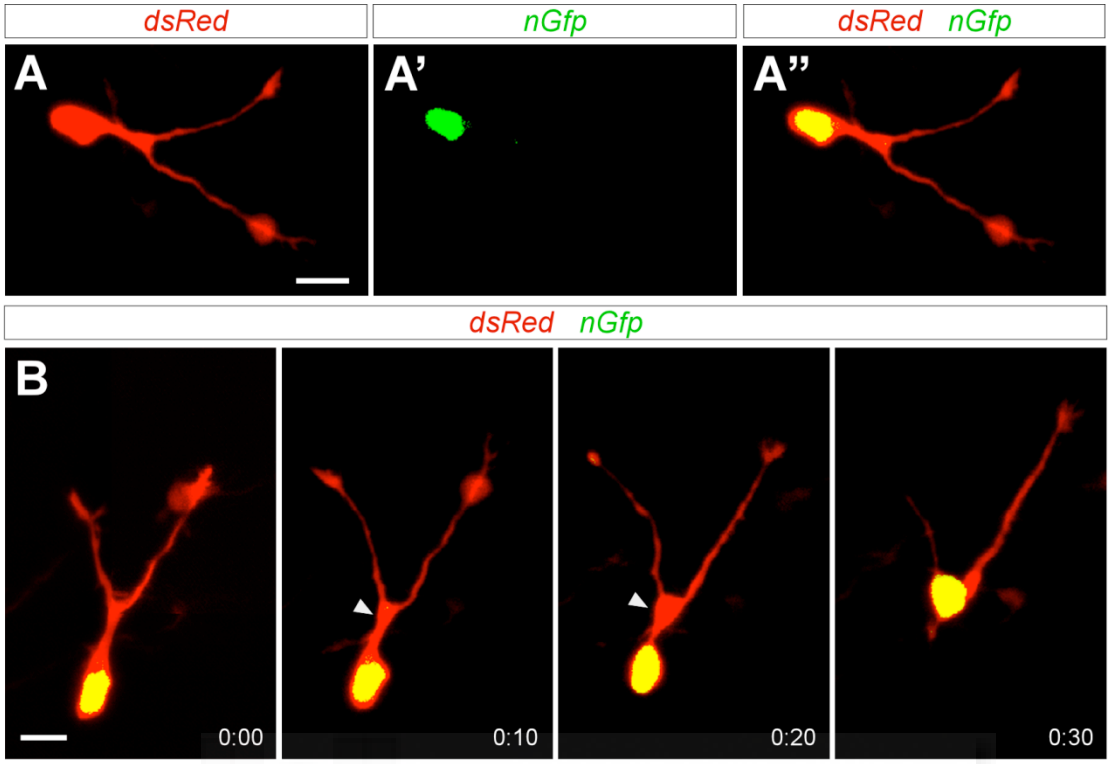

Figure S2. Nuclear movement in tangentially migrating interneurons. (A-A") An E13.5 tangentially migrating interneuron electroporated with dsRed and nuclear Gfp (nGfp). (B) Time-lapse sequence of an E13.5 dsRed-electroporated interneuron migrating from the subpallium to the cortex in a slice culture. Time is depicted in hours. Note that nuclear movement is preceded by the formation of a swelling in front of the nucleus (arrowhead). Scale bar: 10 µm

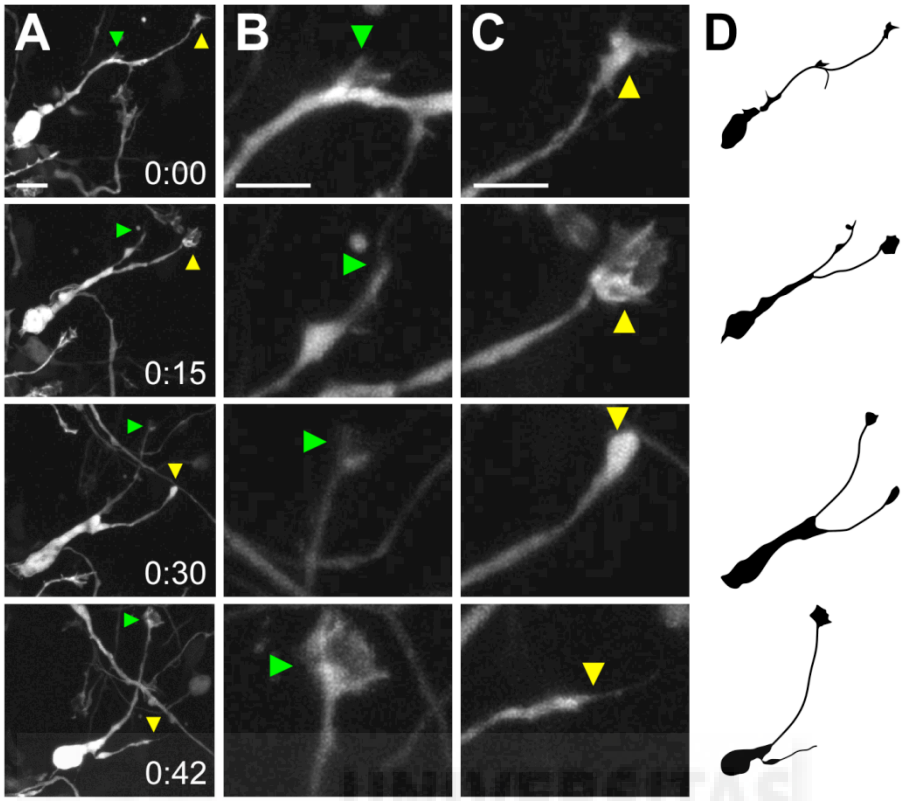

Figure S3. Leading process branch selection during tangential migration is preceded by coordinated growth cone structural modifications. (A) Time-lapse sequence of an E13.5 dsRed-electroporated interneuron migrating from the subpallium to the cortex in a slice culture. Time is depicted in hours. Only selected frames from the entire sequence are shown. Color-coded arrowheads indicate individual growth cones tipping each of the branches of the leading process. (B,C) High-magnification images of the tips of the leading process branches shown in A, illustrating the morphology of individual growth cones as the branch growths (B) or retracts (C; t=0:30, t=0:42). (D) Drawings illustrating the morphology of the migrating neuron for each of the frames shown in A. Scale bars: 10 µm in A; 5 µm in B,C.

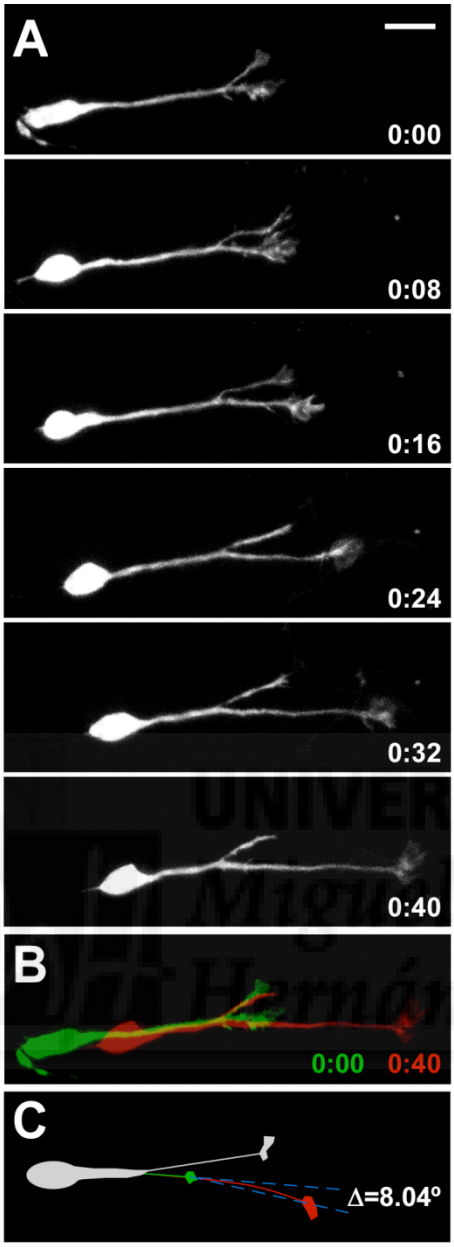

Figure S4. Leading process branches do not steer much during elongation. (A) Time-lapse sequence of a dsRed-electroporated interneuron migrating from the subpallium to the cortex in a slice culture. Time is depicted in hours (h). (B) Superimposed images of the frame t=0:00 h (green) and t=0:52 h (red). Despite prominent growth, there is very little variation in the direction followed by both leading process branches. (C) Schematic of an average cell, showing the angular shift of a process between incipient (green) and final stages (red). A total of 27 processes were measured, corresponding to 14 nucleokinetic phases in 10 different cells. The average angular shift of a process during its lifetime (birth to retraction/branching) ±s.e.m. was 8.04±1.46°. The average angular shift of a process after 10-15 minutes was only 1.86±0.31°. Scale bar: 10 µm.

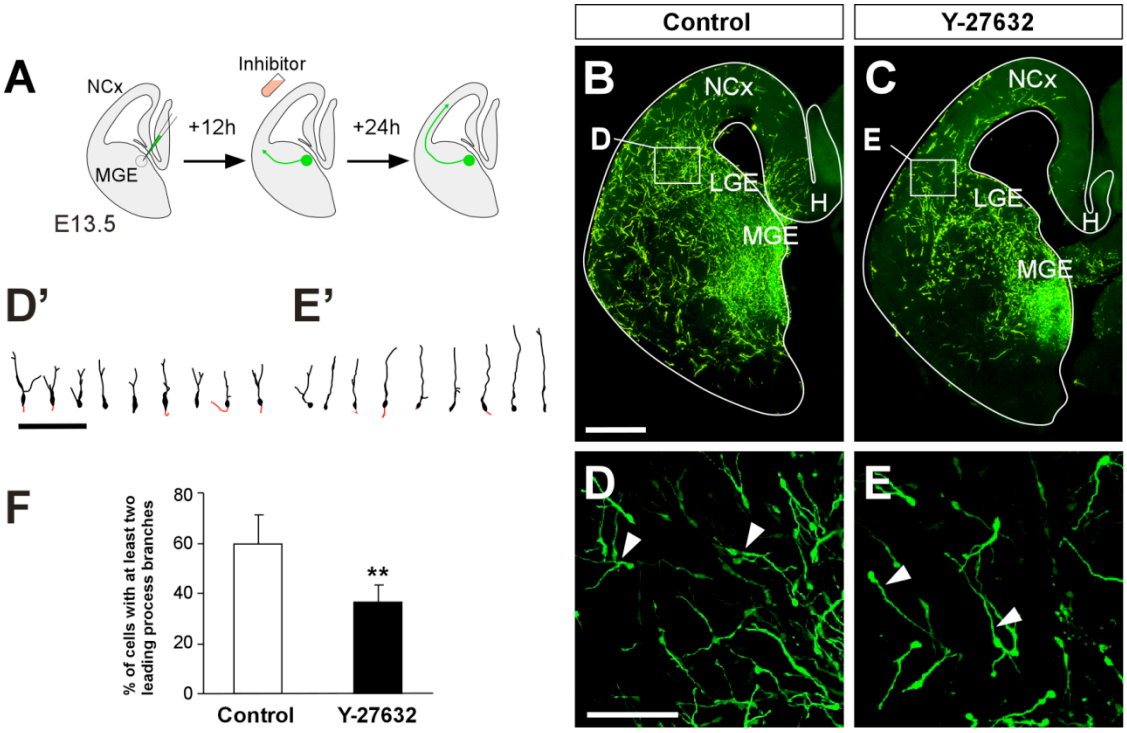

Figure S5. The migration of cortical interneurons depends on ROCK function. (A) Experimental paradigm for the pharmacological inhibition of ROCK in organotypic slices. Gfp-electroporated interneurons have migrated for 12 hours before the addition of vehicle solution or inhibitor to the culture medium. (B,C) Representative examples of Gfp-electroporated interneurons migrating from the medial ganglionic eminence (MGE) to the neocortex (NCx) in organotypic slices cultured in medium containing vehicle solution (B) or the ROCK inhibitor Y27632 (30 µM) (C). After 36 hours in culture, migrating interneurons reach the cortex in large numbers in control slices than in slices treated with Y27632 (control: 75% of electroporated slices with at least 100 cells in the cortex, n=8; Y27632: 21% of electroporated slices with at least 100 cells in the cortex, n=19). (D,E) High-magnification images of neurons migrating in slices treated with vehicle solution (D) or the ROCK inhibitor Y27632 (E). Images were obtained from the boxed regions in B and C. Arrowheads indicate representative cells for each case. (D',E') Drawings of representative neurons migrating in slices cultures treated with vehicle solution (D') or the ROCK inhibitor Y27632 (E'). (F) Quantification of the effect of the pharmacological inhibition of ROCK in the relative abundance of neurons with a branched leading process (n=35). X^2 test, **P<0.01. H, hippocampus; LGE, lateral ganglionic eminence. Scale bar: 300 µm in B,C; 100 µm in D,D',E,E'.

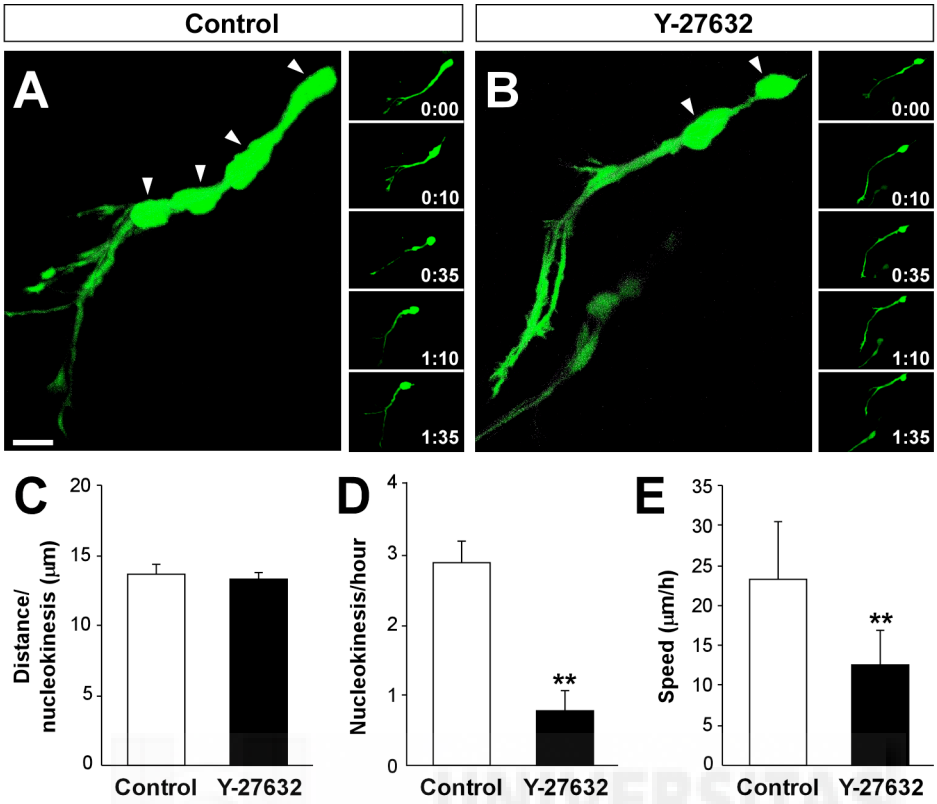

Figure S6. ROCK function is required for nucleokinesis in migrating interneurons. (A,B) Time-lapse sequences of Gfp-electroporated interneurons migrating in organotypic slices cultured with vehicle solution (A) or in the presence of the ROCK inhibitor Y27632 (30 µM) (B). Superimposed images of selected cumulative frames from a recording lasting 1:35 hours for both conditions. Selected individual frames are also shown for each case. Arrowheads indicate the position of the nucleus at the end of discrete active periods of nucleokinesis. (C) Quantification of the distance migrated by the nucleus in each nucleokinesis. Histograms show mean±s.e.m.: 13.66±0.75 µm (control, n=5), 13.27±0.46 µm (Y27632, n=6); t-test, P=0.412. (D) Quantification of the nucleokinesis frequency. Histograms show mean±s.e.m.: 2.91±0.31 µm (control, n=5), 0.77±0.30 µm (Y27632, n=6); t test, **P<0.01. (E) Quantification of the migration speed. Histograms show mean±s.e.m. 23.34±7.13 µm/hour (control, n=5), 12.52±4.33 µm/hour (Y27632, n=6); t-test, **P<0. 01. Scale bar: 10 µm.

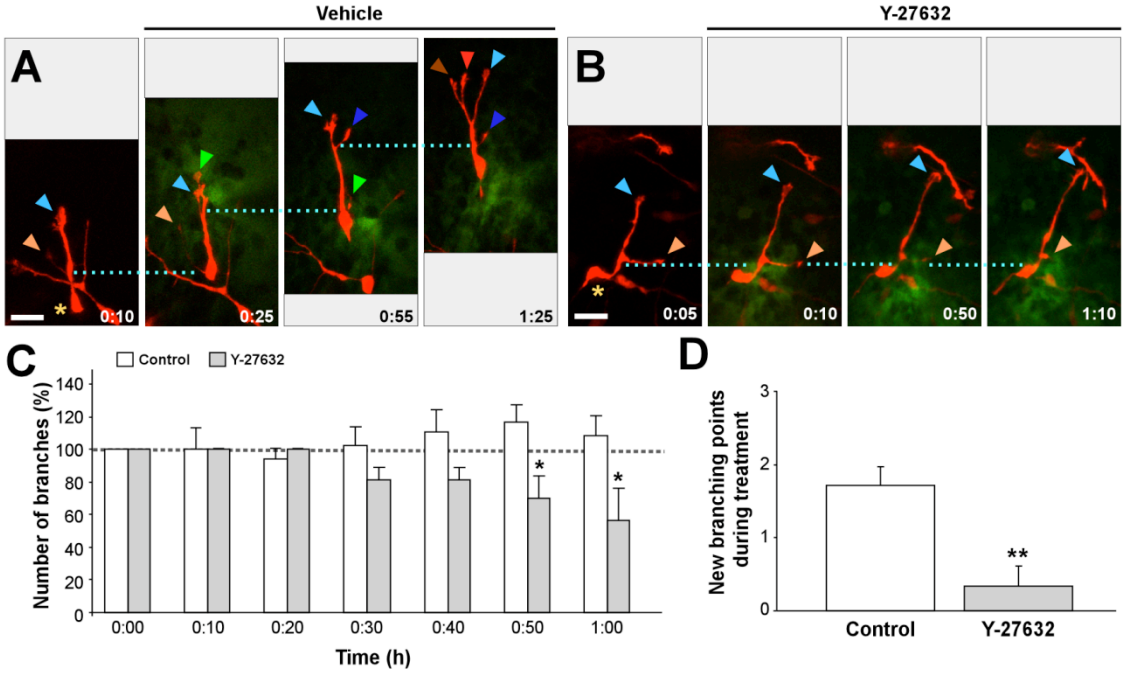

Figure S7. ROCK inhibition perturbs leading process branching in migrating interneurons. (A,B) Time-lapse sequences of E13.5 dsRed-electroporated interneurons (asterisks) migrating from the subpallium to the cortex in slice cultures. Time is depicted in hours. The broken lines indicate the position of the bifurcations from frame to frame, and each leading process branch is coded with color arrowheads. In A, vehicle solution containing Alexa 488 was applied through a glass micropipette from t=0:25 hours to t=1:25 hours, whereas in B, 3 mM Y27632 was applied through a glass micropipette from t=0:10 hours to t=1:10 hours. (C) Quantification of the relative number of branches in control and Y27632-treated neurons during micropipette application, represented in relative proportion to the initial state for each case. Histograms show mean±s.e.m. t-test, *P<0.05 (n=6). (D) Quantification of the number of new branching points in the leading process of control and Y27632-treated neurons during micropipette application. Histograms show mean±s.e.m.: 1.33±0.52 (control, n=6), 0.33±0.52 (Y27632, n=6); t-test, **P<0.01.

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Part 2. Focal adhesion kinase controls neuronal radial migration by regulating connexin-mediated adhesion to radial glia.

Manuel Valiente, Beatriz Rico, and Oscar Marín.

Submitted.

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Summary

FAK is an intracellular kinase and scaffold protein that regulates cell-matrix and cell-cell interactions in many different cellular contexts, but whose function in neuron migration remains controversial. While FAK has been shown to regulate microtubule dynamics during radial migration, current genetic evidence suggests that neuronal FAK is dispensable for this process. Here, we have analyzed the function of FAK in cortical pyramidal neuron migration and found that this protein cell-autonomously regulates cell-matrix and cell-cell adhesions during neuronal migration. Loss of FAK function prevents neuron-glia interactions, disrupts the normal morphology of migrating neurons, delays migration and increases the tangential dispersion of neurons arising from the same radial unit. Remarkably, FAK signaling is not only involved in regulating classical integrin-based adhesions, but it also controls the formation of connexin cell-cell contact points. These results indicate that FAK plays a fundamental role in the dynamic regulation of adhesions during glial-guided neuronal migration.

Introduction

The mammalian neocortex is a six-layered structure in which different cohorts of neurons are precisely organized according to their time of neurogenesis. The earliest cortical neurons, the Cajal-Retzius cells, colonize the surface of the cortex through tangential migration at the time when the pallial ventricular zone (VZ) begins to generate the first cohort of pyramidal neurons, which will eventually populate the subplate (Allendoerfer and Shatz, 1994; Bielle et al., 2005; Takiguchi-Hayashi et al., 2004). Subsequent cohorts of newborn neurons migrate past their predecessors to settle in the progressively more superficial positions within the cortical plate (CP), in which the different layers of the neocortex will differentiate (Gupta et al., 2002; Marín and Rubenstein, 2003). This “inside-out” pattern of cortical lamination is then refined during the differentiation of pyramidal neurons to conform the adult architecture of the neocortex (Caviness et al., 2008).

It is now well established that pyramidal neurons derive from radial glial cells, multifunctional progenitors that also provide the required scaffold for the migration of many cortical neurons (Campbell and Gotz, 2002; Ever and Gaiano, 2005). Radial glial cells reside in the VZ and have a long basal process that extends to the pial surface, where it anchors into the basement membrane. During cortical development, the first cohorts of newborn pyramidal neurons reach the CP through a mechanism known as somal translocation, which is independent of their interaction with the processes of radial glial cells (Kriegstein and Noctor, 2004; Miyata et al., 2001; Nadarajah et al., 2001; Nadarajah and Parnavelas, 2002). As the cortex grows, however, pyramidal neurons migrate progressively longer distances to reach the CP, and this requires the support of radial glial cells. This process of glial-guided migration involves a dynamic interaction between migrating neurons and the basal process of radial glial cells, which serves as the main substrate to support their movement (Kriegstein and Noctor, 2004; Nadarajah and Parnavelas, 2002; Rakic, 1972; Rakic et al., 1974).

Molecules controlling the adhesion of migrating neurons to radial glial cells are thought to play a prominent role in glial-guided migration. Along with other adhesion molecules (Hatten, 2002), extracellular matrix (ECM) proteins and their integrin family of surface receptors have been implicated in regulating the migration of pyramidal neurons (Anton et al., 1999; Schmid et al., 2004). Although their precise contribution to cortical lamination in vivo remains controversial (Belvindrah et al., 2007), integrin-based adhesions are thought to support the movement of pyramidal neurons along radial glial fibers. Moreover, regulation of the disassembly of integrin-based adhesions seems critical for cortical lamination (Sanada et al., 2004). In addition to ECM adhesion, recent work has shown that cell-cell contacts are essential for glial-guided migration (Cina et al., 2009; Elias et al., 2007). These contacts points between migrating neurons and radial glial fibers take the form of Gap junction adhesions, in which only the adhesive properties of connexins are required to support migration. Thus, current evidence suggests that glial-guided locomotion of pyramidal neurons involves the dynamic regulation of cell-matrix and cell-cell adhesions.

Although much is known about the molecules that mediate the interaction between migrating neurons and radial glial fibers, our understanding of the intracellular mechanisms controlling this dynamic process is very limited. In other cell types, the intracellular kinase and scaffold protein Focal Adhesion Kinase (FAK) regulates the formation and disassembly of cell-matrix and cell-cell during adhesion, spreading and migration (Avizienyte and Frame, 2005; Mitra et al., 2005). In migrating neurons, however, FAK function remains controversial. While current genetic evidence suggests that FAK function is only indirectly required for neuronal migration through its function in radial glia cells (Beggs et al., 2003), other experiments have implicated FAK in regulating microtubule dynamics during nucleokinesis in migrating neurons (Xie et al., 2003). Here, we show that FAK cell-autonomously controls neuronal migration, but it does so by primarily regulating adhesion between migrating neurons, ECM proteins and radial glial cells. Beyond its classical function in regulating integrin-based adhesions, our results indicate that FAK is an important regulator of connexin adhesions, which are thought to provide dynamic contact points between migrating neurons and radial glial fibers. These results demonstrate that FAK plays a fundamental role in the dynamic regulation of adhesions during neuronal migration.

Results

FAK is required for cortical pyramidal neuron migration in vivo

To unravel the function of FAK in the migration of pyramidal neurons, we knocked down FAK protein levels in neocortical cells by using RNA interference (RNAi). In brief, plasmids encoding for control or short hairpin RNA (shRNA) constructs that produced significant knockdown of mouse FAK by western blot (Figure S1), along with a plasmid encoding for the enhanced Green fluorescent protein (GFP), were co-transfected in progenitor cells in the dorsal pallium by in utero electroporation at embryonic day (E) 14.5, and the distribution of migrating neurons was analyzed at E18.5 (Figure 1A). Consistent with previous reports (Bai et al., 2003; Hand et al., 2005; Kawauchi et al., 2003; Shu et al., 2004), analysis of mouse embryos electroporated with control plasmids (*shLuc*) revealed that most cortical neurons had reached the cortical plate (CP) at this stage (40.60 ± 2.66 %, *n* = 11; Figures 1C and 1F). By contrast, FAK knock down using two different plasmids encoding for FAK shRNA led to a pronounced migration defect, with accumulation of transfected cells in the intermediate zone (IZ) and a marked reduction in the proportion of neurons reaching the CP compared to controls [*shFak(1)*: 20.43 ± 3.74 %, *n* = 3. *shFak(2)*: 30.62 ± 2.16 %, *n* = 11; Figures 1D and 1F, and Figure S2]. To verify the target specificity of the shRNA effect and demonstrate that the observed phenotype was specifically due to loss of FAK, we took advantage of the fact that one of the shRNA constructs used in our assays targets a sequence that is not conserved in chicken *Fak* (four base-pairs mismatches, see Experimental Procedures). In utero electroporation of E14.5 mouse embryos with plasmids encoding for *Gfp*, *shFak* and chicken *Fak* resulted in a complete recovery of the migration phenotype by E18.5, with a similar proportion of transfected neurons invading the CP than in controls (43.40 ± 6.07 %, *n* = 10; Figures 1E and 1F).

To further ensure that the phenotype observed in FAK knockdown experiments was exclusively due to loss of FAK, we next deleted Fak from cortical progenitors by electroporating a plasmid encoding for the recombinase Cre (Cre-i-Gfp; where i stands for internal ribosome entry site, IRES) in the dorsal pallium of E14.5 mouse embryos harboring conditional Fak alleles (Fakflox/flox mice, Beggs et al., 2003b). As in RNAi experiments, Cre-mediated deletion of Fak reduced the proportion of neurons reaching the CP compared to control embryos (Gfp: 52.42 ± 0.85 %, n = 6; Cre-i-Gfp: 35.19 ± 1.22 %, n = 4; Figures 1B, 1G, 1H and 1I). Of note, genetic deletion of Fak caused a milder migratory phenotype than Fak knockdown (compare Figures 1D, 1F and 1H, 1I). This disparity is likely due to the differential timing of FAK removal between the two types of experiments, since conditional deletion of Fak must be preceded by expression of Cre and the subsequent recombination of the targeted locus. Consistent with this idea, when the analysis of the migratory phenotype was restricted to the population of neurons born one day after Cre electroporation (as assayed by BrdU incorporation, see Figure S3), the migratory phenotype was very similar to that found in Fak knockdown experiments (compare Figures 1F and S3). Altogether, these experiments demonstrated that FAK function is required for the migration of pyramidal neurons in vivo.

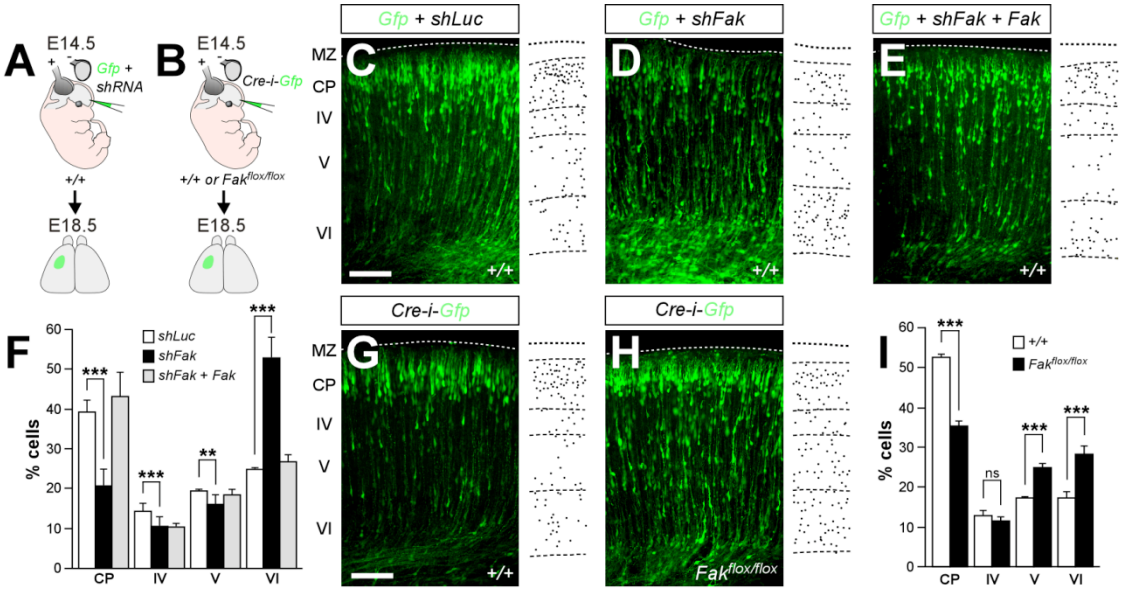

Figure 1. Removal of FAK in cortical projection neurons disrupts neuronal migration. (A and B) Schematic diagrams of experimental designs. (C-E) Coronal sections through the somatosensory cortex of E18.5 wild type embryos after E14.5 in utero electroporation with plasmids encoding for *Gfp* and *shLuc* (C), *shFak* (D), or *shFak* + *Fak* (E). Schemas with dots depict representative distributions of GFP-expressing neurons in each condition. (F) Quantification of the relative distribution (%) of GFP-expressing cells in different cortical layers for each condition. Histograms show mean ± s.e.m. from at least 3 different brains. *shLuc*: CP, 40.60 ± 2.66; layer IV, 14.39 ± 2.01; layer V, 19.71 ± 1.55; layer VI, 25.04 ± 0.94. *shFak*: CP, 20.43 ± 3.74; layer IV, 10.71 ± 2.54; layer V, 16.22 ± 2.23; layer VI, 52.44 ± 4.77. X^2-test for *shLuc* and *shFak* comparison: CP, $P < 0.001$; IV, $P < 0.001$; V, $P < 0.01$; VI, $P < 0.001$. *shFak* + *Fak*: CP, 43.40 ± 6.07; layer IV, 10.51 ± 1.27; layer V, 18.62 ± 1.41; layer VI, 26.95 ± 1.99. X^2-test for *shFak* and *shFak* + *Fak* comparison: CP, $P < 0.001$; IV, ns; V, $P < 0.01$; VI, $P < 0.001$. (G and H) Coronal sections through the somatosensory cortex of E18.5 wild type (G) and *Fak*$^{flox/flox}$ (H) embryos after E14.5 in utero electroporation with a plasmid encoding for *Cre-i-Gfp*. Schemas with dots depict representative distributions of GFP-expressing neurons in each condition. (I) Quantification of the relative distribution (%) of GFP-expressing cells in different cortical layers for each condition. Histograms show mean ± s.e.m. from at least 3 different brains. Control (*Cre-i-Gfp* in wild type embryos, *Gfp* in *Fak*$^{flox/flox}$ embryos or *Gfp* in wild type embryos): CP, 52.42 ± 0.85; layer IV, 12.72 ± 1.23; layer V, 17.18 ± 0.33; layer VI, 17.21 ± 1.61. *Cre-i-Gfp* in *Fak*$^{flox/flox}$ embryos: CP, 35.19 ± 1.22; layer IV, 11.37 ± 1.04; layer V, 24.77 ± 1.10; layer VI, 28.05 ± 2.12. X^2-test for the comparison: CP, $P < 0.001$; IV, ns; V, $P < 0.001$; VI, $P < 0.001$. See Figure S8 for additional statistical analyses. Scale bar: 100 µm.

Loss of FAK impairs the morphology of cortical migrating neurons

To get insight into the mechanisms through which FAK may contribute to the migration of pyramidal neurons, we examined the morphology of migrating neurons prior to their entrance in the CP, where most FAK-deficient neurons accumulated. As part of their migratory cycle, migrating neurons form a cytoplasmic dilatation in the leading process that contains the centrosome and the Golgi apparatus, towards which the nucleus translocates forward in a subsequent stage (Bellion et al., 2005). Consistent with previous reports, control transfected neurons were found to contain either one or none dilatations in the leading process, depending on the stage of their migratory cycle at which they were fixed (n = 176 cells analyzed from 3 different embryos; Figures 2C and 2E). In contrast, we observed that migrating neurons transfected with *shFak* frequently contained several of such dilatations (n = 173 cells analyzed from 3 different embryos; Figures 2D and 2E). In addition, migrating neurons transfected with *shFak* were found to harbor elaborated leading processes (Figures 2G, 2I and 2J), whereas control transfected neurons typically had a single or bifurcated leading process (Figure 2F, 2H and 2J). Of note, these

phenotypes were not unique to RNAi experiments, as similar morphological defects were found in migrating neurons after conditional deletion of *Fak* (Figure S4). Thus, loss of FAK leads to profound modifications in the morphology of migrating pyramidal neurons.

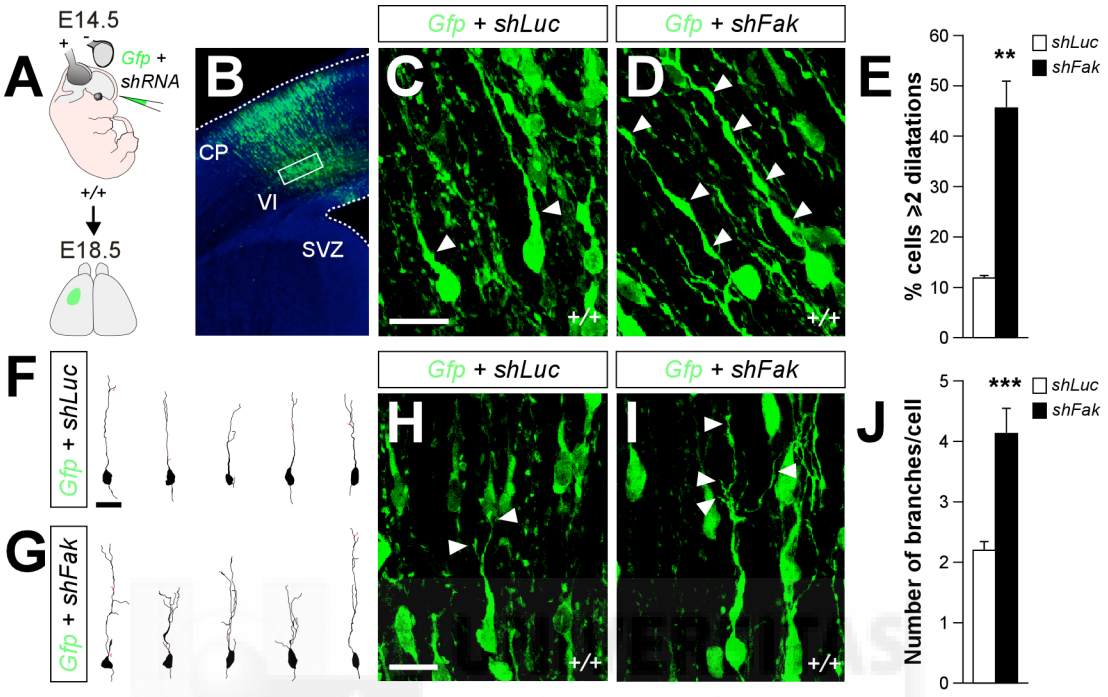

Figure 2. Migrating FAK-deficient projection neurons display important morphological defects. (A) Schematic diagram of experimental design. (B) Representative example of a coronal section through the somatosensory cortex of an E18.5 wild type embryo after E14.5 in utero electroporation showing the approximate region containing the neurons analyzed. Cell morphology was analyzed in layer VI, where the highest amount of cells accumulated upon *Fak* knockdown. (C and D) Representative examples of GFP-expressing migrating cells after in utero electroporation with plasmids encoding for *Gfp* and *shLuc* (C) or *shFak* (D). Note the increased number of cytoplasmic dilatations in FAK-deficient neurons. (E) Quantification of the percentage of cells with two or more dilatations in the leading process. Histograms show mean ± s.e.m. from at least 3 different brains. *shLuc*: 10.83 ± 0.36, n = 176 cells. *shFak*: 45.33 ± 5.21, n = 173 cells. **t-test , P = 0.0027. (F and G) Drawings illustrate the morphology of migrating cells expressing *Gfp* and *shLuc* (F) or *shFak* (G). Note the increased number of leading process branches in FAK-deficient neurons (G). (H and I) Representative examples of GFP-expressing migrating cells after in utero electroporation with plasmids encoding for *Gfp* and *shLuc* (H) or *shFak* (I). Note the increased number of leading process branches in FAK-deficient neurons (I). (J) Quantification of the number of leading process branches. Histograms show mean ± s.e.m. from at least 3 different brains. *shLuc*: 2.2 ± 0.13, n = 30 cells; *shFak*: 4.13 ± 0.37, n = 30 cells. ***t-test, P = 7,46432E-06. Scale bars equal 15 µm (C, D, H and I) and 25 µm (F and G).

FAK is cell-autonomously required for cortical pyramidal neuron migration

Since in utero electroporation primarily targets progenitor cells contacting the ventricle (i.e. radial glial cells, Noctor et al., 2001b), loss of FAK function could impair cortical migration by affecting radial glia, migrating neurons or both. Previous work has suggested that targeted deletion of *Fak* in the dorsal telencephalon perturbs neuronal migration by disrupting radial glia and the cortical basement membrane, but not by directly affecting migrating neurons (Beggs et al., 2003). In our experiments, however, radial glia morphology seems normal (Figure S5), suggesting that loss of FAK in migrating neurons may indeed perturb migration. To test this hypothesis, we generated conditional mutants embryos in which *Fak* was deleted using *NEX-Cre* mice (Goebbels et al., 2006). In this strain, the promoter region of the

transcription factor *Math2* drives the expression of *Cre*, which is thereby confined to a subset of subventricular zone (SVZ) progenitors and most migrating cortical pyramidal neurons, but excluded from radial glial cells (Wu et al., 2005). To analyze the migration of pyramidal neurons, we electroporated in utero a plasmid encoding for *Gfp* in either *Fak*$^{flox/flox}$ (control) or *NEX-Cre;Fak*$^{flox/flox}$ (mutant) embryos (Figure 3A). We observed that loss of *Fak* function in migrating neurons leads to migratory defects similar to those found in our initial experiments, with relatively fewer cells reaching the CP in mutant embryos than in controls (controls: 46.50 ± 2.52 %, *n* = 4; mutants: 37.19 ± 2.82 %, *n* = 4; Figures 3B–3D). These experiments demonstrated that FAK function is cell-autonomously required in pyramidal neurons for their correct migration to the CP.

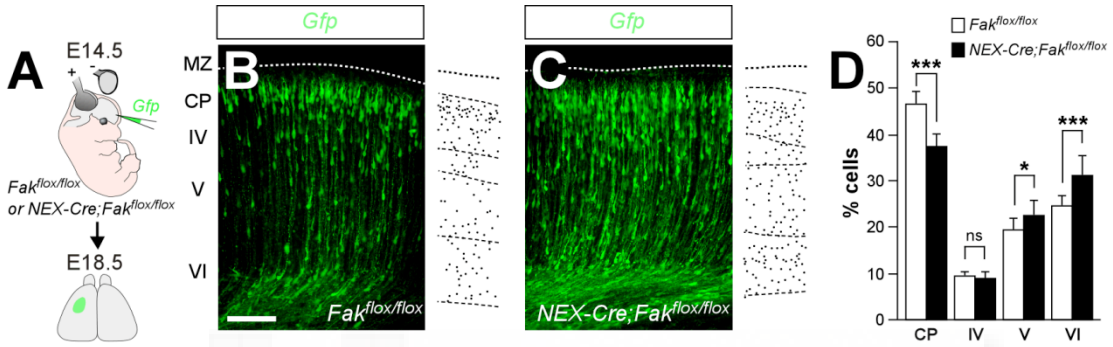

Figure 3. FAK cell autonomously disrupts cortical neuronal migration. (A) Schematic diagram of experimental design. (B and C) Coronal sections through the somatosensory cortex of E18.5 *Fak*$^{flox/flox}$ (B) and *NEX-Cre;Fak*$^{flox/flox}$ (C) embryos after E14.5 in utero electroporation with a plasmid encoding for *Gfp*. Schemas with dots depict representative distributions of GFP-expressing neurons in each condition. (D) Quantification of the relative distribution (%) of GFP-expressing in different cortical layers for each condition. Histograms show mean ± s.e.m. from at least 4 different brains. *Fak*$^{flox/flox}$: CP, 46.50 ± 2.52; layer IV, 9.39 ± 0.64; layer V, 19.32 ± 2.31; layer VI, 24.42 ± 2.10. *NEX-Cre;Fak*$^{flox/flox}$: CP, 37.19 ± 2.82; layer IV, 8.86 ± 1.19; layer V, 22.39 ± 3.01; layer VI, 8.86 ± 1.19. X^2-test for comparison: CP, $P < 0.001$; IV, ns; V, $P < 0.05$; VI, $P < 0.001$. See Figure S8 for additional statistical analyses. Scale bar equals 100 µm.

Disrupted radial glia-neuron interaction in the absence of FAK

FAK has been shown to dynamically regulate adhesion to a variety of substrates during migration of different cell types (Mitra et al., 2005; Parsons, 2003). Since the glial scaffold is the main substrate supporting the migration of neurons to the superficial layers of the cortex (Hatten, 1999; Rakic, 2007), we hypothesized that FAK could regulate the adhesion of neurons to radial glial fibers.

To begin to test the hypothesis, we first analyzed the consequences of loss of FAK function at early stages of corticogenesis, when migrating neurons arrive to the CP by somal translocation without interacting with the radial glia (Hatanaka et al., 2004; Miyata et al., 2001; Nadarajah et al., 2001). To this end, we electroporated the dorsal pallium of wild type embryos at E12.5 with plasmids encoding for *Gfp* and either *shLuc* or *shFak*, and the distribution of transfected neurons was analyzed at E16.5 (Figure 4A). We found no differences in the percentage of neurons that reached the CP between control and *shFak*-electroporated embryos (*shLuc*: 71.16 ± 2.69 %, *n* = 3; *shFak*: 67.72 ± 0.84 %, *n* = 3; Figures 4B–4D), suggesting that FAK function in radial migration might depend on the interaction between cortical neurons and radial glial cells. In addition, we did not detect important morphological differences between control and *shFak*-transfected neurons (Figures 4E and 4F), indicating that the disruption of cell morphology

found at later stages (E14.5 electroporation; Figure 2) might be due to a defect in the interaction between migrating neurons and radial glia.

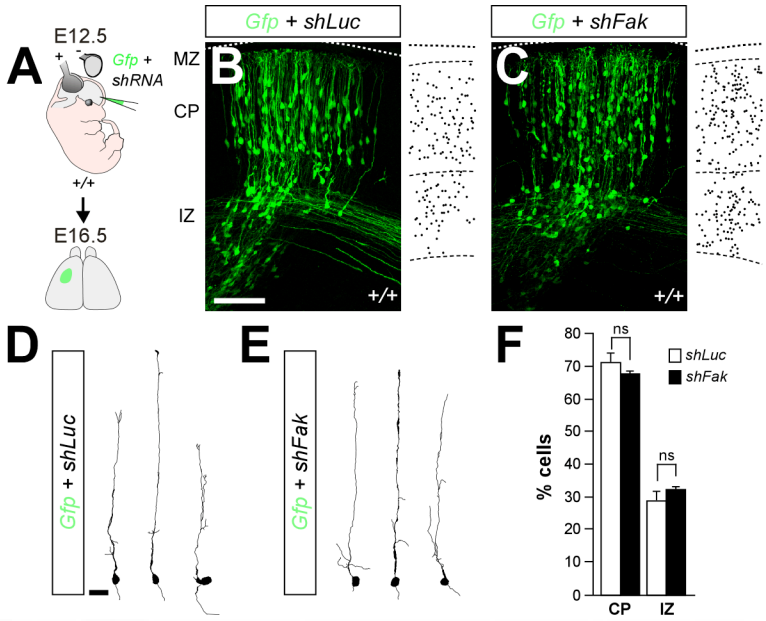

Figure 4. Somal translocation occurs normally in the absence of FAK. (A) Schematic diagram of experimental design. (B and C) Coronal sections through the somatosensory cortex of E16.5 wild type embryos after E12.5 in utero electroporation with plasmids encoding for *Gfp* and *shLuc* (B) or *shFak* (C). Schemas with dots depict representative distributions of GFP-expressing neurons in each condition. (D) Quantification of the relative distribution (%) of GFP-expressing in different cortical compartments for each condition. Histograms show mean ± s.e.m. from at least 3 different brains. *shLuc*: CP, 71.16 ± 2.69; IZ, 28.84 ± 2.69. *shFak*: CP, 67.72 ± 0.84; IZ, 32.10 ± 0.89. X^2-test for *shLuc* and *shFak* comparison: CP, ns; IZ, ns. See Figure S8 for additional statistical analyses. (E and F) Drawings illustrate the morphology of migrating cells expressing Gfp and shLuc (E) or shFak (F). Scale bars equal 100 µm (B and C) and 20 µm (E and F).

To directly test this hypothesis, we analyzed the adhesive properties of control and FAK-deficient cortical neurons. In brief, we electroporated the dorsal pallium of wild type embryos at E14.5 with plasmids encoding for *Gfp* and either *shLuc* or *shFak*. Four days later, migrating neurons were disaggregated from the electroporated region and plated on Laminin-coated plates (Figure 5A). We found a 40 % reduction of *shFak*-transfected cells adhered to the substrate compared with control neurons (Figures 5B–5D). Thus, loss of FAK function in embryonic cortical neurons reduces their ability to adhere in vitro to ECM components that resembles their normal substrate in vivo (Chen et al., 2009).

We reasoned that if loss of FAK function disrupts the adhesion of pyramidal neurons to radial glial cells in vivo, then migrating neurons might have a tendency to disperse tangentially away from the basal processes of their parental radial glial cell as they migrate towards the CP. To test this idea, we examined the spatial relationship between individual radial glial processes and their progeny of migrating neurons by

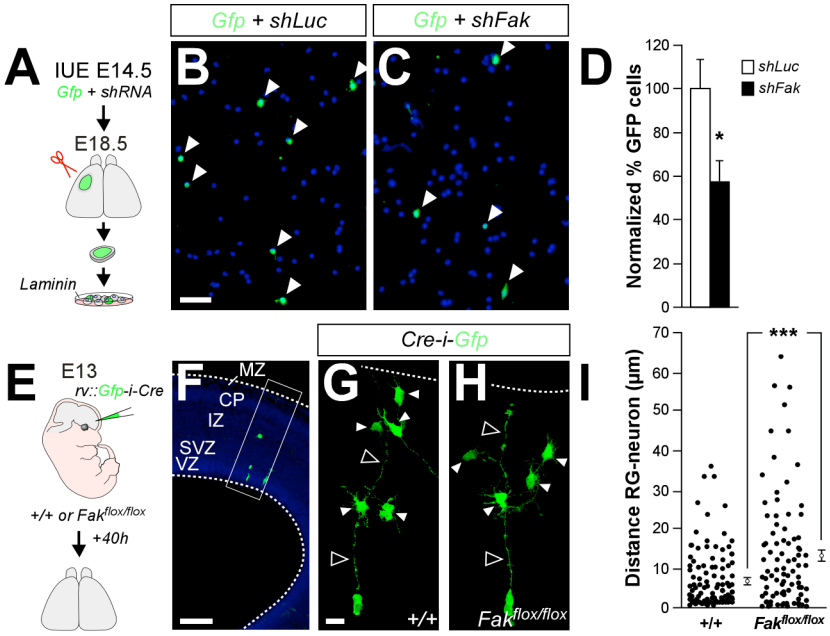

Figure 5. Perturbed adhesion in migrating pyramidal cells in the absence of FAK. (A) Schematic diagram of experimental design. Wild embryos were in utero electroporated with *Gfp* and *shRNA* (either *shLuc* or *shFak*) at E14.5. Four days later, the electroporated areas were dissected under a fluorescence scope, cells disaggregated and subsequently plated on 48 well plates coated with Laminin (2 µg/µl). Cells were washed and fixed after 150 minutes of incubation. (B and C) Distribution of adhered GFP-expressing cells transfected with *shLuc* (B) or *shFak* (C) after incubation and washing. (D) Quantification of the percentage of adhered GFP-expressing cells transfected with *shFak* relative to control (*shLuc*). Histograms show mean ± s.e.m. from 10 different brains for each condition. *shLuc*: 100 ± 12.94; *shFak*: 57.45 ± 9.05. *t-test, P = 0.01479. (E) Schematic diagram of experimental design. (F) Representative example of a coronal section through the somatosensory cortex of an ~E14.5 wild type embryo after E13 infection with retroviruses expressing *Gfp-i-Cre*, showing the approximate region containing the clones analyzed in (G) and (H). (G and H) Representative examples of single clones containing GFP-expressing cells in the developing cortex 40 h after infection of wild type (G) and *Fak*$^{flox/flox}$ (H) embryos with retroviruses encoding for *Gfp-i-Cre*. Note that FAK-deficient cells are often located at greater distance from the parental radial glial cell than wild type cells. (I) Quantification of the distance between migrating neurons and the corresponding parental radial glia fiber. Dots represent individual cells from multiple experiments. Histograms show mean ± s.e.m. from 3 different brains. Wild type: 7.14 ± 0.76; *Fak*$^{flox/flox}$: 13.46 ± 1.52. ***t-test, P = 1.4483E-04. Scale bars equal 50 um (B, C and F) and 15 um (G and H).

performing clonal analyses. To this end, low titer *Gfp-i-Cre*-expressing retroviruses were injected in the lateral ventricles of E13 wild type or *Fak*$^{flox/flox}$ mouse embryos to sporadically label radial glial cells throughout the telencephalon (Figure 5E). Forty hours after infection, labeled radial glial units typically comprised a radial glial cell in the VZ, a few multipolar cells located between the SVZ and the IZ, and some cells near or within the CP (Figures 5F and 5G). We found that wild type GFP-expressing migrating neurons were located in a range between 0.29 µm and 35.67 µm from the basal process of their parental radial glial cells (Figures 5G and 5I). In contrast, FAK-deficient GFP-expressing migrating neurons were dispersed across a wide territory, with distances from the basal process of their parental radial glial cells ranging from 0.29 µm to 63.68 µm (Figures 5G and 5H). Consequently, the average distance between neurons and their parental radial glial processes was significantly higher in FAK-deficient clones (13.46 ± 1.52 µm; *n* = 115 cells from 3 embryos; Figure 5I) than in wild type clones (7.14 ± 0.76 µm; *n* = 146 cells from 3 embryos; Figure 5I). Together with the in vitro adhesion assays, these experiments indicated that FAK is required to support the normal interaction between migrating neurons and radial glial cells during glial-guided locomotion.

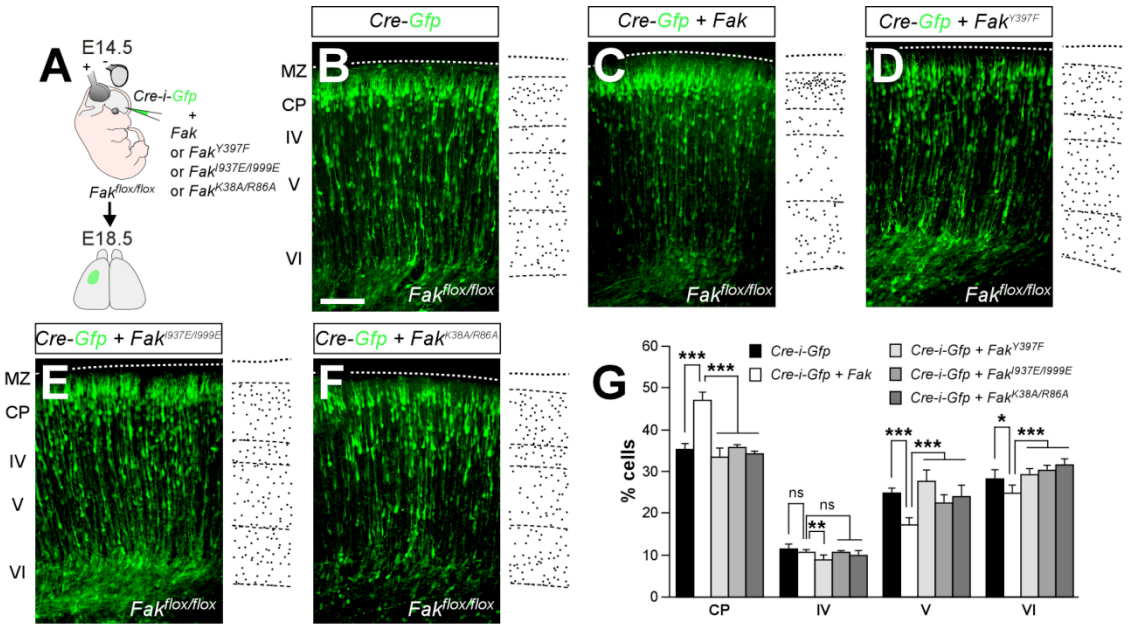

Figure 6. FAK regulation of adhesion is required for cortical neuronal migration. (A) Schematic diagram of experimental design. (B-F) Coronal sections through the somatosensory cortex of E18.5 $Fak^{flox/flox}$ embryos after E14.5 in utero electroporation with plasmids encoding for *Cre-i-Gfp* (B), *Cre-i-Gfp* + *Fak* (C), *Cre-i-Gfp* + Fak^{Y397F} (D), *Cre-i-Gfp* + $Fak^{I937E/I999E}$ (E), or *Cre-i-Gfp* + $Fak^{K38A/R86A}$ (F). Schemas with dots depict representative distributions of GFP-expressing neurons in each condition. (G) Quantification of the relative distribution (%) of GFP-expressing in different cortical layers for each condition. Histograms show mean ± s.e.m. from at least 3 different brains for each condition. *Cre-i-Gfp*: CP, 35.19 ± 1.22; layer IV, 11.37 ± 1.04; layer V, 24.77 ± 1.10; layer VI, 28.05 ± 2.12. *Cre-i-Gfp* + *Fak*: CP, 47.05 ± 1.84; layer IV, 10.79 ± 0.64; layer V, 17.25 ± 1.43; layer VI: 24.85 ± 2.00. X^2-test for *Cre-i-Gfp* and *Cre-i-Gfp* + *Fak* comparison: CP, $P < 0.001$; IV, ns; V, $P < 0.001$; VI, $P < 0.05$. *Cre-i-Gfp* + Fak^{Y397F}: CP, 33.45 ± 2.07; layer IV, 8.93 ± 1.18; layer V, 27.62 ± 2.56; layer VI: 29.13 ± 1.45. X^2-test for *Cre-i-Gfp* and *Cre-i-Gfp* + Fak^{Y397F} comparison: CP, ns; IV, $P < 0.01$; V, ns; VI, ns. X^2-test for *Cre-i-Gfp* + *Fak* and *Cre-i-Gfp* + Fak^{Y397F} comparison: : CP, $P < 0.001$; IV, $P < 0.05$; V, $P < 0.001$; VI, $P < 0.001$. *Cre-i-Gfp* + $Fak^{I937E/I999E}$: CP, 35.73 ± 0.41; layer IV, 10.67 ± 0.26; layer V, 22.49 ± 1.69; layer VI, 30.35 ± 1.14. X^2-test for *Cre-i-Gfp* and *Cre-i-Gfp* + $Fak^{I937E/I999E}$ comparison: CP, ns; IV, ns; V, ns; VI, ns. X^2-test for *Cre-i-Gfp* + *Fak* and *Cre-i-Gfp* + $Fak^{I937E/I999E}$ comparison: : CP, $P < 0.001$; IV, ns; V, $P < 0.001$; VI, $P < 0.001$. *Cre-i-Gfp* + $Fak^{K38A/R86A}$: CP, 34.12 ± 0.61, layer IV: 9.89 ± 0.93; layer V: 23.93 ± 2.61; layer VI: 31.64 ± 1.36. X^2-test for *Cre-i-Gfp* and *Cre-i-Gfp* + $Fak^{K38A/R86A}$ comparison: CP, ns; IV, ns; V, ns; VI, $P < 0.05$. X^2-test for *Cre-i-Gfp* + *Fak* and *Cre-i-Gfp* + $Fak^{K38A/R86A}$ comparison: : CP, $P < 0.001$; IV, ns; V, $P < 0.001$; VI, $P < 0.001$. See Figure S8 for additional statistical analyses. Scale bar equals 100 µm.

Regulation of adhesion by FAK is required for pyramidal neuron migration

The previous experiments strongly suggested that FAK is required to regulate the normal interaction of migrating neurons with radial glia. In other cell types, FAK is recruited to sites of integrin activation or cell-cell junctions, where it regulates the assembly and disassembly of adhesion contacts through multiple signaling pathways. Several landmark molecular events underlie the function of FAK in adhesion contacts, including its activation through autophosphorylation at Y397, recruitment to the membrane by interaction with proteins such as Paxillin and Talin, and the nucleation of actin-filament networks through its interaction with actin regulators like the Arp2/3 complex and the Wiskott–Aldrich syndrome protein, N-WASP (Mitra et al., 2005). To test their involvement in the migration of pyramidal neurons, we performed experiments in which we attempted to rescue the migration phenotype caused by loss of FAK function by expressing specific point mutants interfering with each of these signaling pathways (Figure 6A). In brief, we electroporated *Cre* in the dorsal pallium of E14.5 $Fak^{flox/flox}$ mouse embryos, alone or in combination

with plasmids encoding wild type *Fak* or several *Fak* mutants: Fak^{Y397F} (autophosphorylation site, Schaller et al., 1994), $Fak^{I937E/I999E}$ (Paxillin interaction, Hayashi et al., 2002), or $Fak^{K38A/R86A}$ (Arp2/3 interaction, Serrels et al., 2007). As expected, expression of wild type *Fak* rescued the migration phenotype observed in *Cre*-electroporated $Fak^{flox/flox}$ pyramidal neurons (Figures 6B, 6C and 6G). In contrast, disrupting FAK autophosphorylation site or those residues required for its interaction with Paxillin and the Arp2/3 complex prevented FAK from restoring pyramidal neuron migration (Figures 6D, 6E, 6F and 6G). These results demonstrate that FAK signaling pathways involved in the dynamic assembly and disassembly of adhesions are required for neuronal migration.

Aggregation of Connexin puncta in migrating neurons requires FAK

It is well established that migrating pyramidal neurons form transient interstitial junctions with radial fibers (Cameron and Rakic, 1994; Gadisseux et al., 1990), but the molecular architecture of these adhesions have remained largely unknown. Recent studies have shown that several connexins, the components of Gap junctions, localize in neurons to the regions of contact with radial fibers (Elias et al., 2007; Nadarajah et al., 1997). In addition, it has been recently shown that the adhesive properties of connexins are required during glial-guided migration (Cina et al., 2009; Elias et al., 2007). In particular, Connexin-26 (Cx26) puncta are abundant in the perinuclear region and in the cytoplasmic dilatation formed in the proximal leading process prior to nucleokinesis (Figures 7A, 7C and 7C', see also Elias et al., 2007). This later region, which contains the centrosome (Figure 7A and 7B), is also enriched in Paxillin and FAK (Figures 7A, 7D and 7D', and data not shown).

Since FAK is known to regulate cell-cell adhesions in other cellular contexts (Schaller, 2004; Yano et al., 2004), we next tested the hypothesis that FAK function is required for the correct targeting of connexins to adhesion contacts in migrating neurons. To this end, we electroporated progenitor cells in the dorsal pallium with control or *Fak* shRNA at E14.5 and analyzed Cx26 protein in migrating pyramidal neurons at E18.5 (Figure 7E). We found that the number of Cx26-containing puncta in migrating neurons transfected with *shFak* was dramatically reduced compared to control neurons (Figures 7F–7H). This result indicated that FAK mediate its function during neuronal migration at least in part by regulating neuron-glia interactions through connexins.

To shed light on the molecular mechanism through which FAK regulates connexin puncta in migrating pyramidal neurons, we performed in vitro experiments using a C6 cell line that contains FAK but lacks endogenous expression of connexins (Hwang et al., 2006; Naus et al., 1991). Forty hours after transfection of C6 cells with a plasmid encoding for *Cx26* fused to *Gfp* (*Cx26-Gfp*) and *shLuc*, we observed many cells with two or more GFP+ puncta close to the plasma membrane (Figures 8A and 8E). In contrast, we found fewer cells containing two or more GFP+ puncta when *Cx26-Gfp* was co-transfected along with *shFak* in C6 cells (Figures 8B and 8E). In most cases, FAK-deficient cells contained a single GFP+ aggregate, which was located far from the plasma membrane (Figures 8B and 8E). These experiments strongly supported the idea that FAK function is required for Cx26 assembly or transport, and suggested that post-transcriptional mechanisms may regulate this process.

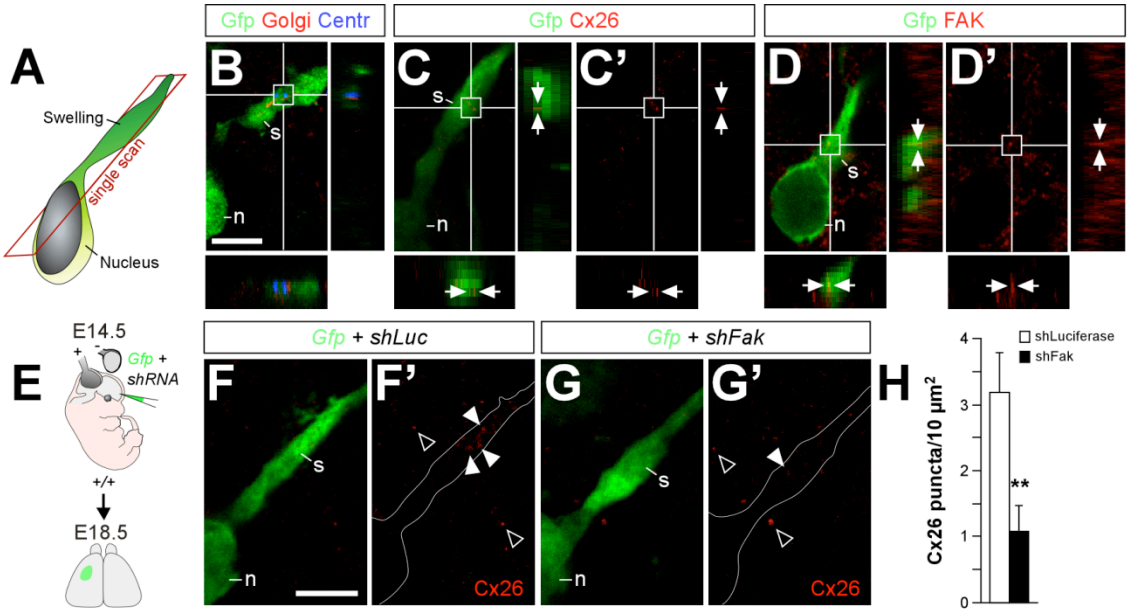

Figure 7. Reduced number of Cx26 adhesions in FAK-deficient neurons. (A) Schematic drawing showing the location of images displayed in (B) to (D'). (B-D') Distribution of Cx26 (C and C') and FAK (D and D') puncta (arrows) in the cytoplasmic dilatation (swelling, s) preceding the nucleus (n) in migrating GFP-expressing pyramidal neurons. The location of the Golgi apparatus and the centrosome is shown in (B) as a reference. (E) Schema of experimental paradigm. (F-G) Distribution of Cx26 puncta (arrowheads) in the cytoplasmic dilatation preceding the nucleus in migrating control (F and F') and FAK-deficient (G and G') pyramidal neurons. (H) Quantification of the number of Cx26 puncta in migrating neurons. Histograms show mean ± s.e.m. from 3 different brains. *shLuc*: 3.18 ± 0.59, n=30 cells; *shFak*: 1.08 ± 0.37, n=30 cells. **t-test, P = 0.0039961. Scale bar equals 5 µm.

The mechanisms controlling trafficking and assembly of connexins are complex and often subunit-dependent. Src-mediated phosphorylation has been shown to modulate this process for several connexins (Solan and Lampe, 2005), and Src activity is tightly regulated by FAK Y397 autophosphorylation (Mitra et al., 2005). Although the short C-terminal tail of Cx26 makes this region unlikely to undergo phosphorylation, recent studies suggest that Cx26 can be phosphorylated at the N-terminal region (Locke et al., 2006). To test whether FAK/Src interactions could at least in part contribute to the location of Cx26 to the membrane, we repeated the previous experiments in the presence of the Src kinase inhibitor PP2 (10 µM). We found that blocking Src activity in control cells did not modify the pattern of Cx26 expression (Figures 8C and 8E). In contrast, inhibition of Src in *shFak*-transfected cells rescued

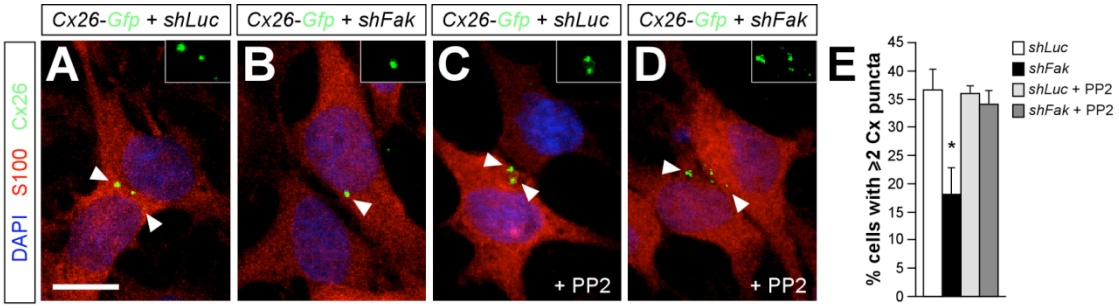

Figure 8. FAK regulates Cx26 adhesions through Src. (A-D) Distribution of Cx26-GFP puncta (arrowheads) in *shLuc*- (A and C) and *shFak*- (B and D) transfected C6 cells, cultured in the presence of vehicle (A and B) or the Src inhibitor PP2 (10 µM) (C and D). (E) Quantification of the percentage of C6 cells containing two or more Cx26-GFP puncta. Histograms show mean ± s.e.m. from 3 different wells. 2 or more puncta: *shLuc*, 36.86 ± 3.73; *shFak*, 18.08 ± 4.60; *shLuc* + PP2, 36.09 ± 1.25; *shFak* + PP2, 34.22 ± 2.30. *t-test for *shLuc* and *shFak*, P = 0,033738. *t-test for *shFak* and *shLuc* + PP2, P = 0,01945438. *t-test for *shFak* and *shFak* + PP2, P = 0,03491544. Scale bar equals 10 µm.

the phenotype observed in FAK-deficient cells (Figures 8D and 8E). These results suggested that the deficiency of Cx26 puncta caused by loss of FAK function is, at least in part, a consequence of abnormal Src activation.

Discussion

The basal processes of radial glial cells, which expand from the ventricular zone to the pial surface throughout the entire brain and spinal cord, represent the most predominant cellular substrate for neuronal migration. The existence of close appositions between neurons and radial glial fibers has long been documented (Gadisseux et al., 1990; Gregory et al., 1988; Rakic, 1971, 1972; Sidman and Rakic, 1973), but the molecular pathways regulating their dynamic interaction during migration are still poorly understood. In this study, we demonstrate that FAK plays a critical role in glial-guided locomotion by regulating the adhesive properties of migrating neurons. In pyramidal neurons, FAK function is cell-autonomously required to efficiently established Gap junction adhesions with radial glial fibers, which in turn are necessary to support glial-guided locomotion. Based on these and previous results, we hypothesize that pyramidal neuron migration requires the dynamic regulation of cell-matrix and cell-cell adhesions during cell locomotion, and that this function is likely to be integrated by FAK.

FAK function in neuronal migration

Two previous studies have explored the function of FAK in neuronal migration (Beggs et al., 2003; Xie et al., 2003), with conflicting results. Beggs and colleagues (2003) used a genetic loss of function approach to study the consequences of conditionally ablating *Fak* from cortical progenitors (with an *Emx1-Cre* mouse strain) or migrating neurons (with the same *NEX-Cre* mouse strain used in this study). They reported that loss of *Fak* in radial glial cells causes cortical ectopias, caused by a disruption of the integrity of the pial basement membrane (Beggs et al., 2003). By contrast, they did not observe obvious cortical abnormalities after conditional deletion of *Fak* in migrating pyramidal neurons (Beggs et al., 2003). While we have not specifically explored the function of FAK in radial glial cells, our results strongly suggest that FAK is indeed required in cortical neurons for their normal migration. Several factors may account for this apparent discrepancy, including the analytical methods (Nissl staining versus *Gfp* electroporation). We found that loss of FAK in *NEX-Cre;Fak*$^{flox/flox}$ embryos causes a relatively small migratory phenotype compared to early deletion of FAK by Cre electroporation or, even more so, to knockdown of *Fak* using shRNA. Differences in these three different approaches are likely explained by the timing of FAK elimination (*NEX-Cre* > *Cre* in VZ > *Fak* shRNA in VZ). Moreover, our analysis of the distribution of FAK-deficient neurons in postnatal mice revealed that most of them managed to reach the superficial layers of the cortex (Figure S6), suggesting that FAK deficiency delays rather than blocks the migration of pyramidal neurons. Of note, some FAK-deficient neurons accumulated in ectopic locations in the postnatal brain (Figure S6), and these neurons displayed abnormal dendritic morphology (Figure S6), as described by Beggs and colleagues (2003). Thus, FAK function appears to be required in both radial glial cells and migrating pyramidal neurons for their migration.

FAK function has been previously implicated in the migration of pyramidal neurons by regulating the organization of the perinuclear microtubule cytoskeleton in a Cdk5-dependent manner (Xie et al., 2003). Overexpression of a non-phosphorylatable mutant FAK at the site of Cdk5 phosphorylation (FAK-S732A) impairs the migration of pyramidal neurons, probably by disrupting the microtubule network that is important for nucleokinesis (Xie et al., 2003). Interestingly, expression of FAK-S732A in a FAK-deficient background partially rescues the migratory phenotype observed in the complete absence of FAK (Figure

S7), indicating that although FAK-S732 plays a role in neuronal migration, additional signaling pathways mediate the function of FAK in this process. Consistent with this notion, we found that FAK autophosphorylation and association with Paxillin and Arp2/3 are essential for cortical neuronal migration. Since all of these pathways are required for FAK function in regulating cell-matrix and cell-cell interactions in other cellular contexts (Mitra and Schlaepfer, 2006; Serrels et al., 2007), our results strongly suggest that the modulation of adhesion by FAK is an important mechanism through which this protein influences neuronal migration.

The analysis of the distribution of migrating pyramidal neurons in E18.5 embryos revealed that FAK-deficient delayed neurons accumulated primarily in the upper intermediate zone and lower cortical layers. In addition, although FAK-deficient neurons displayed an abnormal morphology with multiple branches in the leading process, cells remained highly polarized. These observations suggest that the transition from the multipolar to bipolar stage, a critical step during pyramidal neuronal migration (LoTurco and Bai, 2006), is not dramatically affected by the loss of FAK. Instead, loss of FAK function seems to primarily affect pyramidal neurons as they engage in the main phase of glial-guided migration.

Cell-matrix adhesions during neuronal migration

Cell migration involves the dynamic control of adhesions established between migrating cells and their substrate, which includes other cells and ECM proteins (Kunwar et al., 2006; Thiery et al., 1985). In the cerebral cortex, the interaction between migrating pyramidal neurons, radial glial cells and ECM proteins secreted by both cell types is crucial for glial-guided locomotion and neuronal positioning (Hatten, 2002; Marín and Rubenstein, 2003). Our study suggests that FAK is required during pyramidal neuron migration for the regulation of cell-matrix interactions, by modulating the assembly of canonical Paxillin-based adhesions at sites of integrin activation, as well as cell-cell interactions, by controlling the assembly of connexin-based adhesions.

FAK is recruited to sites of integrin activation known as focal contacts. The molecular architecture and size of adhesion contacts differs among cell types, but they characteristically include integrin-related proteins (e.g. Paxillin) and signaling (e.g. Src family kinases) proteins necessary for adhesion and migration (Mitra et al., 2005). Neuronal adhesion contacts has only been previously analyzed in growth cones, where they take the shape of small, discrete point contacts with a high turnover rate (Bechara et al., 2008; Gomez et al., 1996; Robles and Gomez, 2006). In migrating pyramidal neurons, point contacts are enriched in the perinuclear region, in particular in the cytoplasmic dilatation of the leading process that precedes the nucleus. The existence of discrete sites of adhesion in this later location has been hypothesized as a mechanism to regulate the saltatory movement of migrating neurons (Schaar and McConnell, 2005). Interestingly, while FAK activity is primarily involved in the disassembly of adhesion contacts in most cell types, focal adhesion signaling is crucial for the formation of adhesion points both during growth cone extension (Bechara et al., 2008; Robles and Gomez, 2006) and in neuronal migration (this study), suggesting that fundamental differences exist in adhesion complex formation between neuronal versus non-neuronal cells.

The function of integrin-based adhesion sites in pyramidal neuron migration remains controversial. Although early evidence indicated an important role for several integrins in glial-guided migration, in

particular α3β1 (Anton et al., 1999; Schmid et al., 2004), it is presently unclear whether integrin signaling is required in migrating neurons, radial glial fibers or both. Multiple studies have shown that mutations in several ECM proteins and integrin receptors impair cortical migration. In most cases, however, this defect has been associated with disruptions of the pial basement membrane and/or alterations in the anchorage of radial glial fibers to it, which ultimately leads to a disorganized radial glial scaffold that disrupts glial-guided migration (De Arcangelis et al., 1999; Georges-Labouesse et al., 1998; Graus-Porta et al., 2001; Halfter et al., 2002; Miner et al., 1998; Moore et al., 2002). Moreover, conditional deletion of β1 integrins in migrating neurons using *NEX-Cre* mice does not seem to disrupt cortical lamination, suggesting that integrin signaling in migrating neurons is not essential for glial-guided migration (Belvindrah et al., 2007). This seems consistent with the idea that functional integrins might not play a major role in migration through three-dimensional environments (Lämmermann et al., 2008). However, our study demonstrate that in vivo defects in the motility of pyramidal neurons may go unattended in conditional knockout mutants if the analysis focuses on the global examination of large population of cells (i.e. using markers for specific layers), because some alterations are relatively subtle and may only cause a delay in migration. Moreover, recent work suggests that neuron-specific deletion of laminin γ1 indeed impairs the migration of pyramidal neurons, a defect that correlates with decreased phosphorylation of Paxillin and FAK in vivo (Chen et al., 2009). In sum, these results, along with our experiments disrupting Paxillin binding to FAK, indicate that integrin-based adhesion plays a role – even if minor – during pyramidal neuron migration, and that FAK regulates this process.

Regulation of connexin adhesions by FAK

Although best known for its role in integrin-based adhesions, FAK is also involved in the regulation of cell-cell junctions during migration (Avizienyte and Frame, 2005; Mitra et al., 2005). In HeLa cells, for example, FAK activity has been associated with the formation or turnover of contacts in N-cadherin-based cell–cell junctions (Schaller, 2004; Yano et al., 2004). The interaction of migrating neurons with the basal processes of radial glial fibers does not seem to be mediated by cadherin-based cell–cell junctions, but instead appears to rely on the dynamic regulation of Gap junction adhesions. Gap junctions are best known for their role in intercellular communication by electrically and chemically coupling cells or by forming hemichannels for the extracellular release of substrates such as ATP (Söhl et al., 2005). However, recent work has shown that connexin-mediated adhesion, and not channel function, is required for pyramidal neuron migration (Cina et al., 2009; Elias et al., 2007). Our study suggest that FAK is involved in the dynamic regulation of this novel type of cell-cell adhesions, since loss of FAK in migrating neurons causes a reduction in the number of connexin puncta and impairs migration.

We have begun to explore the mechanisms through which FAK function may contribute to the assembly of Cx26 adhesions. In other cell types, several kinases control the trafficking, assembly and degradation of connexins (Solan and Lampe, 2005). One of the best characterized is Src, whose function is known to regulate several connexins, most notably Cx43 (Pahujaa et al., 2007). Src function is strongly linked to FAK, since autophosphorylation of FAK at Y397 promotes Src binding and the formation of a Src-FAK activated complex (Mitra and Schlaepfer, 2006). Interestingly, loss of FAK function in some cellular contexts leads to exuberant Src-mediated phosphorylation of multiple substrates, including connexins (Sachdev et al., 2009). Our experiments are consistent with this later possibility, since inhibition of Src rescued the loss of Cx26 puncta observed in *Fak*-deficient cells. It should be noted, however, that

additional mechanisms might mediate the regulation of Cx26 assembly by FAK. For example, FAK has been shown to interact with Cx26 in prostate cells (Tate et al., 2006), suggesting that this connexin might be a direct substrate of FAK activity. Alternatively, FAK might be directly required to recruit Cx26 to adhesions acting as a scaffold protein. In addition, intracellular transport of Cx26 seems to depend on intact actin filaments (Thomas et al., 2001), and loss of FAK function likely destabilizes the actin cytoskeleton assembled around adhesions (Mitra et al., 2005). In conclusion, although FAK-Src interactions appears to be important for the assembly of Cx26 adhesion points, additional studies should address in detail the mechanisms through which FAK regulates this process.

Dynamic control of neuron-glia interactions during migration

Signals regulating neuronal positioning and laminar formation in the cortex are likely to play a major role in regulating adhesion during glial-guided migration. This is the case of Reelin, a large glycoprotein that binds to receptors Apolipoprotein E receptor-2 (ApoER2) and very low-density lipoprotein receptor (VLDLR) in migrating neurons (Rice and Curran, 2001). Genetic studies have positioned Reelin, ApoER2, VLDLR and the intracellular adaptor protein Disabled-1 (Dab1) into a common signaling pathway that leads to the phosphorylation of Dab1 in migrating neurons, an event that is required for normal pyramidal neuron migration (Howell et al., 2000). Dab1 mediates its function, at least in part, by regulating the adhesion of migrating neurons to radial fibers. Thus, clonal analysis of radial units revealed that *Dab1* (*scrambler* homozygous) mutant neurons fail to detach from the basal process of their parental radial glial cells at the end of their migration (Sanada et al., 2004). Interestingly, Dab1 functions in this process by regulating α3 integrin levels (Sanada et al., 2004), which suggests that phosphorylated Dab1 disrupts integrin-based adhesion. Since FAK contributes to glial-guided migration by participating in the assembly of adhesions between migrating neurons and their substrates, it is tempting to speculate that the normal detachment of migrating neurons from radial glial fibers may also involve the regulation of this intracellular kinase.

Disruption in the pattern of cortical lamination is the most direct consequence of a defect in glial-guided migration. However, it is well established that radial migration also contributes to the delineation of distinct cortical areas by faithfully transferring positional information from the VZ to the CP (Rakic, 2007). In the absence of FAK function, however, neurons disperse tangentially away from the parental radial glial cell due to loss of neuron-glia adhesions, and it is conceivable that this may distort cortical area boundaries. Thus, under certain circumstances such as defects in adhesion dynamics, disrupted radial migration may have additional consequences beyond defects in cortical lamination. Similarly, it is tempting to speculate that regulation of FAK function is a potential mechanism for pyramidal neurons to escape, in certain situations, the control of glial-guided migration (for example, during the tangential expansion required for the colonization of large gyrus in complex brains). In conclusion, our results reinforce the view that the dynamic control of cell adhesion is essential for glial-guided migration, and that FAK plays a major role in regulating this process.

Experimental Procedures

Mouse strains. Fak$^{flox/flox}$ (Beggs et al., 2003), *NEX-Cre* (Goebbels et al., 2006) and *NEX-Cre;Fak* $^{flox/flox}$ were maintained in a CD1 background. The day of vaginal plug was considered as embryonic day (E) 0.5. Mice were kept at the Instituto de Neurociencias in accordance with Spanish and EU regulations.

DNA Constructs. Fak chicken cDNA (kindly provided by T. Parsons) was subcloned into a pCAGIG vector (#11159, Addgene) and all point mutations were based on this construct (GenScript). All shRNA were expressed from U6 promoter containing vectors. *shLuc* was obtained from Ambion, *shFak(1)* was kindly provided by J. L. Guan and *shFak(2)* was cloned in the pSilencer™ vector (Ambion) using the sequence described by Tighman and colleagues (2005). *Gfp* was cloned into the β-actin promoter expression vector (pCAGGS). The *Cre-i-Gfp* plasmid was kindly provided by F. Guillemot. The *Cx26-Gfp* expression plasmid has been described elsewhere (Falk, 2000).

Cell lines. N2A and C6 cell lines were co-transfected (Fugene, Roche) with the indicated plasmids at 0.9 µg/µl and incubated for 72 hours. PP2 (10 µM; Calbiochem) was added 5 hours before fixation of cells.

Western blotting. Cells or tissue were homogenized in TBS-T (50 mM Tris pH 7.4, 150 mM NaCl, 10% Glicerol, 5 mM EDTA, 1% Triton X-100, and a mixture of proteases inhibitors). Proteins were resolved by SDS-PAGE on 10% or 12.5% gels (samples were not boiled prior to loading when Cx26 detection was desired) and blotted to 0.2 mm nitrocellulose in transfer buffer, pH 8.3 (25 mM Tris, 192 mM glycine, and 20% v/v methanol). Immunoblots were blocked for 1 hour in TBS-T (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.2% Tween 20) containing 5% BSA and incubated overnight at 4°C with primary antibodies diluted in TBS-T containing 1% BSA powder. The following primary antibodies were used: rabbit anti-FAK 1:3000 (06-543, Upstate), rabbit anti-Cx26 1:500-1000 (51-2800, Zymed), and mouse anti-Actin 1:3000 (ab11003, Abcam). Membranes were then washed in TBS-T, incubated for 1 hour with secondary antibodies (goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG, 1:25000 (31444 & 31460, Pierce) diluted in TBS-T, washed and incubated for 1 min with ECL chemiluminescence reagents (Immobilon Western, Millipore).

Immunohistochemistry. Embryos and postnatal mice were perfused with 4% PFA, postfixed overnight at 4°C, equilibrated in 15% and 30% sucrose in PBS and cut on a freezing microtome (HM450, Microm) into 40 µm thick coronal sections. Alternatively, brains were postfixed 30 min at room temperature + 90 min at 4°C, and then 40-80 µm thickness coronal sections were cut on a vibratome (VT1000S, Leica). Free floating sections were processed for immunohistochemistry by blocking with 0.3% Triton X-100 and 1% BSA in PBS for 1 hour, following by overnight incubation with primary antibody diluted in 0.1% Triton X-100 and 0.5% BSA in PBS. The following primary antibodies were used: rabbit anti-GFP 1:1000 (A11122, Molecular Probes), chicken anti-GFP 1:1000 (GFP-1020, Aves Labs), rabbit anti-FAK 1:300 (06-543, Upstate), rabbit anti-Cx26 1:25 (51-2800, Zymed), rabbit anti-Pericentrin 1:100 (ab4448, Abcam), mouse anti-GM130 1:100 (610822, BD), rabbit anti-S100 1:1000 (S2644, Sigma). Secondary antibodies diluted in 0.1% X-100 and 0.5% BSA in PBS were incubated for 90 min. The following secondary antibodies were used: donkey anti-rabbit 488 1:500 (A21206, Invitrogen), goat anti-chicken 488 1:500 (A11039,

Invitrogen), donkey anti-rabbit Cy5 1:100 (AP1285, Chemicon), donkey anti-mouse 555 1:300 (A31570, Invitrogen), donkey anti-rabbit 555 1:400 (A31572, Invitrogen). Cell nuclei were stained using Bis-Benzamide 1:1000 (Sigma) and sections mounted with Mowiol (Sigma) with NPG (Calbiochem). Cx26 immunohistochemistry was performed as described before (Elias et al., 2007). For GFP/BrdU double immunohistochemistry, sections were first processed for GFP immunohistochemistry, fixed in 4% PFA for 50 min, and then processed for BrdU staining.

In utero electroporation and retroviral infection. Pregnant females were anesthetized with isofluorane, their uterine horns were exposed and the lateral ventricles of embryos were injected through the uterus wall using pulled capillaries (1B120F-4, World Precision Instruments) filled with either DNA (0.8–2.25 µg/µl) or retrovirus stock (10^7 c.f.u./ml) diluted in PBS and colored with 0.5% Fast Green (Sigma). DNA-injected embryos were electroporated using a 35-45 V/50 ms/950 ms/5 pulses program (CUY21/CUY650P3/CUY650P5, Nepa Gene Co Ltd). Retroviruses were prepared as described elsewhere (Tashiro et al., 2006). After appropriate survival times, mice were deeply anesthetized and sacrificed using cervical dislocation. In some cases, pregnant females were injected with a 40 mg/kg BrdU solution (Sigma).

Adhesion assays. Adhesion assays were performed as described previously (Koirala et al., 2009). In brief, brains were dissected and *Gfp*-electroporated areas were isolated in cold Hanks medium under a fluorescence scope. Cells were then trypsinized, filtered (0.2 µm pore), centrifuged for 5 min and resuspended in DMEM with no serum. $30x10^3$ cells were plated on Laminin coated (2 µg/µl) 48 well plates, incubated for 150 minutes, gently washed in PBS, and fixed with 4% PFA and 4% sucrose for 20 minutes.

Imaging. Images were acquired using fluorescence microscopes (DM5000B/CTR5000 and DMIRB, Leica) coupled to digital cameras (DC500 or DFC350FX, Leica) or a confocal microscope (DMIRE2/CTRMIC/TCS SP2, Leica). Neurolucida™ software (MBF Bioscience) was used for the reconstruction of cell morphologies using a fluorescence microscope with a 63x oil objective (DM4000B, Leica) coupled to a digital camera (QICAM Fast 1394, QImaging).

Quantification. Quantification of images was done using Canvas (ACD Systems), ImageJ (NIH, http://rsb.info.nih.gov/ij/) or Neurolucida Explorer (MBF Bioscience) software. For quantification of the migration phenotypes, layers were drawn following nuclear staining and GFP-expressing cells were quantified in a common boxed region through the somatosensory cortex at the same rostro-caudal level for each brain. Swelling quantification was performed acquiring photos every 3 µm from electroporated cortices and identifying cytoplasmic dilatations in the region of the leading process preceding the nucleus. Neurolucida Explorer™ was used to quantify the number of branches (>5 µm in length) in drawn cells. Adhesion assays were analyzed by acquiring 6 images per well from different fields. GFP-expressing cells and total nuclei were counted in every field using ImageJ. In retroviral experiments, GFP+ clones were acquired using an inverted confocal microscope. For each clone, the radial glia process was localized and the shortest distance from the centroid of every clonally related migrating neuron to the apical process of their mother glia cell was calculated using ImageJ. Images of GFP/BrdU double labeled cells were acquired using a confocal microscope. The cortical plate was defined using BrdU staining and cells were quantified as located in or out of this layer. In postnatal brains, layers were drawn according to

nuclear staining and the total numbers of cells located below layer IV were quantified. Images of centrosomes, Golgi apparatus, and FAK and Cx26 puncta were acquired using an inverted confocal microscope. X- and Y-axis sectioning of the acquired images was performed using Leica confocal software. Cx26 puncta were quantified using ImageJ, and their number normalized with the total area of the cell. Western blots were quantified using Quantity One (BIORAD). A two-tailed *t*-test was used for single pair-wise comparisons to examine differences in number of swellings, number of processes, adhesion assays, distance to radial glial process following retrovirus injection, and number of Cx26 puncta. A χ^2-test was used to examine differences between subpopulation of neurons following in utero electroporation.

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Supplemental data

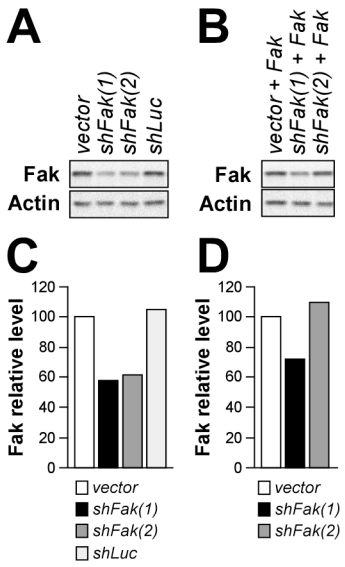

Figure S1. FAK RNAi decreases FAK levels in cell lines. (A) Representative immunoblots of FAK protein levels in N2A cells 72 h after transfection with a control plasmid (empty vector *pCAGIG*), two different types of *shRNA* against *Fak* [*shFak(1)* and *shFak(2)*], or *shRNA* against *Luciferase* (*shLuc*). (B) Representative immunoblots of FAK protein levels in N2A cells 72 h after transfection with a control plasmid (empty vector *pCAGIG*) + a plasmid encoding for *Fak*, *shFak(1)* + *Fak*, or *shFak(2)* + *Fak*. Full length *Fak* was obtained from chicken, and it is not effectively target by *shFak(2)* due to four mismatched base pairs. (C) Quantification of FAK protein levels from the experiment shown in (A). FAK endogenous levels were reduced about 40% with both *shFak(1)* and *shFak(2)* (higher concentration of *shFak* (1.5 µg/µl, used in electroporation assays) reachs until 80% reduction in protein level, data not shown) , but not with *shLuc*. (D) Quantification of FAK protein levels from the experiment shown in (B).

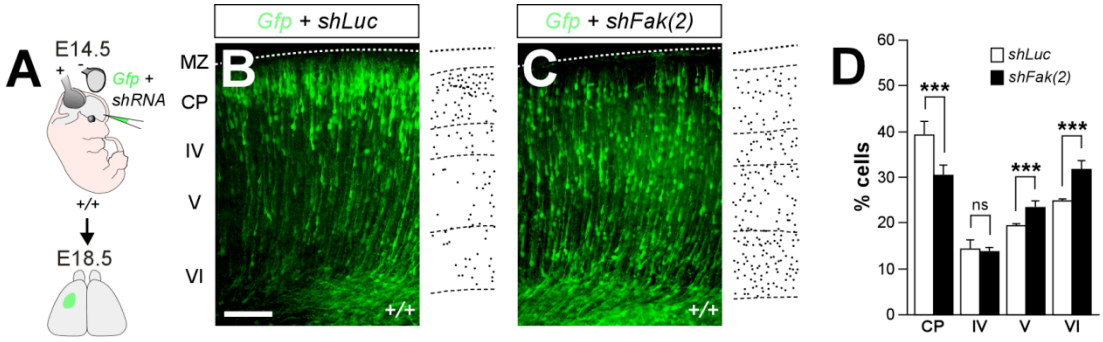

Figure S2. *shFak(2)* also disrupts radial migration. (A) Schematic diagram of experimental design. (B and C) Representative examples of GFP-expressing migrating cells after in utero electroporation with plasmids encoding for *Gfp* and *shLuc* (B) or *shFak(2)* (C). Schemas with dots depict representative distributions of GFP-expressing neurons in each condition. (F) Quantification of the relative distribution (%) of GFP-expressing in different cortical layers for each condition. Histograms show mean ± s.e.m. from at least 3 different brains. *shLuc*: CP, 40.60 ± 2.66; layer IV, 14.39 ± 2.01; layer V, 19.71 ± 1.55; layer VI, 25.04 ± 0.94. *shFak*: CP, 30.62 ± 2.16; layer IV, 13.81 ± 0.72; layer V, 23.43 ± 1.36; layer VI, 31.77 ± 1.73. X^2-test for *shLuc* and *shFak* comparison: CP, $P < 0.001$; IV, ns; V, $P < 0.001$; VI, $P < 0.001$. See Figure S8 for additional statistical analyses. Scale bar equals 100 µm.

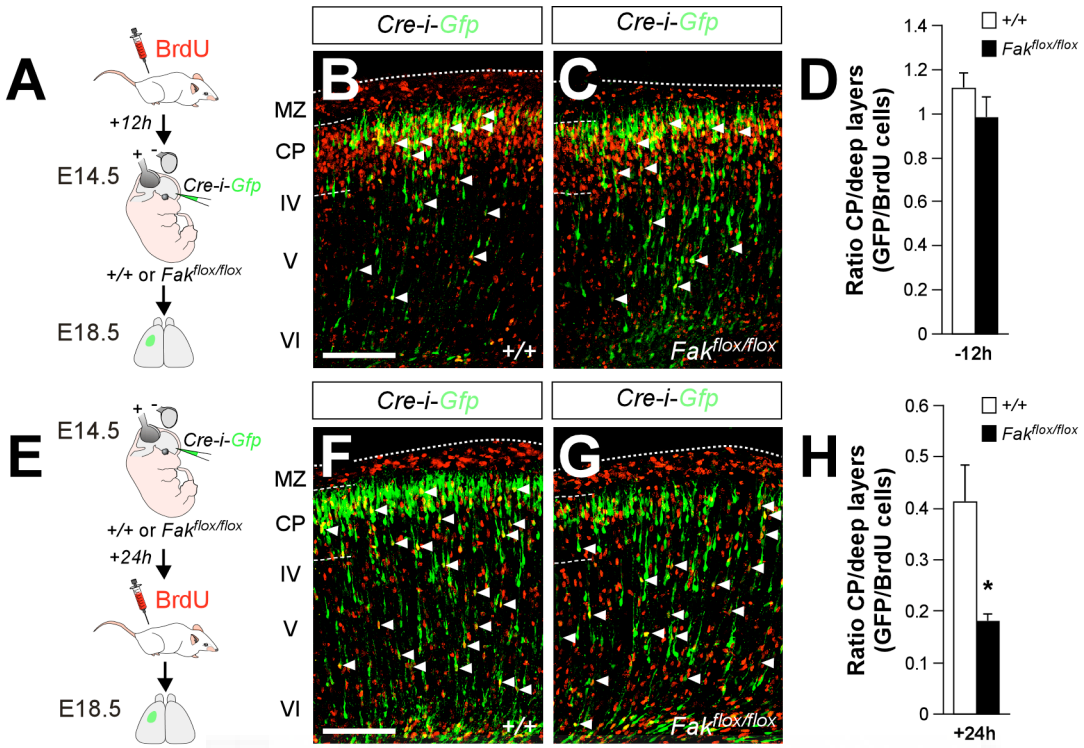

Figure S3. Genetic deletion of *Fak* in cortical projections primarily affects late born neurons. (A and E) Schematic diagrams of experimental designs. BrdU pulses were given to pregnant females using two different protocols. To label early-generated layer II-III neurons (A), BrdU was injected 12 hours before performing in utero electroporation with *Cre-i-Gfp* DNA in both wild type and *Fak*$^{flox/flox}$ mice. To label late-generated layer II-III neurons, BrdU was injected 24 hours after in utero electroporation (E). In both cases, brains were fixed and processed at E18.5. (B and C) Coronal sections through the somatosensory cortex of E18.5 wild type (B) and *Fak*$^{flox/flox}$ (C) embryos that received a BrdU pulse 12 hours before in utero electroporation at E14.5. Double labeled cells (white arrows) correspond to a subpopulation from the total GFP-expressing cells. (D) Quantification of the ratio between cells that have arrived to the cortical plate (CP) and those still located in deep layers. Following this protocol, similar numbers of cells were found inside and outside the CP (ratio approximates to 1), in both control and *Fak*$^{flox/flox}$ mice. Histograms show mean ± s.e.m. from 3 different brains. Wild type: 1.11 ± 0.14. *Fak*$^{flox/flox}$: 0.98 ± 0.09. t-test, P = 0.47310. (F and G) Coronal sections through the somatosensory cortex of E18.5 wild type (B) and *Fak*$^{flox/flox}$ (C) embryos that received a BrdU pulse 24 hours after in utero electroporation at E14.5. Double labeled cells (white arrows) correspond to a subpopulation from the total GFP-expressing cells. (H) Quantification of the ratio between cells that have arrived to the cortical plate (CP) and those still located in deep layers. Following this protocol, this ratio is much higher in controls than in *Fak*$^{flox/flox}$ mice, indicating a prominent delay in the population of BrdU/GFP cells in the absence of FAK. Histograms show mean ± s.e.m. from 4 different brains. Wild type: 0.41 ± 0.07. *Fak*$^{flox/flox}$: 0.18 ± 0.01. *t-test, P = 0.02280. Scale bars equal 100 µm.

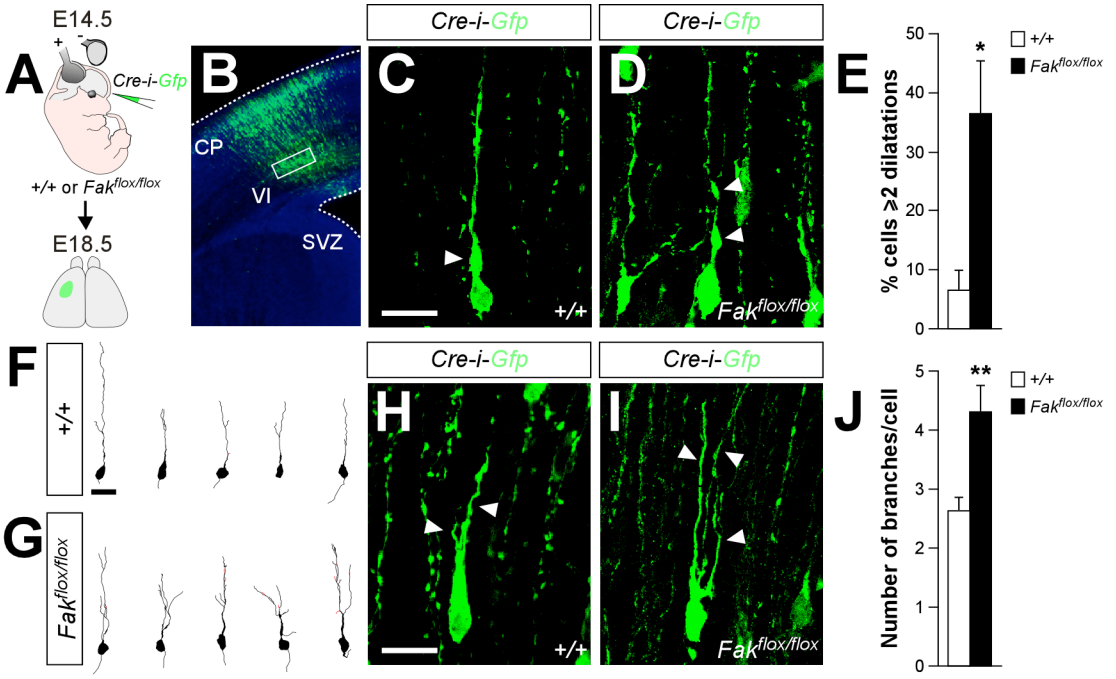

Figure S4. Morphological defects in migrating neurons after genetic ablation of FAK. (A) Schematic diagram of experimental design. (B) Representative example of a coronal section through the somatosensory cortex of an E18.5 wild type embryo after E14.5 in utero electroporation showing the approximate region containing the neurons analyzed. Cell morphology was analyzed in layer VI, where the highest amount of cells accumulated upon *Fak* deletion. (C and D) Representative examples of GFP-expressing migrating cells after in utero electroporation with *Cre-i-Gfp* in wild type (B) or in *Fak*$^{flox/flox}$ (D) embryos. Note the increased number of cytoplasmic dilatations in FAK-deficient neurons. (E) Quantification of the percentage of cells with two or more dilatations in the leading process. Histograms show mean ± s.e.m. from at least 3 different brains. Wild type: 6.67 ± 3.33, n = 30 cells. *Fak*$^{flox/flox}$: 36.67 ± 8.82, n = 30 cells. *t-test , P = 0,03347174. (F and G) Drawings illustrate the morphology of migrating cells transfected with *Cre-i-Gfp* in wild type (F) or in *Fak*$^{flox/flox}$ (G) embryos. Note the increased number of leading process branches in *Fak*-deficient neurons (G). (H and I) Representative examples of GFP-expressing migrating cells after in utero electroporation with *Cre-i-Gfp* in wild type (H) or in *Fak*$^{flox/flox}$ (I) embryos. Note the increased number of leading process branches in *Fak*-deficient neurons (I). (J) Quantification of the number of leading process branches. Histograms show mean ± s.e.m. from at least 3 different brains. Wild type: 2.63 ± 0.21, n = 30 cells. *Fak*$^{flox/flox}$: 4.3 ± 0.43, n = 30 cells. **t-test , P = 1,00362E-03. Scale bars equal 15 µm (C, D, H and I) and 25 µm (F and G).

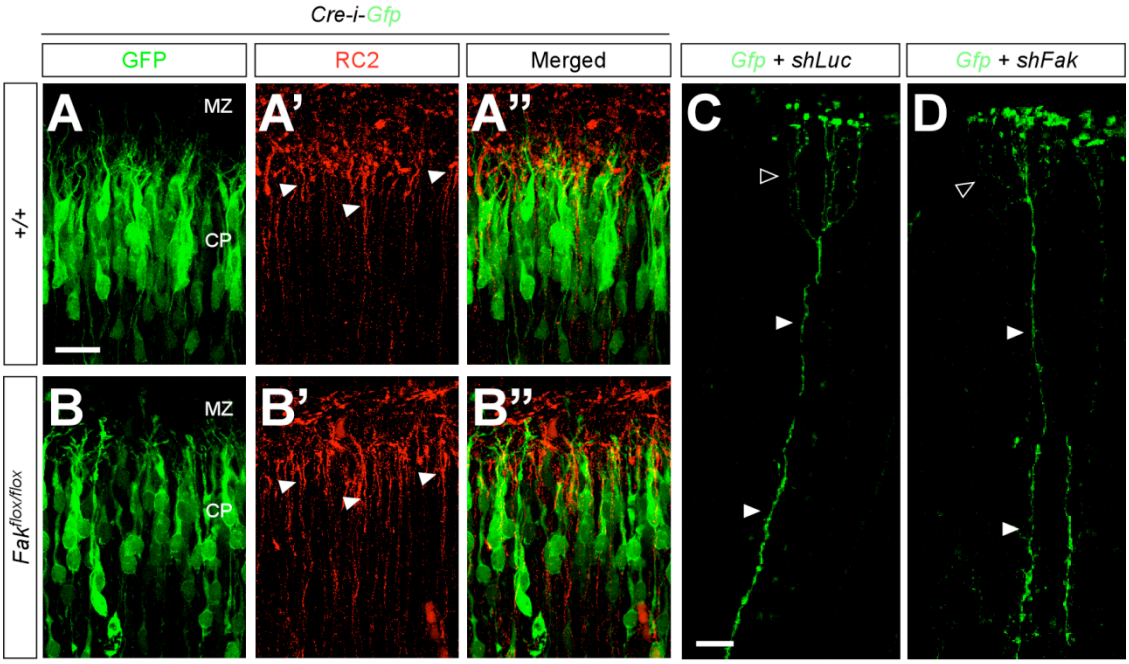

Figure S5. Removal of FAK does not impair radial glia morphology. (A-B“) High magnification images of the CP in the somatosensory cortex of E18.5 wild type (A-A”) and *Fak*$^{flox/flox}$ (B-B“) embryos electroporated with *Cre-i-Gfp* at E14.5, stained with antibodies against GFP (A, A”, B and B“) and RC2 (A’, A”, B’ and B“). Radial glia morphology (revealed with RC2) was similar in wild type (A’) and *Fak*$^{flox/flox}$ (B’) embryos (white arrowheads). (C and D) Images of isolated GFP+ radial glia cells transfected with *Gfp* and *shLuc* (C) or *shFak* (D). The morphology of basal processes (arrowheads) and glial endfeets (open arrowheads) was similar in both conditions. Scale bars equal 20 µm.

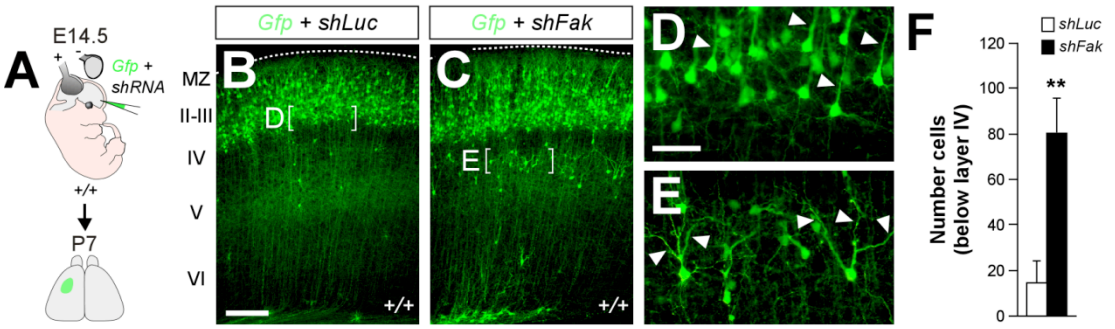

Figure S6. Dendrite abnormalities in ectopic postnatal pyramidal neurons after embryonic *Fak* knockdown. (A) Schematic diagram of experimental design. (B and C) Coronal sections through the somatosensory cortex of P7 wild type mice after E14.5 in utero electroporation with plasmids encoding for *Gfp* and *shLuc* (B) or *shFak* (C). At this age, most cells were found in a similar position in both conditions, although ectopic cells were also present in deep layers of the cortex (below layer IV) in *Gfp* + *shFak* electroporated brains. (D and E) High magnification images of the boxed areas shown in (B) and (C), respectively. The morphology and orientation of dendrites in *Gfp* + *shFak* electroporated pyramidal cells is very disorganized compared to control cells. (F) Quantification of the number of ectopic cells (below layer IV) for both conditions. Histograms show mean ± s.e.m. from 7 different brains in every condition. *shLuc*: 14.57 ± 9.33. *shFak*: 80.22 ± 15.11. **t-test, P = 0.0040. Scale bars equal 100 µm (B and C) and 50 µm (D and E).

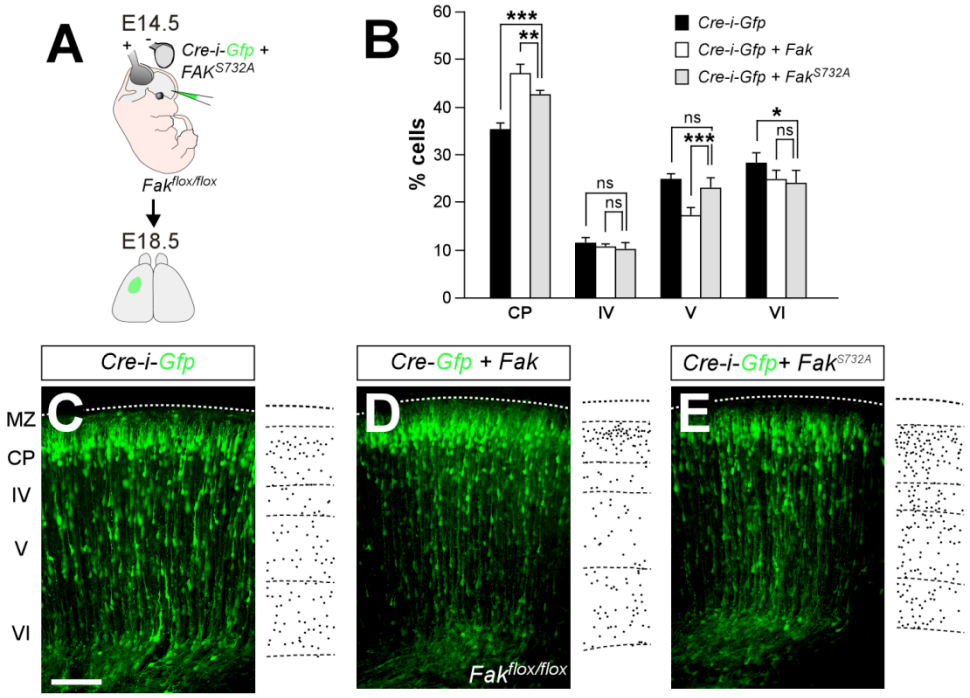

Figure S7. S732 only mediates part of the function of FAK in the migration of cortical neurons. (A) Schematic diagram of experimental design. (B) Quantification of the relative distribution (%) of GFP-expressing in different cortical layers for experiments shown in (C), (D) and (E). Histograms show mean ± s.e.m. from at least 3 different brains for each condition. *Cre-i-Gfp*: CP, 52.42 ± 0.85; layer IV, 12.72 ± 1.23; layer V, 17.18 ± 0.33; layer VI: 17.21 ± 1.61 *Cre-i-Gfp* + *Fak*: CP, 47.05 ± 1.84; layer IV, 10.79 ± 0.64; layer V, 17.25 ± 1.43; layer VI: 24.85 ± 2.00. *Cre-i-Gfp* + *Fak*^{S732A}: CP, 42.46 ± 0.88; layer IV, 10.12 ± 1.40; layer V, 23.02 ± 1.85; layer VI: 24.09 ± 2.61. X^2-test for *Cre-i-Gfp* and *Cre-i-Gfp* + *Fak*^{S732A} comparison: CP, $P < 0.001$; IV, ns; V, ns; VI, $P < 0.05$. X^2-test for *Cre-i-Gfp* + *Fak* and *Cre-i-Gfp* + *Fak*^{S732A} comparison: : CP, $P < 0.01$; IV, ns; V, $P < 0.001$; VI, ns. (C-E) Coronal sections through the somatosensory cortex of E18.5 *Fak*$^{flox/flox}$ embryos after E14.5 in utero electroporation with plasmids encoding for *Cre-i-Gfp* (C), *Cre-i-Gfp* + *Fak* (D), *Cre-i-Gfp* + *Fak*^{S732A} (E). Schemas with dots depict representative distributions of GFP-expressing neurons in each condition. Scale bar equals 100 µm.

shLuc vs shFak(1)

Layers	CP	IV	V	VI	
	42,479245	3,7081528	2,406887	88,971896	
	140,81158	12,291905	7,9784274	294,92694	
Result	183,29083	16,000058	10,385314	383,89883	
P value	***	***	**	***	1 d.f.

shLuc vs shFak(2)

Layers	CP	IV	V	VI	
	58,540656	0,4829723	12,864229	32,029332	
	49,617038	0,4093506	10,903276	27,146955	
Result	108,15769	0,8923229	23,767505	59,176287	
P value	***	ns	***	***	1 d.f.

shLuc vs shFak(2)+Fak

Layers	CP	IV	V	VI	
	1,4143952	9,2917005	0,4990818	1,1507619	
	2,7317897	17,946167	0,963936	2,2226035	
Result	4,1461849	27,237868	1,4630177	3,3733654	
P value	*	***	ns	ns	1 d.f.

shFak(1) vs shFak(2)+Fak

Layers	CP	IV	V	VI	
	126,03815	0,0339444	2,7155824	149,09008	
	73,437341	0,019778	1,5822602	86,868767	
Result	199,47549	0,0537224	4,2978425	235,95885	
P value	***	ns	**	***	1 d.f.

shFak(2) vs shFak(2)+Fak

Layers	CP	IV	V	VI	
	36,003647	6,4737961	7,993563	5,8123112	
	82,044552	14,752386	18,215607	13,245005	
Result	118,0482	21,226182	26,209171	19,057316	
P value	***	***	***	***	1 d.f.

Wt vs Cre in Fak$^{flox/flox}$

Layers	CP	IV	V	VI	
	23,768064	0,5539891	10,919335	21,06224	
	47,281319	1,1020391	21,721608	41,898679	
Result	71,049383	1,6560283	32,640943	62,96092	
P value	***	ns	***	***	1 d.f.

Cre in Fak$^{flox/flox}$ vs Cre in Fak$^{flox/flox}$ + Fak

Layers	CP	IV	V	VI	
	24,543445	0,2314703	21,64738	2,9914517	
	12,088132	0,1140037	10,661764	1,4733492	
Result	36,631577	0,345474	32,309144	4,4648008	
P value	***	ns	***	*	1 d.f.

Wt vs Cre in Fak$^{flox/flox}$ + Fak

Layers	CP	IV	V	VI	
	4,9492467	2,7003546	0,0019539	23,548277	
	4,8490713	2,645698	0,0019144	23,071647	
Result	9,7983181	5,3460526	0,0038683	46,619924	
P value	**	*	ns	***	1 d.f.

Cre in Fak$^{flox/flox}$ vs Cre in Fak$^{flox/flox}$ + Fak^{Y397F}

Layers	CP	IV	V	VI	
	0,6840062	4,6803548	2,3061417	0,306779	
	0,332883	2,2777726	1,1223223	0,1492991	
Result	1,0168892	6,9581274	3,428464	0,4560781	
P value	ns	**	ns	ns	1 d.f.

Cre in Fak$^{flox/flox}$ + Fak vs Cre in Fakff + Fak^{Y397F}

Layers	CP	IV	V	VI	
	39,68779	3,0516935	41,319516	5,8634242	
	39,216138	3,015427	40,828473	5,7937429	
Result	78,903929	6,0671205	82,147989	11,657167	
P value	***	*	***	***	1 d.f.

Cre in Fak$^{flox/flox}$ vs Cre in Fak$^{flox/flox}$ + Fak$^{I937E/I999E}$

Layers	CP	IV	V	VI	
	0,0586888	0,3448216	1,6897352	1,3367316	
	0,029119	0,1710861	0,8383763	0,6632306	
Result	0,0878078	0,5159077	2,5281114	1,9999623	
P value	ns	ns	ns	ns	1 d.f.

Cre in Fak$^{flox/flox}$ + Fak vs Cre in Fak$^{flox/flox}$ + Fak$^{I937E/I999E}$

Layers	CP	IV	V	VI	
	26,201766	0,0127359	11,695809	9,2855748	
	26,395337	0,01283	11,782214	9,3541739	
Result	52,597103	0,0255659	23,478023	18,639749	
P value	***	ns	***	***	1 d.f.

Cre in Fak$^{flox/flox}$ vs Cre in Fak$^{flox/flox}$ + Fak$^{K38A/R86A}$

Layers	CP	IV	V	VI	
	0,2342499	1,4641287	0,2045312	2,9088037	
	0,1318048	0,8238177	0,1150831	1,6366895	
Result	0,3660547	2,2879464	0,3196143	4,5454932	
P value	ns	ns	ns	*	1 d.f.

Cre in Fak$^{flox/flox}$ + Fak vs Cre in Fak$^{flox/flox}$ + Fak$^{K38A/R86A}$

Layers	CP	IV	V	VI	
	30,306112	0,5916084	16,268663	12,220423	
	34,622499	0,675869	18,585748	13,960932	
Result	64,928611	1,2674775	34,854411	26,181354	
P value	***	ns	***	***	1 d.f.

Cre in Fak$^{flox/flox}$ vs Cre in Fak$^{flox/flox}$ + Fak^{S732A}

Layers	CP	IV	V	VI	
	8,0594331	0,8992565	0,7904177	3,7033755	
	5,3697572	0,5991475	0,5266315	2,4674474	
Result	13,42919	1,4984039	1,3170491	6,1708229	
P value	***	ns	ns	*	1 d.f.

Cre in Fak$^{flox/flox}$ + Fak vs Cre in Fak$^{flox/flox}$ + Fak^{S732A}

Layers	CP	IV	V	VI	
	2,8788171	0,2677518	10,398385	0,1445493	
	3,8943997	0,3622087	14,066704	0,195543	
Result	6,7732168	0,6299604	24,46509	0,3400923	
P value	**	ns	***	ns	1 d.f.

Fak$^{flox/flox}$ vs *NEX-Cre*;Fak$^{flox/flox}$

Layers	CP	IV	V	VI	
	11,816264	0,1757954	2,6014394	9,0514322	
	12,522867	0,1863078	2,7570035	9,5927007	
Result	24,339131	0,3621032	5,3584429	18,644133	
P value	***	ns	*	***	1 d.f.

E12 shLuc vs shFak(1)

Layers	CP	IZ			
	0,4697593	0,9178225			
	0,122348	0,2390452			
Result	0,5921073	1,1568677			
P value	ns	ns	1 d.f.		

Figure S8. Statistical parameters and X^2 tests used to analyze the different migratory phenotypes described in this study.

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Summary of results and general discussion

Different migratory mechanisms in the cerebral cortex are sustained by specific leading process dynamics

Migrating cortical neurons are generated in different areas of the forebrain and therefore use different migratory routes. For projection neurons, the interaction with radial glia fibers largely defines the direction of migration with the exception of early-born pyramidal cells, which reach their final position in a process that is independent of the radial glia scaffold. By contrast, the number of extracellular cues that seem to guide migrating cortical interneurons is very large, much higher than that described for projection neurons. These differences suggest that the migratory behavior of both cell types might be very different. In fact, migratory cells of both classes have very different morphologies. In particular, differences in the leading process illustrate particularly well that projection neurons and interneurons use different mechanisms to adapt to their respective migratory requirements. Thus, while the leading process of projection neurons generally consists of a single branch, migratory interneurons develop a much more complex leading process, which branches continuously as part of the migratory cycle. Experimental evidence from our studies and from others suggests that the specific dynamic of each type of leading process is crucial for the migration of both cell types (Kawauchi et al., 2003; Polleux et al., 2002; Ward et al., 2005).

Time-lapse assays of migrating interneurons revealed that their leading process is continuously remodeled, dynamically adapting to specific requirements of the migratory pathway. For instance, during subpallial migration, interneurons randomly generate and select alternatively processes in a binary manner, which leads to a rectilinear trajectory. The same mechanism is also used during their intracortical tangential migration within the different cortical streams. In contrast, rapid tangential to radial changes in direction, for example during CP invasion, are preceded by the generation and selection of a new branch that is better oriented towards the final destination of the cell. We have also shown that leading process branches do not steer much, or at least that the reduced steering activity they display does not sustain rapid turnings. In other words, the angle formed between the existing leading process branch and the newly generated branch remains stable until one of the branches retracts in the subsequent cycle. Hence, the two branches define the two possible directions followed by the cell.

Our experiments suggest that leading process branches extend and retract in response to extracellular cues that interneurons encounter during their migration. This dynamic behavior is perturbed when the activity of ROCKI/II is blocked, as this causes migrating interneurons to loose all branches except one, which is extended. In the absence of multiple branches, the ability of interneurons to change direction is severely compromised. In vitro, MGE derived interneurons fail to reorient their migratory direction in response to COS cells aggregates secreting the chemoattractant Ig-Nrg1. In organotypic culture assays, the inability of interneurons to generate new processes perturbed their switch from tangential to radial migration, which causes an overall reduction in the number of cells invading the CP. Thus, leading process branching is the fundamental cellular mechanism underlying directional guidance of cortical interneurons.

In contrast to interneurons, a single leading process seems sufficient to sustain the migration of projection neurons after they have left the multipolar stage at the SVZ/IZ (Noctor et al., 2004; Tabata and Nakajima, 2003). Others and we have found that pyramidal cell radial migration is blocked in a multipolar morphology when they fail to polarize and generate a single leading process (Chen et al., 2008; Elias et al., 2007; Heng et al., 2008; Ohshima et al., 2007). *Fak*-deficient neurons, for example, have multiple leading process branches, and the distance between migrating neurons and their parental radial fiber is increased. Interestingly, similar defects were found in mouse mutants for the p35 regulatory unit of Cdk5 (Gupta et al., 2003). Thus, *p35* mutant projection neurons also display increased number of leading process branches and increased distance from RGC fibers. These observations suggest that the two phenotypes (e.g. increased leading process branches and decrease adhesion to RGCs) might be linked. In other words, that defects in leading process branching are a consequence of decrease adhesion to RGC rather than a completely cell autonomous regulation of the dynamic behavior of the leading process. To address this question, we analyzed the migration of *Fak*-deficient early-born projection neurons, whose migration is independent of RGCs. The normal morphology and migratory behavior of these cells in absence of FAK function suggests that this protein is primarily implicated in regulating the interaction between neurons and RGCs, and that probably the morphological defects observed in late-born projection neurons derive from their inability to attach to the glial scaffold. These results also suggest that glial-guided migration somehow seems to restrict leading process branching in projection neurons.

The specific molecular requirement of FAK during glial-guided migration, but not during early somal translocation, is common to Cdk5. Loss of Cdk5 also disrupts the migration of late-born projection neurons without affecting the migration neurons undergoing somal translocation (Hatanaka et al., 2004). These observations suggest that FAK and Cdk5 might act in the same signaling pathway regulating glial-guided migration. In fact, it has been shown that Cdk5 phosphorylates FAK at a specific residue in the serine 732, and mutation of this specific residue disrupts migration of projection neurons when expressed in a wild type background (Xie et al., 2003). Our results, however, suggest that expression of this form of mutant FAK (FAK^{S732A}) can partially rescue the loss of FAK function, which indicates that phosphorylation at S732 by Cdk5 does not completely account for the phenotype observed in *Fak*-deficient neurons. Accordingly, point mutations blocking FAK interaction with Paxillin and Arp2/3, two proteins involved in adhesion dynamics and actin nucleation, respectively, severely disrupt radial migration. In sum, although we confirmed the role of Cdk5-FAK signaling in radial migration, our results suggest that additional FAK related molecular pathways are involved in this process.

Integration of guidance information in branched leading processes

Neuronal migration was initially studied in the developing cerebral cortex (Rakic, 1972), and for this reason the typical bipolar morphology of cortical projection neurons has been systematically used to illustrate neuronal migration in the CNS. Work over the past decade has revealed that the migratory behaviors of neuronal precursors throughout the nervous system are very diverse, and that bipolar morphologies are rather uncommon for migrating neurons. Instead, the use of branched leading process seems to represent the main strategy employed by neuronal precursors to move throughout the nervous system. For example, cortical interneurons (Marín and Rubenstein, 2001), precerebellar neurons (Komuro et al., 2001), mossy-fiber projecting precerebellar nuclei neurons (Watanabe and Murakami,

2009), neuronal subpopulations of the thalamus and spinal cord (Martini et al., 2009), and neuronal precursors of the enteric nervous system (Hao et al., 2009), they all seem to display branched leading processes as part of their migratory cycle. Since the discovery of the subpallial origin of cortical interneurons (Anderson et al., 1997), extracellular guidance cues have been considered as major players in their guidance. However, the particular morphology of the leading process in these cells suggests that the mechanisms used to integrate extracellular signals must be different than those used by projection neurons or growing axons.

Leading process branches in tangentially migrating neurons are tipped with growth cones that are similar to those described for axons. Axonal growth cones are highly motile structures that are responsible for sensing the surrounding microenvironment during navigation. In response to extracellular cues, the growth cone of axons undergoes differential growth and retraction that leads to steering of the axon in a particular direction (Wen and Zheng, 2006). In contrast, although the growth cones found in migrating interneurons are also highly dynamic, and display extended and collapsed morphologies during the migratory cycle, they do not seem to modify their trajectory during their lifetime. Consequently, interneurons do not change direction through growth cone steering, but rather by maintaining the leading process branch that is best aligned with the guidance signal. We have also observed that interneurons may adapt the angle at which they branch the leading process depending on the environment. During the subpallial migration and subsequent tangential dispersion through the pallium, interneurons branch their leading process at relatively small angles and tend to maintain left or right branches with similar probability. As a result, neurons follow rectilinear trajectories during these phases of the migration. The leading process branching mechanism used by migrating interneurons has been mathematically modeled as a stochastic birth-death process in which the migratory interneuron efficiently searches an unexplored territory through a directed but still random exploration (Britto et al., 2009). Of note, this mathematical model has only taken into consideration the influence of motogenic factors in leading process branching, but not that of chemoattractive or chemorepellent cues. To explain the ability of interneurons to undergo rapid changes in direction, we presented migrating interneurons with a sharp gradient of a chemoattractant molecule. The results of these experiments, which indicate that cells rapidly respond to guidance cues by generating branches that are better aligned with the source of the signal, discard the possibility that sharp changes in direction are autonomously fixed in the migratory program of the cell. Thus, extracellular cues may exert their guidance function by regulating the direction of branch generation. In addition, we found that under the influence of guidance cues, migrating interneurons tend to maintain branches that are better aligned towards the source of the signals, which suggest that guidance cues also influence the process of selection/removal of branches. Thus, the later process might be stochastic in shallow gradients, but not in the presence of profound differences in guidance cues.

We believe that the mechanism described here for cortical interneurons might be used by many other long-range migrating cell types which guidance is highly dependent on diffusible molecules rather than on physical guiding substrates. In this type of guidance, cellular mechanisms that allow optimization of metabolic resources, time and cell displacement are critical to reach their destination properly. In that sense, one could compare different hypothetical migrating morphologies with the one describe above. For example, cells with a branched leading process might be able to explore a wider territory in search for guidance cues than cells with a single leading process that steers for guidance information. Another advantage of cells with branched leading processes could be the ability of computing data simultaneously

from the broad territory occupied by both branches (up to 50 microns in the case of migrating cortical interneurons). This will be much more limited in migrating cells with a single leading process, because integration of information occurs only in the growth cone. These limitations would likely increase the time needed by cells to find their way, especially when sharp changes in direction are required. Thus, for long-range migrations, neurons with a single leading process will be less efficient than those with branched leading processes

If having two branches is good, why not having more? Cells with three or more branches should be able to recognized extracellular cues faster than cells with two leading process branches simply because their branches are oriented in more directions. However, having too many branches might become inefficient if the time and resources necessary to compute very subtle differences in the extracellular territory also increases. From an evolutionary point of view, the use of leading process branches by migrating neurons may recapitulate the same mechanisms used by other organisms and cell types, such as *Dictyostelium*, neutrophils and fibroblasts, in which directed movement in shallow gradients relies on the differential stabilization of protrusions within the leading edge (Andrew and Insall, 2007). In fact, branches are very dynamic and consistently behave as binary elements, as every migratory cycle always finish with the collapse of one of the growth cones and its corresponding branch in benefit to the other one. This observation could be interpreted as the ability of these cells to compute differences between at least two values within the explored field at every point in which they must decide the direction to be followed. Such a mechanism seems appropriate for providing accurate guidance in highly complex extracellular territories.

Substrates of migrating cortical neurons

Cell migration involves the generation of forces against a substrate that allow movement in a certain direction. For this reason, migration is largely dependent on the type of substrate used by migrating cells. In the CNS, migrating neurons are found in very different environments along the neural tube, which suggests that different neuronal precursors must adapt to the substrate on which they migrate and modify the basic migratory cycle as part of this process. In the case of the radial migration of projection neurons in the cerebral cortex, these cells have adopted a mode of migration that involves interaction with radial glia fibers for most of the migratory pathway. This requires that migrating neurons acquire the ability to recognize and interact with RGCs, which involves specific molecular requirements. The molecular regulators of this interaction have been shown to belong to the integrin family (Dulabon et al., 2000; Sanada et al., 2004), and more recently to connexin proteins (Cina et al., 2009; Elias et al., 2007). Integrin receptors expressed by projection neurons recognize different components of the extracellular matrix, and therefore are thought to mediate cell-matrix interactions (Legate et al., 2009). Connexins typically travel to the plasma membrane and accumulate forming plaques (Thomas et al., 2001). Subsequently, connexin plaques from adjacent cells interact to form large, channeled structures known as Gap junctions. There are, however, increasing evidences suggesting that Gap junctions also have functions that are independent of channel formation, and one of those would be their implication in adhesion processes (Prochnow and Dermietzel, 2008). This seems to be the case in radial migration, where connexins are expressed in both migrating neurons and RGC fibers, and are though to mediate transient adhesions between both cells (Elias et al., 2007).

The first evidence that integrins mediate neuron-glia interactions was obtained from in vitro experiments in which blocking α3 integrin receptors resulted in a reduction in the speed of migration and the dissociation of neurons from glia in aggregation assays (Anton et al., 1999). Further analysis of mouse mutants for this receptor revealed a clear disorganization of the cerebral cortex, indicative of a possible implication in migration (Anton et al., 1999; Schmid et al., 2004). In line with these results, it was also shown that detachment from radial glia fiber at the end of the migration requires the endocytosis of integrins (Dulabon et al., 2000). However, recent analysis of conditional knockout mice for integrin in postmitotic neurons have not revealed major abnormalities in the cerebral cortex, thus arguing against a possible cell-autonomous role for these receptors in the migration of projection neurons (Belvindrah et al., 2007). The prominent cortical defects observed in mice in which β1 receptors were ablated in neuronal progenitors argue in turn to a role for all α integrin receptors (which must dimerize with β1) in the development of RGCs (Radakovits et al., 2009). To further complicate this issue, a recent study has shown that conditional deletion of laminin γ1 from postmitotic neurons in the cerebral cortex leads to migratory defects without affecting the morphology of RGCs (Chen et al., 2009). Laminin γ1 seems to cover the processes of RGCs hypothetically after being secreted by projection neurons, so it is possible that migrating neurons fail to properly adhere to RGCs in the absence of this ECM component. Interestingly, activation of FAK and Paxillin is dramatically impaired in laminin γ1 mutant mice, suggesting that laminin deficiency disrupts integrin downstream events that involve the formation of adhesion points (Chen et al., 2009). Since no direct evidence on the implication of integrin receptors in the phenotype has been shown, it remains possible that the interaction with laminin γ1 is mediated by other receptors. Our own results also support a role for integrin downstream effectors in the migration of projection neurons. Disruption of FAK in migrating projection neurons through in utero electroporation using RNAi against *Fak* or expression of *Cre* in $Fak^{flox/flox}$ mouse embryos disrupts radial migration. We also showed that this defect does not derive from physical disruption of RGCs and that it is, at least in part, cell autonomous. In vitro, adhesion assays showed that *Fak*-deficient cells fail to attach to laminin coated plates, reinforcing the possibility that integrin downstream effectors would in fact regulate the interaction of projection neurons with their substrate during migration.

What is the basis for such a divergence in experimental results on the implication of integrins in the radial migration of projection neurons? One possibility is related to the different analytical methods that have been used in the assessment of different mouse mutants. Since the final role of migration is to assure the arrival of neuronal precursors to their final position, evaluation of the function of different molecular regulators of neuronal migration have been mostly addressed by analyzing the final picture that emerge after the end of migration, which in the case of the cerebral cortex is the formation of a six-layered structure. Although many molecules that severely disrupt this layered structure have shown to play an important role in migration, it is also true that not all molecules implicated in the regulation of migration lead to disruption of layering. In some cases this is due to redundancy, as other proteins may overcome the loss of function of a single protein. This is not surprising, given the relevance of migration in building up the nervous system. This is well illustrated with the role of DCX in cortical development. *Dcx* mutations cause prominent layering defects in humans, while mouse mutants for *Dcx* do not have major defects in the cerebral cortex (Corbo et al., 2002). However, acute knockdown of *Dcx* in rats reproduces the human phenotype (Ramos et al., 2006), suggesting that other proteins could compensate for the loss of *Dcx* in the mutant. This protein exists and is named doublecortin like kinase (DCLK). *Dclk* mutants do

not have defects in the migration of projection neurons, but *Dcx;Dclk* double mutants have severe impairment of the development of the cerebral cortex in part derived from neuronal migration arrest (Deuel et al., 2006; Koizumi et al., 2006b).

In addition to compensatory mechanisms, the absence of a major disruption in the layering of the cortex should not exclude by principle a possible function for a protein in the migratory cycle of projection neurons. This seems to be the case for FAK, which seems to be involved in the migration of projection neurons as shown by our results and previous studies (Xie et al., 2003), but whose genetic disruption in postmitotic neurons (*NEX-Cre*;*Fak*$^{flox/flox}$ mice) did not reveal substantial defects in cortical structure (Beggs et al., 2003). Our analysis of the same mutant mice revealed a subtle but consistent defect in neuronal migration, which reinforces the view that FAK plays a cell-autonomous role in this process. Moreover, although most neurons were able to reach their proper layer in the cortex after acute knockdown of *Fak* at E14.5, a small population of projection neurons was found arrested in infragranular layers. Interestingly, these cells showed a defect in dendritic pattern that is similar to that described previously in *NEX-Cre*;*Fak*$^{flox/flox}$ mice (Beggs et al., 2003), suggesting that knockdown experiments and genetic ablation have very similar consequences. In conclusion, we believe that in addition to the histological analysis of the cortex, the migration of projection neurons must be carefully assessed with modern techniques that allow the analysis of the different phases of the migratory cycle before ruling out the involvement of specific genes in this process (Noctor et al., 2001; Tabata and Nakajima, 2001).

Recent studies have shown that the interaction of migrating neurons with radial glia fibers also relies on connexin dependent cell-cell adhesions. Connexin 43 (Cx43) and Connexin 26 (Cx26) have been both involved in this process through RNAi knockdown and genetic ablation in vivo (Cina et al., 2009; Elias et al., 2007). The role of these proteins seems to be independent on channel formation, since point mutations in certain residues of Cx43 and Cx26 necessary for the formation of channels do not prevent radial migration (Elias et al., 2007). In other cell types, Cx43 and Cx26 do not localize to the same membrane plaques, which suggests that they may complement each other rather than interacting among them (Falk, 2000). Accordingly, specific connexins seem to be enriched at specific different subcellular localizations within migrating projection neurons: Cx43 is mainly located at the leading process, whereas Cx26 is enriched at the perinuclear dilatation, in close proximity to the centrosome (Elias et al., 2007). FAK is also localized at the swelling in a dotted pattern similar to Cx26. We found that knockdown of *Fak* leads to a decrease in the number of detectable dots of Cx26 in migrating neurons, suggesting that this scaffold protein could also be implicated in the regulation of adhesions between migrating cells and radial glial fibers. Interestingly, Cx26 seems to directly interact with FAK in a prostate human cancer cell line (Tate et al., 2006), which indicate that a possible role for FAK in this process may involve the stabilization of Cx26 close to the membrane of migrating neurons.

Although the structure and regulation of Cx43 are well characterized this is not the case for Cx26. Its structure is very different from Cx43, with a shorter C-terminus, and its regulation is largely unknown (Hervé et al., 2004; Maeda et al., 2009). Src is one of the most important regulators of connexins, as it induces connexin degradation via the lysosome and proteosome pathways (Leithe and Rivedal, 2007; Pahujaa et al., 2007). The process of degradation is mediated by Src-dependent phosphorylation, although other proteins such as MAPK and PKC have also been implicated in this process. This role of Src is well characterized for Cx43, but the implication of this kinase in Cx26 degradation remains

unknown. In our experiments, however, we were able to block the reduction in Cx26 plaques following *Fak* knockdown by blocking Src activity. This result is surprising, not only because it suggests that Src could also be implicated in the disruption of Cx26 containing junctions, but also because Src blocking alone has no effect on the number of Cx26 plaques. This suggest that FAK could be playing a role in the stabilization of Cx26 plaques at the membrane and that Src will be only active in this context after loss of FAK function, leading to the disruption of connexin junctions. Since Src activation is tightly coupled to FAK autophosphorylation and activation of the kinase domain (Mitra et al., 2005), it is more plausible that this mechanism implicate intermediate proteins. One of the candidates to play a role in the process is occludin. This protein is implicated in the establishment of the blood-testis barrier tight junctions in a FAK dependent manner (Siu et al., 2009). Depletion of *Fak* reduces tyrosine and serine phosphorylation in occludin which results in disassembly of zona occludens-1 (ZO-1)/occludin complexes leading to tight junction disruption. Interestingly, occludin is one of the proteins that are known to interact with Cx26 (Nusrat et al., 2000). Future experiments should address the specific mechanism through which FAK regulates Cx26 plaque formation and the involvement of Src and other proteins in this process.

The substrates supporting the migration of cortical interneurons are largely unknown, although several candidates have been proposed. The close apposition of tangentially migrating interneurons with thalamocortical and corticofugal axons was considered suggestive of a potential interaction. Although different laboratories have performed in vitro experiments favoring this hypothesis (Denaxa et al., 2001; McManus et al., 2004), in vivo perturbation of molecules thought to be involved in this interaction does not perturb the migration of interneurons (Denaxa et al., 2005). Consequently, additional experiments would be required to clarify this interaction. For example, time-lapse videomicroscopy analysis of interneurons migration in mouse models in which these axonal projections are disrupted should shed light into this question. Once interneurons have arrived to the cerebral cortex, the processes of RGCs become potential substrates supporting their migration. During their tangential dispersion, interneurons transfer from one stream to another in an oblique manner, which suggest that this process is not dependent on the radial glia scaffold. However, interneurons invade the CP following a radial direction (López-Bendito et al., 2008), and it is possible that they may interact with the basal processes of RGCs during this phase of their migration. Dynamic analysis of this phase of the migratory process suggests that the interaction of migrating interneurons with RGCs is not as permanent as for projection neurons (Yokota et al., 2007), but its requirement in interneuron migration remains unknown. Recent experiments have described that interneurons invade the CP only after projection neurons have reached their final position (López-Bendito et al., 2008), which indicates that it would be possible to disrupt radial glia morphology without affecting the positioning of projection neurons, thereby testing to what extent the final phase of interneuron migration really depends on radial glia.

Radial and tangential migrations are not mutually exclusive

There are several examples throughout the CNS that support the idea that the mechanisms underlying radial and tangential migrations are not mutually exclusive. In other words, it is rather frequent that the same population of neurons uses radial and tangential movements during different phases of the migration, with the later mechanism typically taking place after radial migration. This could be interpreted as suggestive that tangential migration may allow cells to travel long distances, and that this requires a

certain independence of the physical restrictions for movement that the radial glial scaffold imposes. Subsequently, neurons would use radial migration to integrate into their final position. For instance, precerebellar neuronal precursors migrate tangentially through the external granule layer of the cerebellum until they reach a certain position, and then they switch their orientation to migrate radially in close apposition to the scaffold provided by Bergmann glial cells (Komuro et al., 2001; Kumada et al., 2009). A similar routine is used by mossy-fiber projecting precerebellar nuclei neurons in their way through the hindbrain. These cells migrate tangentially towards the midline until they reach the region close to where the pontine grey nucleus and reticulotegmental nucleus will be formed. At this position, the cells undergo a tangential to radial switch that allow them to interact with the radial scaffold and find their final position in their correspondent nuclei (Watanabe and Murakami, 2009). Finally, migrating cortical interneurons use tangential migration to reach and disperse through the cortex, and then invade the CP through radial migration in close association with the fiber scaffold provided by RGCs.

As mentioned above, radial migration of interneurons do not seem to follow the same rules that apply for the radial migration of projection neurons. Thus, the persistence of contacts, direction of migration and morphology seem to be different. Moreover, it is clear that interneurons have the ability to invade the CP from the MZ, which indicates that they can travel radially in the opposite direction than projection neurons or other interneurons coming from the subplate or SVZ streams (López-Bendito et al., 2008; Yokota et al., 2007). Since the molecular specificities of the radial glia scaffold within the CP are thought to induce the detachment of migrating neurons to facilitate the process of lamination (Gongidi et al., 2004), it is unclear how interneurons coming from the MZ would begin to interact with radial glial fibers precisely at that location to start their radial migration in the direction to the ventricle. Recent findings suggest that although interneurons seem to express Dab1, their integration into specific layers of the cortex seems independent of Reelin signaling (Pla et al., 2006). Interestingly, migrating cortical interneurons express α3 integrin (Stanco et al., 2009). In projection neurons, it has been suggested that interaction of α3 integrin with Reelin leads to Dab1 phosphorylation and induce the detachment of migrating projection neurons from radial glia through a process that involves the removal of α3 integrins from the membrane (Dulabon et al., 2000; Sanada et al., 2004). In migrating interneurons, in contrast, α3 integrin receptors have been shown to bind to Netrin-1, which is expressed by cells at the MZ. Conditional deletion of both α3 and Netrin-1 from migrating interneurons accelerates the switch from tangential to radial migration, which suggests that radial migration of interneurons does not depend on the same regulatory mechanisms than projection neurons. Intriguingly, the authors also reported a substantial increase in the reversal of polarity of migrating cells in the MZ, which could indicate that a population of interneurons fails to undergo radial migration in the absence of α3 integrin (Stanco et al., 2009).

If tangentially migrating interneurons can switch to radial migration, it is conceivable that radially migrating projection neurons may also undergo tangential dispersion before reaching their final position. Such a mechanism would be appropriate in gyrencephalic species, in which the cerebral cortex is folded to cope with its massive tangential expansion (Van Essen and Drury, 1997). Since the ventricular wall does not expand in parallel to the overlying CP (Mayhew et al., 1996), projection neurons that were born in similar locations must cover vast territories in the cerebral cortex. Thus, it is reasonable that, among other mechanisms, projection neurons may have acquired through evolution the ability to disperse tangentially during the last phase of their migration (Torii et al., 2009). Our experiments showed that regulation of molecules that control the interaction between migrating neurons and radial glial fibers, such as FAK,

might indeed provide a mechanism for the tangential dispersion of projection neurons. Downregulation of *Fak* leads to lateral movement of projection neurons, away from the parental radial glial fiber. Moreover, the morphology of these cells was similar to that found in tangentially migrating cortical interneurons, which suggest that disruption of neuron-glia interactions through *Fak* knockdown may not abort radial migration but also induce tangential dispersion of projection neurons. The contribution of tangential dispersion to the final positioning of projection neurons needs to be further explored.

Role of FAK in tangential migration

Our studies suggest that FAK regulates the adhesive properties of projection neurons during radial migration in the cerebral cortex. It remains to be solved, however, whether this kinase also participates in controlling the dynamics of adhesions in other migrations. We addressed this possibility by examining the function of FAK in migrating cortical interneurons, since these cells also express this protein during migration. Conditional deletion of *Fak* from subpallial-derived interneurons in *Dlx5,6-Cre;Fak*$^{flox/flox}$ mouse mutants does not seem to perturb the migration of cortical interneurons. Mutant cells migrate normally out of MGE explants and reach the pallium in organotypic cultures, with no apparent defects in their tangential dispersion or eventual switch from tangential to radial migration. Of note, these mice develop a cleft palate, which prevented us from analyzing these mice after birth. Nevertheless, analysis of mice in which *Fak* was specifically deleted from MGE-derived interneurons (*Nkx2.1-Cre;Fak*$^{flox/flox}$ mice) confirmed that loss of FAK does not seem to affect the migration of cortical interneurons.

To discard the possibility that the function of FAK in migration of interneurons could be masked by a compensatory mechanism, we simultaneously knocked down the structurally-related protein PYK2. In other experimental models, PYK2 has been shown to take over FAK function in the absence of this protein (Lim et al., 2008; Weis et al., 2008). Downregulation of *Pyk2* in the MGE by RNAi did not prevent the tangential migration of interneurons at E13.5. When RNAi against *Pyk2* was electroporated in *Dlx5,6-Cre;Fak*$^{flox/flox}$ mice, we detected a decrease in the number of interneurons reaching the pallium, suggesting that PYK2 could be playing a role in the compensation of FAK loss of function. *Pyk2* is not normally expressed at E14.5, but it begins to be expressed within the next two days, making plausible the hypothesis that upon depletion of *Fak*, *Pyk2* is expressed and compensate for its function.

FAK may play a role in regulating the adhesion of cortical interneurons, but since tangentially migrating neuronal precursors do not use radial glia as a substrate, the function of FAK in this context would be different. Consistently, connexins are not likely to play a role in the migration of cortical interneurons (Elias and Kriegstein, 2007). Since conditional deletion of certain integrins from interneurons perturbs their migration (Stanco et al., 2009), and FAK activation is one of the main downstream events following integrin activation (Legate et al., 2009), it is plausible that FAK and Src play a role downstream of integrins during the tangential migration of interneurons. As described earlier, interneurons deficient in α3 integrins have a tendency to prematurely switch from tangential to radial migration (Stanco et al., 2009).

FAK as an integrator of adhesion-dependent functions in cortical neurons

FAK has been shown to be implicated in a wide variety of biological processes in the nervous system, including proliferation, migration and differentiation (Rico et al., 2004; Xie et al., 2003; Yutaka et al., 2003). Our results have shed light on the specific role of this scaffold protein in the migration of cortical neurons. The function of FAK during cortical development was previously investigated, but results were contradictory. Disruption of FAK from proliferative areas severely disrupts cerebral cortex development mainly through its implication in the maintenance of basal membrane integrity. In the absence of FAK, the basal membrane looses its continuity, which disrupts the attachment of radial glia endfeet and leads to a cobblestone lissencephaly-like phenotype, characterized by excessive migration of neurons out of the pial basal membrane (Beggs et al., 2003). In this study, however, the authors did not perform a careful analysis of the developmental stages in which *Fak* was specifically deleted in projection neurons.

Analysis of the phenotype of a non-phosphorylatable mutation of FAK in neuronal migration was also previously addressed (Xie et al., 2003). This study revealed a role for FAK in the migration of projection neurons, but the mechanisms through which this process was regulated were only minimally explored. Our results demonstrate that the function of FAK in neuronal migration does not exclusively involved phosphorylation by Cdk5, as previously suggested (Xie et al., 2003). We found that interactions of FAK with proteins related to membrane targeting and actin cytoskeleton nucleation are very important in this process. Moreover, we showed that interaction with these proteins activates an important signaling pathway regulating the adhesive properties of migrating neurons. Both cell-matrix and cell-cell interactions are likely to be affected in projection neurons lacking FAK, which leads to delayed migration. However, disrupting the adhesive properties of migrating neurons does not completely abrogate their ability to move. Consistent with this idea, analysis of single clones after *Fak* depletion suggest that neurons deviate from the radial glia, but they still retain the ability to migrate. Finally, our finding of the regulation of connexin function during radial migration describes a new role for FAK in the developing nervous system. The precise mechanism underlying this process remains to be fully elucidated.

Our analysis of the function of FAK in regulating the migration of different classes of cortical neurons nicely illustrates the important differences that exist in the mechanisms controlling the migratory behavior of interneurons and projection neurons. These not only relate to the migratory pathways, substrates, or morphologies adopted, but also to the intracellular signaling pathways used by each class of neuron to successfully adopt a specific migratory program.

Conclusions

1. The migration of cortical interneurons is dependent on their ability to generate a branched leading process which allows their adaptation to distinct guidance requirements along the migratory pathway.

2. The RhoA effector ROCK seems to be implicated in the regulation of leading process dynamics, although the underlying mechanisms remain unknown.

3. Focal adhesion kinase (FAK) plays an important role in radial migration by regulating the adhesive properties of migrating projection neurons to radial glial fibers. This process involves FAK autophosphorylation and interaction with Paxillin and Arp2/3 complex.

4. FAK controls cell-cell adhesion during radial migration at least in part by regulating the distribution of connexins at adhesion points in migrating neurons.

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