THE EXTRACELLULAR ENVIRONMENT IN CONTROLLING NEURONAL MIGRATION DURING NEOCORTICAL DEVELOPMENT

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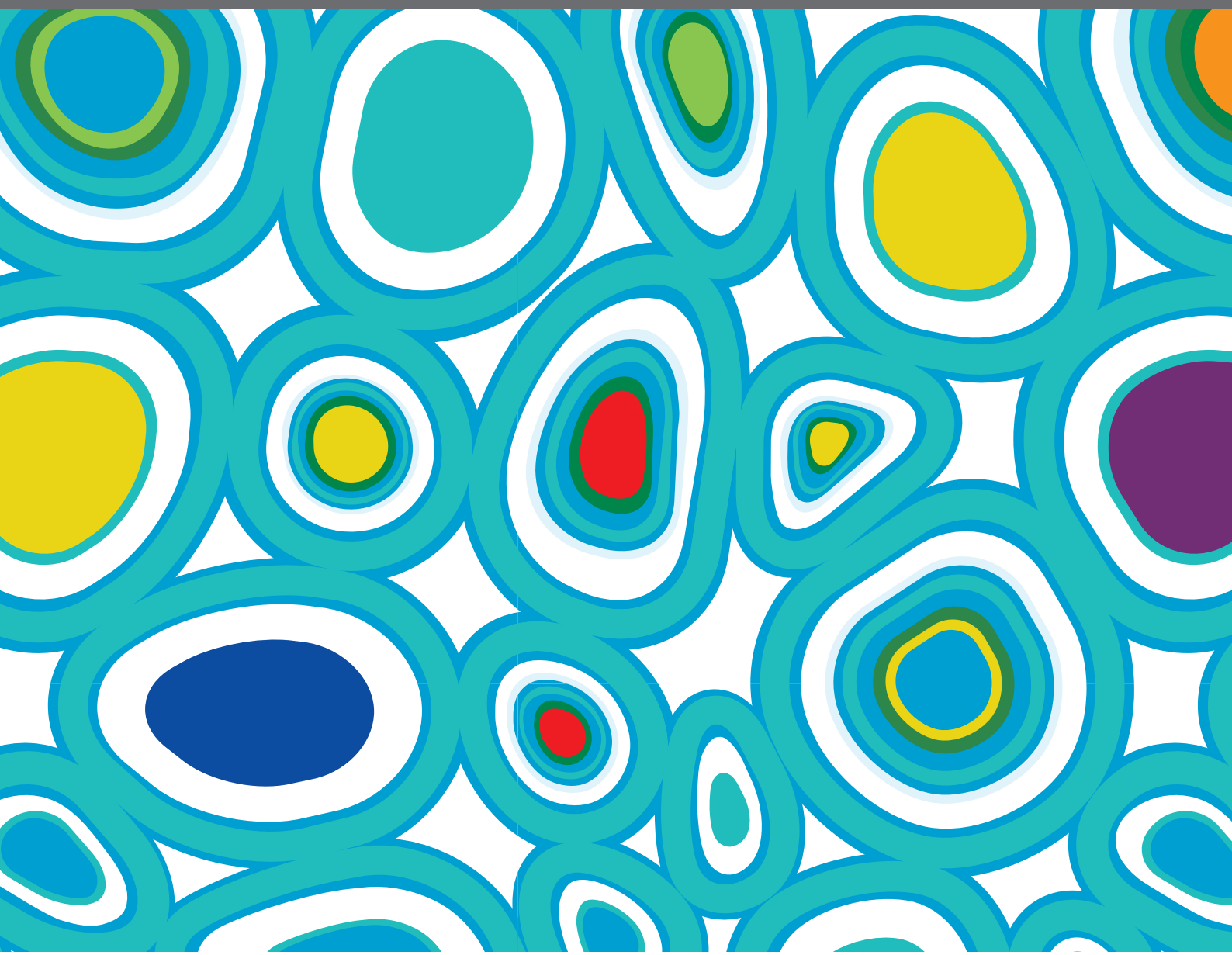

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THE EXTRACELLULAR ENVIRONMENT IN CONTROLLING NEURONAL MIGRATION DURING NEOCORTICAL DEVELOPMENT

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EDITORIAL
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Editorial: The Extracellular Environment in Controlling Neuronal Migration During Neocortical Development

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Editorial on the Research Topic

The Extracellular Environment in Controlling Neuronal Migration During Neocortical Development

In the developing brain, new neurons are generated at specific locations before migrating to their final destination, where they perform their adult functions. The cerebral cortex comprises neurons born in different regions and at different developmental times, but these are eventually organized into six layers according to their birth date. During this process, excitatory neurons undergo dynamic changes in their morphology and migration modes, including multipolar migration, locomotion, and terminal translocation. During development, neuronal migration impairment causes abnormal organization of the neocortex, resulting in various functional disorders such as epilepsy and neurological disability. Understanding the precise mechanisms underlying neuronal migration is a fundamental basis for understanding both neocortical development in the healthy brain and pathophysiological mechanisms underlying developmental neurological disorders.

During migration, neurons sense and respond to a broad range of extracellular environmental signals, including biochemical and mechanical cues, which serve as scaffolds to guide and support them. Genetic and molecular analyses have revealed signaling networks that precisely control the biochemical cellular microenvironment during neocortical development, including extracellular matrix (ECM) proteins, cell surface proteins, and gradients of diffusible guidance cues. Recent studies using live imaging and physical measurement techniques, such as atomic force microscopy (AFM), have revealed the essential role of the mechanical environment, including tissue stiffness, dynamic forces, and scaffolds, in neuronal migration. This Research Topic aims to highlight the critical relevance of the extracellular environment to various modes of neuronal migration in the mammalian brain under both physiological and pathological conditions.

Radial glial (RG) cells lining the ventricular surface function as primary neural progenitor cells, generating various neurons and glial cells. RG cells also serve as a physical scaffold supporting the radial migration of newborn neurons. In this Research Topic, Ferent et al. review the extracellular factors controlling the formation and maintenance of RG scaffolding. The first step of neuronal migration is cell delamination, in which neuronally differentiating cells generated from apical RG cells retract their apical processes and depart from the ventricular surface. Kawaguchi reviews the cellular and molecular mechanisms that regulate this process.

Cell adhesion molecules located on the cell surface play essential roles in the interaction between migrating neurons and their environment. The review by Martinez-Garay summarizes the function of cadherins during cortical migration, mainly focusing on CDH2, which plays critical roles in multiple steps of migration, including the apical delamination of newborn neurons, acquisition of polarity in the intermediate zone, RG-guided locomotion, and terminal soma translocation. More specifically, Peregrina and del Toro focus on reviewing the functions of the FLRT family of cell adhesion proteins to control the radial migration and tangential spread of migrating neurons by regulating the balance between cell adhesion and repulsion. Down syndrome cell adhesion molecules (DSCAMs), a small group of transmembrane proteins of the immunoglobulin superfamily, are also known to be involved in neuronal migration during cortical development. In their original article, Mitsogiannis et al. show that an increased dosage of DSCAMs, which is observed in human disorders, affects morphology and migration of cortical interneurons.

Extracellular signaling components, which are known as axon guidance molecules, also regulate neuronal migration. Yamagishi et al. and Gonda et al. review the involvement of two well-known axon guidance families of proteins, Netrin and Robo/Slit, respectively, in neuronal migration during neocortical development and further discuss their participation in human developmental pathologies.

The termination of neuronal migration is the final essential step for the formation of the fine laminar structure of the cerebral cortex, where neurons detach from the RG scaffold and initiate differentiation. Hatanaka and Hirata summarize the critical roles played by multiple microenvironmental factors, including the ECM, Cajal–Retzius cells, RG cells, and neighboring neurons, during the terminal phase of neuronal migration.

In addition to the signaling action of molecular factors, recent studies have begun to reveal the central importance of mechanical forces in controlling neuronal migration during cortical development. In their review, Minegishi and Inagaki discuss the relevance of forces created between neurons and their surrounding environment and those produced within cells in controlling neuronal migration and molecular mechanisms underlying these forces. Novel insights into these forces are provided by Iwashita et al. who present a novel strategy of measuring tissue stiffness using glyoxal as a fixative combined with AFM. They then used this method to investigate the link between mechanical properties and species-specific brain structures, comparing mice, chicks, turtles, and ferrets. In addition to mechanical forces, the electrical properties of migrating neurons and their cellular environments also influence neuronal migration. Medvedeva and Pierani review the fascinating mechanisms underlying the electric field-mediated control of neuronal migration and maturation.

The evolution of the mammalian cerebral cortex is considered fundamental for the acquisition of higher brain functions. Accumulating evidence indicates that the specific adaptation of progenitor cell proliferation and neuronal migration mechanisms play critical roles in neocortical evolution. Amin and Borrell review the role of ECM molecules in progenitor cell proliferation, neuronal migration, and the evolution of cortical folding. Cortay et al. provide new data from *ex vivo* analysis of the embryonic macaque cortex, demonstrating that the radial migration speed and trajectory vary significantly between cortical areas and layers in primates, exhibiting unique features in an area- and layer-specific manner.

Our Research Topic ends with a review by Hansen and Hippenmeyer. They discuss the different extracellular elements that define the microenvironment that radially migrating neurons encounter, as well as experimental assays and methods to study non-cell-autonomous mechanisms of neuronal migration and brain development.

By assembling these articles together, this special issue provides an updated picture of the field and highlights the importance of the interplay between extrinsic signals and migrating neurons during neocortical development. We are confident that this collection will be of interest to both new and established scientists and help promote research in this fascinating field.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Forces to Drive Neuronal Migration Steps

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To establish and maintain proper brain architecture and elaborate neural networks, neurons undergo massive migration. As a unique feature of their migration, neurons move in a saltatory manner by repeating two distinct steps: extension of the leading process and translocation of the cell body. Neurons must therefore generate forces to extend the leading process as well as to translocate the cell body. In addition, neurons need to switch these forces alternately in order to orchestrate their saltatory movement. Recent studies with mechanobiological analyses, including traction force microscopy, cell detachment analyses, live-cell imaging, and loss-of-function analyses, have begun to reveal the forces required for these steps and the molecular mechanics underlying them. Spatiotemporally organized forces produced between cells and their extracellular environment, as well as forces produced within cells, play pivotal roles to drive these neuronal migration steps. Traction force produced by the leading process growth cone extends the leading processes. On the other hand, mechanical tension of the leading process, together with reduction in the adhesion force at the rear and the forces to drive nucleokinesis, translocates the cell body. Traction forces are generated by mechanical coupling between actin filament retrograde flow and the extracellular environment through clutch and adhesion molecules. Forces generated by actomyosin and dynein contribute to the nucleokinesis. In addition to the forces generated in cell-intrinsic manners, external forces provided by neighboring migratory cells coordinate cell movement during collective migration. Here, we review our current understanding of the forces that drive neuronal migration steps and describe the molecular machineries that generate these forces for neuronal migration.

Keywords: neuronal migration, mechanobiology, traction force, adhesion force, mechanical tension, shootin1, actomyosin, dynein

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INTRODUCTION

Neuronal migration is a fundamental process to establish and maintain the nervous system (Hatten, 2002; Ayala et al., 2007; Ghashghaei et al., 2007; Marin et al., 2010; Kaneko et al., 2017), and defects in neuronal migration cause a number of disorders including brain malformation, intellectual disability, epilepsy and psychiatric diseases (Valiente and Marin, 2010; Evsyukova et al., 2013; Moffat et al., 2015; Stouffer et al., 2016). Decades of intensive analyses of mouse mutants and human brain malformations have yielded substantial progress in our understanding of the

molecular bases for controlling neuronal migration (Hatten, 2002; Govek et al., 2011; Hirota and Nakajima, 2017). In addition, imaging analyses have uncovered spatiotemporal molecular and cellular events underlying neuronal migration (Famulski et al., 2010; Yanagida et al., 2012; Cooper, 2013; Hatanaka et al., 2016; Ohtaka-Maruyama et al., 2018; Saito et al., 2019). Although these studies have significantly advanced our understanding of neuronal migration on the molecular and cellular levels, cell movement ultimately depends on the generation of driving forces. Therefore, one of the major goals of current research is understanding the molecular machineries required to generate forces for neuronal migration.

Migrating neurons exhibit bipolar morphology, with a long leading process and a short trailing process (Tsai and Gleeson, 2005; Marin et al., 2006); the tip of the leading process bears a highly motile structure, the growth cone (Marin et al., 2006; Ayala et al., 2007; Marin et al., 2010; Cooper, 2013; Evsyukova et al., 2013; **Figure 1A**). Typically, neurons migrate in a saltatory manner by repeating two distinct steps, namely extension of the leading process and translocation of the cell body (Edmondson and Hatten, 1987; O'Rourke et al., 1992; Komuro and Rakic, 1993, 1995; Wichterle et al., 1997; Nadarajah et al., 2001, 2002; Schaar and McConnell, 2005; Tsai and Gleeson, 2005; Marin et al., 2006; **Figure 1A**). Neurons must therefore generate forces to extend the leading process as well as to translocate the cell body. In addition, they need to switch these forces alternately in order to orchestrate their saltatory movement. This review outlines recent findings and mechanobiological approaches that are beginning to uncover the forces required to drive neuronal migration. Spatiotemporally organized forces produced between neurons and the extracellular environment play key roles in driving these migration steps (blue box, **Figure 1B**). Namely, the driving force for leading process extension (white arrow, **Figure 1B**) is produced as a counter force of the traction force on the environment (yellow arrow, **Figure 1B**) generated by the growth cone. On the other hand, a decrease in the adhesion force at the cell body (smaller green arrow, **Figure 1B**) propels somal translocation. In addition, intracellular forces (red box, **Figure 1B**), including leading process tension (black arrows, **Figure 1B**) and pushing and pulling forces exerted on the nucleus (red and blue arrows, **Figure 1B**), contribute to somal translocation. Furthermore, forces provided by neighboring migratory cells (brown arrow, **Figure 1B**) coordinate cellular movement during collective migration. Concerning the mechanical regulation of nuclear translocation, readers are also referred to other reviews (Tsai and Gleeson, 2005; Marin et al., 2010; Trivedi and Solecki, 2011; Nakazawa and Kengaku, 2020).

FORCES FOR LEADING PROCESS EXTENSION

Traction Force at the Growth Cone Extends the Leading Process

Neurons migrate within tightly packed environments, including glial cells, other neurons and the extracellular matrix (ECM) (Franco and Muller, 2011; Solecki, 2012), which serve as adhesive substrates. To migrate through these environments, neurons need to produce forces against the adhesive substrates. Indeed, using traction force microscopy (**Figure 2A**), recent studies detected traction forces produced by migrating cerebellar granule cells in 2D conditions (Jiang et al., 2015; Umeshima et al., 2019) and by olfactory interneurons in a semi-3D condition (Minegishi et al., 2018). In all cases, prominent traction forces were observed at the growth cone of the leading process (yellow arrows, **Figure 2B** and **Supplementary Video S1**). The direction of the traction forces at the growth cone was oriented toward the rear of the cell (Minegishi et al., 2018; Umeshima et al., 2019). In addition, the magnitude of the forces showed a positive correlation with the speed of growth cone advance (Minegishi et al., 2018), indicating that the traction forces generated at the growth cone drive leading process extension.

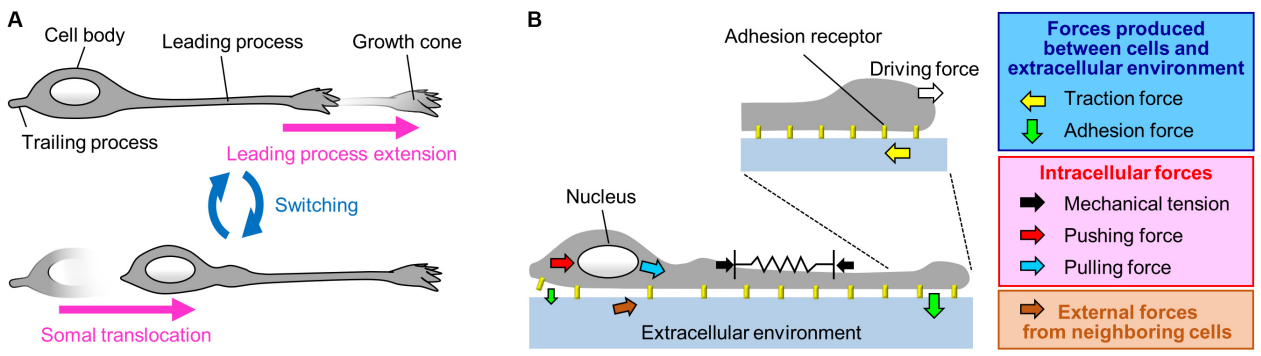

FIGURE 1 | A Mechanical model for neuronal migration. **(A)** Neurons migrate in a saltatory manner by repeating the two distinct steps: leading process extension and somal translocation. **(B)** The driving force for leading process extension (white arrow) is produced as a counter force of the traction force on the adhesive substrate produced by the growth cone (yellow arrow). Somal translocation is likely to be driven by multiple forces, including mechanical tension along the leading process (black arrows), a decrease in the adhesion force at the cell body (smaller green arrow), and pushing (red arrow) and pulling (blue arrow) forces exerted on the nucleus. In addition, forces provided by neighboring cells (brown arrow) coordinate cell movement during collective migration.

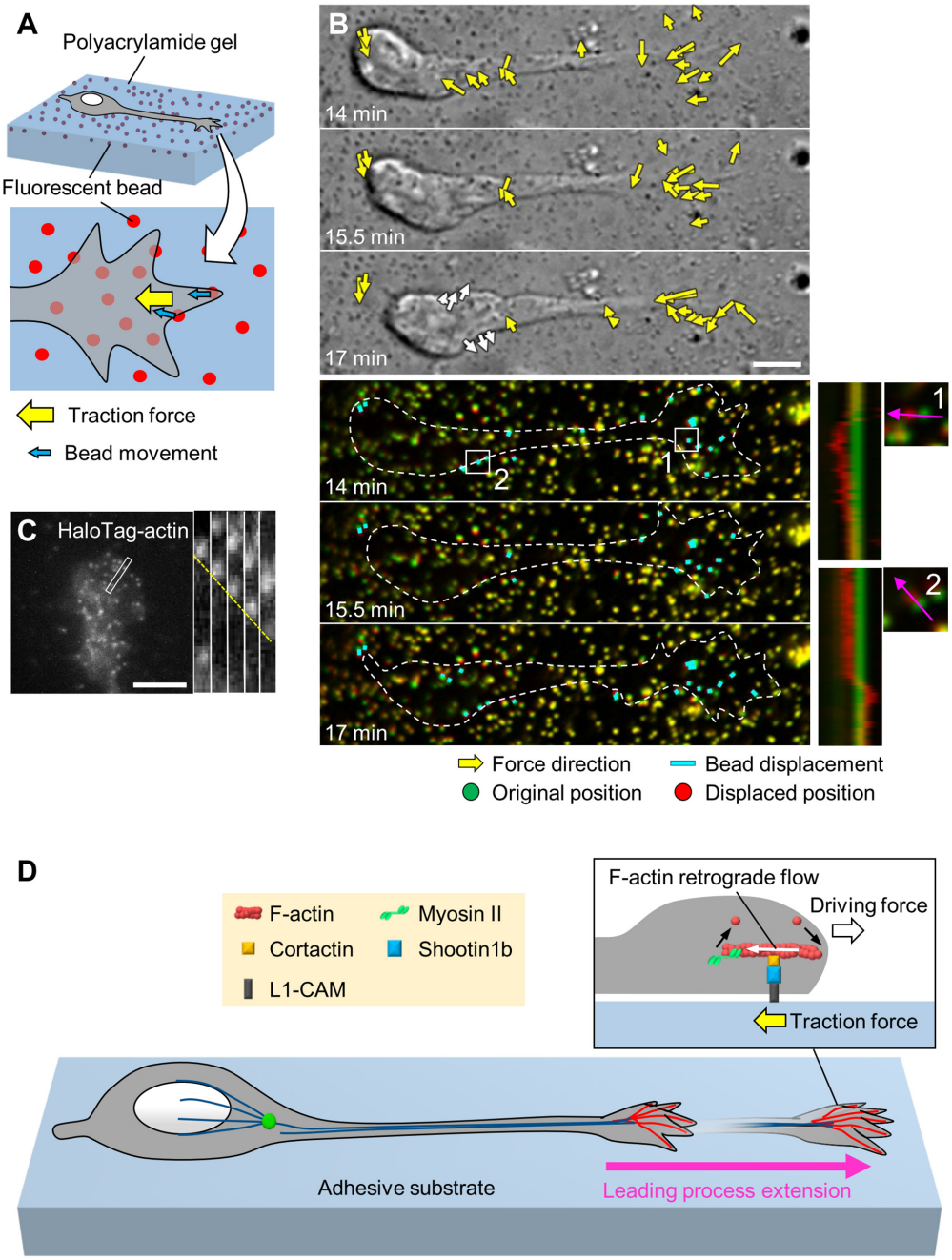

FIGURE 2 | Generation of traction force for leading process extension by actin–adhesion coupling. **(A)** Schema of traction force microscopy to monitor force generated by migrating neurons. Neurons are cultured on polyacrylamide gel coated with adhesive substrates such as L1-CAM; fluorescent beads are embedded in the gel. Traction forces under the cell (yellow arrow) are monitored by visualizing force-induced deformation of the gel, which is reflected by the movement of beads under the neuron (blue arrows). **(B)** Force mapping of a migrating neuron. Differential interference contrast (DIC, upper panels) and fluorescence (lower panels) time-lapse images of a migrating olfactory interneuron (see **Supplementary Video S1**). The original and displaced positions of the beads are indicated by green and red, respectively, while the bead displacements are indicated by cyan rectangles. Yellow arrows in DIC images indicate the magnitude and direction of traction forces. Dashed lines indicate the boundary of the cell. The kymographs (lower right) along the axis of bead displacement (pink arrows) at the boxed areas 1 and 2 of the neuron show movement of beads. Note that the gel under the cell body deformed forward during the somal translocation step (white arrows in the bottom DIC image and box 2). Modified from Minegishi et al. (2018) (This work is licensed under the CC BY license, https://creativecommons.org/licenses/by/4.0/) with permission. **(C)** Fluorescent speckle image of HaloTag-actin at the leading process growth cone of an olfactory interneuron, and kymograph of the boxed area at 3 s intervals (right) (see **Supplementary Video S2**). The dashed line indicates the retrograde flow of speckles. Reproduced from Minegishi et al. (2018) with permission. **(D)** Molecular machinery for generation of traction force in migrating olfactory interneurons. At the leading process growth cone, shootin1b mediates actin–adhesion coupling, through its interactions with cortactin and L1-CAM. This coupling generates traction force under the growth cone (yellow arrow). The driving force for leading process extension (forward white arrow) is generated as a counterforce to the traction forces exerted on the adhesive substrate. Scale bars: 5 μm.

Shootin1b Mediates Actin–Adhesion Coupling for Generation of Traction Force at the Leading Process Growth Cones

The tip of an extending axon also bears a growth cone (Lowery and Van Vactor, 2009), and axonal growth cones produce traction forces for axon outgrowth and guidance (Chan and Odde, 2008; Koch et al., 2012; Abe et al., 2018; Baba et al., 2018). Decades of analyses of axonal growth cones have revealed a key machinery to generate traction forces for growth cone migration. At the leading edge of the axonal growth cone, actin filaments (F-actins) polymerize and disassemble proximally, which, in conjunction with myosin II activity, induces retrograde flow of F-actins (Forscher and Smith, 1988; Katoh et al., 1999; Medeiros et al., 2006). Mechanical coupling between F-actin retrograde flow and adhesive substrates through clutch and adhesion molecules generates traction forces on the substrates (Mitchison and Kirschner, 1988; Suter and Forscher, 2000; Toriyama et al., 2013). Namely, the actin–adhesion coupling transmits the force of F-actin retrograde flow to the adhesive substrate, producing traction force on the substrate. Concurrently, actin–adhesion coupling reduces the speed of the F-actin retrograde flow, thereby converting actin polymerization into force that pushes the leading-edge membrane. To date, shootin1a is one of the best-characterized clutch molecules involved in the generation of traction forces at the axonal growth cone (Toriyama et al., 2006; Shimada et al., 2008). Shootin1a interacts with F-actin retrograde flow through its association with the F-actin-interacting protein cortactin (Kubo et al., 2015). Shootin1a also interacts with the cell adhesion molecule L1-CAM (Baba et al., 2018), which binds to the ECM protein laminin (Abe et al., 2018) as well as to L1-CAM expressed on neighboring cells (Lemmon et al., 1989), thereby mechanically coupling the F-actin retrograde flow with the adhesive substrates. The shootin1a-mediated actin–adhesion coupling generates traction forces for axon outgrowth (Kubo et al., 2015) and axon guidance induced by diffusible and substrate-bound chemical cues (Abe et al., 2018; Baba et al., 2018). N-cadherin–catenin complexes were also reported to mediate actin–adhesion coupling at the axonal growth cone (Bard et al., 2008; Garcia et al., 2015).

As in the case of axonal growth cones, F-actins also undergo retrograde flow at the tip of leading process growth cones (**Figure 2C** and **Supplementary Video S2**; He et al., 2010; Minegishi et al., 2018). A recent study reported that shootin1b, a splicing variant of shootin1a (Higashiguchi et al., 2016), functions as a clutch molecule at the leading process growth cone of migrating olfactory interneurons (Minegishi et al., 2018; **Figure 2D**). During neuronal migration, shootin1b undergoes dynamic accumulation in the leading process growth cone; this accumulation positively correlates with leading process extension. Shootin1b at the growth cone couples F-actin retrograde flow and cell adhesions via cortactin and L1-CAM, thereby generating traction force on the adhesive substrate (Minegishi et al., 2018; yellow arrow, **Figure 2D**). In addition, a recent study reported that shootin1b directly interacts with F-actin and promotes actin polymerization *in vitro* (Zhang et al., 2019). The driving force for leading process extension (forward white arrow) is produced as a counter force of the traction force. Shootin1 knockout (KO) decreased the magnitude of the traction force produced by the growth cone and reduced the extension of the leading process as well as the speed of neuronal migration. Furthermore, shootin1 KO led to abnormal positioning of olfactory interneurons and dysgenesis of the olfactory bulb (Minegishi et al., 2018). These data indicate that traction force generated by shootin1b-mediated actin–adhesion coupling promotes leading process extension for migration of olfactory interneurons.

In addition, shootin1 KO results in ectopic accumulation of mitral cells (Minegishi et al., 2018), olfactory excitatory neurons that undergo radial migration (Hinds, 1968; Blanchart et al., 2006). Recent studies also reported that shootin1 knockdown inhibits the radial migration of cortical neurons (Sapir et al., 2013) and that shootin1 KO delays the collective cell migration of zebrafish posterior lateral line primordium (PLLP), a cluster of progenitor cells destined to form a mechanosensory organ called the neuromast (Urasaki et al., 2019). These data suggest that traction force generated by shootin1-mediated actin–adhesion coupling may propel the migration of multiple types of neurons.

FORCES FOR SOMAL TRANSLOCATION

Tension Along the Leading Process for Somal Translocation

The cell body is the swollen part of migrating neurons; therefore, its translocation against the mechanical barriers of the surrounding environment must rely on the generation of robust forces. One of the candidate forces for mediating somal translocation is tension along the leading process (**Figure 3A**). He et al. (2010) reported that severing the leading process of cerebellar granule cells arrested somal translocation, demonstrating that the leading process is required for somal translocation. Consistently, in cerebellar granule cells and gonadotropin-releasing hormone (GnRH)-expressing neurons, F-actins located along the leading process move forward in correlation with somal translocation (Solecki et al., 2009; He et al., 2010; Hutchins et al., 2013; Hutchins and Wray, 2014). In addition, traction force microscopy demonstrated that the gel substrate under the cell body of olfactory interneurons deformed forward during the somal translocation step (Minegishi et al., 2018; white arrows and Box 2, **Figure 2B** and **Supplementary Video S1**). These data suggest that the leading process pulls the cell body for somal translocation (Solecki et al., 2009; He et al., 2010; Hutchins et al., 2013; Hutchins and Wray, 2014; Minegishi et al., 2018; **Figure 3A**). As described above, shootin1b promotes leading process extension of olfactory interneurons (white arrows, **Figure 3A**) by producing traction force at the growth cone (yellow arrows); on the other hand, shootin1b is also involved in somal translocation (Minegishi et al., 2018). A previous study with chick sensory neurons demonstrated that mechanical tension along neurites increases according to neurite extension (Lamoureux et al., 1989). Therefore, the leading process extension driven by traction force at the growth

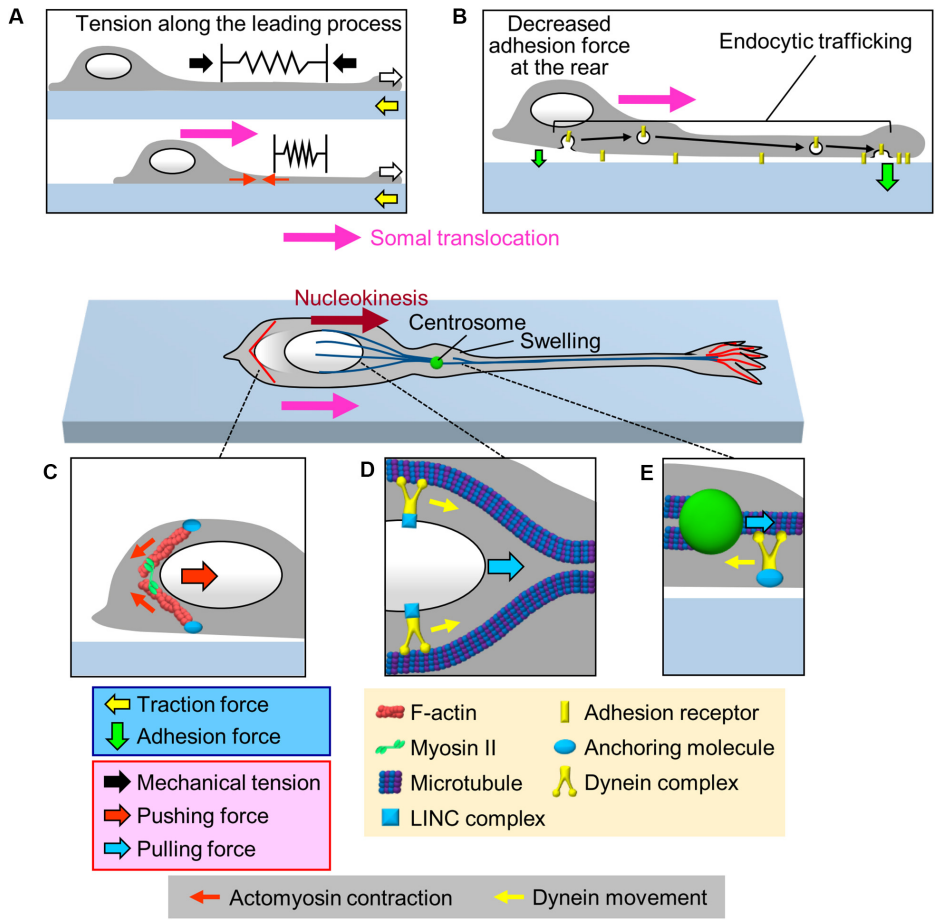

FIGURE 3 | Multiple forces that cooperate for somal translocation. **(A)** Leading process extension (white arrows) increases the mechanical tension along the leading process (black arrows), which in turn pulls the cell body for somal translocation. In addition, actomyosin contraction at the proximal region of the leading process (red arrows) increases the tension along the leading process. **(B)** Decrease in the adhesion force at the cell body (smaller green arrow) propels somal translocation. Adhesion receptors are transported from the cell body to the leading process via endocytic pathways (black arrows), resulting in an increase in the adhesion force at the leading process and a decrease in adhesion force at the cell body (smaller green arrow). **(C)** Actomyosin, which may be anchored to the cell cortex, contracts at the rear of the nucleus (small red arrows), thereby squeezing the nucleus and generating pushing force (large red arrow) for nucleokinesis. **(D)** The dynein complex mechanically interacts with the nucleus via the LINC complex, and its movement (yellow arrows) along perinuclear microtubules generates pulling force (blue arrow) for nucleokinesis. **(E)** In the swelling of the proximal part of the leading process, the dynein motor complex may be immobilized on a cellular component via an anchoring molecule. The force of dynein movement (yellow arrow) slides microtubules forward, thereby pulling (blue arrow) the centrosome forward.

cone (Jiang et al., 2015; Minegishi et al., 2018; Umeshima et al., 2019) would increase tension along the process (black arrows, **Figure 3A**), which in turn pulls the cell body for somal translocation.

In the case of migrating cerebellar granule cells which extend F-actin-enriched leading process (Rivas and Hatten, 1995; Solecki et al., 2009), Myosin II and myosin light chain kinase (MLCK) accumulate at the proximal region of the leading process (Solecki et al., 2009; Umeshima et al., 2019). Consistently, traction force analyses detected a myosin II dependent contraction center at the proximal region of the leading process during somal translocation step (Jiang et al., 2015; Umeshima et al., 2019). Thus, actomyosin contraction would actively contribute to increase the tension along the leading process of cerebellar granule cells (red arrows, **Figure 3A**), thereby pulling the soma (Solecki et al., 2009; Trivedi and Solecki, 2011; Jiang et al., 2015; Umeshima et al., 2019). Recent studies have developed fluorescent tension probes that are applied in fluorescence lifetime imaging (FLIM) (Colom et al., 2018) and fluorescence resonance energy transfer (FRET) imaging (Li et al., 2018). Detailed analyses of mechanical tension along the leading process remains an important issue for future studies.

Decrease in Adhesion Force at the Soma for Somal Translocation

As described above, cell adhesion is required for the generation of traction force for neuronal migration. On the other hand, it was also proposed that a decrease in the adhesion force at the rear of the migratory cells facilitates forward movement of cells (Sheetz et al., 1998). Indeed, overexpression or knockdown of cell adhesion molecules, such as N-cadherin or L1-CAM,

inhibits neuronal migration (Kawauchi et al., 2010; Shikanai et al., 2011; Kishimoto et al., 2013; Tonosaki et al., 2014; Mestres et al., 2016), implying that coordinated regulation of adhesion forces generated between neurons and adhesive substrates is required for neuronal migration. To assess the spatial dynamics of adhesion force in migrating cerebellar granule cells, Jiang et al. (2015) performed a cell detachment assay. They mechanically pulled the middle region of the leading process of cerebellar granule cells using a micropipette, and examined the first detachment point of the neurons. In neurons whose soma was stationary, the growth cone was detached first from the substrate. In contrast, in neurons whose soma was moving forward, the cell body was detached first (Jiang et al., 2015). These data suggest that a decrease in the adhesion force at the cell body is important to propel somal translocation (**Figure 3B**). Although this assay is qualitative, other recent studies have reported a quantitative cell detachment assay: using femtosecond lasers combined with atomic force microscopy (AFM), controlled impulsive forces to induce cell detachment can be applied to estimate the adhesion force of cells (Hosokawa et al., 2011; Iino et al., 2016). In addition, cell adhesion molecules tagged with pH-sensitive GFP could enable analyses of spatiotemporal dynamics of adhesions in migrating neurons (Famulski et al., 2010). Quantification and spatiotemporal analyses of adhesion forces in migrating neurons are important issues for future research.

Several studies have reported an involvement of endocytic trafficking of cell adhesion molecules in regulation of spatiotemporal dynamics of cell adhesion during neuronal migration (Kawauchi et al., 2010; Wilson et al., 2010; Shieh et al., 2011; Mestres et al., 2016; **Figure 3B**). Kawauchi et al. (2010) performed loss-of-function assays and proposed that N-cadherin is internalized at the cell body by Rab5-dependent endocytic pathways and transported to the leading process by a Rab11-dependent recycling pathway; disruption of these trafficking pathways led to migration defects in cortical projection neurons. Similarly, Shieh et al. (2011) reported that inhibition of endocytosis by loss-of-function of dynamin led to an accumulation of integrin β1 at the cell rear, leading to disruption of the migrations of olfactory interneurons and cortical projection neurons. On the other hand, knockdown of the early endosomal protein SARA (Smad anchor for receptor activation), increased surface expression of L1-CAM and delayed radial migration of cortical neurons (Mestres et al., 2016). These findings suggest that endocytic trafficking (black arrows, **Figure 3B**) decreases the number of adhesion receptors at the cell body for somal translocation.

Pushing Forces for Nucleokinesis Generated by Actomyosin Contraction

As somal translocation occurs in a saltatory manner, this step would not be explained simply in terms of a balance between the leading process tension and somal adhesion. Since the nucleus is the largest organelle in the cell body, its movement, nucleokinesis, is critical for somal translocation (Tsai and Gleeson, 2005). Accumulating evidence indicates that actomyosin contraction contributes to nucleokinesis by squeezing the nucleus (**Figure 3C**). During nucleokinesis of olfactory and medial ganglionic eminence (MGE)-derived interneurons, F-actin and myosin II accumulate at the rear of the nucleus, where myosin II is activated (Bellion et al., 2005; Schaar and McConnell, 2005; Martini and Valdeolmillos, 2010). Live imaging analyses demonstrated that the F-actin accumulation at the rear precedes nuclear movement. Furthermore, inhibition of myosin II activity by blebbistatin abolishes F-actin accumulation at the rear, thereby inhibiting nuclear squeezing as well as nuclear translocation (Martini and Valdeolmillos, 2010). These findings indicate that actomyosin at the rear squeezes the nucleus (small red arrows, **Figure 3C**) and exerts pushing force (large red arrow) to drive nucleokinesis in interneurons (Bellion et al., 2005; Schaar and McConnell, 2005; Martini and Valdeolmillos, 2010).

On the other hand, in the case of cerebellar granule cells, F-actins do not accumulate at the rear of the nucleus (Solecki et al., 2009; He et al., 2010; Umeshima et al., 2019). In addition, traction force analyses failed to detect pushing forces at the rear of these neurons (Jiang et al., 2015), thereby suggesting that actomyosin dynamics at the rear differs depending on the cell types (Trivedi and Solecki, 2011).

Pulling Force for Nucleokinesis Generated by Dynein Motor Complex

Live-cell imaging analyses demonstrated that somal translocation is preceded by a swelling of the proximal part of the leading process and forward movement of the centrosome into this swelling (Bellion et al., 2005; Schaar and McConnell, 2005; Tsai and Gleeson, 2005; Marin et al., 2010; Shinohara et al., 2012). Accumulating data suggest that coupling between the centrosome and the nucleus plays an important role in somal translocation (Tsai and Gleeson, 2005; Marin et al., 2010; Cooper, 2013; Kaneko et al., 2017). In migrating neurons, the centrosome acts as a microtubule organizing center and extends microtubules to the leading process and to the nucleus; therefore, centrosome-organized microtubules are oriented with their minus end toward the centrosome (Tsai and Gleeson, 2005; Marin et al., 2010). Previous studies reported that minus-end-directed Lis1/Ndel1/dynein motor complex is responsible for both nucleokinesis and centrosomal movement (Shu et al., 2004; Tanaka et al., 2004; Tsai et al., 2007; Zhang et al., 2009). Perinuclear microtubules act as the scaffold for dynein-mediated nucleokinesis (Shu et al., 2004; Tanaka et al., 2004; Tsai et al., 2007). The dynein complex pulls the nucleus forward via the LINC complex (blue arrow, **Figure 3D**), which is formed by the transmembrane SUN and KASH proteins (Zhang et al., 2009). On the other hand, less is known about the molecular mechanism by which the dynein complex drives the forward movement of the centrosome. Tsai et al. (2007) proposed that dynein motor slides microtubules forward, thereby driving the centrosome movement (blue arrow, **Figure 3E**). This idea is supported by live-cell imaging data that demonstrate forward movement of microtubules in the proximal leading process (Rao et al., 2016). To underpin the centrosomal movement, the dynein complex

must be immobilized on a cellular component; however, the molecular linkage for dynein immobilization remains unclear. Recent studies reported that actomyosin accumulates in front of the nucleus of migrating cerebellar granule cells, suggesting that actomyosin contraction may also contribute to pulling the nucleus for somal translocation of these cells (Solecki et al., 2009; Jiang et al., 2015; Umeshima et al., 2019). Further analyses are required to uncover the detailed molecular mechanics of nuclear translocation.

Forces Externally Provided by Neighboring Cells During Collective Cell Migration

Some of neuronal progenitor cells, for example the cranial neural crest and zebrafish PLLP, migrate as cell clusters during development (Ghysen and Dambly-Chaudiere, 2007; Nogare et al., 2017; Shellard and Mayor, 2019). In addition, neonatal and adult olfactory interneurons undergo a stream-type collective cell migration called chain migration (Marin and Rubenstein, 2003; Rorth, 2009; Kaneko et al., 2017). In such cases, migrating cells receive forces from neighboring migratory cells (brown arrow, **Figure 1B**), and thus their migration is affected by the movements of the neighboring cells. For example, a recent study reported that contraction of "supracellular" actomyosin ring localized at the rear of the neural crest cell cluster drives collective migration of the cell group (Shellard et al., 2018). In the case of endothelial cells, it is proposed that cadherin-mediated cell-cell junctions between leader and follower cells orient cellular movement during collective migration (Hayer et al., 2016). Similarly, chain migration is thought to be associated with the efficient and coordinated movement of olfactory interneurons (Kaneko et al., 2017).

CONCLUDING REMARKS

With the aid of emerging mechanobiological approaches, the molecular mechanics underlying neuronal migration is beginning to be elucidated. In this review, we have described a current view of the forces that drive the two neuronal migration steps, leading process extension and somal translocation. Spatiotemporally organized forces produced between neurons and the extracellular environment, as well as intracellular forces, play pivotal roles to drive these migration steps. Diverse molecules may contribute to the generation of these forces, depending on the neuronal cell type. As an important question, it remains unknown how neurons can switch between these processes. Molecular and mechanical interactions between the leading process and the cell body should coordinate the two processes to achieve saltatory movement. Ca^{2+} imaging analyses demonstrated transient increases in Ca^{2+} concentration in the cell body of cerebellar granule cells, which positively correlated with their somal translocation (Komuro and Rakic, 1996; Kumada and Komuro, 2004). In addition, treatment with BAPTA, a calcium chelator, abolished the transient Ca^{2+} increases and F-actin accumulation at the rear of the nucleus, resulting in inhibition of somal translocation (Martini and Valdeolmillos, 2010). These reports support the notion that transient Ca^{2+} increases are involved in the activation of actomyosin to trigger nucleokinesis. Similarly, shootin1b underwent dynamic accumulation at the leading process growth cone of migrating olfactory interneurons, which positively correlated with leading process extension (Minegishi et al., 2018). In addition, shootin1 KO inhibited the leading process extension, suggesting that the shootin1b accumulation triggers leading process extension (Minegishi et al., 2018). To understand how migratory neurons produce forces for their pathfinding, it is also important to link guidance cues in the extracellular environments with the regulation of the machineries involved in force generation. Such extracellular cues would include diffusible and substrate-bound chemical cues and mechanical properties of the environment. Further detailed measurement of forces, in combination with molecular and cell biological approaches, will enhance our understanding of the mechanics underlying neuronal migration.

AUTHOR CONTRIBUTIONS

TM and NI contributed cooperatively to the conceptual development, literature research, and writing of the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00863/full#supplementary-material

VIDEO S1 | A force-mapping video of a migrating neuron. This video shows DIC (upper) and fluorescence (lower) time-lapse imaging of a migrating olfactory interneuron. Arrows in the DIC image indicate strength and direction of traction forces (force magnitude is shown by the length of the arrows, which are 4.5 times longer than bead displacements). The original and displaced positions of the beads are indicated by green and red, respectively. The bead displacements are also indicated by cyan rectangles. Images were acquired every 30 s for 24.5 min. Scale bar, 5 μm. Modified from Minegishi et al. (2018) with permission (see **Figure 2B**).

VIDEO S2 | Fluorescent speckle imaging of HaloTag-actin at the leading process growth cone of an olfactory interneuron. Images were acquired every 3 s for 42 s. Scale bar, 5 μm. Reproduced from Minegishi et al. (2018) with permission (see **Figure 2C**).

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Comparative Analysis of Brain Stiffness Among Amniotes Using Glyoxal Fixation and Atomic Force Microscopy

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Brain structures are diverse among species despite the essential molecular machinery of neurogenesis being common. Recent studies have indicated that differences in the mechanical properties of tissue may result in the dynamic deformation of brain structure, such as folding. However, little is known about the correlation between mechanical properties and species-specific brain structures. To address this point, a comparative analysis of mechanical properties using several animals is required. For a systematic measurement of the brain stiffness of remotely maintained animals, we developed a novel strategy of tissue-stiffness measurement using glyoxal as a fixative combined with atomic force microscopy. A comparison of embryonic and juvenile mouse and songbird brain tissue revealed that glyoxal fixation can maintain brain structure as well as paraformaldehyde (PFA) fixation. Notably, brain tissue fixed by glyoxal remained much softer than PFA-fixed brains, and it can maintain the relative stiffness profiles of various brain regions. Based on this method, we found that the homologous brain regions between mice and songbirds exhibited different stiffness patterns. We also measured brain stiffness in other amniotes (chick, turtle, and ferret) following glyoxal fixation. We found stage-dependent and species-specific stiffness in pallia among amniotes. The embryonic chick and matured turtle pallia showed gradually increasing stiffness along the apico-basal tissue axis, the lowest region at the most apical region, while the ferret pallium exhibited a catenary pattern, that is, higher in the ventricular zone, the inner subventricular zone, and the cortical plate and the lowest in the outer subventricular zone. These results indicate that species-specific microenvironments with distinct mechanical properties emerging during development might contribute to the formation of brain structures with unique morphology.

Keywords: mechanical property, brain morphology, force spectrometry, tissue mechanics, glyoxal fixation

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INTRODUCTION

Although the vast majority of molecular machinery to generate neurons from progenitors are commonly conserved in amniotes (Englund et al., 2005; Martínez-Cerdeño et al., 2016; Nomura et al., 2016; Turrero García et al., 2016; Yamashita et al., 2018), the alignment of neurons in matured brains exhibits remarkable diversity (Medina and Abellán, 2009; Jarvis et al., 2013;

Puelles et al., 2017; Cárdenas and Borrell, 2019; Pessoa et al., 2019). For instance, the mammalian brain has a six-layered structure, while the avian brains consist of compartmentalized nuclear slabs. During brain formation, newly generated neurons in the proliferative region [the ventricular zone (VZ) and subventricular zone (SVZ)] migrate to their final destinations. The mammalian neocortex (NCx) is originated in the most dorsal part of embryonic telencephalon (Puelles, 2013; Nieuwenhuys, 2017). In the mammalian telencephalon, most glutamatergic projection neurons are born in the dorsal proliferative region and migrate into the cortical plate (CP) radially (Nadarajah and Parnavelas, 2002; Noctor et al., 2004; Tabata et al., 2009), whereas GABAergic interneurons are born in the ventral proliferative region and migrate into the CP tangentially, resulting in a highly organized six-layered structure (Anderson et al., 1997; Batista-Brito and Fishell, 2009). Migrating neurons respond not only to biochemical signals but also to mechanical cues from distinct extracellular environments on the way to their destinations (Park et al., 2002; Huang, 2009; Honda et al., 2011; Long and Huttner, 2019). Indeed, intensive research using atomic force microscopy (AFM) has revealed the spatiotemporal diversity and crucial roles of the mechanical properties of the extracellular environment, especially stiffness, in the developing central nervous system (Elkin et al., 2007, 2010; Christ et al., 2010; Iwashita et al., 2014; Nagasaka et al., 2016; Thompson et al., 2019; Kjell et al., 2020). However, it remains unclear how stiffness controls cellular behavior to form species-specific brain structures.

To understand the role of stiffness in organizing diverse brain structures, a comparative analysis of stiffness in several animal brains is required. In this study, we examined the stiffness of pallia among amniotes: mice, turtles, songbirds, chicks, and ferrets. In general, living tissue should be used for stiffness measurements to obtain physiological profiles close to *in vivo*. However, there are practical difficulties in handling several kinds of living animals, such as breeding and shipping. Furthermore, stiffness measurements should be performed under identical experimental conditions and an identical AFM system to minimize deviations. Therefore, we examined whether fixed tissues could substitute for living tissues for stiffness measurements. For fixatives, we chose 4% paraformaldehyde (PFA), a common fixative, and 3% glyoxal, a novel fixative. Recent studies have demonstrated the powerful ability of glyoxal to preserve tissue and cellular structures (Bussolati et al., 2017; Richter et al., 2018).

Here, we confirmed that the macroscopic structure of a glyoxal-fixed brain was maintained as well as a PFA-fixed one. Surprisingly, our AFM measurements revealed that glyoxal-fixed brains showed much lower stiffness than PFA, conserving stiffness profiles similar to living brains, indicating that glyoxal fixation could be applicable to studying tissues' mechanical properties. Based on this method, we found diverse stiffness patterns among amniote brains. The distinct mechanical properties of tissue microenvironments might provide different cues and scaffolds for neural cells and regulate their migrations to form diverse brain structures during development.

MATERIALS AND METHODS

Animals

Animal protocols for mice and songbirds, including breeding and experiments, were approved by and performed according to guidelines of the Committee of Korea Brain Research Institute (KBRI). Pregnant ICR mice were purchased from Core Tech and bred in KBRI. The noon on which the virginal plug was detected was defined as embryonic day 0.5 (E0.5). The day of birth was defined as postnatal day 0 (P0). E16.5 embryos and 4 weeks juvenile mice were used in this study. Songbirds (*Taeniopygia guttata*) were raised in KBRI. Juvenile birds (30–40 days post hatch, dph) were used in this study. Other brains [turtle (*Pelodiscus sinensis*), chick (*Gallus gallus*), and ferret (*Mustela putorius furo*)] were obtained according to the guidelines of each institute (turtles and chicks, Kyoto Prefectural University of Medicine; ferrets, RIKEN, Center for Biosystems Dynamics Research).

Preparation of PFA and Glyoxal Fixative

Paraformaldehyde (Merck, #8.18715) was dissolved in PBS (pH 7.4) at a final concentration of 4%. The glyoxal fixative solution was prepared according to published protocol (Richter et al., 2018). Briefly, 28 ml of ddH_2O, 7.89 ml absolute of ethanol (analysis grade), 3.13 ml of glyoxal (Sigma-Aldrich, #128465), and 0.3 ml of acetic acid (Sigma-Aldrich, #A6283) were mixed well by vortex. After adjusting to pH 4.0 with 1 N of NaOH, the solution was filled up to 40 ml with ddH_2O. The final concentration of glyoxal was 3%. Both fixative solutions were prepared on the day of the experiment and kept cool until use.

Preparation of Fixed Brain Slices

Immersion fixation was applied in the embryonic stage for the mice and chicks, at 4 months and 2.5 years for the turtles, and at E35 and P0 for ferrets. Mouse embryos were taken from uteruses following the cervical dislocation of the mothers and kept in ice-cold PBS. Chick embryos were taken from fertilized eggs and fixed at 7 and 10 days of incubation at 37°C (E7 and E10, respectively) (Nomura et al., 2013). These stages correspond to the Hamburger and Hamilton stages (HH31-33 and HH36, respectively) (Hamburger and Hamilton, 1951). A ferret embryo was taken from the uterus, as previously described (Tsunekawa et al., 2016). The brains were dissected in ice-cold PBS and then transferred to the ice-cold fixative solution immediately. The turtles and one ferret at P0 were deeply anesthetized with isoflurane, and their brains were taken out. The brains in the fixative solution were put on a rotator in a cold room (4°C) overnight and then kept in PBS at 4°C until sectioning. The brains were cut into 300-μm-thick coronal sections in ice-cold PBS using a vibratome (Leica, VT1200S).

The transcardial perfusion was applied to juvenile animals. ICR mice were deeply anesthetized with intraperitoneal injections of pentobarbital (Entobar, HanLim Kharm, Co. Ltd., South Korea) and then perfused transcardially with either 4% PFA or 3% glyoxal (pH 4.0) fixative followed by PBS. The

songbirds were deeply anesthetized with isoflurane (Hana Pharm Co. Ltd., South Korea) and then perfused like the mice. The brains were dissected out and post-fixed with fixative with the same perfusion overnight at 4°C and then kept in PBS at 4°C until vibratome sectioning.

Preparation of Acute Brain Slices

All procedures were performed in ice-cold media according to a previous publication with slight modifications (Iwashita et al., 2014). Briefly, embryonic brains were dissected out in ice-cold DMEM/F12 (Sigma-Aldrich) containing D (+)-glucose and then embedded in 2% agar (Nacalai) in PBS. Embedded brains were cut into 300-μm-thick coronal sections in DMEM/F12/D (+)-glucose using a vibratome. Sections with agar frames were placed on a plastic dish coated with BD Cell Tak (BD Bioscience) and kept on ice until measurement. Before measurement, the dish containing the slices and media was allowed to reach room temperature.

To obtain the acute slices of juvenile mouse and songbird brains, artificial cerebrospinal fluid containing sucrose (slicing ACSF) was used to dissect and make vibratome sections. Brains were immediately dissected into ice-cold slicing ACSF and glued directly on the stage with cyanoacrylate glue. The brains were cut into 300-μm-thick slices in ice-cold slicing ACSF using a vibratome. Acute slices were incubated in slicing ACSF for 45 min and then transferred to measurement ACSF. The ACSF composition was described in a previous study (Kojima and Aoki, 2003). **Supplementary Tables 1, 2** describe the media components.

Measurement of Stiffness Using AFM

The measurement method was slightly modified from our previous publication (Iwashita et al., 2014) to optimize for fixed samples. The measurements were carried out using AFM (Bioscope Resolve, NanoScope 9.4, Bruker), which was mounted on an inverted microscope (Nikon, ECLIPSE Ti2). A tipless silicon cantilever with a 20-μm borosilicate bead (Novascan) was used. The spring constant of the cantilever was calibrated using the thermal noise method in air. We chose cantilevers with the same spring constant (nominal value: 0.03 N/m; actual value: 0.07 N/m) and used them for acute and fixed slices individually to avoid cross-contamination of the remaining fixative in acute condition. The applied force was 10 nN. The measurement was done under physiological conditions for the acute slices (37°C) and at room temperature (25°C) for fixed slices. The force curves were acquired using the contact mode. Bright field images were acquired by a CMOS camera (Hamamatsu, ORCA-Flash4.0, C13440-20CU) to determine the measured region. The obtained force curves were analyzed to calculate the stiffness fit with the Hertzian model (spherical) using NanoScope Analysis 1.9 software (Bruker). **Supplementary Table 3** describes the parameters for measurement.

Immunostaining

To confirm the measured regions, the acute slices were immediately fixed with 4% PFA for 1 h at room temperature after measurement for DAPI staining. For immunohistochemistry, the adjacent cryosections of stiffness measured slices were incubated in 0.5% Triton-X 100/PBS for permeabilization for 10 min and then a 2% BSA/0.1% Triton-X-100 solution for 2 h for blocking followed by washing with PBS. Subsequently, the cryosections were incubated with primary antibodies for overnight at 20°C and then incubated with secondary antibodies for 2 h at room temperature followed by washing with PBS. The primary and secondary antibodies used in this study were rabbit anti-Tbr1 (1:500; Abcam, ab31490), rat anti-Ctip2 (1:500; Abcam, ab18465), and mouse anti-Satb2 (1:100; Abcam, ab51502). The secondary antibodies were Alexa 488-, 555-, and 647-conjugated (1:500; Molecular probes). DAPI was used to counter stain nuclei. Stained samples were mounted with PermaFluro (Thermo Fischer Scientific) and then observed using an upright confocal laser microscopy (Nikon, A1R-MP) and Panoramic Scan II (3DHISTECH).

Statistical Analysis

All statistical analyses were performed using Prism 8 (GraphPad). A two-tailed unpaired *t*-test was applied to compare two conditions, and one-way analysis of variance (ANOVA) and the Tukey *post hoc* test were applied to compare more than three conditions. Differences were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. Error bars in graphs are represented as the mean ± SEM.

RESULTS

Glyoxal Fixative Maintained Brain Structures as Well as PFA

To obtain reliable stiffness values in post-fixed brains, the tissue structure itself, including the macroscopic architecture and microenvironment, must be maintained like living conditions. Therefore, we investigated effective fixative solutions to maintain brain structure *in situ*. For this purpose, we chose 4% PFA and 3% glyoxal solutions as fixatives. PFA is common in histological studies, and glyoxal is a small dialdehyde molecule that is reported to provide better morphological preservation and strong fixation of both proteins and RNAs at cellular resolution because of its rapid penetration (Bussolati et al., 2017; Richter et al., 2018). We tested the immersion fixation for embryonic brains, transcardial perfusion for juvenile brains, and as a control, acutely prepared brains without fixation. The sizes of the fixed brains were slightly smaller than the acute brains because of the shrinkage following fixation in both methods. There was no difference at macroscopic resolution between either fixative except the color of the fixed brains (**Figure 1**). The brains fixed with glyoxal exhibited a white color, while the PFA-fixed brains exhibited a pale pink color. The acutely prepared juvenile brains showed a red color because of blood cells. Sectioned glyoxal-fixed brains also exhibited a white color, low contrast, and low transparency (**Figures 2A**, **3A**, **4A**, **6**). The nuclei stained by DAPI showed similar brain cytoarchitectures in both fixative solutions (**Figures 2A**, **3A**). These results show that glyoxal has an ability equivalent to PFA

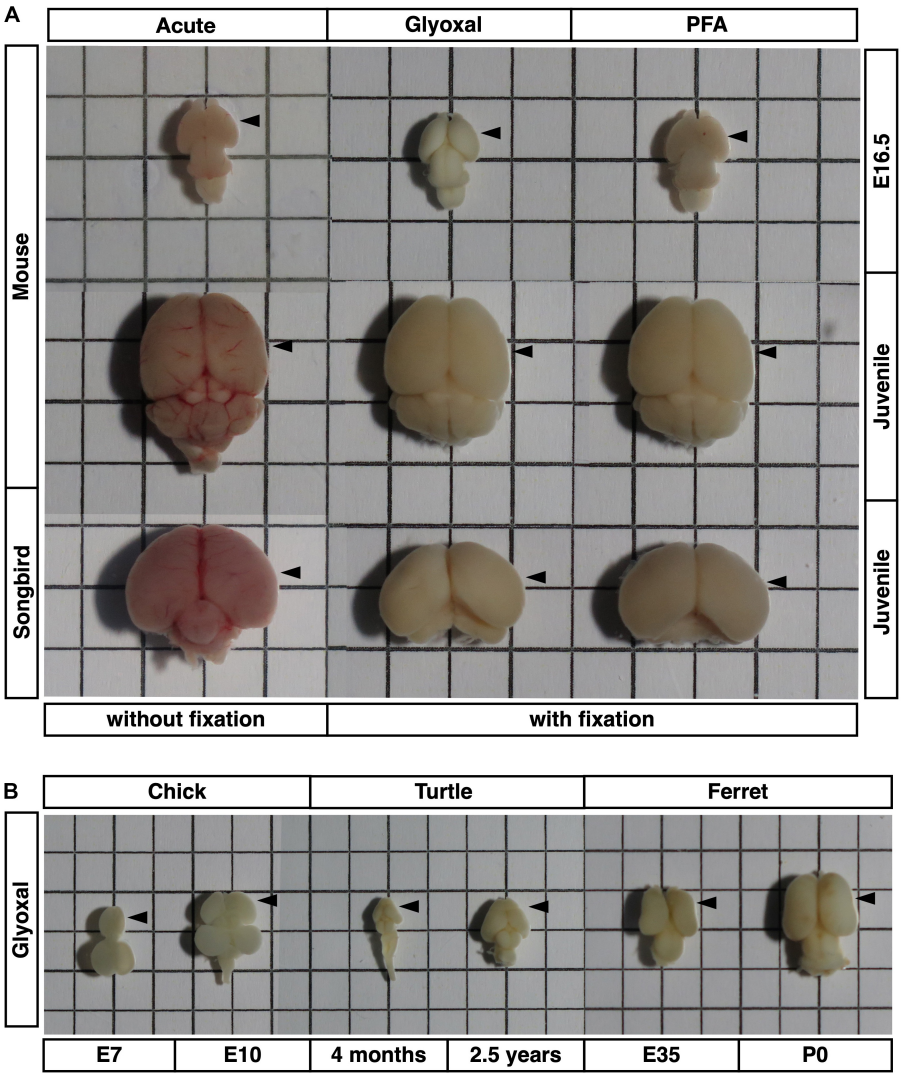

FIGURE 1 | Brains used in this study. Brain sizes and morphologies of five animals (mouse, songbird, chick, turtle, and ferret). **(A)** Embryonic mouse brains at E16.5, 4 weeks mouse brains, and 30–40 dph songbird brains used in **Figures 2–5**. Fixation methods (PFA or glyoxal) are indicated. Acute: brains without fixation. **(B)** Chick brains at E7 and E10, turtle brains at 4 months and 2.5 years old, and ferret brains at E35 and P0 used in **Figure 6**. All brains were fixed with glyoxal. The grid has a resolution of 5 mm. Arrow heads: positions of stiffness measured in slices.

to preserve brain structures using both immersion and perfusion-fixation methods.

Glyoxal-Fixed Brains Remained Much Softer Than PFA-Fixed Ones and Maintained the Relative Stiffness Profile of Living Tissue

Next, we examined differences in brain tissue stiffness between PFA and glyoxal fixation in comparison to acutely prepared living brains using AFM. We initially measured the stiffness of the mouse embryonic brains at E16.5. We prepared coronal slices from PFA- and glyoxal-fixed brains and, as a reference, acutely from living brains. The dorsal cortices were divided into three regions: the CP, intermediate zone (IZ), and proliferative region, including SVZ and VZ (SVZ+VZ), based on the phase-contrast images (**Figure 2A**). Subsequently, the stiffness in each region was measured using AFM (**Figure 2B**). Consistent with our previous results using a different AFM system (Iwashita et al., 2014), the stiffness in the IZ showed significantly higher values than other regions in acutely prepared living slices. In both PFA- and glyoxal-fixed brains, the stiffness in the IZ was significantly higher than in the CP but like that in the SVZ+VZ. Notably, although overall stiffness values increased with fixation, the glyoxal-fixed brains showed much lower stiffness than PFA-fixed brains (**Figure 2C**).

We further compared the stiffness tendencies of PFA- and glyoxal-fixed brains and acutely prepared living brains using 4-week-old juvenile mice. We made coronal slices, including the hippocampus, from each condition (**Figure 3A**) and measured

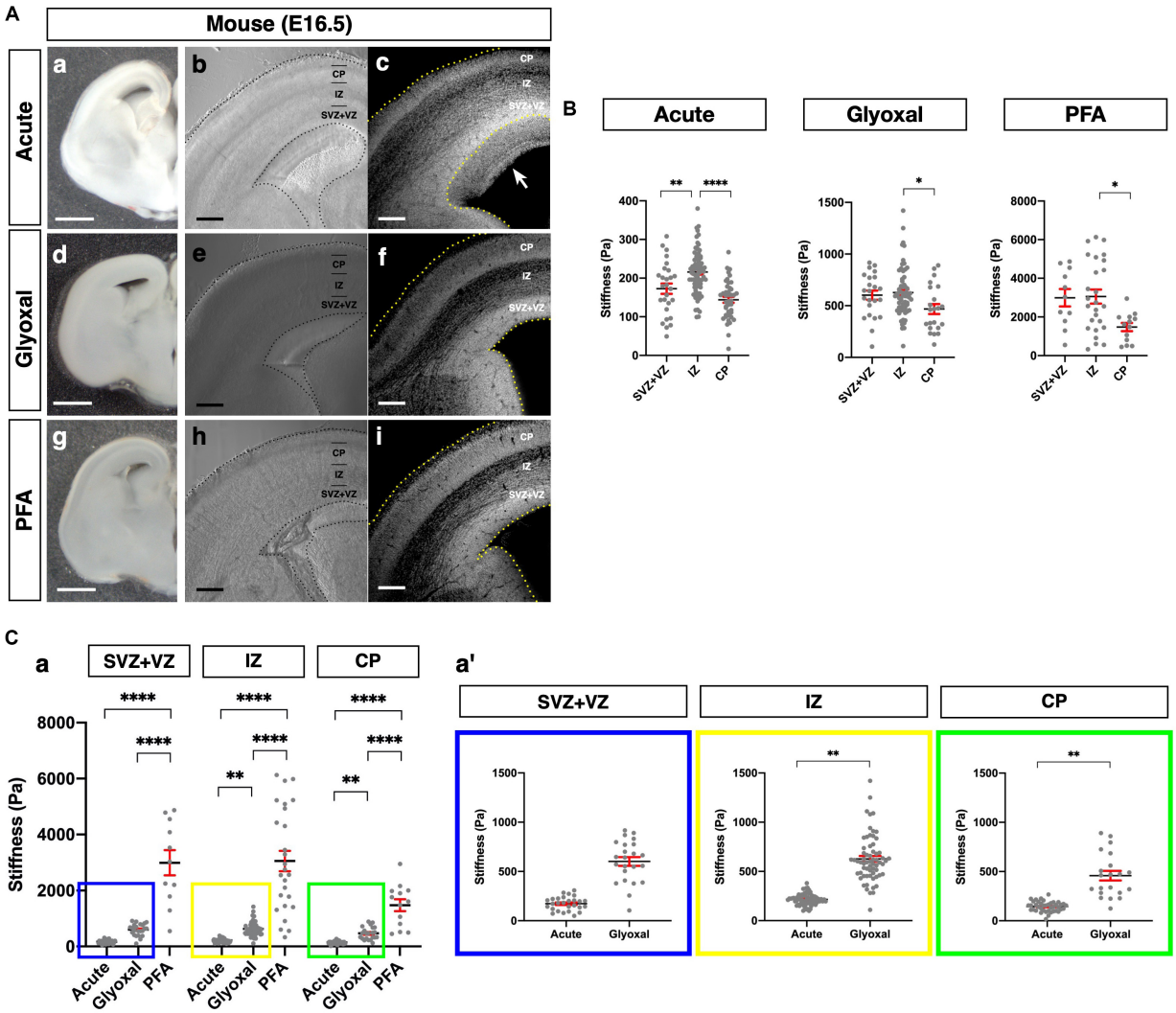

FIGURE 2 | Comparison of tissue stiffness in different fixative conditions. **(A)** (a, d, and g) Representative images of brain slices: (a) acute, (d) glyoxal-fixed, and (g) PFA-fixed. (b, e, and h) Phase-contrast images of brain slices set on AFM: (b) acute, (e) glyoxal-fixed, and (h) PFA-fixed. (c, f, and i) DAPI images after measurement. Acute slice was fixed with PFA: (c) acute, (f) glyoxal-fixed, and (i) PFA-fixed. **(B)** Cortical stiffness measured by AFM. **(C)** (a) Comparison of stiffness in 3 measurement regions (SVZ+VZ, IZ, and CP). (a′) Magnified views of insets in (a). Each color corresponds to the measurement region. Blue, SVZ+VZ; yellow, IZ; green, CP. SVZ, subventricular zone; VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate; scale bar: 1 mm for **(A)** (a, d, and g); 200 μm for **(B)** (b, c, e, f, h, and i). An arrow indicates an expanded proliferative region during measurement. One-way ANOVA with Tukey post hoc test; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) for **(B)** and **(C)**. Error bars in graphs are represented as the mean ± SEM.

the stiffness in the NCx and corpus callosum (CC) (**Figure 3B**). Like the embryonic brains, the PFA-fixed slices showed the highest stiffness in both the NCx and CC, while the glyoxal-fixed slices showed much lower stiffness in both regions. We also found that the stiffness in the NCx was relatively higher than in the CC in acute slices (294 ± 10 and 271 ± 19 Pa, respectively), although the statistical significance was not identified (**Figure 3C**). The relative difference in stiffness between the NCx and CC became strikingly higher in PFA (4761 ± 232 and 956 ± 129 Pa, respectively) but only moderate in the glyoxal-fixed condition (998 ± 56 and 678 ± 127 Pa, respectively). Our results from embryonic and juvenile mouse brains indicate that fixation by glyoxal can fairly maintain the relative stiffness profile of the NCx, keeping the overall softness of brain tissue adequately compared to PFA fixation.

Stiffness in Glyoxal-Fixed Brains Had Tendencies Like Living Brains in Songbirds

Subsequently, we investigated the effect of fixative solutions on tissue stiffness using other species. For this purpose, we measured stiffness in juvenile songbird brains (30–40 dph). Juvenile songbirds can begin to feed themselves around this age, and this developmental period is considered relevant to the weaning stage of mice around 4 weeks after birth. We prepared

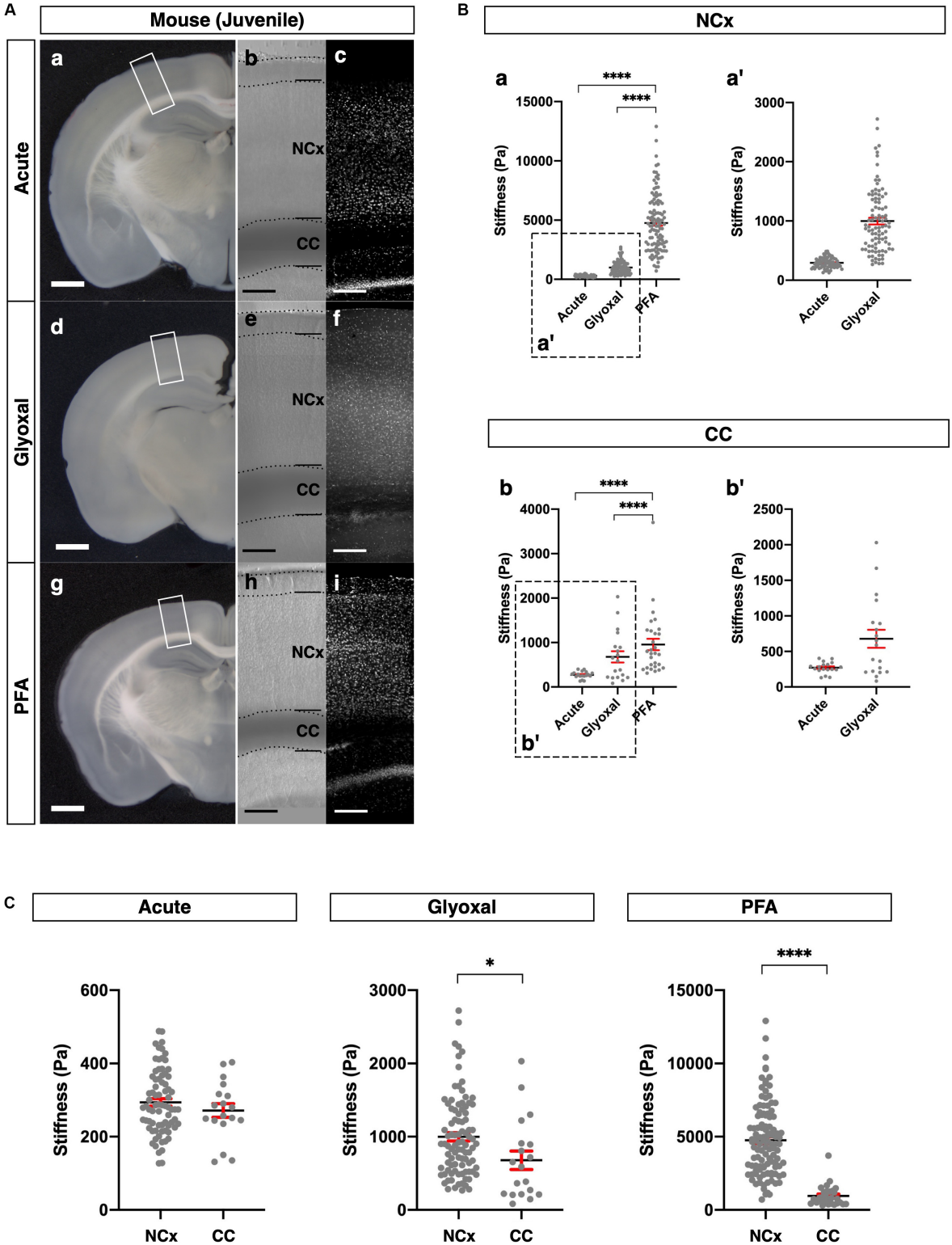

FIGURE 3 | Comparison of tissue stiffness in juvenile mouse brains in different fixatives. **(A)** (a, d, and g) Representative images of 4-week-old mouse brain slices: (a) acute, (d) glyoxal-fixed, and (g) PFA-fixed. (b, e, and h) Phase-contrast images of brain slices set on AFM: (b) acute, (e) glyoxal-fixed, and (h) PFA-fixed. Note that CC shows dark color because of an optical filter setting. (c, f, and i) DAPI images: (c) acute, (f) glyoxal-fixed, and (i) PFA-fixed. **(B)** Comparison of stiffness in NCx (a) and CC (b) in different fixatives. (a′ and b′) Magnified views of insets in (a and b), respectively. **(C)** Comparison of stiffness between NCx and CC in each fixative. NCx, neocortex; CC, corpus callosum; scale bar: 1 mm for **(A)** (a, d, and g); 200 μm for **(A)** (b, c, e, f, h, and i). One-way ANOVA with Tukey post hoc test; $P < 0.0001$ (****) for **(B)**. Two-tailed unpaired *t*-test; $P < 0.05$ (*) and $P < 0.0001$ (****) for **(C)**. Error bars in graphs are represented as the mean ± SEM.

coronal slices containing pallium in the dorsal part and striatum (Str) in the ventral part of the brains and measured the stiffness in both regions (**Figure 4A**). As with the mouse brains, the stiffness in the PFA-fixed brain slices was dramatically higher than in the other conditions, while glyoxal-fixed brain slices did not show any significant differences from the acute slice (**Figure 4B**). Notably, the pallium was significantly stiffer than the Str in both the acute and glyoxal-fixed slices (**Figure 4C**). However, the Str was stiffer than the pallium in PFA-fixed brains (**Figure 4C** and **Table 1B**). Furthermore, the range of stiffness values per measured point tended to be extremely broad in many PFA-fixed brains. Altogether, these results imply that fixation by PFA is not suitable for obtaining consistent tissue-stiffness profiles. With the results of the mouse and songbird brains, we conclude that glyoxal fixation might provide stiffness that relatively fits living tissue.

Distinct Neuronal Subtypes in the Mouse and Songbird Pallia Exhibited Different Stiffness Profiles

The mammalian cerebral cortex consists of six layers, whereas the avian telencephalon consists of neuronal nuclei. Despite this difference in brain structure, mammalian and avian brains share representative neuronal markers, such as Satb2, Ctip2, and Tbr1 (Britanova et al., 2008; Nomura et al., 2013; Briscoe et al., 2018; García-Moreno et al., 2018; Tosches et al., 2018), although the conserved and diversified characteristics of these marker-positive neurons have not been fully addressed. To confirm whether glyoxal-fixed brains are eligible for comparatively analyzing the tissue stiffness of the pallium in distinct neuronal subtypes of different species, we compared the stiffness of tissue composed of cells expressing different neuronal markers in the mouse NCx and songbird pallium.

As reported previously (Molyneaux et al., 2007; Srinivasan et al., 2012; Hanashima and Toma, 2015), cortical layers in mice were divided as follows: Satb2 single-positive neurons in the upper layer (layers II–IV), Ctip2 strongly positive neurons in the middle part of the NCx (layer V), and Tbr1-positive neurons in the deeper layer (layer VI) (**Figure 5A**). The stiffness in layers II–IV was relatively higher than the other layers, but no significant differences existed between the layers. Interestingly, the stiffness in the NCx was based on the criterion of marker expression showing a monotonical increase along the apico-basal axis, that is, from the ventricular to the pial surface (**Figure 5A**).

The pallium of songbirds consists of the hyperpallium (HP), mesopallium (M), nidopallium (N), and entopallium (E) (**Figure 5B**; Reiner, 2005; Medina and Abellán, 2009). The songbird pallium shares neuronal markers with the mouse brain. Satb2 was strongly expressed in the HP and M, while Tbr1 was strongly expressed in the M and N. Ctip2 was expressed in the entire brain, including the ventral part (Str), while it was relatively weaker in the HP. We compared the regional stiffness in the pallium based on the expression level of the neuronal marker and found that the HP was stiffer than the M region (**Figure 5B**). These results indicate that the neuronal populations distinguished by subtype-specific markers exhibited different mechanical properties, particularly stiffness, in the mouse NCx and songbird pallium.

Pallium in Amniotes Exhibited Species-Specific Stiffness Profiles

Finally, we applied our glyoxal brain-fixation method to comparatively analyze the stiffness of various species using chicks (E7 and E10), turtles (4 months and 2.5 years), and ferrets (E35 and P0). These brains were fixed by glyoxal immediately after dissection and shipped overseas. Subsequently, slices were made to measure their stiffness in one place using the same conditions and an AFM system. We prepared coronal brain slices of each animal and then measured the stiffness in the pallium along the apico-basal axis (**Figure 6**). The embryonic chick HP was divided into three areas (1, 2, and 3) corresponding to the VZ and the lower and upper neuronal zones based on the phase-contrast image (**Figure 6A**). We found a gradual increase of stiffness along the apico-basal axis at E10, the softest region being the VZ, which is occupied by neural progenitor cells (Nomura et al., 2016). Compared to E10, no stiffness gradient was observed at E7. The turtle dorsal cortex (DC) was divided into four areas corresponding to the VZ and cortical layers III, II, and I (**Figure 6B**). The major cell type in the turtle DC is neurons at these stages, especially Satb2-positive and Ctip2-positive neurons, which are intermingled in layers II and III (Areas 2 and 3 in **Figure 6B**; Nomura et al., 2013, 2018; Suzuki and Hirata, 2014; Tosches and Laurent, 2019). We found significantly higher stiffness in Area 4 at 4 months. However, the stiffness of Areas 1 and 3 had no significant difference. The 2.5 years turtle DC showed gradually increasing stiffness along the apico-basal axis, the softest region at the VZ, the most apical region. We also examined the stiffness of the ferret NCx, which was divided into seven areas along the apico-basal axis at E35 and P0 (**Figure 6C**). The NCx stiffness exhibited a much different profile in ferrets, showing a higher stiffness in Areas 6 and 7, a region corresponding to the CP, and a flattened pattern of stiffness from Areas 1–5 at E35. Intriguingly, a parabolic stiffness pattern with the lowest valley in Area 3, a region corresponding to the outer SVZ (OSVZ) (Reillo et al., 2011; Reillo and Borrell, 2012), was observed at P0. In embryonic mouse brains, the IZ was stiffer than other regions (**Figure 2**), but the IZ in P0 ferret brain (Areas 4–5) was softer, while the VZ and the inner SVZ (ISVZ) (Areas 1 and 2, respectively) and the CP (Areas 6 and 7) were stiffer. These differences in mechanical properties during development might provide distinct physical cues that contribute to the species-specific morphologies of the respective animals' mature brains (see section "Discussion").

DISCUSSION

The highlighted findings of this study are as follows: (1) Glyoxal-fixed brains can fairly maintain the relative stiffness of living tissue, and (2) brains fixed with glyoxal exhibit species-specific stiffness profiles.

Regarding (1), glyoxal has several advantages in fixation. It was reported several decades ago as an alternative fixative

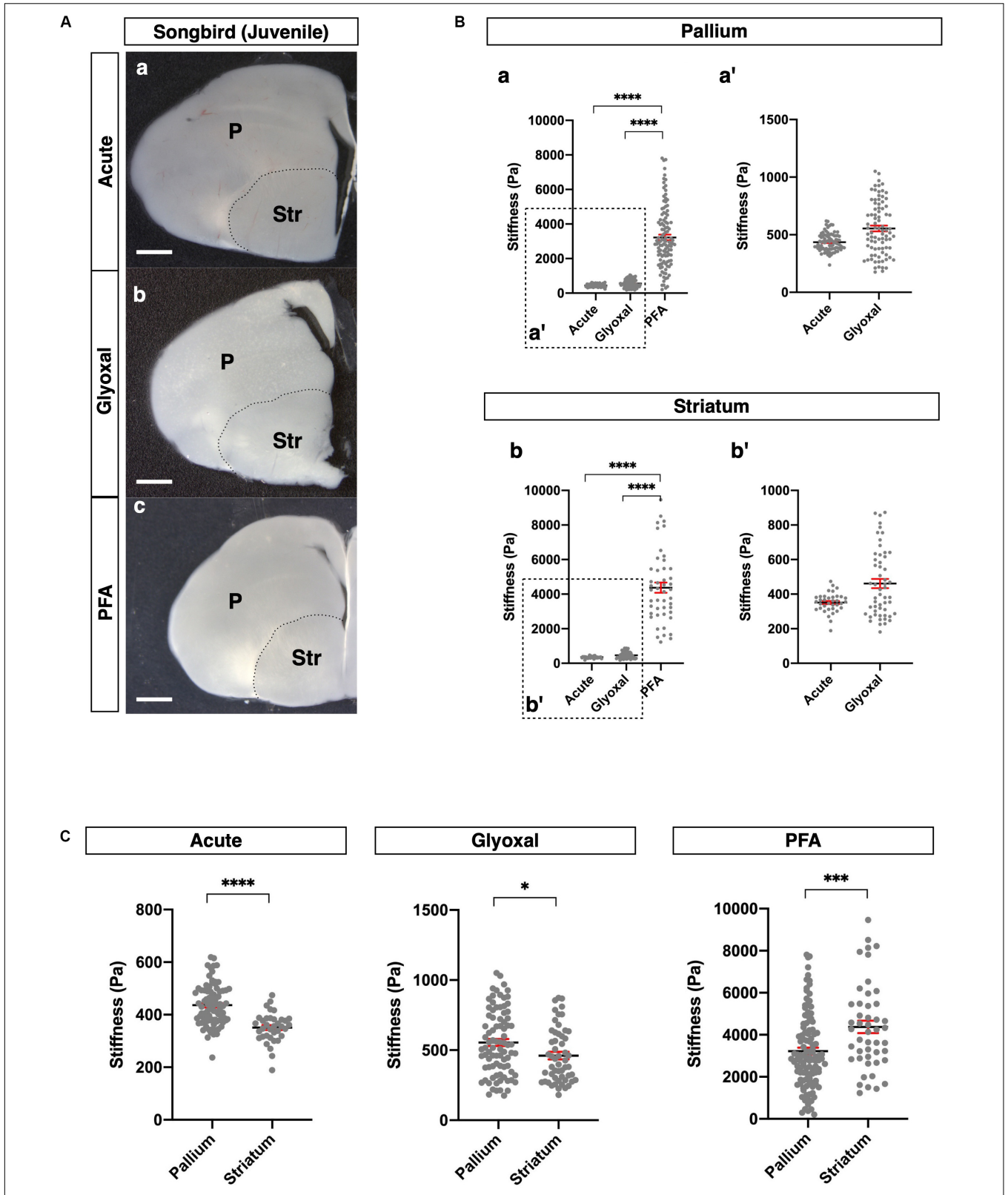

FIGURE 4 | Comparison of tissue stiffness in juvenile songbird brains in different fixatives. **(A)** Representative images of juvenile songbird brain slices: (a) acute, (b) glyoxal-fixed, and (c) PFA-fixed. **(B)** Comparison of stiffness in P (a) and Str (b) in different fixatives. (a′ and b′) Magnified views of insets in (a and b), respectively. **(C)** Comparison of stiffness between P and Str in each fixative. P, pallium; Str, striatum; scale bar: 1 mm. One-way ANOVA with Tukey post hoc test; $P < 0.0001$ (****) for **(B)**. Two-tailed unpaired *t*-test; $P < 0.05$ (*), $P < 0.001$ (***), and $P < 0.0001$ (****) for **(C)**. Error bars in graphs are represented as the mean ± SEM.

TABLE 1 | Summary of stiffness.

(A) Stiffness in mouse (Figures 2, 3)

	E16.5			Juvenile	
	SVZ+VZ	**IZ**	**CP**	**CC**	**NCx**
Acute	173 ± 13 Pa (27 points)	216 ± 7 Pa (71 points)	144 ± 8 Pa (42 points)	271 ± 19 (18 points)	294 ± 10 (76 points)
Glyoxal	601 ± 44 Pa (23 points)	628 ± 30 Pa (64 points)	468 ± 48 Pa (22 points)	678 ± 127 (19 points)	998 ± 56 (93 points)
PFA	2993 ± 448 Pa (11 points)	3054 ± 361 Pa (26 points)	1475 ± 212 Pa (13 points)	956 ± 129 (29 points)	4761 ± 232 (111 points)

E16.5: Four acute brains, four glyoxal-fixed brains, and two PFA-fixed brains.Juvenile: Three acute brains, four glyoxal-fixed brains, and three PFA-fixed brains.

(B) Stiffness in songbird (Figure 4)

	Pallium	**Striatum**
Acute	436 ± 8 Pa (83 points)	351 ± 10 Pa (36 points)
Glyoxal	554 ± 25 Pa (85 points)	461 ± 26 Pa (54 points)
PFA	3224 ± 165 Pa (114 points)	4373 ± 298 Pa (47 points)

Three brains for each condition.

(C) Stiffness in mouse and songbird categorized by expression of neuronal markers (Figure 5)

	Mouse (Juvenile)			Songbird (Juvenile)	
	Layer VI	**Layer V**	**Layers II-IV**	**HP**	**M**
Glyoxal	879 ± 105 Pa (20 points)	972 ± 90 Pa (22 points)	1167 ± 85 Pa (49 points)	632 ± 38 Pa (45 points)	466 ± 26 PA (40 points)

Four mouse brains and three songbird brains.

(D) Stiffness in chick, turtle, and ferret fixed with glyoxal (Figure 6)

(a) Chick

E7			E10		
Area 1	**Area 2**	**Area 3**	**Area 1**	**Area 2**	**Area 3**
630 ± 44 Pa (60 points)	614 ± 41 Pa (67 points)	609 ± 47 Pa (51 points)	568 ± 41 Pa (30 points)	612 ± 43 Pa (46 points)	771 ± 66 Pa (25 points)

(a) Two brains (five slices) at E7 and one brain (two slices) at E10.

(b) Turtle

	Area 1	**Area 2**	**Area 3**	**Area 4**
4 months	612 ± 45 Pa (23 points)	641 ± 32 Pa (23 points)	543 ± 36 Pa (22 points)	836 ± 97 Pa (22 points)
2.5 years	571 ± 30 Pa (18 points)	590 ± 19 Pa (28 points)	706 ± 42 Pa (17 points)	730 ± 58 Pa (17 points)

(b) One brain (two slices) at 4 months and one brain (two slices) at 2.5 years.

(c) Ferret

	Area 1	**Area 2**	**Area 3**	**Area 4**	**Area 5**	**Area 6**	**Area 7**
E35	396 ± 32 Pa (34 points)	472 ± 37 Pa (34 points)	455 ± 23 Pa (63 points)	402 ± 26 Pa (31 points)	487 ± 46 Pa (41 points)	610 ± 72 Pa (32 points)	578 ± 78 Pa (15 points)
P0	721 ± 58 Pa (16 points)	841 ± 69 Pa (18 points)	423 ± 19 Pa (44 points)	538 ± 49 Pa (22 points)	768 ± 51 Pa (23 points)	743 ± 85 Pa (19 points)	943 ± 136 Pa (13 points)

(c) One brain (four slices) at E35 and one brain (three slice) at P0.

to formalin (Wicks and Suntzeff, 1943). Its simple dialdehyde structure enables it to penetrate cells rapidly and preserve the immunoreactivity of proteins (Richter et al., 2018). Its preservation of nucleic acid is also of acceptable quality for fluorescent *in situ* hybridization and next-generation sequencing analysis (Bussolati et al., 2017). Importantly, glyoxal is easily handled due to its low toxicity—that is, its lack of evaporation from solution. We tested the standard fixation methods, that is, immersion fixation and transcardial perfusion. Glyoxal-fixed brains turned white during fixation in both methods

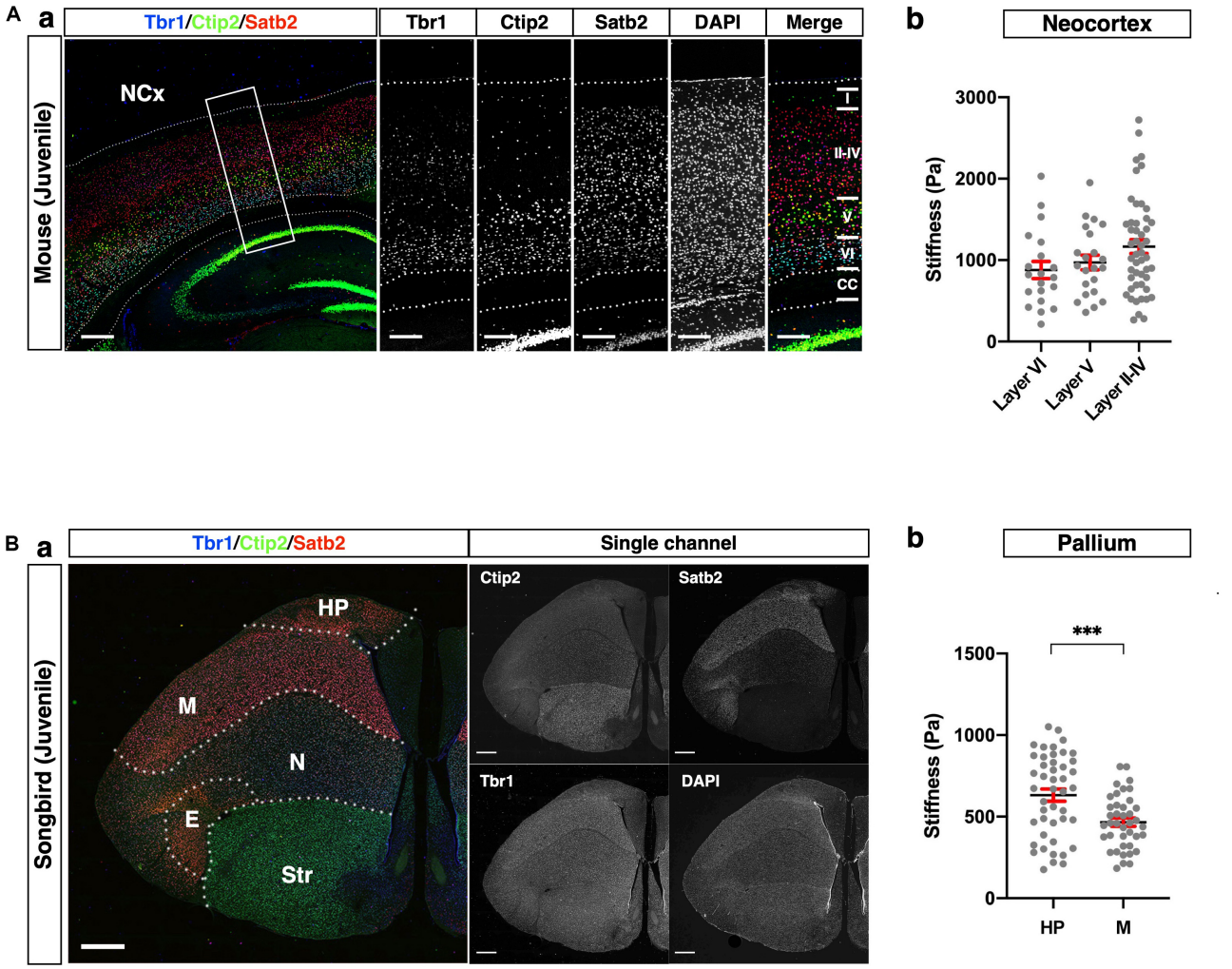

FIGURE 5 | Comparison of stiffness in homologous regions between mouse and songbird. **(A)** (a) Expression of Satb2, Ctip2, and Tbr1 in juvenile mouse NCx. Satb2 was expressed broadly but relatively strongly in layers II–IV. A strong Ctip2 signal was observed in layer V. Tbr1 was expressed in layer VI. (b) Stiffness in each layer. **(B)** (a) Expression of Satb2, Ctip2, and Tbr1 in juvenile songbird brain. A strong Satb2 signal was observed in HP and M, while the Ctip2 signal was observed in the entire brain. Tbr1 was expressed in the M and N. (b) Regional stiffness in the pallium. NCx, neocortex; HP, hyperpallium; M, mesopallium; E, entopallium; Str, striatum; scale bar: 300 μm for **(A)**; 1 mm for **(B)**. Two-tailed unpaired *t*-test; $P < 0.001$ (***) for **(B)**. Error bars in graphs are represented as the mean ± SEM.

(**Figure 1**), a color that was maintained in the sectioned brains and resulted in low contrast and transparency compared to the acute and PFA-fixed brain slices (**Figures 2–4**, **6**). This effect sometimes creates difficulty distinguishing tissue structures under a microscope. The glyoxal-fixed brains also became brittle, so trimming the region of interest in advance when using tissue with uniform structure and large, thick sections might be necessary.

The stiffness in the fixed tissue was higher than that of living tissue irrespective of brain size and fixation method, that is, PFA or glyoxal fixation. Remarkably, however, brain tissue fixed with glyoxal remained much softer than PFA and maintained relative stiffness like living conditions in most cases (**Figures 2–4**). Since glyoxal is a small molecule, non-cross-linked molecules might be washed out by PBS replacement. In contrast, the stiffness in the PFA-fixed brains was approximately 10 times higher than in living brains. Interestingly, the stiffness in the juvenile mouse CC was not so high, even in the PFA-fixed brain (**Figure 3**). The previously reported stiffness of white matter in rat cerebellums was 294 ± 74 Pa in living tissue (Christ et al., 2010). The stiffness of the CC measured in this study was 271 ± 19 Pa in acute slices, which fits the range found by a previous report (Christ et al., 2010), while it was 678 ± 127 Pa in the glyoxal solution and 956 ± 129 Pa in the PFA solution. The NCx, however, showed much larger differences in stiffness, with 294 ± 10 Pa in the acute, 998 ± 56 Pa in the glyoxal, and 4761 ± 232 in the PFA solutions (**Table 1A**). It is unclear why the CC showed lower stiffness in the fixed brains. Axon bundles in the CC are tightly wrapped with myelin sheaths consisting of lipids, so the lipid-rich myelin structure might affect the cross-linking of fixatives, lowering overall stiffness.

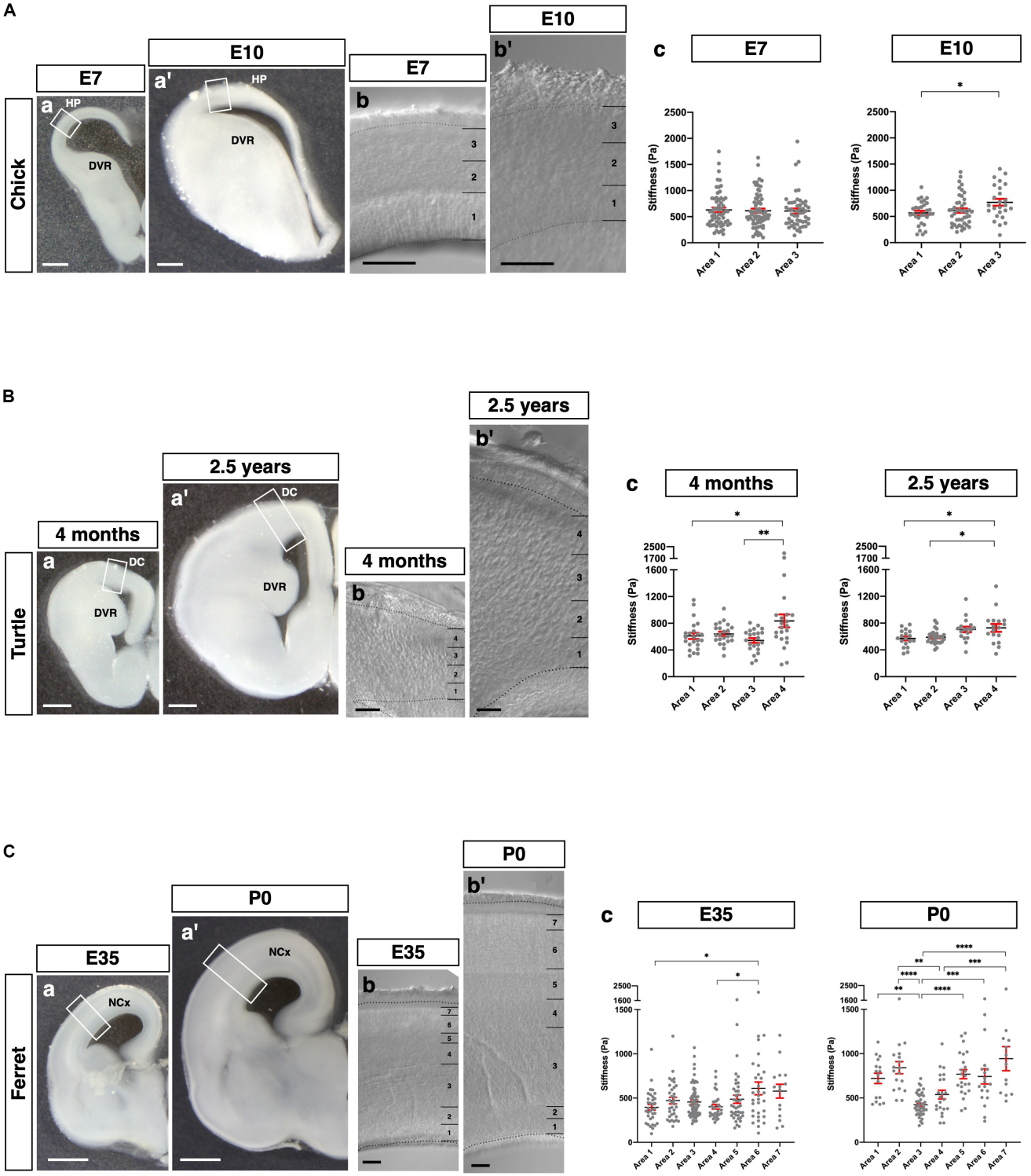

FIGURE 6 | Species-specific stiffness profiles in amniote brains. **(A)** (a and a′) Representative images of the chick brains (E7 and E10, respectively) fixed with glyoxal. (b and b′) Phase-contrast image of the HP (E7 and E10, respectively), which was divided into three areas. (c) Stiffness in the HP along the apico-basal axis. **(B)** (a and a′) Representative images of the turtle brains (4 months and 2.5 years, respectively) fixed with glyoxal. (b and b′) Phase-contrast image of the DC (4 months and 2.5 years, respectively), which was divided into four areas. (c) Stiffness in the DC along the apico-basal axis. **(C)** (a and a′) Representative images of the ferret brains (E35 and P0, respectively) fixed with glyoxal. (b and b′) Phase-contrast image of the NCx (E35 and P0, respectively). The NCx was divided into seven areas. (c) Stiffness in the NCx along the apico-basal axis. HP, hyperpallium; DVR, dorsal ventricular ridge; DC, dorsal cortex; NCx, neocortex; scale bar: 500 μm for **(A)** (a and a′) and B (a and a′); 1 mm for **(C)** (a and a′); 200 μm for **(A)** (b and b′), **(B)** (b and b′), and **(C)** (b and b′). One-way ANOVA with Tukey post hoc test; $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) for **(A–C)**. Error bars in graphs are represented as the mean ± SEM.

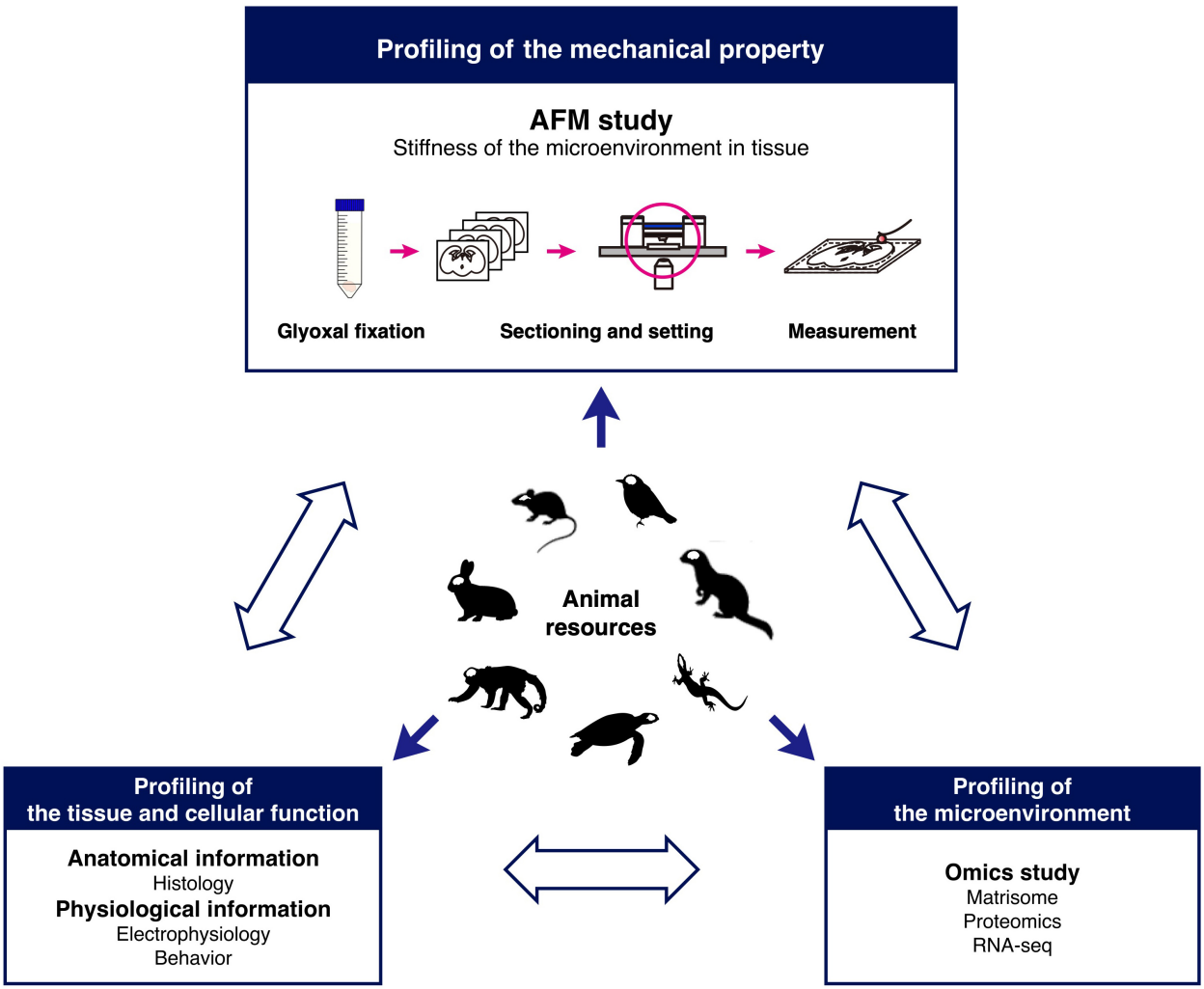

FIGURE 7 | Research model for comparative analysis of tissue mechanics. The schematic shows a model to investigate tissue stiffness in animals to understand the role of the microenvironment in development.

Regarding (2), we examined the stiffness profiles of homologous structures across species (**Figure 5** and **Table 1C**) and stage-dependent stiffness profiles in the extant amniotes (**Figure 6** and **Table 1D**).

First, we compared the stiffness of the homologous structure shared by the mouse NCx and songbird HP to investigate whether the pallial cytoarchitecture affected the tissue stiffness (**Figure 5**). In the mammalian NCx, each cortical layer consists of specific neural subtypes distinguished by layer-specific transcription factors, such as Ctip2, Satb2, and Tbr1 (Molyneaux et al., 2007; Britanova et al., 2008; Srinivasan et al., 2012; Hanashima and Toma, 2015). In contrast, the songbird HP, a homologue of the mammalian NCx, does not exhibit layer structures. Since the expression of orthologous genes does not discriminate between structural differences, we did not divide further areas in the songbird HP. Instead, neural subtype-specific genes allowed us to distinguish each pallial compartment in the songbird brain (**Figure 5B**). However, we could not find conserved stiffness patterns in neuronal populations expressing specific neuron-subtype markers; rather, neuronal subtypes in distinct pallial regions exhibited species-specific stiffness. This result is consistent with our previous report (Nomura et al., 2018): the expression of cell type-specific transcription factors does not confer evolutionarily conserved cellular characteristics, which disputes the theory of cell-type homology based on the expression of orthologous gene expression. Nevertheless, integrating the histological and functional analyses of neurons may be necessary for direct interspecies stiffness comparisons in the future.

Second, we applied glyoxal fixation to several amniotes to investigate the stiffness of their pallia exhibiting different morphologies. We chose representative species based on phylogenetic and histological reasons (Puelles et al., 2000, 2016, 2019; de Juan Romero and Borrell, 2015; Medina et al., 2017, 2019; Desfilis et al., 2018; Pessoa et al., 2019; García-Moreno and Molnár, 2020). We successfully obtained species-specific

stiffness profiles using chick, songbird, turtle, mouse, and ferret brains, which are classified as birds, reptiles, and mammals. Although birds and turtles are Sauropsids, the structure of their pallium exhibits a different cytoarchitecture. The DC in the reptile pallium has a layered structure (Connors and Kriegstein, 1986; Crockett et al., 2015; Tosches et al., 2018), whereas the HP in the bird pallium consists of neuronal nuclei (Reiner, 2005; Medina and Abellán, 2009). Mice and ferrets have an enlarged NCx with distinct complexities, as the mouse NCx is lissencephalic, while the ferret's exhibits remarkable gyrification (de Juan Romero et al., 2015), although six-layered cortical lamination is extensively shared by both species.

We found stage-dependent and species-specific stiffness in pallia among amniotes. For instance, a monotonous pattern of stiffness was observed in chicks at E7 (**Figure 6A**). This tendency corresponds to our previous report (Iwashita et al., 2014), showing that no significant differences among cortical layers were detected in early neurogenesis in mice (E12.5–14.5). In contrast, the embryonic ferret NCx showed a different tendency, indicating a monotonous pattern in the proliferative region, including the VZ, ISVZ, OS, IZ, and SP but with a relatively higher stiffness in the CP (**Figure 6C**). Notably, the ferret NCx at P0 exhibited a catenary stiffness pattern. In contrast to embryonic mouse brains, the CP, ISVZ, and VZ showed higher stiffness values than other layers, while the IZ showed lower stiffness. The OSVZ, a distinctive layer in species with a folded NCx, including ferrets, monkeys, and humans (Smart, 2002; Fietz et al., 2010; Reillo et al., 2011), showed the lowest stiffness. These differences in mechanical properties might affect cellular behavior during brain development. The extracellular matrix (ECM) determines the stiffness of tissues; in fact, recent studies have identified tissue-specific ECMs and their effects on cellular behavior, including proliferation, fate determination, and migration (Fietz et al., 2012; Long and Huttner, 2019; Ueno et al., 2019). Distinct stiffness might control cellular behavior during development and contribute to the different morphologies of brains, such as lissencephalic surfaces in mice, gyrencephalic surfaces in ferrets, and neuronal slabs in birds.

In comparing the matured pallia, gradually increasing stiffness along the apico-basal axis was observed in the 2.5 years turtle DC, which was derived from the dorsal pallium, a homologous region of the mammalian NCx (**Figure 6B**). This gradient pattern was also observed in the mouse juvenile NCx (**Figure 5A**). The outer layer of the NCx in mice is terminated with apical dendrites, while the DC in turtles is occupied by densely packed dendrites (Connors and Kriegstein, 1986; Crockett et al., 2015). Cytoskeleton-rich processes might contribute to determining regional stiffness. Further systematic studies using different stages and animals are required to confirm that this stiffness gradient is common in pallia with laminar structures.

CONCLUSION

In conclusion, glyoxal fixation can be applicable to the study of the mechanical properties of the brain in combination with AFM. Our findings based on our method strongly suggest that species-specific microenvironments might exist in the brain and that distinct mechanical properties could provide different cues to neural cells to form diverse brain structures as a result of migration during development. To identify the interactions between cells and microenvironments, further systematic analysis is required. Therefore, we propose a novel research model for brain development based on the mechanical properties of microenvironments (**Figure 7**). Combining stiffness data with histological and omics (Naba et al., 2016) analysis enables systematically and quantitatively analyzing correlations between mechanical properties and molecules in developing brains. This research model using glyoxal-fixed brains could help elucidate the diversity of brain structures.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee of Korea Brain Research Institute.

AUTHOR CONTRIBUTIONS

MI and YK designed the research concept and wrote the manuscript. MI prepared the mouse brains, measured the stiffness, and analyzed the results. TN prepared the turtle and chick brains and wrote the manuscript. TS and FM prepared the ferret brains. SK prepared the songbird brains. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.574619/full#supplementary-material

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MINI REVIEW
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Molecular Mechanisms of Cadherin Function During Cortical Migration

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During development of the cerebral cortex, different types of neurons migrate from distinct origins to create the different cortical layers and settle within them. Along their way, migrating neurons use cell adhesion molecules on their surface to interact with other cells that will play critical roles to ensure that migration is successful. Radially migrating projection neurons interact primarily with radial glia and Cajal-Retzius cells, whereas interneurons originating in the subpallium follow a longer, tangential route and encounter additional cellular substrates before reaching the cortex. Cell-cell adhesion is therefore essential for the correct migration of cortical neurons. Several members of the cadherin superfamily of cell adhesion proteins, which mediate cellular interactions through calcium-dependent, mostly homophilic binding, have been shown to play important roles during neuronal migration of both projection neurons and interneurons. Although several classical cadherins and protocadherins are involved in this process, the most prominent is CDH2. This mini review will explore the cellular and molecular mechanisms underpinning cadherin function during cortical migration, including recent advances in our understanding of the control of adhesive strength through regulation of cadherin surface levels.

Keywords: cerebral cortex, neuron, migration, cell surface, adhesion molecules, CDH2, molecular mechanism

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INTRODUCTION

Whilst cellular movements are an essential developmental feature of most organs, the orchestration of such movements in the nervous system is particularly relevant, as much of the central nervous system is organized in distinct layers that typically share functional properties. In the brain, neurons are generated in proliferative areas close to the ventricles and subsequently migrate to reach their definitive positions in different regions. Neocortical projection neurons migrate radially from the pallial ventricular or subventricular zone (Haubensak et al., 2004; Noctor et al., 2004), whereas cortical interneurons migrate tangentially from the ventral telencephalon (de Carlos et al., 1996; Anderson et al., 1997; reviewed in Marín and Rubenstein, 2001; Ayala et al., 2007; see also **Figure 1**). During their journey, they all navigate through complex extracellular environments that include other cells, which play critical roles in ensuring a successful migration. Interactions between Cajal-Retzius cells in the marginal zone of the cortex and migrating projection neurons are crucial for somal and terminal translocation (Gil-Sanz et al., 2013). Likewise, locomoting neurons use the basal processes of radial glia progenitors as a scaffold to migrate along (Rakic, 1972). Contact between migrating neurons and their cellular substrates is mediated by different cell-cell adhesion molecules, including the cadherin superfamily, with over 100 members expressed preferentially or exclusively in the nervous system in vertebrates. Cadherins are calcium-dependent cell-cell

adhesion molecules, characterized by the presence of a variable number of extracellular cadherin repeats, that can be broadly subdivided into three main subfamilies (Sotomayor et al., 2014): the classical cadherins and the clustered and non-clustered protocadherins. Members of all three subfamilies have been shown to play a role in cortical migration. Alpha clustered protocadherins regulate radial migration through a pathway involving WAVE, Pyk2 kinase and the small GTPase Rac1 (Fan et al., 2018). Interfering with protocadherins DCHS1 and FAT4 also affects neuronal positioning (Cappello et al., 2013) and leads to migration defects in human projection neurons (Klaus et al., 2019). Protocadherin 20 determines the final position of layer 4 neurons in mice (Oishi et al., 2016), while another protocadherin, Celsr3, is required for interneuron migration (Ying et al., 2009). However, the cadherin with the best characterized role in cortical migration is the classical cadherin CDH2 (N-cadherin), which will therefore be the focus of this review.

CDH2 ROLES DURING NEURONAL MIGRATION

After initial bipolar migration to the subventricular zone and lower intermediate zone, newborn neurons adopt a multipolar morphology before developing leading and trailing processes and becoming bipolar in the upper intermediate zone to progress into the cortical plate (Nadarajah et al., 2001; Tabata and Nakajima, 2003; Noctor et al., 2004). The multipolar to bipolar (MBP) transition is a crucial step that involves many different proteins (Cooper, 2014). Failure to form a leading process impedes migration into the cortical plate, which proceeds first through somal translocation for early born neurons and then by glia-guided locomotion (Kawauchi, 2015) followed by terminal translocation as the cortical wall grows in thickness (Nadarajah et al., 2001; **Figure 1**). Interneurons first migrate tangentially from the ganglionic eminences into the cortex and subsequently switch to radial migration to invade the cortical plate (Marín and Rubenstein, 2001).

CDH2 has been involved in most of these steps. As one of the main components of adherens junctions keeping radial glia endfeet connected (Kadowaki et al., 2007), CDH2 first needs to be downregulated to allow detachment of newborn neurons from the adherens junction belt and their subsequent delamination (Rousso et al., 2012). Once neurons reach the subventricular zone/intermediate zone, CDH2 is needed for correct polarization in the radial direction and for the specification of the leading and trailing processes (Jossin and Cooper, 2011; Gärtner et al., 2012; Xu et al., 2015). Further to its role in MBP transition, CDH2-mediated adhesion between migrating neurons and radial glia fibers is needed for the locomoting phase (Shikanai et al., 2011), with adhesion sites providing traction for the forward movement of the nucleus (Martinez-Garay et al., 2016). Similarly, attachment of the leading process to radial glia endfeet and Cajal-Retzius cells in the marginal zone for somal or terminal translocation is also dependent on CDH2 (Franco et al., 2011; Gil-Sanz et al., 2013). The following sections will focus on the molecular mechanisms known to operate around CDH2 during those different roles, with a significant part of the review devoted to regulation of CDH2 surface levels, as this topic has been more extensively studied.

REGULATION OF CDH2 LEVELS DURING RADIAL MIGRATION

The strength of cadherin-mediated adhesions depends mainly on the levels of cadherins at the cell surface. These levels are, in turn, regulated by the balance between the rate of biosynthesis and of delivery to the plasma membrane, and turnover of cadherin molecules by endocytic processes. Recent reviews provide a comprehensive account of cadherin trafficking (Cadwell et al., 2016; West and Harris, 2016), so this section will mainly focus on the specifics of CDH2 regulation in migrating neurons (**Table 1**).

Newly synthesized CDH2 in the endoplasmic reticulum binds to β-catenin and p120-catenin (Wahl et al., 2003), which regulates subsequent transport to the Golgi and the plasma membrane through interaction with kinesin motors (Mary et al., 2002; Chen et al., 2003; Teng et al., 2005; Wehrendt et al., 2016). Internalization of surface CDH2 is mediated by clathrin-dependent and independent endocytic mechanisms (Sharma and Henderson, 2007; Tai et al., 2007; Chen and Tai, 2017) and binding of β-catenin and p120-catenin to CDH2 regulates this process, partly by masking key residues needed for endocytosis (Chen and Tai, 2017). Internalized receptors can be either recycled back to the cell membrane or routed to the lysosome for degradation in an endosomal sorting pathway mainly controlled by Rab GTPases and their effectors (Wandinger-Ness and Zerial, 2014).

An intriguing possibility to further modulate cadherin adhesion is allosteric regulation. This form of inside-out signaling, well characterized in integrin-mediated adhesion (Hynes, 2002), has so far been demonstrated for CDH1 (Shashikanth et al., 2015), but whether it could provide a way to quickly control CDH2 adhesive strength is not yet known.

Regulation of CDH2 Total Levels

In migrating neurons, one way to adjust CDH2 levels is through regulation of *Cdh2* mRNA. FMRP binds *Cdh2* mRNA and in $Fmr1^{-/-}$ animals, which display increased numbers of multipolar neurons in the intermediate zone, *Cdh2* mRNA levels are reduced and the migration defect is rescued by overexpression of CDH2 (La Fata et al., 2014).

Similarly, downregulation of the small GTPase Rab23 also leads to a decrease of CDH2 levels in newborn neurons (Hor and Goh, 2018). This reduction, which is apparent at contact surfaces between interacting neurons, affects both the full-length protein and its processed cytoplasmic fragment, and is accompanied by a decrease in mRNA levels. Phenotypically, it leads to an increase in the number of multipolar neurons in the intermediate zone that fail to progress into the cortical plate. At the molecular level, diminished Rab23 levels seem to impair activation of PDGFRα and its subsequent phosphorylation of ERK1/2; indeed, pharmacological inhibition of ERK1/2 also led to a reduction in CDH2 levels (Hor and Goh, 2018).

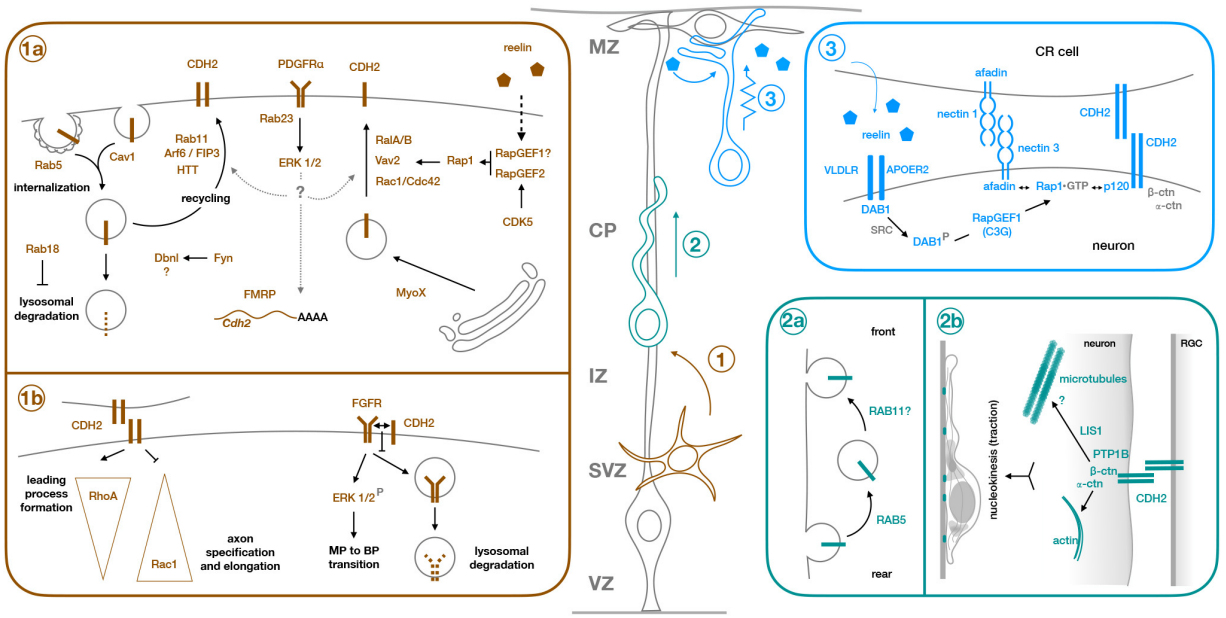

FIGURE 1 | Molecular mechanisms of CDH2 during the different steps of radial neuronal migration. Projection neurons are born from Radial Glia progenitors (gray cell, RGC) in the ventricular zone (VZ) or from intermediate progenitors in the subventricular zone (SVZ). They undergo multipolar migration while in the SVZ and intermediate zone (IZ) (step 1, brown cell), before developing a leading process and an axon, and acquiring bipolar morphology. They enter the cortical plate (CP) as bipolar cells and use the processes of the Radial Glia progenitors as scaffold to migrate along (step 2, green cell). Once they are close enough to the marginal zone (MZ) for their leading process to make contact with RGC endfeet and Cajal-Retzius cells (CR cell), neurons switch to terminal translocation for the last phase of their migration (step 3, blue cell). Early born neurons do not need a locomoting step because the cortical wall is still very thin at the time of their migration, so they ascend directly through somal translocation after becoming bipolar. Box **1a** shows the molecular mechanisms regulating CDH2 total or surface expression during multipolar migration and multipolar to bipolar transition (step 1), while box **1b** shows downstream effects of CDH2 at this stage. Similarly, boxes **2a** and **2b** show regulation of CDH2 and downstream effects during glia-guided locomotion (step 2), and box **3** shows regulation of CDH2 surface levels during somal translocation (step 3). For details, see text.

Another Rab GTPase involved in the control of total CDH2 levels is Rab18. Knockdown of this GTPase or interference with its function leads to a migration defect in the intermediate zone that can also be partially rescued by overexpression of CDH2. In this case, reduction of surface CDH2 levels as revealed by TIRF microscopy are correlated with a global decrease in CDH2 through lysosomal degradation rather than protein expression (Wu et al., 2016).

Regulation of CDH2 Surface Levels

In addition to adjusting global CDH2 levels, migrating neurons also dynamically regulate CDH2 surface levels through several pathways, allowing for more flexible responses to varying extracellular environments (Solecki, 2012; **Figures 1,1a,2a,3**).

Regulation by Rab GTPases

As main regulators of endosomal trafficking, Rab GTPases are ideal candidates to control CDH2 surface levels. Kawauchi et al showed that Rab11 and Rab5 play opposing but essential roles in the trafficking of CDH2 to and from the plasma membrane (Kawauchi et al., 2010; Kawauchi, 2011; **Figures 1,1a,2a**). Knockdown of Rab5 reduces Rab5-mediated endocytosis, leading to an accumulation of cells in the intermediate zone with abnormal morphology and increased adhesion between neurons and radial glia processes. This migration defect is partially rescued through slight downregulation of CDH2 levels, implicating Rab5 in the internalization of CDH2 (Kawauchi et al., 2010). This same study showed that Rab11 is needed for correct recycling of CDH2 back to the cell membrane after endocytosis. Electroporation of dominant negative (DN)-Rab11 results in redistribution of CDH2 from the cell surface to perinuclear regions and its accumulation in transferrin positive vesicular compartments, concomitant with a delay in radial migration.

Two other proteins have been shown to regulate CDH2 surface levels through an interplay with Rab11. Lack of HTT, which is expressed in the upper intermediate zone and the cortical plate, results in migration defects with neurons failing to acquire a bipolar morphology and showing abnormal interaction with radial glia fibers. HTT and CDH2 normally co-localize in the leading process of migrating neurons, but in the absence of HTT, CDH2 is relocated to transferrin positive vesicles in the perinuclear region and CDH2 surface levels are significantly reduced. Interestingly, co-expression of Rab11 or its constitutively active form rescues the migration defect of HTT depleted cells, as does overexpression of CDH2 (Barnat et al., 2016; **Figures 1,1a**).

The small GTPase Arf6 localizes to a subpopulation of early and recycling endosomes and interfering with its function disrupts migration and increases intracellular CDH2. This increase is not due to changes in the internalization of CDH2,

TABLE 1 | Regulators of CDH2 levels in migrating neurons.

Protein	Paradigm[1]	Effect on CDH2 levels	Migration stage	References
Afadin	KD (shRNA) (*in vitro*)[2]	↓ surface	ST	Gil-Sanz et al., 2013
ARF6	KD (shRNA) iuEP	↑ intracellular	MBPT	Hara et al., 2016
Caveolin 1	KD (shRNA) (*in vitro*)	↑ surface	MBPT	Shikanai et al., 2018
CDK5	*Cdk5* ko (*in vitro*) DNCDK5 (K33T) (293T cells)	↓ levels = total levels ↑ intracellular	MBPT MBPT	Ye et al., 2014 Lee et al., 2019
DBNL	KD (shRNA) (*in vitro*) FIP3 ΔABD (*in vitro*) FIP3 ΔRBD (*in vitro*)	↓ surface	MBPT	Inoue et al., 2019
ERK 1/2	Inhibition of phosphorylation with PD98059, U0126 (*in vitro*)	↓ levels	MBPT	Hor and Goh, 2018
FIP3	KD (shRNA) iuEP	↑ intracellular	MBPT	Hara et al., 2016
FMRP	*Fmr1* ko (WB, RT-qPCR)	↓ Cdh2 mRNA ↓ CDH2 protein	MBPT	La Fata et al., 2014
HTT	$Htt^{lox/lox}$;$Nex^{CRE/+}$ ND:CRE-GFP iuEP into $Htt^{lox/lox}$ (WB, *in vitro*)	= total levels ↓ surface ↑ perinuclear	MBPT	Barnat et al., 2016
MYO10	KD (shRNA) (293T cells)	↓ surface ↑ cytoplasmic	MBPT	Lai et al., 2015
Nectin 1/3	Primary neurons cultured on Nectin-1 vs. PLL	↑ surface	ST	Gil-Sanz et al., 2013
RAB5	DNRAB5 (S34N) (*in vitro*) KD (shRNA) (*in vitro*)	↑ surface	MBPT GGL	Kawauchi et al., 2010
RAB11	DNRAB11 (S25N) (*in vitro*, iuEP) KD (shRNA) (*in vitro*, iuEP) DNRAB11 (S22N) (*in vitro*, iuEP)	↑ perinuclear ↑ perinuclear	MBPT GGL MBPT	Kawauchi et al., 2010 Barnat et al., 2016
RAB18	KD (shRNA) (*in vitro*)	↓ surface	MBPT	Wu et al., 2016
RAB23	KD (shRNA) (*in vitro*, iuEP) $Rab23^{lox/lox}$;$Emx1^{CRE/+}$ (WB)	↓ protein (FL and cytopl. fragment) ↓ Cdh2 mRNA	MBPT	Hor and Goh, 2018
RAP1	conditional KO (Rap1a + b) (IHC of EP brain slices) RAP1GAP OE (*in vitro*, iuEP) RAPGEF2 KD (shRNA), RAPGEF2 S1124A (*in vitro*)	↓ levels ↓ surface ↓ surface	MBPT MBPT MBPT	Shah et al., 2016 Jossin and Cooper, 2011 Ye et al., 2014
REELIN	Application of reelin to *reeler* neurons cultured on Nectin-1	↑ surface	ST	Gil-Sanz et al., 2013

[1]Note that in some of the references in the table, other experimental paradigms were used in addition to the ones listed. However, the table reflects the ones used when assessing effects on CDH2 levels, rather than migration as a whole. [2]In vitro refers to cortical primary neurons cultured in vitro, either from mutant brains, electroporated brains, or obtained from control brains and transfected. KD, knockdown; shRNA, short hairpin RNA; iuEP, in utero electroporation; MBPT, multipolar to bipolar transition; GGL, glia-guided locomotion; ST, somal or terminal translocation; FL, full length.

but rather to its defective recycling back to the cell surface. Only Arf6 capable of interacting with its effector FIP3 and with Rab11 can rescue the migration defect of Arf6 knockdown (Hara et al., 2016; **Figures 1,1a**).

Regulation by Rap1

The small GTPase Rap1 is one of the major regulators of CDH2 surface levels, both during multipolar migration and the MBP transition, and during somal and terminal translocation (**Figures 1,1a,3**). Jossin and Cooper showed that interfering with normal Rap1 function delays the progression of neurons to a bipolar state (Jossin and Cooper, 2011). This cell-autonomous defect is due to reduced surface levels of CDH2 and can be rescued by CDH2 overexpression. The molecular mechanism linking Rap1 to CDH2 involves Vav2, an activator for Rac1, and other small GTPases (RalA/B and Rac1/Cdc42). Interestingly, reelin signaling acts upstream of Rap1 to regulate CDH2 membrane levels in the intermediate zone, as it does in the upper cortical plate during somal or terminal translocation (Franco et al., 2011; Jossin and Cooper, 2011). In translocating neurons, reelin activates Rap1 through RapGEF1 (C3G) (Franco et al., 2011), but CDH2 recruitment to the cell surface is dependent on nectin-mediated adhesion between migrating neurons and Cajal-Retzius cells (Gil-Sanz et al., 2013). CDH2 overexpression rescues migration defects caused by downregulation of nectin 3 or its effector Afadin, which interacts with Rap1 and p120 catenin, providing a link between Rap1 activation and CDH2 (Gil-Sanz et al., 2013; **Figures 1,3**). Recently, the reelin-induced increase in CDH2 surface levels in translocating neurons has been shown to be transient rather than sustained, but the mechanism behind it remains unknown (Matsunaga et al., 2017).

Another molecule acting upstream of Rap1 to regulate CDH2 surface levels is CDK5. Despite initial reports of CDK5 negatively regulating cadherin-mediated adhesion and the interaction between β-catenin and CDH2 (Kwon et al., 2000), this negative effect of CDK5 on CDH2 adhesion has not been reproduced *in vivo*. CDK5 phosphorylates RapGEF2 in the developing cortex, enhancing its GEF activity toward Rap1

(Ye et al., 2014). RapGEF2 is strongest expressed in the upper intermediate zone and is required for MBP transition, since its knockdown leads to multipolar neurons that accumulate in the lower intermediate zone. Membrane CDH2 levels are reduced in the *Cdk5*$^{-/-}$ cortex and in neurons electroporated with either shRNA against RapGEF2, or its non-phosphorylatable form S1124A, and migration defects caused by RapGEF2 inhibition can be rescued by moderate overexpression of CDH2 (Ye et al., 2014). Similar results were reported in a recent study that looked at the link between CDK5 and CDH2 in the developing cortex, which showed that migration defects caused by *in utero* electroporation of DNCDK5 at E14.5 could be partially rescued by co-electroporation with CDH2 (Lee et al., 2019; **Figures 1,1a**).

Regulation by Other Proteins

In addition to small GTPases, other proteins linked to endocytosis and the actin cytoskeleton are involved in the control of CDH2 surface levels in migrating neurons. The actin motor MYO10 interacts with CDH2 through its FERM domain and seems to mediate its transport from the Golgi to the plasma membrane. Downregulation of this unconventional myosin reduces surface CDH2, but not its total levels. This leads to accumulation of cells in the intermediate zone that display disrupted interaction with radial glia fibers and decreased locomoting speed in those neurons that make it to the cortical plate. MYO10 also colocalizes with markers for early, late and recycling endosomes, suggesting that it might play a role in the trafficking of CDH2-containing endosomes (Lai et al., 2015). Drebrin-like (DBNL) is an adaptor protein that binds F-actin and Dynamin 1 and is thus involved in receptor-mediated endocytosis and remodeling of the actin cytoskeleton. As in the case of MYO10, knockdown of DBNL reduces CDH2 levels at the cell surface. How this reduction is brought about at the molecular or cellular level is not known, but it seems to involve phosphorylation of two Tyr residues in Dbnl by Fyn. Dbnl-deficient neurons complete the MBP transition despite defects in neurite extension and polarization, but do not enter the cortical plate (Inoue et al., 2019). In both cases, overexpression of CDH2 partially rescues the migration defects (**Figures 1,1a**).

Despite neurons not displaying caveolae, caveolin 1 is expressed in the developing cortex, particularly in the neurites of multipolar cells in the intermediate zone, where it is involved in clathrin-independent endocytosis. Downregulation of caveolin 1 increases the ratio of surface to total levels of two adhesion proteins: CDH2 and L1CAM, while decreasing their levels in early endosomes, suggesting that caveolin 1 is needed for their internalization (Shikanai et al., 2018). Neurons deficient in caveolin 1 acquire bipolar morphology, but their leading processes are shorter and more branched than in control neurons, with increased immature neurites that are retained even after leading process formation (**Figures 1,1a**).

MECHANISMS DOWNSTREAM OF CDH2

Compared to the wealth of information about the regulation of CDH2 surface levels in migrating neurons, much less is known about the mechanisms operating downstream of CDH2. With regards to the initial specification of neuronal processes, *in vitro* experiments suggest that opposing gradients of active RhoA at the leading process and Rac1 in the axon are established as a consequence of CDH2-mediated contact (Xu et al., 2015). Although the exact mechanism by which this is accomplished remains unclear, work in C2C12 fibroblasts has shown that CDH2 engagement decreases Rac1 and Cdc42 activity and increases RhoA activity (Charrasse et al., 2002). This could explain the formation of the gradients, as sites of CDH2 adhesion between neurons and radial glia fibers provide a positional cue for the development of the leading process while directing axonal formation to the opposite pole of the cell (Gärtner et al., 2012; Xu et al., 2015; **Figures 1,1b**).

A second mechanism at play during CDH2-mediated neuronal polarization involves its interaction with FGFRs to prevent their ubiquitination and subsequent lysosomal degradation. This interaction happens in *cis* and, surprisingly, does not require CDH2-mediated adhesion. As a result of higher levels of FGFR at the cell membrane, the ERK1/2 signaling pathway is activated. Since similar results in ERK1/2 activation are obtained by long treatment with reelin *in vitro*, FGFR and ERK1/2 can be considered downstream components of the reelin – Rap1 – CDH2 axis (Kon et al., 2019; **Figures 1,1b**).

Glia-guided locomotion requires CDH2-mediated adhesion and its connection to the actin cytoskeleton through alpha-N-catenin. When CDH2 (and CDH4) adhesion is weakened by electroporation of different dominant negative forms, neurons can still polarize and extend leading processes into the cortical plate. However, nucleokinesis fails and the leading processes become twice as long as in control neurons. The collapse of the processes upon induced actomyosin contraction indicates that CDH2-mediated contacts between neurons and radial glia fibers probably act as sites for traction generation to allow nuclear movement. In addition, neurons electroporated with DNCDH also show an abnormal accumulation of LIS1 in the leading process, hinting to a potential mechanism involving microtubules downstream of CDH2 in migrating neurons (Martinez-Garay et al., 2016; **Figures 1,2b**).

ROLE OF CDH2 IN INTERNEURON MIGRATION

CDH2 is also involved in the generation and migration of cortical interneurons. As well as maintaining the organization of the neuroepithelium through adherens junctions, CDH2 engagement stimulates interneuron motility (Luccardini et al., 2013). Interneurons expressing DNCDH *in vitro* show disrupted centrosome dynamics and non-muscle myosin IIB localization, while complete elimination of CDH2 reduces their migration speed and impairs polarity. *In vivo*, interneurons lacking CDH2 are less efficient in leaving the medial ganglionic eminence and reaching the cortex, as well as in invading the cortical plate (Luccardini et al., 2013, 2015). Interestingly, the effect of CDH2 on the ability of interneurons to migrate to the cortex and colonize it seems to be cell type specific, as knockout of CDH2

in Dlx5/6 expressing cells selectively reduces the numbers of calretinin and somatostatin positive interneurons, but does not alter other interneuronal types (László et al., 2020). However, the molecular mechanisms underpinning the role of CDH2 in interneuron migration remain to be elucidated.

DISCUSSION

The involvement of CDH2 in every step of radial migration and its function in interneurons underscore the importance of this adhesion molecule in mediating cell-cell interactions during cortical development. However, we still have a fragmented view with different observations that need to be integrated to provide a full picture of CDH2 function during neuronal migration. It is still not known if and how the different players regulating CDH2 levels are coordinated. For example, it remains to be determined whether Rap1 and Rab GTPases act in parallel pathways of if they cooperate to regulate CDH2 surface levels. The molecular signals activated upon CDH2 adhesion are also poorly understood, and the fact that mechanisms downstream of cadherins seem to be context specific means that caution should be exerted when extrapolating from different cellular systems and assumptions should be experimentally verified. It is important to keep in mind that the timepoint of intervention, dependent on age at electroporation but also on the use of different promoters, will influence results. The timing until analysis is also important because delays in polarization might mask roles in later migration phases, and the use of different dominant negative cadherin forms, sometimes at different concentrations, will also impact the phenotypes observed. These factors might explain why locomotion was considered relatively independent of CDH2 in one study (Jossin and Cooper, 2011), while being shown to be needed for this process in others (Shikanai et al., 2011; Martinez-Garay et al., 2016). Similarly, the requirement for CDH2 adhesion during MBP transition is questioned by a recent study (Kon et al., 2019), but this might reflect separate functions of CDH2 at slightly different timepoints during this complex process. Another controversy that might be explained, at least in part, by different experimental conditions is the fact that although reelin-mediated Rap1 activation seems to be required for MBP transition (Jossin and Cooper, 2011), Dab1 deficient neurons polarize correctly, enter the cortical plate and only show defects in somal translocation (Franco et al., 2011).

A final open question is the extent to which CDH2 cooperates with other adhesion molecules. Beyond its cooperation with nectins during somal translocation, no equivalent mechanism has been described for other migration phases. Connexins 43 and 26 also provide adhesion between cortical migrating neurons and radial glia fibers (Elias et al., 2007) and, interestingly, Cx43 directly downregulates CDH2 transcription during neural crest cell migration (Kotini et al., 2018). In addition, CDH2 binds astrotactin in cerebellar migration (Horn et al., 2018), raising the possibility of a similar function in the cortex. These examples highlight the potential for functional interactions between adhesion proteins and the need to expand our studies beyond individual molecules.

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REVIEW
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FLRTing Neurons in Cortical Migration During Cerebral Cortex Development

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During development, two coordinated events shape the morphology of the mammalian cerebral cortex, leading to the cortex's columnar and layered structure: the proliferation of neuronal progenitors and cortical migration. Pyramidal neurons originating from germinal zones migrate along radial glial fibers to their final position in the cortical plate by both radial migration and tangential dispersion. These processes rely on the delicate balance of intercellular adhesive and repulsive signaling that takes place between neurons interacting with different substrates and guidance cues. Here, we focus on the function of the cell adhesion molecules fibronectin leucine-rich repeat transmembrane proteins (FLRTs) in regulating both the radial migration of neurons, as well as their tangential spread, and the impact these processes have on cortex morphogenesis. In combining structural and functional analysis, recent studies have begun to reveal how FLRT-mediated responses are precisely tuned – from forming different protein complexes to modulate either cell adhesion or repulsion in neurons. These approaches provide a deeper understanding of the context-dependent interactions of FLRTs with multiple receptors involved in axon guidance and synapse formation that contribute to finely regulated neuronal migration.

Keywords: FLRT, Latrophilin, adhesion, repulsion, neuronal migration, Teneurin, Unc5

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INTRODUCTION

The cerebral cortex is an evolutionary advanced structure with complex functionality that is organized in two main axes: radial (vertical) and tangential (horizontal) (Geschwind and Rakic, 2013). The radial axis results from the migratory direction of pyramidal neurons in relation to the pial surface. This neuronal migration follows an inside-out pattern and produces distinct cortical layers where specific neurons settle and differentiate based on their time of birth and migration dynamics. The tangential axis reflects the horizontal position of cortical neurons and segregates them into different functional areas that process specific sensory, motor and cognitive information (Herculano-Houzel et al., 2013). The horizontal coordinates of neurons is determined by both, the relative position of their progenitors in the germinal zone lining the lateral ventricles and their tangential dispersion that occurs while migrating radially. This process is limited in lissencephalic species (with smooth cortices such as rodents) (Noctor et al., 2001) but extensive in gyrencephalic species (with folded cortices such as ferrets and most primates) (Reillo et al., 2011).

Cortical neurons migrate through dense environments where they can display complex trajectories. During their journey from germinal zones to the cortical plate (CP), neurons integrate a combinatorial code of receptor and ligand interactions that are presented from three sources: neighboring neurons/radial glia fibers, extracellular matrix components (ECM) and diffusible cues. These interactions can trigger a variety of context-dependent cellular responses based on the arrangement of receptors and expression of signal transducers. Most extracellular cell guidance cues belong to the axon guidance-related protein families that control the wiring of the neural system by guiding axons to their appropriate target, and can act over short (cell-cell/substrate contact) or long range (diffusible cues), triggering either adhesion/attraction or repulsive functional responses (see Seiradake et al., 2016; Bellon and Mann, 2018; for recent reviews). Thus, several cues have a dual role in both axon guidance and cellular migration, where they display similar cooperation and crosstalk between different pathways and stabilization through redundant mechanisms, making both processes remarkably robust despite their enormous complexity.

In this review we will focus on the fibronectin leucine-rich repeat transmembrane proteins (FLRTs) that emerged as the first class of cell adhesion molecules (CAMs) with repulsive functions by heterophilic interactions (and thus also referred to as ReCAMs) (Seiradake et al., 2014). Recent data has revealed a remarkable variety of structural arrangements between FLRTs and their binding partners known to be involved in axon guidance and synapse formation (Seiradake et al., 2014; Jackson et al., 2015, 2016; Lu et al., 2015; del Toro et al., 2020). By manipulating FLRTs binding interactions, cell migration assays have shown that they act as a bimodal guidance system regulating both radial migration by short and long-range repulsive signals and tangential dispersion through adhesive interactions. FLRTs are therefore the first family of ReCAMs to be described as having dual-functionality during the migration of cortical neurons.

FLRTs

The FLRT family protein comprises three members (FLRT1-3), all of which are type-I single-pass transmembrane receptor proteins involved in both repulsion and cellular adhesion depending on the cellular context and their binding partners. FLRT1 was the first member discovered around 20 years ago following an attempt to identify novel ECM components and interactors by screening a human skeletal muscle cDNA library (Lacy et al., 1999). The extracellular N-terminal region of all FLRTs contains 10 LRRs that are flanked by two highly conserved cysteine-rich regions and a fibronectin type III (FNIII) domain located adjacent to the membrane-spanning region by a linker containing a metalloprotease cleavage site (Yamagishi et al., 2011). FLRTs are glycosylated at 2 (FLRT1), 5 (FLRT2), or 4 (FLRT3) sites on their extracellular domains. A conserved sequence of 28 hydrophobic amino acids spans the cytoplasmic membrane (**Figure 1A**). The transmembrane helix connects to a relatively short non-homologous intracellular domain (ICD) that has been shown to interact with small Rho GTPases (Ogata et al., 2007) and modulate canonical fibroblast growth factor receptor (FGFR) signaling through the mitogen-activated protein kinase (MAPK) pathway (Böttcher et al., 2004; Wheldon et al., 2010).

LRR-INDEPENDENT BINDING PARTNERS

FGFRs

Fibroblast growth factors (FGFs) and their receptors FGFRs (1-4) are known to regulate a plethora of developmental processes in the nervous system, including patterning, cell proliferation, survival, guidance, and synaptogenesis (Reuss and Von Bohlen Und Halbach, 2003; Salinas, 2005; Guillemot and Zimmer, 2011). FGFRs structure consists of an extracellular domain comprising of three immunoglobulin (Ig1, 2, and 3) domains, followed by a single transmembrane helix and an intracellular tyrosine kinase (Y-kin) domain (Dai et al., 2019; **Figure 1B**). At the cell surface, FGFRs have been shown to form large complexes involving various CAMs that modulate their signaling (Latko et al., 2019). FLRTs were first identified as modulators of FGF/FGFR signaling after the observation that both show similar expression patterns in many developing tissues of *Xenopus* (*African clawed frog*) (Böttcher et al., 2004) and mouse (Haines et al., 2006). Studies using fusion constructs have shown that all FLRT members interact with FGFR1, albeit with different affinities, by their FNIII domain (Böttcher et al., 2004; Haines et al., 2006), which is similar to the structural case between the Ig (2-3) motifs of FGFR1 and the FNIII domains of the neural cell adhesion molecule (NCAM) (Kiselyov et al., 2003). A later study using the yeast two-hybrid system also suggested that, in addition to the FNIII, both LRR and ICD domains contribute to the interaction between FLRT2 and FGFR2 (Wei et al., 2011; **Figure 1B**). This could explain the interaction between all FLRTs and a constitutively active form of FGFR1 where the entire extracellular region was replaced with the immunoglobulin FC domain (Haines et al., 2006), and the fact that the ICD of FLRT3 alone can promote FGF signaling (Böttcher et al., 2004). However, it is important to note that there is no structural data available for FLRT-FGFR interaction. All FLRTs have been shown to enhance FGF signaling by increasing the levels of phosphorylated ERK via MAPK activation (Böttcher et al., 2004; Wheldon et al., 2010). Interestingly, the ICD of FLRT1 contains three tyrosines that are targets for a FGFR1-dependent phosphorylation, which in turn potentiates the ability of FLRT1 to stimulate the ERK pathway. The functional consequence of such activation is the promotion of neurite outgrowth in mouse primary hippocampal neurons (Wheldon et al., 2010), which is consistent with previous studies showing that FLRT3 is upregulated after nerve injury and enhances neurite outgrowth (Robinson et al., 2004; Tsuji et al., 2004). Like FLRTs, other CAMs can stimulate neurite outgrowth such as neuroligins (NGLs) (Lin et al., 2003), and synaptic adhesion-like molecules (SALMs) (Wang et al., 2008).

Robo

Robo (1-4) receptors are type-I single-pass transmembrane proteins. Their ectodomains are composed of five Ig and three

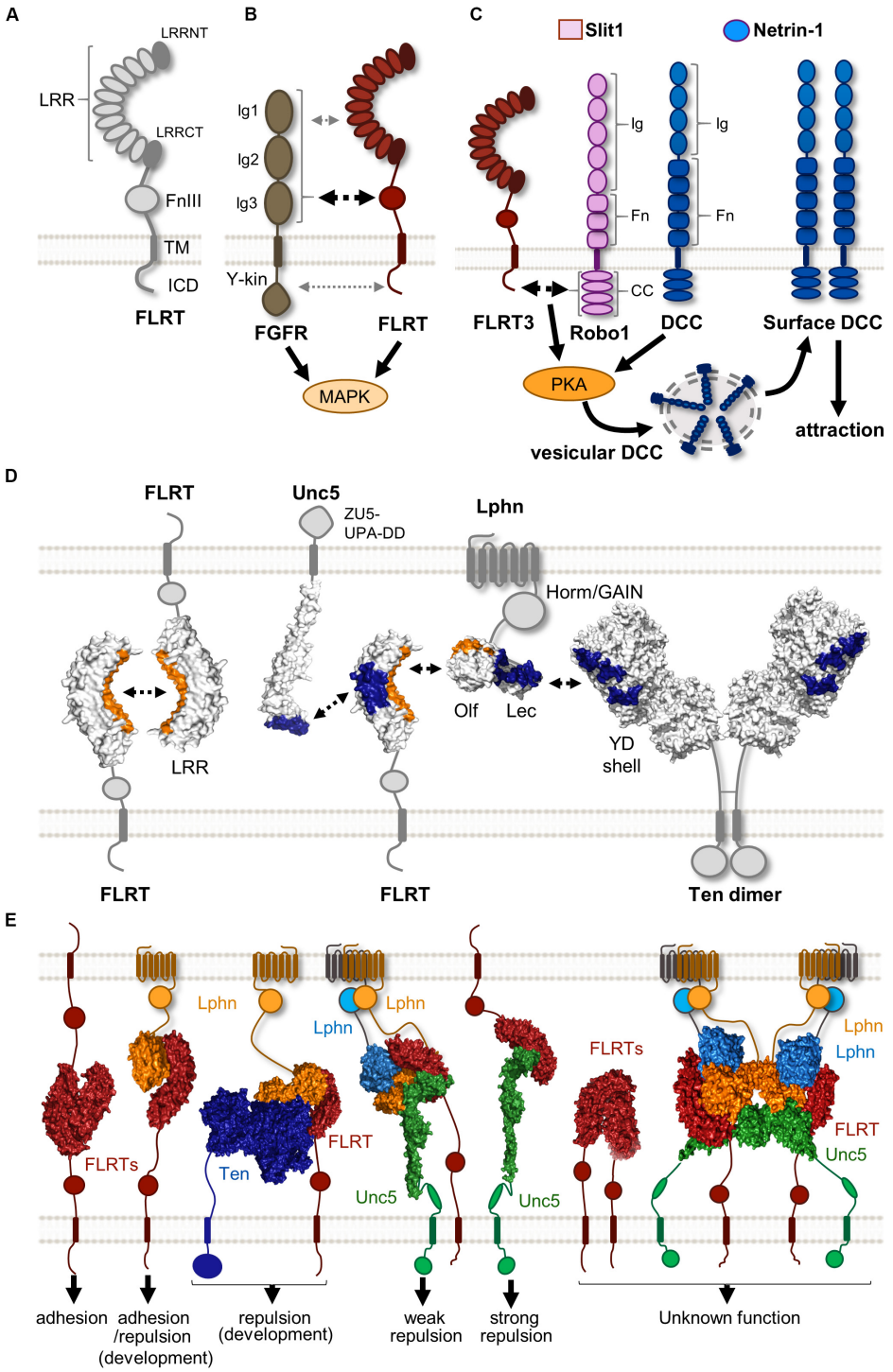

FIGURE 1 | Protein complexes formed by FLRTs and their binding partners. **(A)** Scheme showing the structure of FLRTs proteins and their main domains. **(B)** FLRT interact with FGFR through its FNIII domain (black dashed arrow) regulating MAPK activity. Other interactions through FLRT LRR and ICD have been suggested (gray dashed arrow). **(C)** Scheme representing the mechanism by which responsiveness to Netrin-1 is achieved in rostral TCAs. In the presence of Slit1 and Netrin-1 FLRT-Robo interaction enhances PKA activity, which in turn increases surface levels of DCC, thereby enabling Netrin-1 attraction. **(D)** Scheme illustrating the known surfaces involved in FLRT LRR-dependent interactions. LRR domains participate in homophilic and heterophilic binding with different binding partners. Surfaces interacting with the concave site of the FLRT LRR domain are labeled in orange, whereas those binding the convex site are labeled in blue. **(E)** Overview of the variety of binary and ternary structure arrangements formed by FLRTs and its binding partners. These complexes cover a variety of responses ranging from adhesion to repulsion. The biological function of some of these structures remains largely unknown. These are selected examples of published protein structures (Seiradake et al., 2014; Jackson et al., 2015, 2016; del Toro et al., 2020).

FNIII domains (although Robo4 is smaller, containing only two of each domains), followed by a single transmembrane helix connected to a large ICD showing different conserved cytoplasmic (CC) motifs (Bisiak and McCarthy, 2019). Their ectodomain can trigger both repulsive and adhesive signaling depending on its binding partner. The classical Robo ligand, Slits (1-3), are best known for their function as chemorepellents during neuronal and axon guidance (Wu et al., 1999; Zhu et al., 1999; Ypsilanti et al., 2010) by binding to the Ig (1 and 2) domains of Robo receptors (Liu et al., 2004). This interaction is further stabilized by the addition of heparin sulfate (Fukuhara et al., 2008). However, their ectodomain can also mediate Robo homophilic interactions, mainly through the Ig domains, in a Slit-independent manner (Zakrys et al., 2014). These homophilic bindings trigger cell adhesion and stimulate neurite outgrowth (Hivert et al., 2002). Robo receptors participate in widely diverse functions during development due to their ability to interact with different co-receptors through forming both *cis* and/or *trans* extracellular interactions, as well as intracellular *cis*-interactions, thus creating crosstalk between several distinct signaling pathways (Bisiak and McCarthy, 2019).

One example of such interplay that has been studied in detail is the ability of Slit/Robo to regulate the Deleted in Colorectal Cancer (DCC) receptor signaling in a Netrin-1 dependent manner. In embryonic *Xenopus* spinal axons, activation of Robo1 by Slit leads to binding of its ICD to that of DCC, silencing Netrin-1 attraction (Stein and Tessier-Lavigne, 2001), which is consistent with the *in vivo* finding that postcrossing commissural axons acquire responsiveness to Slit, preventing their attraction to Netrin-1 (Reeber and Kaprielian, 2009). Interestingly, Slit/Robo can also enhance the attractive response to the guidance cue Netrin-1. The mammalian Robo3, which does not bind Slits because of mutations in the Ig1 domain, interacts with DCC via its ICD and is thereby phosphorylated when Netrin-1 binds DCC, thus potentiating Netrin attraction. Pontine neurons, lacking Robo3, phenocopy neurons deficient for DCC in their absence of attraction toward Netrin-1, suggesting that both receptors are required in mediating Netrin-1 dependent attraction of these neurons (Zelina et al., 2014). Further complexity in the regulation of the interplay between Slit/Robo and Netrin/DCC has been found during the development of thalamocortical axons (TCAs). Here, Slit1 enables Netrin-1 attraction in rostral TCAs, but exerts repulsion in intermediate TCAs, suggesting that Slit1 has a context-dependent role in TCA pathfinding and that combination of Slit1 and Netrin-1 differs from their individual effects (Bielle et al., 2011). Interestingly, both subsets of TCAs express similar levels of Robo (1 and 2), and the Netrin receptors, DCC and Unc5C, indicating that other co-receptors could participate in their molecular differences.

FLRT3 was found as a novel Robo1 interacting partner in a yeast two-hybrid screen using its ICD domain as a bait. There is a *cis*-interaction between FLRT3 and Robo1 ICDs and interestingly FLRT3 is expressed in TCAs in a rostral-to-caudal gradient (Leyva-Díaz et al., 2014). In the presence of Slit1 and Netrin-1, rostral TCAs expressing Robo1 and FLRT3 show upregulation of surface DCC through the activation of protein kinase A, which in turn induces Netrin-1 attraction (**Figure 1C**). Loss of FLRT3 or Robo1 in rostral TCAs prevents this effect, suggesting that both proteins are required to enable Netrin-1 sensitivity. Moreover, ectopic expression of FLRT3 in non-responsive intermediate TCAs that normally lack FLRT3, is sufficient to induce the attractive response observed in rostral TCAs in the presence of Slit1 and Netrin-1. This modulation of Netrin-1 responsiveness by FLRT3 is required for the proper navigation of TCAs to target different cortical areas, consistent with evidence of abnormal pathfinding of rostral TCAs in the absence of FLRT3 *in vivo* (Leyva-Díaz et al., 2014).

LIMITED LRR-BINDING PARTNERS STILL FORM MULTIPLE PROTEIN-PROTEIN COMPLEXES

The LRR domain is the most studied ectodomain of FLRTs. It is present in a large number of proteins with diverse structure and function, being commonly found in proteins associated with the immune system and in neural development (Ng et al., 2011). The repeating nature of this domain, where LRR motifs array in tandem, results in a non-globular horseshoe-shape structure with two distinct surface areas: concave and convex, which correspond to the inner and outer circumference of the horseshoe, respectively (Kobe and Deisenhofer, 1994; Kajava, 1998). The increased surface area of this domain facilitates protein-protein interaction, and thus LRR- containing proteins have been implicated in intercellular communication and cell adhesion (Chen et al., 2006; Matsushima et al., 2007). In the nervous system, LRR-enriched proteins are highly expressed during development, showing diverse spatiotemporal expression patterns and roles in processes such as axon guidance, cellular migration and synapse formation (see de Wit et al., 2011; Schroeder and de Wit, 2018; for reviews). Here, we discuss the different LRR-dependent binding partners of FLRTs and the surprising variety of protein complexes that modulate several developmental processes.

FLRT

The first LRR-dependent interaction responsible for mediating cell adhesion and sorting was found through direct FLRT-FLRT homophilic binding (Karaulanov et al., 2006). Structural data suggests that FLRTs dimerize via the concave surface of their LRR domain (Seiradake et al., 2014; **Figure 1D**), which is the common protein interaction surface on LRR domains (Kobe and Kajava, 2001). Supporting this notion, a single mutation in the concave surface reduces FLRT-FLRT interaction, based on multiangle light scattering (MALS) and cellular aggregation assays (Seiradake et al., 2014). We thereby named this mutant protein FLRT^{FF} (no FLRT-FLRT binding). Interestingly, there are different packing arrangements in FLRT-FLRT structures that all use the LRR concave surface, suggesting that FLRTs could multimerize rather than just dimerize. Indeed, the full ecto- and LRR domains of FLRT3 can oligomerize in a concentration-dependent manner (Seiradake et al., 2014), which could enhance the rather low-affinity nature of this interaction,

as has been observed for other multimeric protein complexes (Hagner et al., 2018).

The homophilic *trans*-interaction between LRR domains could participate in different processes where FLRTs have been found to promote cell adhesion *in vivo* (**Figure 1E**). In the mouse, FLRTs are widely expressed in several tissues, except for FLRT1 that is restricted to the nervous system (Lacy et al., 1999). Embryos deficient for either FLRT2 or FLRT3 show lethality at earlier stages of development (around E10-E12) due to a wide range of malformations related to the formation and maintenance of tissue integrity, processes known to depend on cell adhesion mechanisms (Gumbiner, 1996). Absence of FLRT3 induces tissue disturbances that include headfold fusion and ventral closure defects leading to cardia bifida (Egea et al., 2008; Maretto et al., 2008), as well as disruptions to the basement membrane integrity of the anterior visceral endoderm (Egea et al., 2008), which is similar to those found in the basement membrane of the epicardium in FLRT2 mutant embryos (Müller et al., 2011). Interestingly, the phenotypes described in the absence of either FLRT2 or FLRT3 were found to be independent of FGF signaling (Egea et al., 2008; Maretto et al., 2008; Müller et al., 2011), suggesting that FLRTs mediate cell adhesion through other mechanisms or binding partners. Support for this idea comes from the finding that the FLRT LRR domain is dispensable for modulating FGF signaling (Böttcher et al., 2004), but essential for FLRT3-mediated cell sorting and aggregation (Karaulanov et al., 2006; Seiradake et al., 2014).

Unc5

A further indication of the complexity of FLRT function comes from studies in *Xenopus* where FLRT3 was shown to interact with the small GTPase Rnd1, thus promoting cellular de-adhesion via downregulation of the CAM C-Cadherin, and thereby causing detachment of migrating equatorial cells (Ogata et al., 2007). A follow-up study to identify novel partners of the *Xenopus* FLRT3 ectodomain using a mouse embryonic cDNA library, found that the Netrin Uncoordinated-5 (Unc5) receptors, Unc5B and Unc5D, interact with high affinity to the LRR domain of FLRT3 (Karaulanov et al., 2009). Unc5B also interacts with Rnd1 and its expression enhances the de-adhesion effects of FLRT3 and Rnd1, suggesting that Unc5B modulates FLRT3 adhesive properties (Karaulanov et al., 2009). Like FLRTs, Unc5 receptors are type-I transmembrane protein, but their ectodomain structure differs radically, with two Ig (1 and 2) and two thrombospondin-like (TSP1 and 2) domains, followed by a transmembrane and cytoplasmic tail that contains ZU5, UPA and a death domain (DD) (Wang et al., 2009).

Uncoordinated-5 receptors are best known for their role in axon guidance triggering repulsion in response to Netrin-1, mainly through heterodimerization with DCC between their cytoplasmic domains (Hong et al., 1999; Finci et al., 2014), but also binding to Down Syndrome Cell Adhesion Molecule (DSCAM) through their ectodomains (Purohit et al., 2012). In addition to their roles in axon guidance, Unc5 receptors act as dependence receptors for Netrins, inducing apoptosis after cleavage of their intracellular DD domain in the absence of a ligand (Llambi et al., 2001), and also inhibit sprouting angiogenesis in a Netrin-1 dependent manner (Larrivée et al., 2007). The strong link between Unc5 receptor function and Netrin, contrast with the finding that some phenotypes observed in Unc5 null mouse models, such as trochlear nerve misprojetions in Unc5C- (Burgess et al., 2006) or increased vascular branching in the retina of Unc5B-deficient mouse (Koch et al., 2011), are not observed in embryos lacking Netrin-1. These results, together with the finding that Netrin is not present in several Unc5-expressing tissues in the mouse, such as the developing cortex, suggests the presence of other interactors. Supporting this notion is the finding that some Unc5 receptors bind to *Xenopus* FLRT3 promoting cellular de-adhesion (Karaulanov et al., 2009), raising the possibility that similar interactions could provide guidance to migrating cells and/or pathfinding axons in other organisms.

The work of Yamagishi and coworkers provided the first evidence that FLRT/Unc5 signaling regulates both neuronal migration and axon guidance by triggering repulsion in the mouse (Yamagishi et al., 2011). The full ectodomain of all FLRTs is shed from neurons by an unknown metalloprotease that cleaves near the plasma membrane, and binds to all Unc5 (A-D) receptors, albeit with different affinities. Thus, Unc5A/B prefer FLRT1, Unc5D prefers FLRT2 and Unc5B has higher affinity for FLRT3 (Yamagishi et al., 2011; Seiradake et al., 2014). Structural data showed that their binding interface involves the convex surface of the LRR domain of FLRTs and the most N-terminal domain of Unc5 receptors (Ig1 domain) (**Figure 1D**). This was further confirmed in surface plasmon resonance (SPR) and cell-based assays that showed the lack of binding between mutant proteins targeting these interactions domains, thus named as $FLRT^{UF}$ and $Unc5^{UF}$ (no Unc5-FLRT binding) (Seiradake et al., 2014). The FLRT/Unc5 interaction is likely to occur *in trans* (**Figure 1E**) because of the long stretched nature of the entire Unc5 ectodomain, the *in vivo* diffusion of the shed FLRT ectodomains (Yamagishi et al., 2011), and the frequent non-overlapping expression between FLRTs and Unc5 receptors in different tissues such as the cortex, hippocampus (Yamagishi et al., 2011) and retina during development (Visser et al., 2015).

Our studies focusing on FLRT/Unc5 signaling have shown that FLRTs trigger repulsion and growth cone collapse to Unc5-expressing neurons (Yamagishi et al., 2011). This response is induced by the ectodomains of FLRT and $FLRT^{FF}$, but not $FLRT^{UF}$, suggesting that it depends on FLRT-Unc5 interactions (Seiradake et al., 2014). A similar result to that has been observed in classical axon guidance protein families where both partners act as receptors, such as Eph/ephrin. There are, however, important differences between these two systems. Although one study suggested that Unc5C can repel a subpopulation of retinal neurons expressing FLRT2 (Visser et al., 2015), there is so far no evidence for FLRT/Unc5 bidirectional signaling, where Eph/ephrin complex signaling acts upon both Eph- and ephrin-expressing cells (Kania and Klein, 2016). Moreover, co-expression of Ephs and ephrins within the same cellular membrane can result in *cis*-interaction that reduces the number of receptors available for functional interaction, known as "*cis* inhibition" (Egea and Klein, 2007). This is in contrast to the FLRT/Unc5 system where rostral TCA that express both FLRT3 and Unc5B

(Leyva-Díaz et al., 2014), do not show *cis* interaction, but rather parallel signaling where both FLRT3 and Unc5B at the cell surface can bind to exogenous FLRT3, and the adhesive FLRT interaction reduces the repulsive response triggered by FLRT-Unc5 interaction in a combinatorial way (Seiradake et al., 2014).

LATROPHILIN

Latrophilins (Lphn1-3) were first identified as receptors for α-latrotoxin, a black widow spider toxin that results in activation of exocytosis mechanisms causing massive release of neurotransmitters from synaptic terminals (Krasnoperov et al., 1997; Lelianova et al., 1997). Lphn receptors are members of the G protein-coupled receptors (GPCRs) superfamily (Sugita et al., 1998), the largest and most diverse group of mammalian transmembrane proteins (Heifetz et al., 2015). They remained orphan receptors for several years, despite its expression being largely restricted to the brain for Lphn1 and 3 (Ichtchenko et al., 1999), and a proposed role in synaptic function (Südhof, 2001). All Lphns (1-3) show a similar structure comprising a large ectodomain (around 1000 amino acids) with lectin (Lec), olfactomedin (Olf), hormone receptor and GAIN domains, followed by the common feature of all GPCRs – the seven-pass transmembrane domain and the ICD (Lelianova et al., 1997; Sugita et al., 1998).

Fibronectin leucine-rich repeat transmembrane proteins were found to be endogenous ligands for Latrophilins by affinity chromatography coupled with mass spectrometry, using the ectodomain of Lphn3 fused to FC as a bait to identify binding partners from synaptosome extracts (O'Sullivan et al., 2012). All FLRTs were found to bind Lphn1 and 3 in *trans* through their ectodomains, and localized in hippocampal neurons to glutamatergic synapses. Disruption of FLRT3-Lphn3 binding by competition using their ectodomains or by knocking down either Lphn3 or FLRT3 reduced the density of glutamatergic synapses *in vitro* and *in vivo*, suggesting a role in synapse formation or maintenance as heterophilic CAMs (O'Sullivan et al., 2012). A follow-up study found that the Lphn3 Olf domain is required for this synapse-promoting function, as well as for FLRT3 binding (O'Sullivan et al., 2014). Structural data confirmed that the Lphn Olf domain interacts with the concave surface of the FLRT LRR domain (Lu et al., 2015; Ranaivoson et al., 2015), previously known to mediate homophilic FLRT binding, as observed by the lack of binding of $FLRT^{UF}$ mutants to Lphns by SPR and cell binding assays (Jackson et al., 2015; Lu et al., 2015; **Figure 1D**). Stripe assays showed that Lphn3 promotes adhesion of HeLa cells expressing FLRT2, which supports the proposed role of Lphn3 in promoting synapse development (O'Sullivan et al., 2012). Surprisingly, the same experimental approach revealed that Lphn3 induces repulsion of cortical neurons that endogenously express FLRTs (Jackson et al., 2015). This repulsive effect depends on the binding of Lphn3 to FLRTs, since the non-FLRT-binding mutant, $Lphn3^{LT}$, was unable to elicit repulsion. Therefore, this result could reflect the ability of FLRTs in *cis* recruitment of other receptors with repulsive activity, such as Robo1 (Leyva-Díaz et al., 2014) or Unc5 (Yamagishi et al., 2011) upon Latrophilin binding. Indeed, the Lphn3/FLRT3 structure showed that Lphn3 binds FLRT3 at a surface distinct from Unc5, and cell binding assays suggested that Latrophilin and Unc5 could simultaneously bind to FLRT3 (Lu et al., 2015).

Jackson and coworkers obtained the structure of the ternary complex Lphn/FLRT/Unc5 formed by their ectodomains (Lec-Olf/LRR/Ig1), revealing a stoichiometry of 1:1:2 (FLRT2:Unc5D:Lphn3) (Jackson et al., 2016; **Figure 1E**). Stripe assays showed that Lphn3 does not induce adhesion in HeLa cells expressing Unc5D and FLRT2, which contrasts to the strong adhesive response found in cells expressing FLRT2 alone or Unc5D with $FLRT2^{UF}$ mutant that binds Lphn3 but not Unc5 (Jackson et al., 2016). These results suggest that Unc5D acts as a switch modulating the adhesive properties of FLRT2-Lphn3 interaction, resembling the finding that Unc5B regulates FLRT3 adhesive properties (Karaulanov et al., 2009). Interestingly, a complex comprised of FLRT2 LRR, Lphn3 Lec-Olf domains, and a larger ectodomain of Unc5D (Ig1Ig2TSP1), leads to the formation of an octamer through dimerization of the tetramer described above, and was the first example of a super-complex formed by three receptors involved in cell guidance (Jackson et al., 2016; **Figure 1E**). This structure showed a new binding interface between the Unc5D TSP1 and the convex side of FLR2 LRR domain, close to the binding site for Unc5D Ig1 domain. Although the function of the octamer is unclear, it could promote cell adhesion in different scenarios, such as maintenance of synaptic connections, similar to the finding that large protein complexes stabilize cellular contacts with the ECM (Wu, 2007).

Other known Latrophilin ligands involved in cellular adhesion include members of the Neurexin (Boucard et al., 2012) and Teneurin (Silva et al., 2011) protein family. Teneurins (Ten 1-4) are type-II single-pass transmembrane receptor proteins, strongly enriched in the nervous system where they play a role in synapse organization, neuronal migration and axon guidance (see Jackson et al., 2019 for a recent review). These diverse functions are thought to reflect their interactions with different binding partners. Indeed, Teneurins are characterized by a long modular C-terminal extracellular region that contains at least 16 domains. Some of the domains are involved in adhesive Teneurin homophilic interaction, such as the NHL domain (Berns et al., 2018), and others promote heterophilic binding, such as the *trans*-synaptic adhesion by engaging in *trans* interaction with Latrophilin (Silva et al., 2011; Boucard et al., 2014). This large ectodomain is followed by a transmembrane region and an N-terminal ICD. All Teneurins are localized at the cell surface and form *cis*-dimers through a covalent disulfide-link close to the plasma membrane (Feng et al., 2002).

Latrophilin was found to interact with FLRTs and Teneurins through two distinct domains: its Olf domain binds FLRT, while the Lec domain is mostly involved in the interaction with Teneurin (Boucard et al., 2014; O'Sullivan et al., 2014). This finding indicated that Latrophilins could interact simultaneously with FLRTs and Teneurins, similar to how FLRTs form a complex with Latrophilin and Unc5 receptors through distinct surfaces (Jackson et al., 2016). Consistent with this idea, it was found that Teneurin and FLRT located on the pre-synaptic

site interact with the post-synaptic Latrophilin in *trans*. This coincident binding was necessary to induce synapse formation in hippocampal neurons *in vivo* (Sando et al., 2019). Recently, the structure of Latrophilin-Teneurin interaction has been solved, revealing that the Latrophilin Lec, and to a lesser extend Olf domain, bind across a spiraling beta-barrel domain of Teneurin, the YD shell (del Toro et al., 2020; **Figure 1D**). Structural superposition showed that the Latrophilin Olf domain can interact simultaneously with the Teneurin YD shell domain and the concave surface of the FLRT LRR domain (**Figure 1E**), thus suggesting the possibility of the formation of a Ten/Lphn/FLRT ternary complex. Evidence for physical interaction between these three proteins was found in a subset of embryonic cortical neurons expressing FLRT3 and Ten2, where both receptors show coincident binding to externally presented Latrophilin (del Toro et al., 2020). Strikingly, Latrophilin binding to Teneurins and FLRTs is repulsive for cortical neurons but not for their axons (del Toro et al., 2020), which contrasts with the adhesive/attractive nature of this interaction involved in synapse formation (Sando et al., 2019). Such a dual role in repulsive cell guidance and adhesive synaptogenesis has also been observed in the Eph/ephrin protein family (Kania and Klein, 2016; Henderson and Dalva, 2018), but the underlying mechanisms remain poorly understood.

CORTICAL MIGRATION

Neuronal migration is a tightly regulated and coordinated process that is essential for cortex development. During this phase, pyramidal neurons have to translate extracellular signals coming from substrates (neighboring neurons, ECM, radial glia fibers) and guidance cues into cytoskeletal arrangements and signal transduction modifications to follow their proper migratory route. This process is fundamental to establish the different cortical layers and appropriate cellular distribution, and thus alterations can lead to several types of cortical malformations including cortical heterotopias (subcortical and periventricular) and abnormal folding, such as lissencephaly, in humans (see Buchsbaum and Cappello, 2019; Subramanian et al., 2019 for recent reviews), that have been associated with several neuropsychiatric disorders such as schizophrenia and autism spectrum disorders (Fukuda and Yanagi, 2017; Guarnieri et al., 2018). Finally, recent studies in mouse and ferret models have demostrated the impact of neuronal migration on cortical folding. Genetic loss of doublecortin, a microtubule-stabilizing protein regulating radial migration, shows a lissencephalic phenotype in ferrets similar to human patients carrying mutations in this gene (Kou et al., 2015). Local knockdown of Cdk5 in upper cortical neurons, which modulates neuron migration and is mutated in some patients with lissencephaly, impairs radial migration and thereby affects the formation of folds in the ferret cortex (Shinmyo et al., 2017). In the mouse, genetic knockdown of genes modulating tangential dispersion of neurons such as EphAs/ephrinAs (Torii et al., 2009) and FLRTs (del Toro et al., 2017) results in cortical regions with neuronal segregation and heterogeneity inducing an uneven CP with alternating thicker and thinner areas, and in some cases can result in sulcus formation (del Toro et al., 2017).

RADIAL MIGRATION

Newborn pyramidal neurons are initially multipolar while moving radially from the SVZ and through the intermediate zone (IZ) to reach the CP. This initial phase of radial migration was initially described by Tabata and Nakajima (2003) and Tabata et al. (2009) after observing abundant migrating neurons populating the SVZ and lower portion of the IZ, with short processes mainly in the tangential axis without a defined polarity, and thus it was referred as multipolar migration. When moving, one of the thin processes of multipolar neurons elongates and gets thicker, becoming the so-called leading process that anticipates the direction of the movement, which can occur along both the radial and tangential axes and apparently seems independent of radial glial (RG) fibers. The elongation of the leading process is followed by nuclear translocation and retracement of the trail process, completing the locomotion movement (Marín et al., 2010). Multipolar neurons, characterized by random and low speed (1–3 μm/h) movements along the radial axis, transition to a bipolar morphology in the upper portion of IZ close to the subplate (SP), acquiring a fiber-guide locomotion mode characterized by fast migration speeds (9–12 μm/h) and strict radial orientation that causes their displacement to the CP (Tabata and Nakajima, 2003; Noctor et al., 2004). A recent study has shown that SP neurons facilitate such transition by inducing transient glutamatergic synaptic transmission to multipolar neurons that activates the calcium-dependent signaling required to modify their polarity and migration mode (Ohtaka-Maruyama et al., 2018; **Figure 2A**). Bipolar neurons entering the CP will migrate over earlier-born neurons residing in deeper layers to form superficial layers. The proteolytic processing of Reelin, a glycoprotein secreted mainly from Cajal–Retzius cells in the marginal zone (MZ), allows the formation of a gradient through the CP (Jossin et al., 2007). This gradient plays a critical role in the last steps of radial migration, directing the formation of cortical layers in an inside-out fashion, and acting as a stop signal to induce terminal translocation of migrating neurons beneath the MZ (see Hirota and Nakajima, 2017 for a detailed review).

Several systems such as cytoskeletal regulators, transcription factors and ECM molecules, have been identified to regulate radial migration and multipolar to bipolar transition. Due to diversity, and for further reading, we suggest reviews on this topic (Cooper, 2014; Ohtaka-Maruyama and Okado, 2015; Silva et al., 2019).

FLRTS REGULATE RADIAL MIGRATION BY REPULSION

The complexity of radial migration, where neurons transition through different phases and acquire a polarized structure with an established leading process that directs their movement, remarkably parallels the process of axon guidance, where

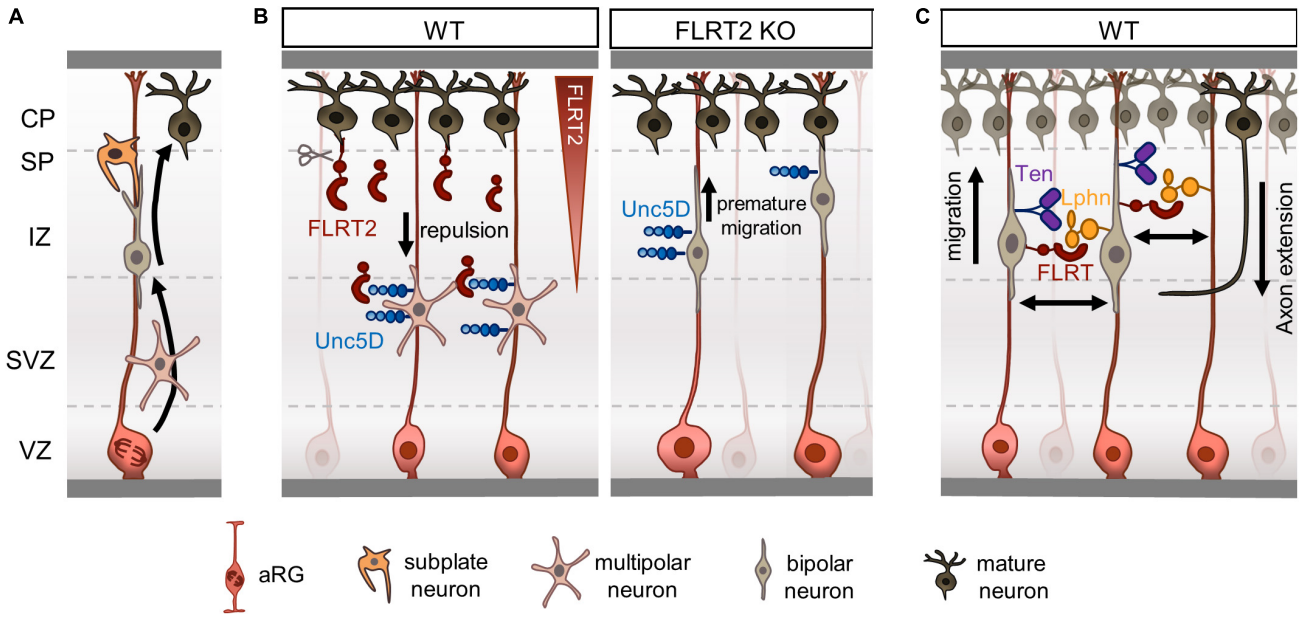

FIGURE 2 | Fibronectin leucine-rich repeat transmembrane proteins (FLRTs) regulate radial migration by repulsion. **(A)** Overview of radial migration. Newborn neurons transition from multipolar to bipolar morphology during their migration to the CP. Subplate neurons facilitate the displacement of bipolar neurons to the CP by direct contacts. **(B)** FLRT2-Unc5D contacts regulate radial migration of neurons by a repulsive mechanism. FLRT2 knockout causes premature migration of Unc5D+ neurons toward the CP. **(C)** Schematic depicting a model where FLRT and Teneurin bind to Latrophilin in *trans* on opposing RG fibers or neurons.

immature neurons with short neurites designed to integrate both extrinsic cues and intrinsic mechanisms induce polarization (dendrites/axon) and axon path-finding. Indeed, several members of the four major classes of axon guidance cues (Eph/ephrins, Semaphorins, Netrins, and Slits) have been shown to participate in either cortical multipolar/bipolar migration or polarity by adhesion/attraction and repulsion mechanisms. Similarly, FLRTs modulate axon pathfinding and radial migration by triggering repulsion.

FLRT-UNC5: LONG-RANGE REPULSIVE INTERACTION

Both DCC and Unc5D are the only known Netrin receptors expressed by pyramidal neurons in the IZ that participate in their migration. Overexpression of DCC delays neuron migration in the IZ, an effect that can be rescued by increasing Unc5D levels, highlighting the need for a balance between levels of both receptors at the cell surface (Miyoshi and Fishell, 2012). Interestingly, Netrin-1 is not expressed in the developing cortex (Braisted et al., 2000), suggesting that other ligands could participate in its place. Unc5D is expressed in a subpopulation of pyramidal neurons that is marked by the expression of the non-coding *Svet1* RNA (Tarabykin et al., 2001). Indeed, *Svet1* RNA was found to be encoded by an intronic region of the unspliced RNA of Unc5D, and thereby a subset of multipolar neurons in the SVZ and IZ express both *Svet1* and Unc5D (Sasaki et al., 2008). Unc5D/*Svet1*-expressing neurons, born around E13.5 in the mouse, reside in the SVZ for an extended period before starting their migration. They begin entering the CP at E18.5 and finish their migration at P2 (Tarabykin et al., 2001). This migration to the CP is slower than other subpopulations born at the same time or even later. Unc5D/*Svet1*-neurons even reach the CP after Stab2-expressing neurons, which are born at around E14-E15, do not remain in the SVZ, and are present in the CP as early as E15.5 (Britanova et al., 2008).

Fibronectin leucine-rich repeat transmembrane protein 2, the main binding partner of Unc5D, is highly expressed in pyramidal neurons located in CP at E15.5. However, the ectodomain of FLRT2 is shed by an unknown metalloprotease and diffuses through the IZ to reach the SVZ where Unc5D/*Svet1* multipolar neurons linger (Yamagishi et al., 2011). Knockdown of FLRT2 accelerates the radial migration of Svet1-expressing neurons, while lack of Unc5D broadens the distribution of Tbr2-expressing cells, which also include Unc5D/Svet1-expressing neurons, toward the CP (Yamagishi et al., 2011; **Figure 2B**). These results are consistent with FLRT2 acting as repulsive cue for Unc5D-migrating neurons and suggests that both receptors participate in the delayed migration of a subset of pyramidal neurons. A follow-up study corroborated this finding by performing *in vivo* gain-of-function experiments using structure-based Unc5D proteins. Unc5D overexpression by *in utero*-electroporation (IUE) in pyramidal neurons born at E13.5 delayed their migration. This effect was partially rescued when expressing Unc5D^{UF} (Seiradake et al., 2014), supporting the notion that FLRTs participate in the radial migration of Unc5D/*Svet1* pyramidal neurons as repulsive cues.

The finding that FLRT2 acts as a diffusible guidance cue regulating radial migration is not without a precedent, and has

been observed in other axon guidance protein families such as Semaphorins. Semaphorin 3A is a classical chemorepellent that regulates axon guidance by inducing growth cone collapse and turning by binding its co-receptors, Neuropilins, and Plexins (Manns et al., 2012; Koncina et al., 2013). During cortical development, Semaphorin 3A is highly expressed in the upper CP where it is secreted forming a gradient that attracts upper layer neurons, and thus promotes radial migration and proper orientation of the leading process of bipolar neurons toward the CP (Chen et al., 2008). Both co-receptors, Neuropilins and Plexins participate in radial migration. Acute knockdown of either Neuropilin-1 or PlexinA3, A4, and D1, which mediate Semaphorin 3A signaling, impairs radial migration (Chen et al., 2008). In addition, silencing of PlexinB2, but not B1, also impairs radial migration by altering RhoA activity that controls cytoskeleton dynamics in migrating neurons (Azzarelli et al., 2014).

FLRT-LATROPHILIN-TENEURIN: CONTACT-REPULSION INTERACTION

We recently showed that Latrophilins and Teneurins, known to promote synapse formation, are expressed in the cortex earlier in development, where they function in a complex with FLRTs to regulate radial migration by a contact-repulsion model (del Toro et al., 2020; **Figure 2C**). Like FLRTs, Teneurins are mainly expressed in pyramidal neurons in the mouse IZ and CP at E15.5, whereas Latrophilins show wider expression including the VZ, where apical RG cells show strong enrichment for Lphn1 and 2 (del Toro et al., 2020). A subset of cortical migrating neurons co-express FLRT3 and Ten2 that bind Latrophilins in *trans* on opposing RG cells or neurons, resembling the configuration proposed for their synaptogenic function (Sando et al., 2019). Stripe assays showed that Lphn1 induces repulsion of cortical neurons but not their axons. A double mutant Lphn1 that cannot bind FLRT and Teneurin, named Lphn1^{TL-FL} (no Ten-Lphn and FLRT-Lphn binding) did not elicit any response. The use of nanofibers mimicking RG fibers allowed the study of Lphn1 function in the context of neuron-RG fiber interaction. In these experiments, cortical neurons were found to migrate slower on nanofibers coated with Lphn1. This impairment was strongly reduced by using the double mutant Lphn1^{TL-FL}. These results suggests that Lphn1 triggers repulsion through an additive or coincident interaction with Teneurins and FLRTs, which in turn affects neuronal migration (del Toro et al., 2020). Supporting the notion that Latrophilins act as a repulsive ligand during development, a recent study showed that Lphn2 repels Ten3-expressing hippocampal axons during target selection (Pederick et al., 2020). Lphn2 and Ten3 show complementary expression in the lateral hippocampal network, and knockdown of Lphn2 in the proximal subiculum results in an ectopic invasion of Ten3-expressing axons (Pederick et al., 2020).

Surprisingly, although FLRT3 and Ten2 show uniform distribution on the cell surface of cortical neurons, axons showed no response toward Lphn1 (del Toro et al., 2020). One possible explanation could be differences in the downstream signaling between the somatodendritic and axonal compartments. Similar results have been shown for Semaphorin 3A that facilitates the polarization of upper pyramidal neurons by attracting their apical dendrite toward the marginal zone (Polleux et al., 2000), while, through a repulsion response, it directs the growth of their axons toward the white matter (Polleux et al., 1998). Moreover, the highly polarized structure of migrating neurons could also contribute to the contrasting response between axons and dendrites. The leading process of migrating neurons preferentially interacts with the RG fibers (Elias et al., 2007). These increased contacts induces the polarized distribution of RhoA to the leading process, and Rac1 to the trail process that will become the axon (Xu et al., 2015).

In vivo overexpression or knockdown of Ten2 in cortical neurons delays their migration toward the CP. This effect is not observed when overexpressing the Ten2 mutant defective in Lphn binding, named Ten2^{LT} (no Lphn-Ten binding), suggesting that this response depends on Latrophilin interaction (del Toro et al., 2020). In support of this, disruption of endogenous interactions by competition using a secreted portion of the ectodomain of Lphn1 that binds FLRTs and Teneurins, but not its double mutant Lphn1^{TL-FL}, also delays cortical migration (del Toro et al., 2020). The delayed migration observed when tampering with Ten2 levels on migrating neurons is reminiscent of other molecules regulating cortical migration, such as Rnd2 (Heng et al., 2008). In addition, FLRT loss- and gain-of-function experiments disturbs cortical migration (Seiradake et al., 2014). These studies suggest that excessive or reduced levels of proteins involved in adhesion or repulsion can be detrimental to cell migration. Indeed, the speed of cell migration can be reduced by modulating either adhesion or repulsion. Increased integrin-mediated cell-ECM adhesion (Haage et al., 2020), or reducing EphB-ephrinB contact repulsion reduces cell motility (Rohani et al., 2011). Conversely, increasing EphB-ephrinB repulsion induces cell detachment (Wen and Winklbauer, 2017), also affecting migration. Previous studies have identified different molecules promoting adhesion of migrating neurons to RG fibers such as connexin26/43 (Elias et al., 2007), focal adhesion kinase (Valiente et al., 2011), and N-cadherin (Shikanai et al., 2011). The molecules that mediate repulsion between neurons and RG fibers are not known, and thus Latrophilins are promising candidates to participate in this process.

Other interactions between Latrophilin, Teneurin, and FLRTs are conceivable. Migrating neurons expressing FLRTs and Teneurins could bind Latrophilins in *trans* on opposing RG fibers or neurons. Although it is possible that a subset of migrating neurons could express all three proteins, our results show that Lphn1 in *cis* does not abolish Ten2 or FLRT3 binding to exogenous Lphn1 (del Toro et al., 2020), which is similar to the finding that co-expression of FLRT3 and Unc5B do not induce *cis*-inhibition (Seiradake et al., 2014). Among all Teneurins, Ten4 showed mild expression in RG cells compared with Latrophilins (del Toro et al., 2020), which opens the possibility for an interaction in *trans* with Latrophilins on migrating neurons. Future studies using cell-specific manipulation of these proteins will help to elucidate

the different context-dependent complexes that form as neurons migrate through their intricate environment.

TANGENTIAL DISTRIBUTION

There is a growing body of evidence showing substantial differences in cortical migration between lissencephalic and gyrencephalic species. Cell lineage analysis in clonal fashion of cortical progenitors have shown a striking diversity of migratory patterns during development. In rats, retroviral-labeling of progenitors at middle stages of development (E15-E16) produced neuronal clones that on average contained four cells spread along 250 μm (Luskin et al., 1993; Mione et al., 1994). In contrast, a similar approach in the ferret showed that neuronal clones labeled at middle-late neurogenesis (E33-35) contained large numbers of neurons with little tendency to cluster that can disperse several millimeters (from 1 mm up to 20 mm) in both the rostro-caudal and medio-lateral axes. These clonal-related neurons were found in different cortical regions such as the prefrontal, motor, somatosensory and visual areas, indicating that these cells are capable to disperse over large distances while acquiring different fates in functionally distinct cortical areas (Reid et al., 1997; Ware et al., 1999). In agreement with these findings, time-lapse experiments have shown that in the mouse, pyramidal neurons mostly migrate radially along a single parent RG fiber with little tangential spread (Noctor et al., 2001), whereas in folded brains like the ferret, migrating neurons do not follow strict radial pathways and instead disperse in the lateral axis leading to more convoluted migration routes concomitant with the start of cortical folding (Gertz and Kriegstein, 2015; **Figure 3A**).

Neurons use RG fibers as guides for migration and therefore changes in the radial fiber scaffold may contribute to neuronal migration differences between lissencephalic and gyrencephalic species. Indeed, gyrencephalic cortices show large numbers of basal RG cells, which account for the "fan-like" divergence of radial fibers, and therefore could facilitate lateral dispersion of migrating neurons (Reillo et al., 2011). Moreover, a recent study has shown that most of the bipolar neurons exhibit a branched leading process in the ferret, and to a lesser extent in the mouse. These branched processes are less parallel to radial fibers, do not affect radial migration, and importantly, seem to be involved in the remarkable lateral dispersion that occurs in folded cortices (Martínez-Martínez et al., 2019). Indeed, both the dynamic branching and filopodia formation that are observed in the leading process are similar features to those found in neurons exploring their environment during axon guidance (Dent et al., 2011). Together, these studies indicate that migrating neurons in folded cortices have increased cellular dynamics, exploratory behavior and lateral dispersion when compared to those in the rodent brain, but the nature of this mechanism remains largely unknown. Two axon guidance family proteins involved in cell adhesion and repulsion, Ephs/ephrins (Torii et al., 2009; Dimidschstein et al., 2013) and FLRTs (Seiradake et al., 2014; del Toro et al., 2017), have been shown to modulate tangential dispersion of migrating neurons in mouse cortices by adhesive mechanisms. Future work will be required to confirm whether changes in the adhesive properties of neurons allows them to acquire wide migratory profiles and tangential spread.

FLRTs MODULATE TANGENTIAL DISPERSION BY ADHESION

The pattern of expression of FLRT3/Unc5B in the mouse cortex at E15.5 is complementary to that of FLRT2/Unc5D, with FLRT3 expressed in migrating neurons in the IZ and Unc5B in the CP. The first insight into FLRT3 function in the cortex came from gain-of-function studies using structure-based FLRT3 proteins. *In vivo* overexpression of FLRT3 or $FLRT3^{UF}$ delays neuron migration and alters their tangential distribution, forming a repeating pattern of aggregates in the IZ (Seiradake et al., 2014; **Figure 3B**). Conversely, overexpression of $FLRT3^{FF}$ partially rescued the delayed migration induced by FLRT3 or $FLRT3^{UF}$, and preserved the regular and homogeneous distribution of migrating neurons in the tangential axis. These results indicate that binding of FLRT3 to other ligands through the concave site of its LRR domain directs tangential distribution. One possible interaction could be FLRT3-FLRT3 homophilic binding, which induces *in vitro* cell aggregation and sorting (Karaulanov et al., 2006; Seiradake et al., 2014). The increased adhesion between overexpressing neurons could cause those cells to aggregate, and thereby result in delayed migration and segregation from surrounding cells. Indeed, ephrinB1 overexpression, which can induce cell homoadhesion (Batlle and Wilkinson, 2012), reduces the horizontal dispersion of multipolar neurons (Dimidschstein et al., 2013). Likewise, EphA/ephrinA gain-of-function experiments show reduced lateral dispersion of multipolar neurons and increased aggregation that alters the proper mixing of pyramidal neurons found in the cortical columns (Torii et al., 2009).

Several studies in CAMs that mediate homophilic binding, support a role for differential adhesion in cell segregation, such as cadherins (Nose et al., 1988; Steinberg and Takeichi, 1994). Interestingly, in *Xenopus*, FLRT3 has been shown to regulate C-cadherin surface expression by binding Rnd proteins through its ICD (Ogata et al., 2007; Karaulanov et al., 2009). A later study found that FLRT3 forms a complex with Paraxial protocadherin and C-cadherin regulating cell adhesion and sorting (Chen et al., 2009). There are therefore other possibilities, such as FLRT3 regulating surface expression and function of N-cadherin that participates in cortical migration (Kawauchi et al., 2010), or Rnd activity, which is known to regulate cell adhesion to the ECM (Guasch et al., 1998; Nobes et al., 1998), as well as radial migration in the developing cortex (Heng et al., 2008; Pacary et al., 2011; Azzarelli et al., 2014). N-cadherin controls cell migration either by regulating actin-myosin contractile forces (Shih and Yamada, 2012) or modulating FGFR-dependent signaling (Nguyen et al., 2019). During cortical migration, N-cadherin interacts in *cis* with FGFR1-3 at the cell surface of multipolar neurons, preventing their degradation. Thus, FGFRs accumulate and enhance their signal to the ERK pathway that is required for proper multipolar neuron migration and transition to bipolar cells (Kon et al., 2019). Given that N-cadherin has been shown to interact with

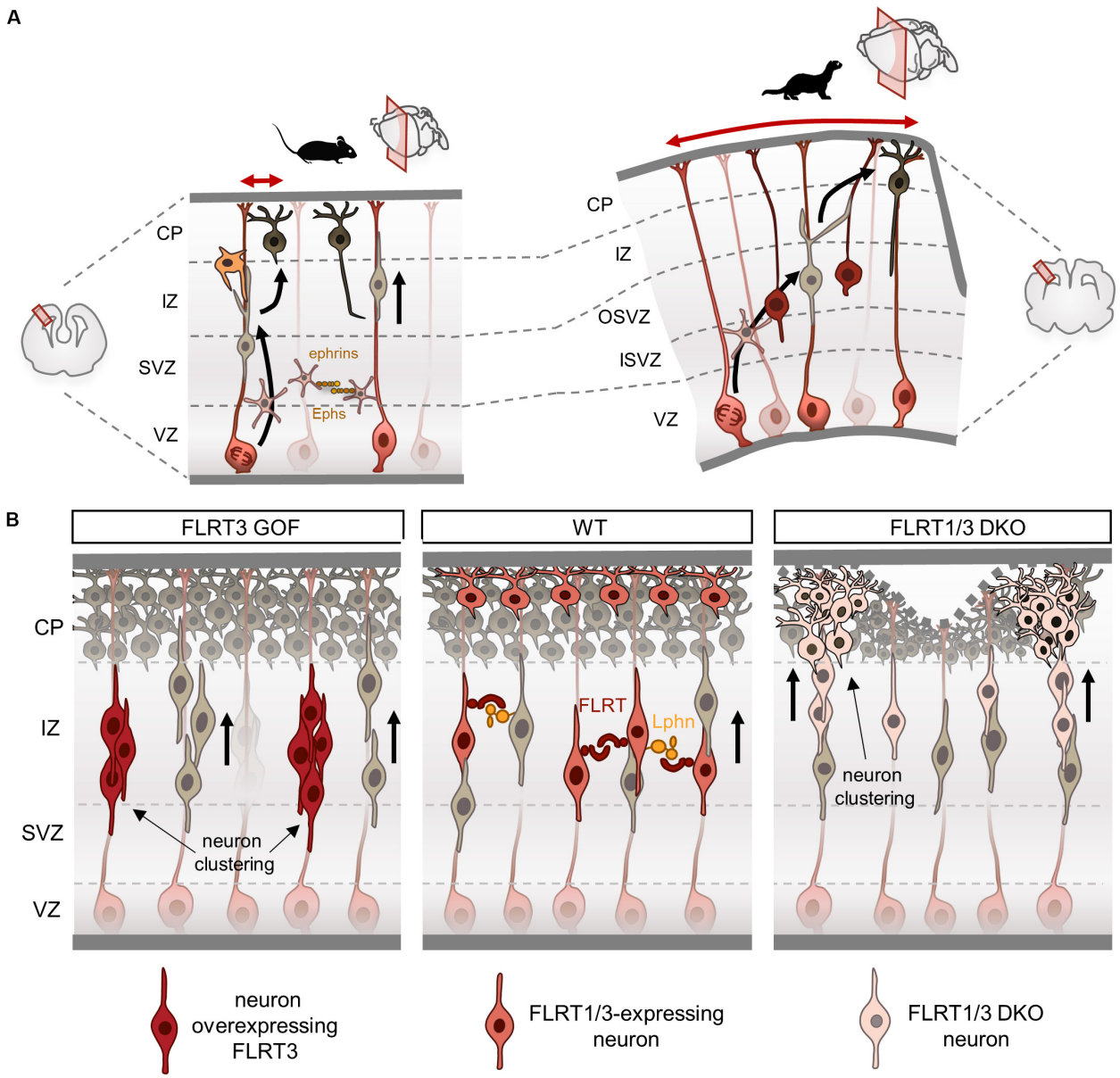

FIGURE 3 | Fibronectin leucine-rich repeat transmembrane proteins (FLRTs) modulate tangential dispersion by adhesion. **(A)** Scheme illustrating tangential dispersion in the lissencephalic mouse and the gyrencephalic ferret during cortical development. In the mouse, pyramidal neurons mostly migrate radially along a single parent RG. Conversely, neurons in the ferret do not follow strict radial pathways and instead disperse in the lateral axis. **(B)** Current model of tangential dispersion modulated by FLRTs. In the wild-type (WT), FLRT1/3+ neurons show homogeneous distribution while migrating to the CP at E15.5. FLRT3 overexpression induces cell clustering in the IZ and delays migration. Loss of FLRT1/3 results in faster migration and clustering in the lower and upper CP. Loss of adhesion may alter tissue elasticity inducing a wavy upper surface of the CP, which can lead to sulcus formation.

the first two Ig domains of FGFR1 (Suyama et al., 2002), it is possible that both FLRT3 and N-cadherin compete for FGFR binding. Thus, the delayed neuron migration observed after FLRT3 overexpression could be due, in part, to altered regulation of N-cadherin-FGFR-dependent signaling. Finally, the recent finding that Latrophilins are expressed in the cortex in both neurons and RG fibers (del Toro et al., 2020), suggests the possible involvement of FLRT3-Lphn interaction directing the lateral distribution of migrating neurons.

Altered tangential distribution is also observed when FLRT3 expression is ablated in migrating neurons (Seiradake et al., 2014). Neurons lacking FLRT3 show abnormal cell clustering in the lateral portion of the cortex within the lower CP. Interestingly, FLRT3-expressing neurons also express FLRT1, which shares similar features in terms of homophilic adhesion and heterophilic binding to Unc5 and Latrophilins (Yamagishi et al., 2011; Seiradake et al., 2014; del Toro et al., 2020). Double deletion of FLRT1 and FLRT3 enhances the clustering effect

observed in FLRT3 mutants, extending into medial and caudal regions of the cortex following a repeated pattern (del Toro et al., 2017; **Figure 3B**). Neurons deficient for FLRT1 and FLRT3 migrate faster and also segregate into clusters in the lower CP that extend into the upper CP as they migrate. This heterogeneity results in a wavy surface of the upper CP that can lead to sulcus formation (del Toro et al., 2017). FLRT-expressing cortical neurons aggregate *in vitro*, but not those deficient for FLRT1 and FLRT3 (del Toro et al., 2017), indicating that the effects of FLRT1/3 ablation *in vivo* are likely non-cell autonomous and may be the result of repulsive interactions with surrounding cells. A similar scenario is seen in the EphB2-ephrinB1 dependent repulsion, where EphB2 cells show increase migration speed during heterotypic repulsion and segregate from those expressing ephrinB1 (Taylor et al., 2017).

The tangential clustering and uneven CP found in FLRT1/3 ablated cortices resembles the phenotype seen in the ephrinA2/A3/A5 mutants, where neuronal segregation in the tangential axis leads to a CP with alternating thicker and thinner areas (Torii et al., 2009). In both mouse models, cell proliferation is not affected and therefore suggests that mechanical factors could influence the morphology of the CP. Supporting this notion, a recent study has shown that manipulation of the ECM can induce folding of the CP by modifying ECM stiffness (Long et al., 2018). Sulcus regions tend to have lower stiffness compared to gyrus areas, suggesting that modulation of local tissue stiffness could participate in the induction of folds in the cortex (Long et al., 2018). Therefore, the segregation and reduced intercellular adhesion of FLRT1/3 ablated neurons could contribute to forming a CP with non-homogeneous tissue elasticity, which in turn favors sulcus formation. Indeed, both FLRT1 and FLRT3 are less abundant in the cortical area that will form the lateral sulcus compared with the splenial gyrus in the ferret (del Toro et al., 2017). The finding that genetic mouse models that alter the morphology of the CP through altered tangential dispersion target at least two genes, such as FLRT1/3 (del Toro et al., 2017) or ephrinA2/A3/A5 (Torii et al., 2009) mutants, suggests the presence of redundant mechanisms that regulate the delicate balance of adhesion/repulsion required for cell migration (Solecki, 2012; Cooper, 2013).

CONCLUDING REMARKS

During the last decade, FLRTs have been found to interact with different ligands governing a wide-repertoire of biological functions such as axon guidance, cell migration and synapse formation. Structural data has revealed a rich variety of protein complexes formed by FLRTs and its binding partners, which modulate the finely tune adhesive and repulsive cellular responses required for nervous system development. The combination of structural biology with cellular assays and the use of conditional knockout mouse models has shed light on how FLRT proteins are mechanistically involved in such a wide range of developmental processes.

The functions of FLRT proteins are best understood in the context of cortical migration during brain development. FLRTs participate in radial migration through at least two distinct mechanisms. FLRT2 acts as a long-range cue, where its ectodomain is shed from the CP triggering repulsion of Unc5-expressing neurons in the SVZ (Yamagishi et al., 2011). FLRT3 and Ten2 act in close contact with another neuron or RG fiber expressing Latrophilin, regulating neural migration by repulsion (del Toro et al., 2020). Conversely, FLRT proteins modulate tangential dispersion by adhesion, where homophilic and perhaps heterophilic interactions with Latrophilin are involved (Seiradake et al., 2014).

These studies elegantly illustrate the full strength of structure-function studies, and how structure-based analysis of mutant proteins can overcome that challenging nature of dissecting the *in vivo* functionality of specific protein complexes. Similar approaches can be used to investigate further interactions, such as the supercomplexes formed by Unc5, FLRT and Latrophilins (Jackson et al., 2016) and the possible role of Unc5 receptors in the context of FLRT-Latrophilin-Teneurin complex.

AUTHOR CONTRIBUTIONS

Both authors wrote and edited the manuscript.

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REVIEW
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How Do Electric Fields Coordinate Neuronal Migration and Maturation in the Developing Cortex?

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During development the vast majority of cells that will later compose the mature cerebral cortex undergo extensive migration to reach their final position. In addition to intrinsically distinct migratory behaviors, cells encounter and respond to vastly different microenvironments. These range from axonal tracts to cell-dense matrices, electrically active regions and extracellular matrix components, which may all change overtime. Furthermore, migrating neurons themselves not only adapt to their microenvironment but also modify the local niche through cell-cell contacts, secreted factors and ions. In the radial dimension, the developing cortex is roughly divided into dense progenitor and cortical plate territories, and a less crowded intermediate zone. The cortical plate is bordered by the subplate and the marginal zone, which are populated by neurons with high electrical activity and characterized by sophisticated neuritic ramifications. Neuronal migration is influenced by these boundaries resulting in dramatic changes in migratory behaviors as well as morphology and electrical activity. Modifications in the levels of any of these parameters can lead to alterations and even arrest of migration. Recent work indicates that morphology and electrical activity of migrating neuron are interconnected and the aim of this review is to explore the extent of this connection. We will discuss on one hand how the response of migrating neurons is altered upon modification of their intrinsic electrical properties and whether, on the other hand, the electrical properties of the cellular environment can modify the morphology and electrical activity of migrating cortical neurons.

Keywords: cerebral cortex, development, electric field, neuronal migration, dendritogenesis

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INTRODUCTION

Construction of the nervous system is achieved through a complex succession of developmental processes. Among them, two are known to predominantly occur at different developmental stages in the cerebral cortex, cell migration largely embryonically and synaptogenesis postnatally.

Research on chemical cues and adhesion molecules guiding neuronal migration, shaping tissue architecture and synapse formation has shed light on the molecular mechanisms underlying migration as well as morphogenesis of dendrites and spines and have been extensively reviewed (Solecki, 2012; Arai and Pierani, 2014; Barber and Pierani, 2016; Ledda and Paratcha, 2017; Chighizola et al., 2019; Lanoue and Cooper, 2019). Besides molecular mechanisms, electric field (EF) is another factor which has been shown to define the morphology and specification of whole tissues and can in certain cases outplay chemical guidance (McCaig et al., 2009; Levin et al., 2017). The field of cortical development is starting to recognize the importance of ion flow for regulation of

early neuronal development: proliferation, migration and differentiation. While nowadays EF is an emerging player in guiding orientation and speed of migrating neurons and in regulating neuronal morphology, it remains to be determined whether and how neuronal migration and morphology establishment are linked.

EFs naturally occur in tissues as a consequence of polarized ion transport inside and outside the cells. Numerous examples of cellular and tissue behavior controlled by bioelectric states are described in amphibians and worms, where altered morphogenesis of whole organs and body parts can occur under ectopic electric stimuli, as well as in mammals during the processes of wound healing, cell proliferation and nerve growth stimulation. Many neural cell types manifest electrotactic behaviors, including neural crest cells and hippocampal neurons (McCaig et al., 2005; Yao et al., 2008; Iwasa et al., 2017). Human neural stem cells migration is also directed by EF while blockade of receptors to classical chemotactic cues does not affect electrotactic responses (Feng et al., 2012, 2017). These results raise the intriguing possibility that cell migration in the developing brain occurs through tissues with steady electrical signals (McCaig et al., 2009; Iwasa et al., 2017) and is guided by them (Li et al., 2014; Feng et al., 2017).

In general, the effects of applied EF on neuronal cells are similar and include changes in length and orientation of cell bodies and leading processes, neurite branching and stimulation of directed migration (Yao and Li, 2016; Bertucci et al., 2019). Indeed, electric stimulation seems to drive all kinds of polarized responses. *In vitro* applications of electric currents to cultured hippocampal cells initiate the cascade of morphological and molecular events. The division cleavage plane turns orthogonally and the mitotic spindle parallel to the EF vector. The Golgi apparatus and centrosome, $MAP2^+$ (dendrite-specific) microtubules and eventually the leading process turns to the cathode and cells migrate in a directed fashion with a leading process at the front (Yao et al., 2009). Examples of EF-induced changes specifically in cortical neurons have been reported: cortical axon length and orientation are a subject to specific electric regulation (Tang-Schomer, 2018). Furthermore, electric stimulation of postnatal prefrontal cortical neurons in culture improves dendritic branching and length as well as synaptic protein amounts in both WT and genetically modified conditions (NRG1-knock-out and DISC1-locus impaired mice), associated with psychiatric disorders (Zhang et al., 2017). This provides, on the one hand, a proof for direct electric regulation of cortical dendrito- and synaptogenesis, and on the other hand, an example of electric cue overriding genetic state.

In adult neural tissue, electrical communication is granted through chemical synapses *via* neurotransmitters, which regulate ion flow through ionotropic receptors. Synaptic connections are canonically at the origin of presynaptic Ca^{2+} influx in response to action potential membrane depolarization and post-synaptic in response to neurotransmitter receptors activation. The same machinery is utilized during post-mitotic neuronal migration and maturation: voltage- and neurotransmitter-gated ion channels are capable of regulating the resting membrane potential, which is usually low in immature neurons (Levin et al., 2017). Neurotransmitters are present throughout developing neural tissues, can be released by cells in close vicinity, i.e., neuroblasts or maturing neurons, and can act on migrating neurons in paracrine, non-synaptic, fashion (Spitzer, 2006; Luhmann et al., 2015; Ojeda and Ávila, 2019). Gap junctions, or electric synapses, undoubtedly also play roles during development, especially in electrically active zones, such as the subplate (SP) (Luhmann et al., 2018; Singh et al., 2019).

Here, we aim to analyze EF-guided migration and maturation in the developing cerebral cortex, with a major focus on radially migrating glutamatergic neurons. We use the term EF to designate a sum of electric currents in the tissue and extracellular environment in general as well as electric activity and responses locally, inside the cell.

Dendritogenesis normally occurs after neurons have completed their migration and is, thus, a post-migratory step of neuronal maturation. In the cortical tissue dendritic development is shaped by extrinsic regulation in destined cortical layers (Martineau et al., 2018). Yet, upon electric activity amplification in migrating cortical neurons, precocious and ectopic dendritogenesis is observed (Bando et al., 2016; Hurni et al., 2017). Here we will review the mechanisms mediating the EF-dependent control of neuronal migration and maturation and we will also touch upon how these two processes can be related to synaptic organizing molecules prior to synaptic formation.

Ca^{2+} IS AN INTRACELLULAR PROXY OF EXTRINSIC EF FLUCTUATIONS IN DEVELOPING NEURONS

EF-triggered receptors displayed on the cell surface activate a number of signaling pathways, such as ERK, PI3K and small Rho GTPases (Yao and Li, 2016). However, the central regulator of neuronal EF-guided processes, both migration and dendritogenesis, is attributed to downstream intracellular Ca^{2+} concentrations, which convert electrical signaling to physiological responses and are used as a readout of electrical activity (Uhlen et al., 2015; Horigane et al., 2019).

In addition to intracellular Ca^{2+} release in migrating neurons, Ca^{2+} enters from the extracellular environment and is mediated by VGCC type Ca^{2+} channels. These channels are sensitive to membrane depolarization and are typically reactive to synapse-triggered action potentials. In young neurons devoid of synapses, these channels are hypothetically capable of responding to low voltage changes (Horigane et al., 2019). The latter can be produced by neurotransmitter- and voltage-gated ion channels, which are well expressed in the developing cortex and are extensively documented as controlling migration and neuritogenesis in cell-autonomous and non-autonomous ways. Clinical importance of ion channels in early brain development is recognized and indicates their role in transmembrane voltage regulation and/or in migration before stable synapse formation (Smith and Walsh, 2020).

Dendritogenesis in general is very sensitive to extrinsic cues and the molecular mechanisms are well studied and summarized in several excellent reviews (Arikkath, 2012; Valnegri et al., 2015;

Ledda and Paratcha, 2017; Lanoue and Cooper, 2019). In cortical neurons, extracellular Ca^{2+} influx is important for dendritic branching, while intracellular Ca^{2+} release affects dendritic branching, axonal growth and density of filopodia (Ramakers et al., 2001). Ca^{2+} events in cortical neurons are localized and may organize dendritic and spine morphology from within: Ca^{2+} waves initiate at dendritic branch points and propagate predominantly at primary apical dendrites. Earlier in development Ca^{2+} events in dendrites are characterized by bigger amplitudes and seem to be dependent mostly by changes of membrane voltage and L type VGCC Ca^{2+} channels (Ross, 2012). Overall, Ca^{2+} signaling in dendritogenesis is well recognized (Konur and Ghosh, 2005).

Intracellular Ca^{2+} fluctuations could thus constitute a convergence point for chemical cues-signaling pathways and EF, summing up in local Ca^{2+} changes that in turn regulate migration, dendritogenesis, and spine formation.

CEREBRAL CORTEX AND ELECTRICALLY ACTIVE ZONES

Direct Studies of EF in the Cortex

In the cerebral cortex, first measurements of tissue endogenous electric current flow were performed in 2013 (Cao et al., 2013) in the walls of the lateral ventricle along the rostral migratory pathway in adult mice. An electric potential gradient measured in the interstitial space along the pathway is of 3–5 mV/mm. It is formed by positively charged ions in the extracellular space, which in turn is supported by currents through cells and tissues. These currents depend on polarized expression of electrogenic pumps (e.g., Na^+/K^+ -ATPases), which are effectively suppressed by selective inhibitors with the subsequent reduction of electric currents in the tissue. Neuroblasts migrating along the pathway rise their migration speed or change direction upon application to the brain slice of higher EF or reversal of the field polarity (Cao et al., 2013). Moreover, pharmacological inhibition of EF effectively suppresses migration in 3D cultures of subventricular zone (SVZ) explants, while EF stimulation, in addition to promoting migration, induces expression of adhesive molecules thus increasing cell-cell contacts (Cao et al., 2014).

Evidence for EF-guidance in cortical tissue *in vivo* are coming from the brain injury field. Increased cell proliferation in the SVZ and directed migration toward the source of the current is observed during motor cortex electrical stimulation (Jahanshahi et al., 2013). Human neural stem cells transplants into the rat rostral migratory stream are efficiently guided by endogenous EF, while applied electric stimulation redirects migration of subpopulation of cells regardless of endogenous tissue cues (Feng et al., 2017).

Electrical Activity During Cortical Development

There are no studies on applied or otherwise manipulated direct EF specifically for the developing cortex and migrating neurons in early stages. Nevertheless, electrical activity of migrating cells in the embryonic cortex is well documented. Individual neurons display spontaneous Ca^{2+} activity (Corlew et al., 2004; Egorov and Draguhn, 2013; Bando et al., 2016; Yuryev et al., 2018; Mayer et al., 2019) and inward Na^+/outward K^+ currents (Picken Bahrey and Moody, 2003). Ca^{2+} waves through the mouse cortical plate are recorded *in utero* (Yuryev et al., 2016). Recordings in the human developing subplate show spontaneous oscillatory activity of GABAergic origin, but almost no synaptic connections (Moore et al., 2011).

The developing cerebral cortex is a rapidly expanding layered structure that mostly relies on highly organized radial migration of newly born glutamatergic neurons. During radial migration neurons undergo morphological changes which are accompanied by the change of migration mode and are influenced by tissue environments (Ohtaka-Maruyama and Okado, 2015; **Figure 1**).

Just born multipolar neurons slowly migrate toward the pial surface through the intermediate zone (IZ) by a multipolar migration mode, with long pauses and frequently changing directionality (Tabata and Nakajima, 2003). This continues until they reach the first structural «border», the SP. The SP is a layer of transient electrophysiologically active and morphologically mature neurons which manifest a high electrical activity as they establish afferent and efferent synaptic connections within the developing cortex (Luhmann et al., 2018). Here multipolar neurons start their polarization process by the formation of tangentially oriented axonal outgrowth (Hatanaka and Yamauchi, 2013). This is well-studied from a biochemical point of view. Reelin, a powerful chemical regulator of radial migration, is present in the IZ and, by initiation of a RAP1-dependent N-cadherin cell surface rise, allows multipolar neurons to sense microenvironmental cues, which in turn can induce polarization (Hansen et al., 2017). Axonal induction is also promoted by $GABA_B$ receptor, and GABA is believed to be present in the IZ due to tangentially migrating interneurons (Bony et al., 2013) (also see *GABA* chapter). Moreover, SP neurons make glutamatergic synaptic contacts with multipolar migrating neurons. This induces NMDAR-dependent Ca^{2+} entry into the migrating neuron and, as a result, facilitates the morphology and migration mode switch to bipolar neurons (Ohtaka-Maruyama et al., 2018; Ohtaka-Maruyama, 2020). Bipolar neurons then migrate by locomotion along radial glia fibers. It is possible that electric stimulation from radial glia is adding to the multipolar-bipolar transition, as Ca^{2+} bursts in both cell types are synchronized during this process (Rash et al., 2016).

Bipolar locomotion is a fast mode of directed migration in which speed and pausing time is managed by the strength of intracellular Ca^{2+} transients (Hurni et al., 2017). The final stage of migration is characterized by a change to terminal translocation, rise of intrinsic frequencies of Ca^{2+} transients and appearence of dendrites, a signature of mature post-migratory neurons (Bando et al., 2016; Hurni et al., 2017). Neuronal dendrites spread out to the marginal zone (MZ), the upper limit «border» for radial migration. The MZ is a low cell density layer at birth, and, as the SP, develops rather early synaptic connections due to a local population of more mature Cajal-Retzius neurons (CRs) (Janušonis et al., 2004) and young post-migratory neurons (Bouwman et al., 2004).

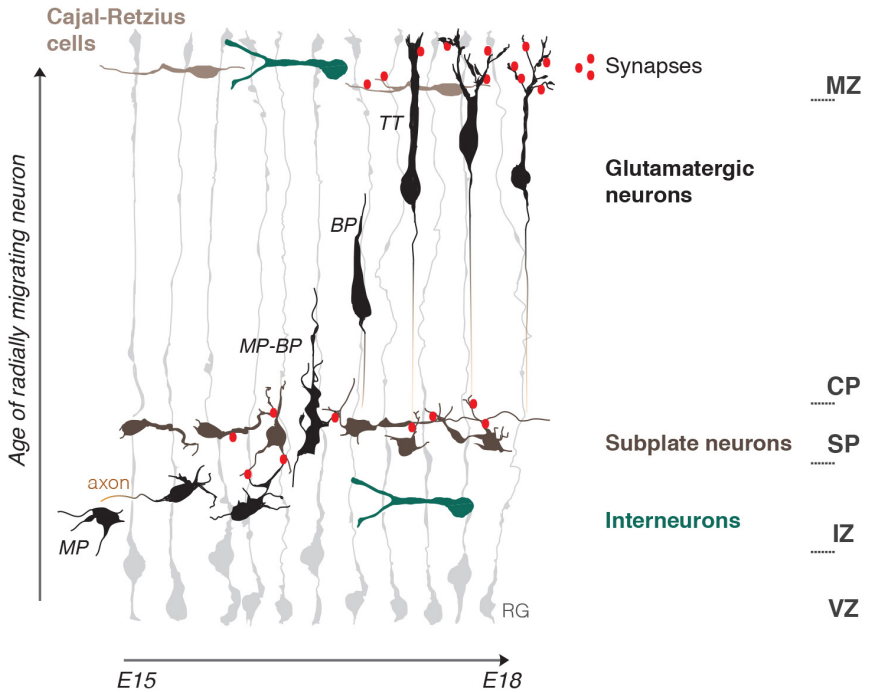

FIGURE 1 | Schematic representation of the embryonic mouse cortex and its electrical zones. Two electrically active borders, the subplate (SP) and the marginal zone (MZ), organize neuronal migration in the developing cerebral cortex. The MZ and SP coincide with morphology and migration mode transformations of radially migrating neurons and represent pathways for tangentially migrating neuronal populations: Cajal-Retzius cells and Interneurons. Note the presence of early functional synaptic contacts in the SP and MZ. Axonogenesis and polarization of migrating neurons occur under the SP and dendritogenesis in the MZ. Neuritogenesis is thus enhanced within the two zones. Radial migration is depicted starting from bipolar neurons (BP) in the intermediate zone (IZ) onwards that represent steps mostly studied in terms of electrical activity. RG, radial glia; MP, multipolar neuron; BP, bipolar neuron; MP-BP, transitional morphology of the neuron going through the SP; TT, neuron undergoing terminal translocation; VZ, ventricular progenitors zone; IZ, intermediate zone; CP, cortical plate.

CRs are early born tangentially migrating neurons which are best known for Reelin expression and its role as a regulator of terminal translocation and dendritogenesis (O'Dell et al., 2015; Hirota and Nakajima, 2017). CRs precise number and distribution have refined functions in cortical circuits organization. Thickness of apical dendritic tufts and of the MZ depend on CR density, as well as the excitation/inhibition ratio of post-migratory neurons. CRs migration is dependent on NMDARs stimulation and therefore is also activity-dependent (de Frutos et al., 2016; Riva et al., 2019). Two key chemical regulators of multipolar-bipolar and terminal translocation steps, Reelin and Dab1 (Hirota and Nakajima, 2017; Zhang et al., 2018) are surprisingly upregulated by electromagnetic field exposure (Hemmati et al., 2014). Altogether these data suggest that electric currents may be well upstream of chemical regulation of radial neuronal migration.

Is There EF in the Developing Cortex?

The presence of an electrically active boundary during cortical development, which organizes neuronal migration was described by Ohtaka-Maruyama (2020). It is possible to further imagine the developing cortex as a stratified structure of variable electric strength. For instance, the SP and the MZ are possibly highly charged compared to the relatively low EF in the IZ and the cortical plate (CP). They, thus, both could serve as electrical guide borders which, together with chemical cues, help attracting migrating glutamatergic neurons in the direction of the pia, orient their polarization and eventually drive corresponding morphological changes: axon initiation under the SP and dendritogenesis in the MZ.

How could this be exerted mechanistically on the migrating cell? As explained by Yao and Li (2016), when a cell is submitted to EF, due to a large membrane resistance, ionic flow is forced mainly around the cell. This creates extracellular current along the cell sides and lateral voltage gradient along the upper and lower membrane surfaces. Charged lipids and proteins, including conductance channels, are redistributed by the electrophoretic force, and form clusters. Voltage-gated ion channels could respond directly, creating local differences in resting membrane potentials and subsequent stimulation of Ca^{2+} influx and signaling activation. For example, increase of the Ca^{2+} influx on one side may signal the cell to form localized lamellipodia (Yao and Li, 2016). The same principle would apply to neurotransmitter-gated ion channels in the presence of neurotransmitter gradients, as discussed in the chapters below.

Some data from related models could support this view. Cao et al. (2014), have shown that EF gradients are present in cultures of SVZ explants from postnatal mice. In their study neuroblasts migration without growth factors is random and cells have multipolar morphologies, but when EF of physiological

strength is applied, cells acquire a bipolar morphology and the directionality of migration significantly increases (Cao et al., 2014) – a situation remarkably similar to the multipolar-bipolar switch in the SP. A wealth of studies on the behavior of neural stem cells upon electric stimulation has established an *in vitro* model that remarkably reminds of the developing cortex organization in the radial axis: in the absence of electric stimulation stem cells self-renew or differentiate into neurons or astrocytes and oligodendrocytes. After EF application they proliferate more efficiently with a shift toward neurons, cells become polarized, migrate toward the cathode and show an increase in intracellular Ca^{2+} (Bertucci et al., 2019). These observations, collected upon direct electric stimulation on immature neurons, in the absence of chemical stimuli, accurately reproduce complex behaviors of intact young neurons in the developing cortex tissue, and therefore suggest that EF variations are capable of inducing a whole panel of elaborated migration and maturation behaviors of glutamatergic cortical neurons.

NEUROTRANSMITTERS, THEIR RECEPTORS AND ION CHANNELS

Migration and dendritogenesis are processes separated both spatially and temporally, suggesting specific mechanisms of regulation. This is true for both tangentially and radially migrating populations. There is a plethora of data on electric modification of cortical neurons leading to migration delays with or without further defects in dendritogenesis. In the following sections, we attempt to dissect possible mechanisms underlying the electric control of migration and dendritogenesis by correlating ion channels distribution and their known effects on these two processes.

Glutamate and Its Receptors

Ambient glutamate concentration in the neonatal cortex is high compared to later postnatal stages, when it is likely uptaken by astrocytes (Hanson et al., 2019). Since neuronal migration and differentiation occurs prior to astrocytic differentiation, which starts at end of the embryonic period, extracellular glutamate concentrations may be even higher at prenatal stages. The source of extracellular glutamate is not exactly known. It can be released when vesicular neurotransmission is blocked and it has been suggested that it acts in a paracrine manner and may be sequestered around migrating neurons (Luhmann et al., 2015). Glutamate has been shown to act as a chemoattractant of cortical neurons *in vitro* (Behar et al., 1999).

Glutamate receptors, namely NMDARs, AMPARs and mGluRs, are expressed in the developing cortex. However, their subunit distribution throughout the developing cortex is uneven and in some cases, they display a clear developmental switch (Luhmann et al., 2015; Mayer et al., 2019; **Figure 2**).

Many studies on the role of NMDARs and AMPARs in migration are summarized in excellent reviews (Rakic and Komuro, 1995; Luhmann et al., 2015; Horigane et al., 2019; Ojeda and Ávila, 2019). In general, NMDAR and AMPAR blockade attenuates the migration of different types of immature neurons and NMDA physiological activation accelerates the movement. Selective manipulations of both NMDARs or AMPARs subunits by genetic and acute invalidation, however, do not exactly reproduce these effects in radially migrating glutamatergic neurons of the developing cortex, leaving the interpretation still unresolved.

NMDARs Subunits

NR1 (a.k.a. Grin1 or GluN1) is absent from the progenitor zone and present in the CP. It is an essential subunit for NMDAR function, but surprisingly its manipulation does not always lead to dramatic alterations in the developing cortex. Luhmann et al. (2015) review a series of studies on cell-type-specific NR1 knockouts (one good example is Iwasato et al., 2000), which indicate either the existence of compensatory mechanisms or extrinsic regulation of migration by non-neuronal target structures, like glial cells. One recent study supports the latter (Pasquet et al., 2019). Here, the authors demonstrate that proper layering of radially migrating neurons relies on NR1 clustering in radial glia fibers at contact sites with the soma and leading process of bipolar neurons (**Figure 2**). On the other hand, acute NR1 downregulation (KD) by *in utero* electroporation (IUE) into neuronal progenitors severely delay the migration of electroporated cells, without changing neuronal fate determination (Jiang et al., 2015). Measurements of dendritic structure of NR1 KD neurons located ectopically in lower cortical layers showed a simplistic morphology, as did controls (siRNA for a motility agent). However, KD neurons which reached the proper layer still showed a simplistic morphology (Jiang et al., 2015). Therefore, NR1 correct expression mediates proper dendritogenesis of post-migratory cortical neurons. Overall it is difficult to uncouple the role for NR1 in both processes. Furthermore, its cell-autonomous role in migrating neurons remains debatable.

NR2A and B (a.k.a. Grin2A,B or GluN2A,B) are modulatory subunits and often undergo a developmental switch in various neural tissues, including the cerebral cortex (**Figure 2**). The switch sharpens the response to glutamate as it yields channels with faster kinetics which are important for regulation of maturation of neuronal circuits (Mayer et al., 2019). In agreement with the expression pattern, NR2B, but not NR2A, cell-autonomous downregulation impedes radial migration, without changes of neuronal identity. Neurons aberrantly located in lower cortical layers develop dendritic trees of abnormally high complexity, and those neurons which reach the targeted position are comparable to controls (Jiang et al., 2015). Therefore, it is possible that NR2B function in physiological dendritogenesis is related to inhibition of the process. Based on these studies it is hard to uncouple its specific roles in dendritogenesis *vs.* migration.

Recent reports further highlight a substantial role for NR2B in neuronal maturation, dendritogenesis and non-synaptic NMDAR function. Human neural progenitors carrying autism spectrum disorder (ASD)-associated NR2B variants show impaired Ca^{2+} influx, membrane depolarization and differentiation failure (Bell et al., 2018). Most of NR2B-containing receptors are found within dendrites and the cortical neurons

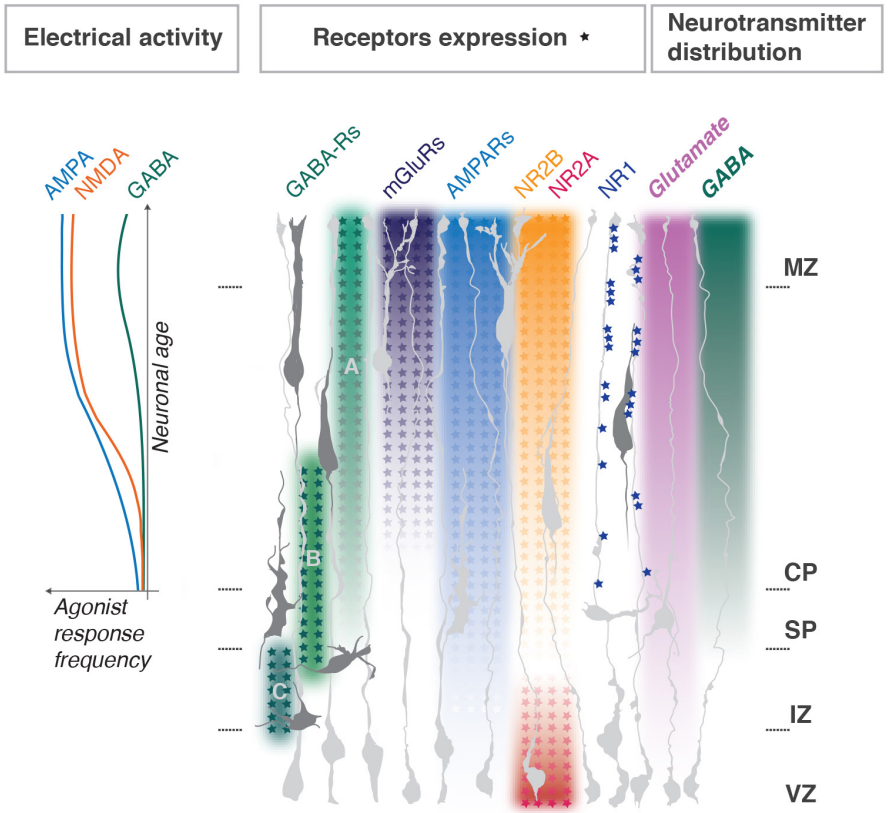

FIGURE 2 | Distribution of neurotransmitters and their receptors during cortical development. Enrichment gradients of ion channels and their specific subunits as neurons develop correlate with Ca^{2+} responses to corresponding agonists (graph to the left, adapted from Mayer et al., 2019) and dendritogenesis in the post-migratory neurons. NMDA NR1 subunits clustering in radial glia fibers regulate neuronal layer positioning; NMDA NR2A and B expression switches as progenitors differentiate into neurons. Metabotropic glutamate receptors gradients are representative for mGluRs1, 3, 4, 5, and 8; for AMPARs – GluR1, 2, 3, 4. $GABA_A$, $GABA_B$, and $GABA_C$ receptors functional switches in radially migrating neurons are shown. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; VZ, ventricular progenitors zone. Major references Hurni et al. (2017), Mayer et al. (2019), and Pasquet et al. (2019).

carrying ASD-associated variants manifest less dendrites, shorter total length and overall dysmorphia, while spine density or morphology is not altered. Mechanistically these mutations abolish channel activity and show no surface expression and reduced delivery to neurites (Sceniak et al., 2019). A more refined mechanism is proposed by a study on hippocampal neurons and cortical spiny stellate cells where dendritic length regulation and branching are uncoupled, with only the latter relying on NR2B (Espinosa et al., 2009).

It is interesting to note that among the known developmental NMDAR-dependent channelopathies it is exactly NR1 and NR2B gain of function variants which cause early developmental migration phenotypes, such as polymicrogyria (Smith and Walsh, 2020). This suggests that gain of function mutations might primarily affect migration in human developmental pathologies.

AMPARs and Metabotropic Glutamate Receptors

AMPA receptors expression increases in cortical neurons throughout development (Hurni et al., 2017; Mayer et al., 2019; **Figure 2**) and, similarly, to NMDARs, have been involved in both migration and dendritogenesis. Pharmacological studies with the AMPA/kainate receptor antagonist CNQX reveal the role in motility dynamics of migrating neurons; enhanced stalling and directionality changes are explained by lack of coordination between soma and leading process extension, possibly due to problems with growth cone dynamics (Jansson et al., 2013). It is important to mention, however, that CNQX is not exclusively selective for AMPARs but acts as well as NMDARs antagonist at glycine site (Lester et al., 1989), so the described effects are hard to dissociate between the two receptors. There is, however, an important argument against a role for AMPARs in radial migration: out of all glutamate receptor ligands only NMDA and L-glutamate (and not AMPA, D-glutamate, kainate or quisqualate) induce chemotactic motility responses in mouse cortical neurons (Behar et al., 1999).

Dendritic arbor development of glutamatergic neurons and interneurons is mediated by distinct AMPA subunits. GluR1, 2 and 3 are involved in dendritogenesis of glutamatergic cortical neurons and their action is associated with spontaneous increase of Ca^{2+} transient amplitudes, but not frequency. Few studies report specifically an increase of dendritic arbor complexity at the third level branches and higher (Chen et al., 2009;

Hamad et al., 2014). Others detect strong upregulation of dendritic length by subunits "flip" isoforms particularly enriched in development (Hamad et al., 2011). GluR1 (and not −2 and −3) is more specific for interneuron dendritogenesis (Hamad et al., 2011). Indeed, interneurons, migrating in the IZ, become more rounded after AMPA exposure and this is mediated by paracrine AMPA receptor activation (Poluch et al., 2001). Therefore, GluRs might participate in guiding the migratory stream, or provide stop signals for migrating interneurons and initiate their maturation.

One hypothesis for AMPA-regulated Ca^{2+} dynamics in immature neurons rests on their deficiency in subunits [e.g., GluR2 (GluA2)] which causes cortical neurons to be permeable to Ca^{2+} , while their gradual enrichment toward birth strengthens Ca^{2+} influx control (Kumar et al., 2002). This is supported by a study in which activation of Ca^{2+}-permeable AMPA receptors induced neural progenitor cells (NPCs) to differentiate to the neuronal lineage and increased their dendritic arbor formation (Whitney et al., 2008). Overall, AMPARs may be a good candidate for preferential regulation of dendritogenesis over the migratory effects on cortical maturing neurons.

AMPARs actions on dendritogenesis interplay with those of metabotropic glutamate receptors, mGluRs (Grms). One example is the disruption of dendritogenesis in mGluR5 knockout cortical neurons associated with an increase of Ca^{2+}-permeable AMPA receptors (Huang and Lu, 2018). However, mGluRs also have a role in stalling during neuronal migration, which is believed to be due to highly localized Ca^{2+} changes and is an important part of migration as it may be rising sensitivity to chemical cues, helping direction searching (Jansson et al., 2013). Although not ionotropic, mGluRs activation seems to be linked to triggering Ca^{2+} high-amplitude waves propagation (typical for developing tissues) only in subregions of the dendrites (Ross, 2012), and therefore in interaction with AMPARs they could contribute to fine dendritic organization.

GABA and Its Receptors

GABA is one of the earliest neurotransmitters expressed in the nervous system and is enriched during early corticogenesis (Lauder et al., 1986; Behar et al., 1996; Bony et al., 2013). GABA influences radial and tangential migration through its various receptors depending on the migration step and exerts some of these actions through Ca^{2+} influx signaling, due to the fact that that it functions as an excitatory neurotransmitter in early development and depolarizes immature neurons. GABA paracrine and chemoattractive actions have been documented by several authors (Horigane et al., 2019; Ojeda and Ávila, 2019).

Glutamatergic neurons migration can be modulated by GABA in a concentration-dependent manner and relies on pharmacologically distinct classes of GABA receptors. VZ/IZ populations, show directed migration in response to femtomolar GABA concentrations. Cells which exit from the proliferative zone to the IZ are blocked by $GABA_C$ ionotropic channels antagonist, and $GABA_C$-R, which has a high affinity to GABA, delivers a signal that maintains migration throughout the IZ (Behar et al., 1998, 2000; Denter et al., 2010). IZ-CP entry is sensitive to G-protein inhibitors, indicating a role for metabotrophic $GABA_B$ G-protein coupled receptors. Neurons migrating in the CP respond to micromolar amounts of GABA with increased cell motility and this response partially relies on G-protein. This is also sensitive to depolarizing agents: glutamate, potassium and $GABA_A$ ionotropic channels activation (Behar et al., 1998, 2000). $GABA_A$-R regulates migration speed in the upper CP and most of all is important for migration termination before the MZ with GABA tonically reducing the speed of cell migration in the upper cortex *via* $GABA_A$-R activation by interfering with Ca^{2+} oscillations (Heck et al., 2007). $GABA_A$-R-dependent regulation of migration may also be mediated by taurine, a compound abundantly present in developing tissues (Furukawa et al., 2014). Therefore, there is an elegant model on the regulation of radial migration by GABA concentration gradients through receptor expression switches depending on the physiologic state of the migrating neuron, crossing each developmental zone (**Figure 2**).

After birth young neurons switch response to GABA from excitatory to canonical inhibitory (Ben-Ari et al., 2012). GABA-induced excitability decreases due to lowering of intracellular Cl^- concentrations *via* developmental upregulation of KCC2, a K^+/Cl^- cotransporter extruder, and downregulation of NKCC1, the chloride-inward Na^+-K^+-Cl^- cotransporter. The $GABA_A$-R-dependent Cl^- flux reverts and becomes hyperpolarizing (Bortone and Polleux, 2009; Horigane et al., 2019). This mechanism is at the basis of the termination of interneurons migration. Before the switch, ambient GABA and Glutamate signals are motogenic, but once interneurons are in the cortex the decrease of Ca^{2+} transients upon $GABA_A$-R activation induces them to stop (Bortone and Polleux, 2009).

For radially migrating neurons excitatory GABA actions exerted through $GABA_A$-R are indispensable for morphological maturation. Premature overexpression of KCC2 as well as downregulation of NKCC1 do not perturb migration (Cancedda et al., 2007; Jiang et al., 2015), but have a dramatic effect on dendritic morphology (Ge et al., 2006). KCC2 overexpression prevents GABA-induced Ca^{2+} elevation and the morphological impairment of properly positioned upper layer neurons comprises pronounced reduction of total dendrite length and branch number, with very few dendritic processes projecting to layer 1 (MZ). The effect worsens with time. Experiment with overexpression of the inward-rectifier K^+ channel Kir2.1 produced similar results, further indicating that reducing membrane depolarization is sufficient to impair dendritogenesis in cortical neurons (Cancedda et al., 2007; Sernagor et al., 2010). However, also direct disruption of $GABA_A$ receptor activity without perturbations of cell polarization produces similar effects. DISC1-KD in young cortical pyramidal neurons leads to perturbations of surface expression of the $GABA_A$-R subunit, while Cl^- cotransporters are unaffected (Saito et al., 2016). Nonetheless, GABA-mediated Ca^{2+} influx is diminished, as demonstrated by $GABA_A$-R antagonist treatment. Acute DISC1-KD in postnatal cortical neurons prevents complex dendritic arborization development and this is accompanied by GABA-mediated post-synaptic currents impairments (Saito et al., 2016). Thus, dendritogenesis is mediated by direct $GABA_A$ receptor activity as well as by the hyperpolarized state of the neuron.

$GABA_B$ metabotropic receptor has been found to be crucial for axon-dendrite polarization and growth. $GABA_B$ downregulation in a dose-dependent manner leads to migration delays and ectopic accumulation of cells with long and thin processes, as well as reduced development of dendrites and pronounced axonogenesis in cells reaching the upper CP. *In vitro* $GABA_B$-R signaling specifically affected axonal initiation. The mechanisms rely on cAMP-dependent phosphorylation of LKB1, a kinase involved in neuronal polarization (Bony et al., 2013). This goes in concordance with the specific receptor role in the IZ-CP transition, which thus may rely on the multipolar-bipolar transition regulation. In tangential migration $GABA_B$ has a pronounced role in a concentration-dependent motility regulation, through regulation of the length of the leading process, and those effects are not accompanied by membrane potential changes, highlighting that mechanism does not rely on electric activity modulation (López-Bendito et al., 2003). Therefore, the primary role of $GABA_B$-R is related to neuronal polarization and subsequent directed migration rather than to dendritogenesis.

Other Ion Channels: Are Migration and Dendritogenesis Uncoupled?

Most studies on neurotransmitter channels do not clearly discriminate whether the effects on dendritogenesis are a direct consequence of those on termination of migration. Few interesting examples below illustrate how ion channels, which modulate electrical activity, can regulate migration and dendritogenesis and possibly help the distinction.

Prokaryotic voltage-gated sodium channel, NaChBac, when overexpressed in cortical radially migrating neurons, dramatically raises excitability and the frequency of spontaneous Ca^{2+} transients. This causes premature migration arrest, but also induce dendritogenesis in ectopic cells. Moreover, the ectopic cells appear to prematurely complete their migration since they loose contact with radial glia fibers. The authors show that migrating neurons have lower Ca^{2+} oscillations parameters than the post-migratory and thus they hypothesize that the difference between neuronal migration and maturation relies on the intensity of spontaneous Ca^{2+} transients (Bando et al., 2016). Another study aiming to stimulate cell-intrinsic activity used an artificial receptor-ligand system (DREADD). The results obtained here were overall similar to those of NAChBAc: cells with raised Ca^{2+} transients frequencies, and not durations, were massively delayed in the IZ-SVZ and lower CP, without changing their birthdate-dependent identity. Moreover, ectopic neurons developed neuritic branching reminiscent of dendrites. The same study, similarly, observed raised Ca^{2+} transients in neurons undergoing migration termination. Moreover, the authors were able to show that DREADD-induced cell migration delays were associated with an increase in pausing time, and not instant migratory speed (Hurni et al., 2017). These studies reinforce the idea that a developmental increase in Ca^{2+} events intensity plays a role in migration arrest and, eventually, maturation.

While the above studies are very illustrative, they depend on artificial expression of non-endogenous channels, which probably overstimulate electrical activity to abnormally high levels and thus cannot exactly reflect the physiological situation in the mammalian cortex. A recent study by Smith et al. (2018) addresses the role of SCN3A, a subunit of voltage-gated sodium channel NaV1.3, which is naturally enriched in migrating neurons of the developing human cortex and is downregulated upon cortical maturation. Few point variants in this gene are associated with rare cases of developmental channelopathies which range from polymicrogyria and intellectual disability to microcephaly and severe seizures. Acute overexpression of the gene and its mutant forms in the ferret cortex highlighted the role in migration and gyrification (additional sulci and gyri) with heterotopic formations exclusively registered in the case of mutants. Overexpression of SCN3A in human cortical neurons promoted dendritic branching and this effect was attenuated in mutant forms. Patch clamping human fetal cortical neurons demonstrated the absence of action potentials, therefore SCN3A likely contribute to Na^+ conductance that modulate other voltage-dependent processes like Ca^{2+} signaling. Altogether, these data suggest, once again, that dendritic branching and migration effects are closely interconnected. However, it is important to point out that the same variant (F1759Y) which aggravates migration phenotypes (more severe gyrification and heterotopia) also attenuates dendritogenesis which goes in the opposite direction to the postulate that simple neuronal activity overstimulation is ultimately responsible for both processes.

Notably, migration arrest due to enhanced neuronal activation is not always accompanied by dendritogenesis. The KCNK family of leak potassium channels conducts potassium currents at resting membrane potential, with little voltage dependence, and is one of the major determinants of neuronal excitability in the cortex. Family members are expressed throughout the cortex and have a role in radial migration with the most prominent phenotypes observed for KCNK9. KCNK9 downregulation as well as overexpression of mutant forms impair radial migration by increasing the frequency of spontaneous Ca^{2+} transients, possibly by controlling resting membrane potassium permeability. The ectopic delayed cells do not die neither they change their identity, but persist in deeper cortical layers showing undeveloped morphology for a prolonged period of time (Bando et al., 2014). Since the correct positioning of neurons is crucial for proper dendritogenesis (Martineau et al., 2018), these results imply that KCNK9-induced Ca^{2+} transients increase is not sufficient to promote ectopic dendritic morphology development, while it is sufficient for the migration arrest.

These few studies demonstrate that although migration, its final termination and dendritogenesis are intimately connected and likely rely on similar mechanisms of electrical activity rise at the maturation stage, the mode of this electrical activity regulation by ion transport type and intensity may vary: from very intense which causes ultimately cell migration arrest and strong ectopic dendritogenesis (like stimulation with DREADD) to milder which only affects migration (like KD of KCNK9). Moreover, it is possible that channel conformation changes due to mutations contribute to its activity and somehow regulates

migration and dendritogenesis in opposing manner, as it seems to happen with SCN3A pathological variants.

SYNAPTOGENESIS AND "SYNAPTIC" PROTEINS

It is generally believed that functional chemical synapses massively appear within the first 2 weeks after birth. However, the exact time of establishment of fully functional synaptic structures is somehow vague. Synapses are complex and comprise many molecules which are supposed to be specific, but have a remarkably variable expression span and, often, functionality (Farhy-Tselnicker and Allen, 2018; Südhof, 2018).

The developing mouse cortex gradually shapes morphologically recognizable synapses. First clearly immature synapses (with pleiomorphic vesicles associating around newly formed terminals, with yet thin pre- and post-synaptic plasmalemmas and narrow gap in between) are identifiable as early as E15 (Li et al., 2010). Functional synapses likely appear in electrically active borders such as the SP and MZ (**Figure 1**). SP neurons forming full synaptic connections with multipolar migrating neurons were observed at E16 in mice (Ohtaka-Maruyama et al., 2018). These connections are defined (i) morphologically by VGLUT2 staining, and the presence of vesicles and electron-dense structures reminiscent of active zones and post-synaptic densities, and (ii) functionally by the presence of Ca^{2+} transients and exocytosis of presynaptic vesicles at the upper IZ. However, data suggest that these functional synapses may be present earlier (Ohtaka-Maruyama et al., 2018). SP neurons may also send electrophysiologically active GABAergic projections to CR cells in the MZ as seen soon after birth (Myakhar et al., 2011), but synaptogenesis on CRs is described already at E17 (Janušonis et al., 2004).

TABLE 1 | Summary of ion channels having differential roles in migration and dendritogenesis of cortical glutamatergic neurons.

	Migration	Dendritogenesis	References
NMDA NR1	Yes	Basic expression levels are needed for proper dendrite arborization	Jiang et al., 2015
NMDA NR2B	Yes	Primary dendrite pruning and patterning	Espinosa et al., 2009; Jiang et al., 2015
AMPA GluR1, 2, 3	Yes*	Arborization length and complexity	Chen et al., 2009; Jansson et al., 2013; Hamad et al., 2014
NKCC1	No	Possibly. Effects on dendritic arborization in young hippocampal granule cells	Ge et al., 2006; Jiang et al., 2015
KCC2	No	Premature expression disrupts dendritogenesis	Cancedda et al., 2007
Kir2.1	No*	Premature expression disrupts dendritogenesis	Cancedda et al., 2007
SCN3A	Yes	Pathological variant attenuates dendritogenesis, but not migration	Smith et al., 2018
KCNK9	Yes	No	Bando et al., 2014

**Alternative interpretations still possible (see text).*

Morphological synapses in the MZ, which are largely formed by two classes of neurons, are registered as early as E16 in mice. Their ultrastructure satisfies the parameters of morphologically well-formed mature synapses: presynaptic vesicles conglomeration, presence of docked vesicles and active zone. By E18, MZ abundantly expresses a list of structural synaptic proteins, including post-synaptic density marker PSD95, synaptic vesicle associated VAMP2, and AMPA subunits 2 and 3. However, the functionality of these synapses may not be fully established yet: in the absence of neurotransmitter release and, thus, synaptic electric activity (Munc18-1 null mice) these synapses are largely preserved (Verhage et al., 2000; Bouwman et al., 2004). A structurally complete synapse, therefore, does not necessarily mean it is electrophysiologically functional and, in fact, may not require electrical activity to be formed.

Many synaptic molecules are expressed in the developing cerebral cortex prior to full synaptic formation. What could be their function? One example is the VAMP-family proteins, controlling migration speed in CRs, as early as E11, possibly through regulation of exocytosis, asymmetric membrane transport and/or endosomal recycling. This function turns out to be dramatically important for regulation of cortical arealization during postnatal development, specifically for primary and secondary sensory cortices and its connection routing (Barber et al., 2015). Another example comprises the whole class of trans-synaptic cell-adhesion molecules (CAMs). CAMs are likely at the basis of primary organization of synaptic junctions, but along with that they are as numerous and efficient for synaptogenesis as they are multifunctional outside this process. CAMs families such as LAR-type RPTPs and their ligands, Slitrks, Cadherins, Teneurins, and Ephrins/Eph receptors are all involved in neuronal morphogenesis, both dendritogenesis and axonal pathfinding (Südhof, 2018). Interesting confluence of CAMs role on both morphogenesis and organization of electrical activity zones, such as the synapses, is reminiscent of gradual Ca^{2+} transients rise and morphological refinement of maturing migrating neurons. Few examples of CAMs regulation of neuronal migration exist (Qu and Smith, 2004; Kirkham et al., 2006; Funato et al., 2011; Sentürk et al., 2011; Puehringer et al., 2013; del Toro et al., 2017, 2020), however, whether the mechanisms involve electrical activity regulation remains an open question.

CONCLUSION

In this review, we have discussed the distribution of ion channels canonically involved in the synaptic ion exchange machinery, in the attempt to decipher the principles of electric regulation of migration and maturation in early cortical development, before functional synaptogenesis occurs. While not all neurotransmitter receptor systems involved in the maturation of developing pyramidal neurons were considered (such as purinergic and cholinergic systems), a basic coupling principle seems to predominate: channels underlining excitatory responses and thus electrical cellular activation (with Ca^{2+} transients as a readout)

contribute to migration pausing, eventual arrest and subsequent dendritogenesis (**Table 1**). Although it is hard to clearly correlate particular channels presence with dendritogenesis *vs.* migration arrest, progressive enrichment of specifically the NR2B subunit of NMDA receptors as well as the appearance of fast excitatory transmission supplied by AMPARs expression seem to be the most promising candidates for electrical regulation of initial neuronal maturation.

Dendritogenesis is concurrent with synaptogenesis, and according to the synaptotrophic hypothesis, synaptogenesis comes first and is initially required for filopodia stabilization. The very first step in this process is recruitment of CAMs which subsequently leads to gradual synapse formation and dendritic stabilization and outgrowth (Chen and Haas, 2011). It is now explicitly demonstrated that functional synaptic contacts in the developing cortex help migration pausing and morphological reorganization during the multipolar-bipolar transition in proximity of the electrically mature zone, the SP (Ohtaka-Maruyama et al., 2018). However, how advanced the synaptic machinery assemblage must really be in order to assert proper electrical regulation of migration and maturation processes is unclear. As well as neurotransmitters and their receptors, CAMs expression throughout the early developing cortex is abundant and there are examples where partners for synaptic binding are expressed in complementary manner in areas formally devoid of synaptic structures. Further investigations are needed to fully answer the intriguing questions of how the congregation of synaptic molecules regulates neuronal maturation, whether this relies on adhesive or electric-organizing properties of CAMs or their combination, and how the neuronal electrical and adhesive machinery cooperate during cortical development.

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VM and AP conceived and wrote the review. Both authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Non-Cell-Autonomous Mechanisms in Radial Projection Neuron Migration in the Developing Cerebral Cortex

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Concerted radial migration of newly born cortical projection neurons, from their birthplace to their final target lamina, is a key step in the assembly of the cerebral cortex. The cellular and molecular mechanisms regulating the specific sequential steps of radial neuronal migration *in vivo* are however still unclear, let alone the effects and interactions with the extracellular environment. In any *in vivo* context, cells will always be exposed to a complex extracellular environment consisting of (1) secreted factors acting as potential signaling cues, (2) the extracellular matrix, and (3) other cells providing cell–cell interaction through receptors and/or direct physical stimuli. Most studies so far have described and focused mainly on intrinsic cell-autonomous gene functions in neuronal migration but there is accumulating evidence that non-cell-autonomous-, local-, systemic-, and/or whole tissue-wide effects substantially contribute to the regulation of radial neuronal migration. These non-cell-autonomous effects may differentially affect cortical neuron migration in distinct cellular environments. However, the cellular and molecular natures of such non-cell-autonomous mechanisms are mostly unknown. Furthermore, physical forces due to collective migration and/or community effects (i.e., interactions with surrounding cells) may play important roles in neocortical projection neuron migration. In this concise review, we first outline distinct models of non-cell-autonomous interactions of cortical projection neurons along their radial migration trajectory during development. We then summarize experimental assays and platforms that can be utilized to visualize and potentially probe non-cell-autonomous mechanisms. Lastly, we define key questions to address in the future.

Keywords: cerebral cortex, radial projection neuron migration, non-cell-autonomous mechanisms, neurodevelopmental migration disorders, single cell analysis

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INTRODUCTION

The mammalian neocortex is built by distinct classes of neurons and glial cells which are organized into six stratified layers. Here we focus on projection neurons, the major neuronal population in the cortex. Projection neurons emerge from radial glial cells (RGCs) in the ventricular zone (VZ), intermediate progenitor cells (IPCs), and outer radial glial cells (oRGs, aka basal radial glia, bRGs) which divide in the subventricular zone (SVZ) (Ayala et al., 2007; Hansen et al., 2010;

Wang et al., 2011; Borrell and Götz, 2014). Nascent projection neurons migrate from their place of origin in the VZ/SVZ to their final target position, a process which is highly regulated (Ayala et al., 2007; Valiente and Marín, 2010; Evsyukova et al., 2013). Concerted migration of sequentially generated projections neurons results in a neocortex which is structured into six distinct layers (I–VI), each with different cellular composition and arranged in an inside-out fashion (McConnell, 1995; Lodato and Arlotta, 2015) (**Figures 1A,B**). In order to establish the correct cortical layering during development, projection neurons exhibit radial migration from the VZ/SVZ to the cortical plate (CP). Around embryonic day 11 (E11), post-mitotic neurons migrate mainly by pulling up the soma in the upright direction by using a basal process that is firmly attached to the pial surface. This migration mode is termed somal translocation (Nadarajah et al., 2001). The first cohort of migrating neurons form the preplate (PP), a structure which only exists transiently (Allendoerfer and Shatz, 1994; Nadarajah et al., 2001). At around E12, consecutive waves of neurons migrate toward the pial surface and establish the CP by splitting the PP into the two distinct structures: the deeper located subplate (SP) and the superficially positioned marginal zone (MZ) (layer I) (Ayala et al., 2007) (**Figure 1A**). The subsequent populations of migrating neurons establish the 'first' layer of projection neurons (i.e., layer VI) in the CP which progressively expands in the vertical direction in an inside-out manner (**Figure 1**). In other words, earlier generated neurons settle in the deeper layers (layers V, VI) whereas later generated neurons migrate through the deep positioned neurons creating more superficial layers (II, IV) (Angevine and Sidman, 1961; McConnell, 1995; Valiente and Marín, 2010).

Studies applying histological and time-lapse imaging techniques have shed some light on the dynamics of the radial migration process and described distinct sequential steps of projection neuron migration (**Figure 1A**) (Nadarajah et al., 2003; Tabata and Nakajima, 2003; Noctor et al., 2004). Newly-born neurons delaminate from the VZ and move toward the SVZ where they accumulate in the lower part and acquire a multipolar shape, characterized by multiple processes pointing in different directions (Tabata et al., 2009). In the SVZ, multipolar neurons move tangentially, toward the pia or toward the VZ (Tabata and Nakajima, 2003; Noctor et al., 2004). Multipolar neurons can remain up to 24 h in the multipolar state in the SVZ. Next, within the SVZ and the lower part of the intermediate zone (IZ) multipolar neurons switch back to a bipolar state with a ventricle-oriented process that eventually develops into the axon. The pial oriented leading process is established by reorienting the Golgi and the centrosome toward the pial surface (Hatanaka et al., 2004; Yanagida et al., 2012). Upon multi-to-bipolar transition, neurons attach to the radial glial fiber in the upper part of the IZ and move along RGCs in a migration mode termed locomotion, while trailing the axon behind and rapidly extending and retracting their leading neurite before reaching the SP (Hatanaka et al., 2004; Noctor et al., 2004). Neurons then cross the SP and enter the CP still migrating along the RGCs until they reach the marginal zone (MZ). Just beneath the MZ neurons stop locomoting and detach from the radial glia fiber to perform terminal somal translocation and settle in their target position where they eventually assemble into microcircuits (Rakic, 1972; Nadarajah et al., 2001; Noctor et al., 2004; Hatanaka et al., 2016). All sequential steps of projection neuron migration are critical and disruption at any stage (e.g., due to genetic mutations in genes encoding core migration machinery) can lead to severe cortical malformations (Gleeson and Walsh, 2000; Guerrini and Parrini, 2010). Therefore each step of projection neuron migration must be tightly regulated. Many genes have been identified as causative factors for cortical malformations (Heng et al., 2010; Valiente and Marín, 2010; Evsyukova et al., 2013) and several of the key molecules involved in neuronal migration, e.g., LIS1, DCX, and REELIN have been investigated in detail by molecular genetics (Kawauchi, 2015). Recently, approaches involving *in vivo* electroporation and time-lapse imaging of brain slice cultures have shed light on crucial roles for the dynamic regulation of the cytoskeleton, extracellular cues and cell adhesion during neuronal migration (Noctor et al., 2004; Schaar and McConnell, 2005; Simo et al., 2010; Franco et al., 2011; Jossin and Cooper, 2011; Sekine et al., 2012). An emerging picture is arising with distinct molecular programs regulating neuronal migration through the different compartments VZ/SVZ, IZ, and CP (Kwan et al., 2012; Greig et al., 2013; Hippenmeyer, 2014; Hansen et al., 2017; Jossin, 2020). However, the precise regulatory mechanisms which coordinate each and every specific step of radial migration are still largely unknown, let alone the effects and interactions with the extracellular environment. Most studies so far have described and focused mainly on intrinsic cell-autonomous gene functions (**Figure 1A**) in neuronal migration (reviewed in Heng et al., 2010; Valiente and Marín, 2010; Evsyukova et al., 2013) but there is accumulating evidence that non-cell- autonomous-, local-, systemic- and/or whole tissue-wide effects (**Figures 1A,C**) substantially contribute to the regulation of radial neuronal migration (Hammond et al., 2001; Yang et al., 2002; Sanada et al., 2004; Youn et al., 2009; Hippenmeyer et al., 2010; Franco et al., 2011; Hippenmeyer, 2014; van den Berghe et al., 2014; Gorelik et al., 2017; Nakagawa et al., 2019).

NATURE OF NON-CELL-AUTONOMOUS MECHANISMS IN RADIAL PROJECTION NEURON MIGRATION

In any *in vivo* context, cells will always be exposed to a complex extracellular environment consisting of (1) secreted factors acting as potential signaling cues, (2) the extracellular matrix, and (3) other cells providing cell–cell interaction through receptors and/or direct physical stimuli (**Figure 1C**). Therefore, most genes controlling radial neuronal migration can potentially, besides cell-autonomous functions, also act through non-cell-autonomous mechanisms. As such, non-cell-autonomous regulatory cues could involve molecular, cellular, or physical components (**Figure 1C**). Hence, the distinction between cell-autonomous gene function and non-cell-autonomous mechanisms is important to be able to define the different facets of a gene function *in vivo* and thus intact tissue context. Below we will describe recent studies and findings which

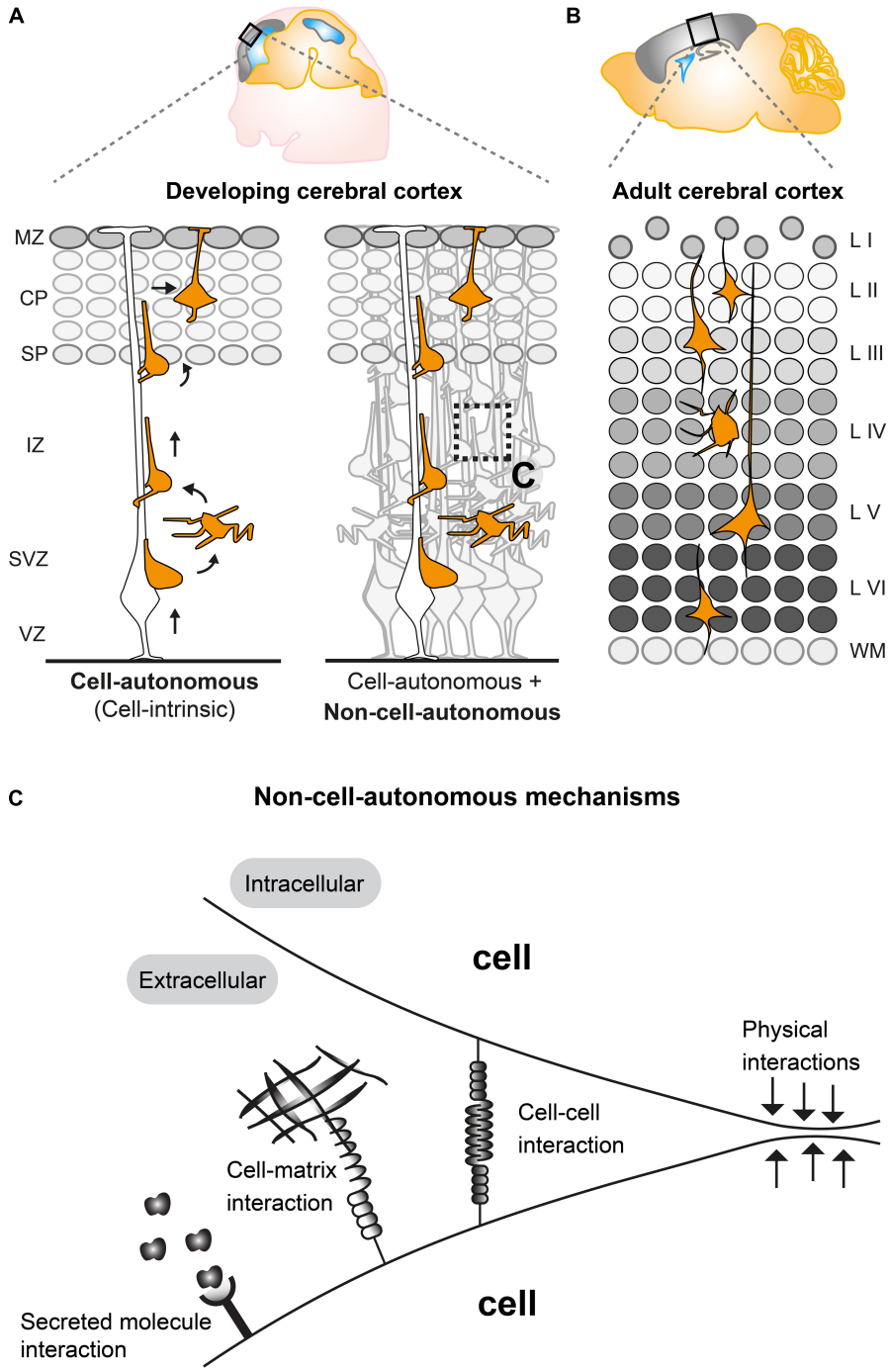

FIGURE 1 | Non-cell-autonomous mechanisms in radial projection neuron migration. **(A)** Migrating cortical projection neurons go through several steps and phases during their journey from their birthplace in the ventricular/subventricular zone (VZ/SVZ) to their final position in the CP. In the left panel, an isolated radially migrating projection neuron is shown to illustrate intrinsic cell-autonomous mechanisms controlling radial migration. The right panel illustrates that radially migrating projection neurons, which are embedded in an environment consisting of many other cells, are potentially influenced (in addition to cell intrinsic cues) through non-cell-autonomous mechanisms (See panel **C**). **(B)** The six layered (I–VI) structure of the adult mouse cerebral cortex. The layers are assembled in an inside out fashion where layers V, VI are the earliest generated and layers II–IV the latest generated cortical projection neurons. **(C)** Possible non-cell-autonomous cellular and molecular interactions during radial projection neuron migration. In any *in vivo* context, cells will always be exposed to a complex extracellular environment consisting of secreted factors acting as potential signaling cues, the extracellular matrix and other cells providing cell–cell interaction through receptors and/or direct physical stimuli. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; WM, white matter; L I-VI, layers 1–6.

have started to describe and characterize non-cell-autonomous effects and mechanisms in projection neuron migration.

Secreted Molecules and the Extracellular Matrix

One of the most apparent non-cell-autonomous interactions includes secreted molecules produced in one cell and eliciting a response in another cell. In addition, interactions with the extracellular matrix are bound to happen for any cell and can occur in various ways. The extracellular matrix provides both structural organization of the cerebral cortex as well as the control of individual neurons. Neuronal migration and lamination is organized by extracellular matrix glycoproteins such as, e.g., laminins, tenascins, proteoglycans, and Reelin (Barros et al., 2011). The specific type of interaction of neurons with secreted molecules and the extracellular matrix and their role in radial neuronal migration have been reviewed recently in detail elsewhere (Franco and Müller, 2011; Maeda, 2015; Long and Huttner, 2019). Here we will briefly elaborate upon a few secreted molecules, mainly Reelin, which play roles in neuronal migration and brain development in general. The Reelin/Dab1 signaling cascade represents one of the best characterized signaling pathways in the developing brain. Reelin is a secreted protein mainly expressed by Cajal-Retzius cells in the MZ of the cortex (Ogawa et al., 1995) and acts via DAB1 in the control of radial projection neuron migration (Rice et al., 1998; Honda et al., 2011). The originally isolated *reeler* mouse mutant and *Dab1* KO mice show a severe disorganization of cortical projection neurons resembling a neocortex layering which is more or less inverted (Caviness and Sidman, 1973). Reelin has been hypothesized to inherit a number of distinct signaling modalities and functions in cortical neuronal migration (Honda et al., 2011; D'Arcangelo, 2014) but the precise role in the local microenvironment of migrating projection neurons is not clear (Jossin, 2020). Yet, Reelin is mainly secreted from the CR-cells in the MZ and processed Reelin fragments has been shown to diffuse from the MZ into the CP and IZ of the developing cortex (Jossin et al., 2007; D'Arcangelo, 2014; Koie et al., 2014). Interestingly, when Reelin is ectopically expressed and secreted by migrating neurons in the IZ, it leads to aggregation of neurons near this ectopic Reelin-rich region resembling the structure of the MZ (Kubo et al., 2010). Furthermore, sequential labeling of migrating neurons revealed that the late-born neurons can still pass by the early-born neurons during the formation of an ectopic Reelin rich aggregate (Kubo et al., 2010). These results indicate that Reelin may have distinct roles in long range versus local signaling. Moreover, a recent study investigating a FMCD-causing (Focal malformations of cortical development) mutation revealed that over activation of AKT3 in a fraction of migrating neurons would lead to misexpression of Reelin in these cells and thereby affect the migration of wild-type neighboring cells in a non-cell-autonomous manner (Baek et al., 2015). Moreover, RNA-seq expression profiling was employed to further investigate the non-cell-autonomous migration defect which could be due to direct physical blockade of the wild-type cells or have a more specific signaling mechanism. The gene ontology enrichment of the 835 significantly deregulated genes identified four main categories for neuronal development, migration, signaling and homeostasis and cell cycle regulation. This suggests that the non-cell-autonomous defect might underlie a more complicated mechanism than just a simple blockade of neurons (Baek et al., 2015). Clearly, the above studies show that global or local expression of a secreted molecule can cause distinct phenotypes, and demonstrating significant non-cell-autonomous impact on projection neuron migration.

Reelin signaling in the control of radial projection neuron migration acts via the intracellular adaptor protein DAB1 (Rice et al., 1998; Honda et al., 2011). Studies applying genetically engineered chimeric mice have suggested that environmental conditions play a role in proper neuronal positioning, and proposed a non-cell-autonomous effect and/or element of *Dab1* function (Hammond et al., 2001; Yang et al., 2002). By using conditional-KO (cKO) mice, in which *Dab1* is specifically deleted after preplate splitting and only in late-born neurons, it was observed that wild-type early born neurons were positioned in the outer layers instead of their usual position in the inner cortical layers. This would suggest that early-born neurons are being "passively" displaced into a deeper position by later-born neurons (Franco et al., 2011). Taken together, the pleiotropy of Reelin-Dab1 loss of function phenotypes could be significantly affected by non-cell-autonomous effects elicited by environmental factors and/or community effects in addition to the cell-autonomous function of Reelin signaling on migrating neurons.

Fibroblast growth factors (FGFs) is a family of secreted molecules and their receptors (FGFRs) were recently shown to play an important role in radial projection neuron migration (Ford-Perriss et al., 2001; Ornitz and Itoh, 2015; Szczurkowska et al., 2018; Kon et al., 2019). A recent study implicated FGFRs in the regulation of the migration orientation of multipolar neurons and the multipolar-to-bipolar transition. It was shown that FGFRs are activated by N-Cadherin when binding in *cis* on the same cell which prevents degradation and results in accumulation of FGFR which stimulate prolonged activation of extracellular signal-regulated kinase (Erk1/2) required for multipolar migration (Kon et al., 2019). In another study, NEGR1, another cell adhesion molecule, was shown to interact with FGFR2 thereby regulating neuronal migration and spine density (Szczurkowska et al., 2018). This study showed that NEGR1 physically interacts with FGFR2 and prevents it from being transported for lysosomal degradation. This accumulation of FGFR2 results in the maintenance of downstream ERK and AKT signaling. These two above studies have shown that FGFR receptors are important in neuronal migration, however the exact response mechanism of secreted FGF ligands is currently unknown. Since a large number of FGFs are expressed in the developing cortex and FGFRs are also activated by heparan sulfate proteoglycans, it is challenging to investigate which and how a specific FGF is involved in neuronal migration (Ford-Perriss et al., 2001; Ornitz and Itoh, 2015). The fact that FGFRs physically interact with different cell adhesion molecules, but act on similar downstream signaling pathways important for neuronal migration, indicates an important general role of FGFR signaling.

Recent findings suggest that alteration of individual neurons might also affect the entire cellular community. As such, a screen identified several potential non-cell autonomous regulators of radial neuronal migration and described autotaxin (ATX*)* to affect the localization and adhesion of neuronal progenitors in a cell autonomous and non-cell autonomous manner (Greenman et al., 2015). In a follow-up study, *Serping1*, a candidate gene identified in the above screen, was found to be expressed and secreted by neurons during brain development and to both affect radial neuronal migration in a cell-autonomous and non-cell-autonomous way (Gorelik et al., 2017). Besides affecting the positioning of the neurons, loss of *Serping1* gene function would also affect the cellular morphology of the neighboring neurons since knockdown neurons exhibited long leading processes which were also observed in the adjacent non-manipulated neurons (Gorelik et al., 2017).

Cell–Cell Interactions Among Migrating Cortical Projection Neurons

It has been observed that migrating neurons can have a positive and negative influence on each other depending on their genetic constitution and the environment. However, the nature of potential positive and/or negative non-cell-autonomous effects and how they affect the migration of mutant and wild-type cortical projection neurons is currently unclear (Hippenmeyer, 2014). Cell–cell interactions during collective cell migration, in a variety of cell types, have indeed been observed previously. Interactions mainly occur when two or more cells that retain their cell–cell contacts move together while coordinating their actin dynamics and intracellular signaling (Friedl and Gilmour, 2009; Tada and Heisenberg, 2012; Londono et al., 2014). Studies looking at collective migration, e.g., in neural crest cells has provided information for the understanding of balanced interaction of cell adhesion and cell signaling between collectively migrating cells. Balancing adhesion and repulsion is one major factor mediating both individual cell and collective migratory coordination (Shellard and Mayor, 2020). Therefore, collective decision making and organization of cells is crucial for the generation of complex tissue and could also apply for the assembly of the cerebral cortex which relies on the migration of neurons. An example of such an collective effect could be physical properties where mutant (which may be less agile) neurons either "piggyback" on adjacent normally migrating neurons or get passively pushed or pulled by a migrating cellular population. Collective influences could also have a negative effect if most or all neurons are mutant and less dynamic, thereby leading to improper migration. Another effect of surrounding neurons could be through signaling, to stimulate or tune down the intrinsic migratory machinery of deficient neurons. This would suggest a mechanism whereby active signaling is utilized through transmembrane receptors and/or extracellular matrix components. Indeed such mechanisms have been described in various cell types where mutant cells negatively affect migration by direct contact inhibition (Huttenlocher et al., 1998; Becker et al., 2013). Upon ectopic expression of cell adhesion molecules, such as N-cadherin, Integrin, Focal adhesion kinase and the focal-adhesion adaptor protein Paxillin in cell culture, direct cell–cell contact inhibited migration. Interestingly, when mutant cells were surrounded by wild-type cells no such effect was seen. Nevertheless, when mutant cells were in direct contact with other mutant cells then the migratory process was inhibited (Huttenlocher et al., 1998; Becker et al., 2013). Although this effect was shown *in vitro* it could also apply to migrating projection neurons *in vivo*. However, in the case of N-cadherin, the cause of inhibited migration could be due to intracellular trafficking and abundance of N-Cadherin rather than expression itself. A study has shown that Rab5-dependent endocytotic-, and a Rab11-dependent recycling pathway regulate N-cadherin trafficking, thereby mediating adhesion between a migrating projection neuron and the radial glial fiber (Kawauchi et al., 2010).

In vivo studies have recently shown that mutant *Ndel1* MADM (mosaic analysis with double markers)-labeled neurons, surrounded by a normal environment, exhibit different migration phenotypes when compared to mutant projection neurons in whole cortex knockout (Youn et al., 2009; Hippenmeyer et al., 2010; Hippenmeyer, 2014). *Ndel1* mutant neurons were incapable of moving in mice with a complete loss of *Ndel1* in the whole cortex, whereas *Ndel1* mutant neurons could migrate through the VZ/SVZ/IZ in a mosaic environment containing wild-type, heterozygous and mutant neurons (Youn et al., 2009; Hippenmeyer et al., 2010). Thus, the comparison of mutant *Ndel1* neurons in mutant versus normal environment clearly suggests a major influence of tissue-wide and/or community effects on radial projection neuron migration. However, the molecular and cellular mechanisms that differentially affect mutant *Ndel1* projection neurons in distinct environments remain unknown. Interestingly, differential gene expression analysis of brains from wild-type mice and full knock out mouse models for *Ndel1* (and *Lis1*, and *Ywhae* acting in the same signaling pathway) have revealed that cell adhesion, and cytoskeleton organization pathways are commonly altered in these mutants (Pramparo et al., 2011). Since cell adhesion is one of the commonly identified deregulated pathways, it would be obvious to speculate that the non-cell-autonomous response could be emerging from cell–cell or cell–matrix interactions and in the end cause the developmental phenotype observed in, e.g., *Ndel1* knockout mice.

P35 is the main activator of CDK5, a serine/threonine kinase mainly expressed in the brain (Su and Tsai, 2011; Kawauchi, 2014). In a study investigating *p35,* it was found that when rescuing *p35* in a subset of neurons in an otherwise *p35*-deficient environment, rescued neurons would migrate 'normally' like wild-type neurons, indicating a prominent cell-autonomous gene function of *p35* (Gupta et al., 2003). However, in a follow-up study using *p35* chimeras (creating a mix of wild-type and *p35* deficient neurons), a partial non-cell-autonomous rescue of *p35* mutant neurons was seen. Interestingly, within the *p35* chimeras it was observed that mutant cells were always present in a higher proportion compared to wild-type cells. These data indicate a certain degree of disadvantage of the wild-type neurons within the mutant cortical landscape, which could be due to non-cell-autonomous effects (Hammond, 2004). While p35/Cdk5 signaling may significantly influence how neurons

interact with one another the nature of these interactions are currently unclear. These interactions however likely involve cell–cell adhesion and/or other community effects (Kwon et al., 2000; Hammond, 2004; Kawauchi, 2012, 2014). Interestingly, the Reelin-DAB1 pathway (see above) has also been shown to control cell-adhesion during neuronal migration (Sekine et al., 2014). Thus a common component of the underlying mechanisms inherent to non-cell-autonomous effects, and as observed in *p35* and *Dab1* mutant, may be acting through similar cell-adhesion signaling modules.

Heterogeneous Cell–Cell Interactions of Migrating Cortical Projection Neurons

The developing brain consists of a heterogeneous mix of different cell types. Therefore, cell–cell interaction between distinct cell types, e.g., a radial glial cell and a migrating neuron, is one such example. Most radially migrating neurons are dependent on the radial glial fiber on which they locomote to move toward the pial surface and surpass earlier born neurons (Rakic, 1972; Nadarajah et al., 2001; Kriegstein and Noctor, 2004). Hence, the migrating neurons are dependent on a proper RGC fiber grid to be able to migrate properly. Indeed, disruption of the proper organization of the RGC fiber grid leads to non-cell-autonomous migration phenotypes because the main substrate of migrating neurons is perturbed (Belvindrah et al., 2007; Cappello et al., 2012; Nakagawa et al., 2019). Such findings initially emerged in a study investigating beta1 integrins in neuronal development. In a KO mouse model which lacks beta1 integrin in the entire central nervous system, consequently in both radial glia cells and neurons, the formation of cortical layers were affected due to perturbations in the radial glial end feet contacting the marginal zone (Graus-Porta et al., 2001). Moreover, the morphology of the apical dendrites of the pyramidal neurons was also perturbed. However, when ablating beta1 integrin specifically in neurons that migrate along radial glial fibers, and not in the radial glia cells themselves, no neurodevelopmental defect was observed (Belvindrah et al., 2007). These findings clearly showed that when one indispensable cell type (in this case the radial glial cell) was impaired, it indirectly affected the migrating neurons and resulted in disrupted layering of the cortex due to non-cell-autonomous effects (Belvindrah et al., 2007). Furthermore, investigation of the interaction of Cajal Retzius (CR) cells and migrating neurons has shown that perturbation of *Nectin1* function in CR cells alone would affect the interaction of CR cells and the leading processes of migrating neurons (Gil-Sanz et al., 2013). This altered interaction non-cell-autonomously disturbed radial glial cell-independent somal translocation of radially migrating neurons in the cortical plate (Gil-Sanz et al., 2013).

A recent study investigating *Memo1* showed that cKO in neurons and glia would cause excessive branching of the basal processes of the RGCs resulting in altered tiling of the RGC scaffolding grid and aberrant lamination of neurons (Nakagawa et al., 2019). However, deletion of *Memo1* only in post-mitotic neurons, and not RGCs, did not affect neuronal migration. Therefore, the altered tiling of the RGCs non-cell-autonomously perturbed neuronal migration and thereby caused abnormal lamination of the cortex (Nakagawa et al., 2019).

In *Flrt1/3* double-knockout mice, which develop macroscopic cortical sulci, it was found that the lack of *Flrt1/3* resulted in reduced intercellular adhesion which lead to a mild acceleration of radially migrating neurons and enhanced clustering of neurons along the tangential axis (del Toro et al., 2017). The clustering of neurons was hypothesized to result from repulsive interactions with neighboring neurons and radial glial cells suggesting a non-cell-autonomous effect of the *Flrt1/3* ablation on radial neuronal migration (Seiradake et al., 2014; del Toro et al., 2017). In a subsequent study it was shown that Teneurins, Latrophillins and FLRTs interact and direct radial neuronal migration by slowing down migration by possible coincidence contact repulsion between the neurons and the radial glia cells (del Toro et al., 2020).

Taken altogether, the above observations suggest that neuronal migration and proper lamination of the developing neocortex are significantly affected by non-cell-autonomous components. However, the precise underlying cellular and molecular mechanisms of non-cell-autonomous effects on radial neuronal migration have yet to be explored by rigorous qualitative and quantitative means. The lack of information on non-cell-autonomous effects is mainly due to the limitation of experimental assays that allow for investigation of such events *in vivo* and with single cell resolution. To this end, in the below section we illustrate contemporary experimental paradigms that have the potential to systematically analyze non-cell-autonomous mechanisms in radial migration of cortical projection neurons.

CELLULAR ASSAYS TO ANALYZE AND GENETICALLY DISSECT NON-CELL-AUTONOMOUS MECHANISMS IN CORTICAL PROJECTION NEURON MIGRATION *IN VIVO*

In this section we will specifically elaborate on the experimental paradigms which can be utilized to dissect non-cell-autonomous mechanisms in cortical projection neuron migration.

Chimeras

A chimera is an animal that has two or more populations of genetically distinct cells. Therefore, chimeric animals allow for the presence of mutant cells in an otherwise wild-type background or *vice versa*. Depending on the degree of chimerism (i.e., ratio of wild-type versus mutant cells) such assay offers one way to distinguish between cell-autonomous gene function and non-cell-autonomous mechanisms *in vivo* (**Figure 2A**) (Gilmore and Herrup, 2001; Hammond et al., 2001; Hammond, 2004). Any phenotypic difference seen between the neurons of the same genotype, but present in distinct genotypic environments indicate non-cell-autonomous effects. However, the degree of chimerism is hard to control, especially in the embryo. Therefore comparative studies across distinct individual animals may be

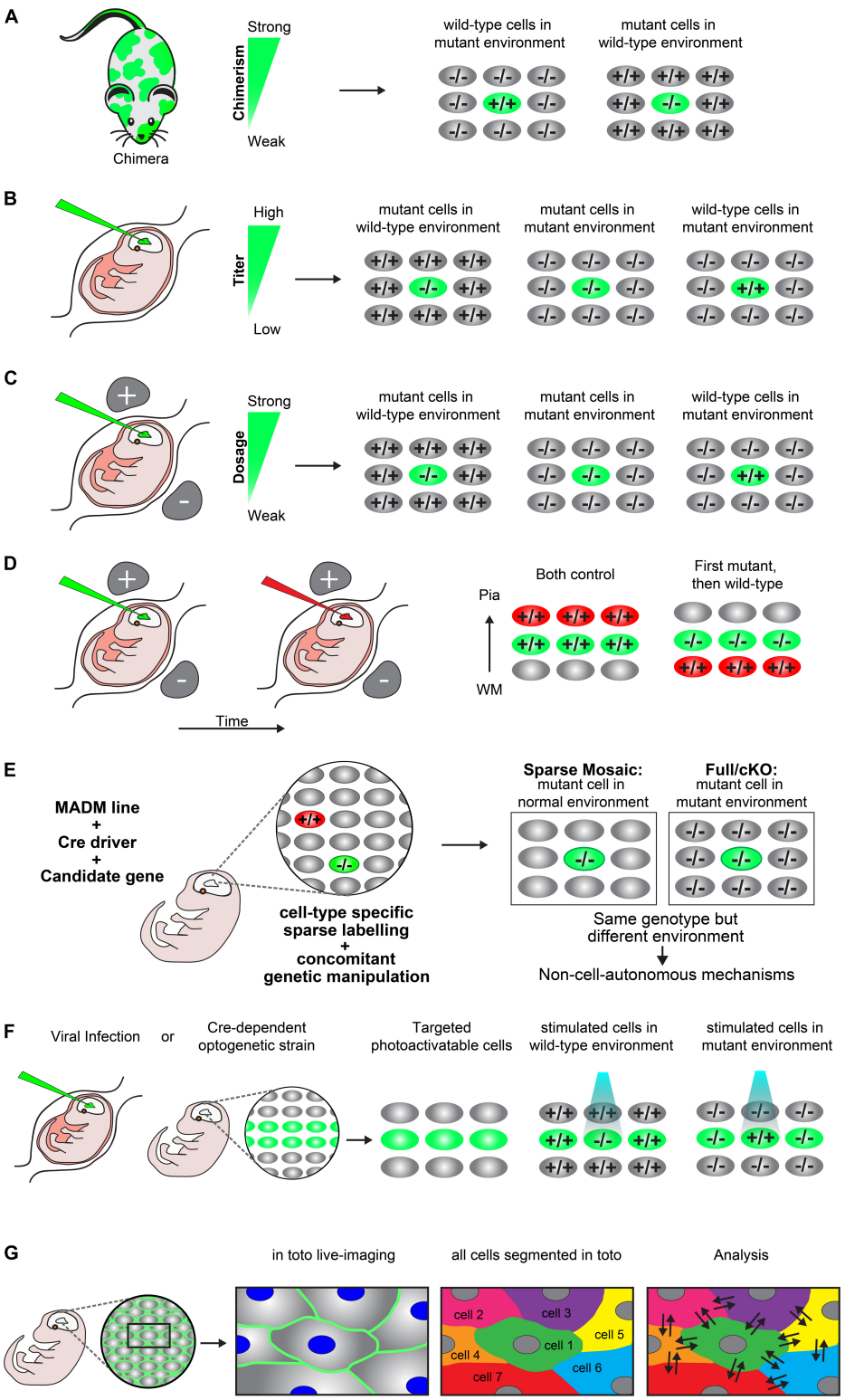

FIGURE 2 | Experimental paradigms to genetically dissect non-cell-autonomous mechanisms in radial cortical neuron migration. **(A)** Chimeras. A chimera is an animal that has two or more populations of genetically distinct cells. Depending on the degree of chimerism (i.e., ratio of wild-type versus mutant cells), such assay offers one way to distinguish between cell-autonomous gene function and non-cell-autonomous mechanisms *in vivo*. Any phenotypic difference seen between the neurons of the same genotype, but present in distinct genotypic environments indicate non-cell-autonomous effects. **(B)** Retroviral infection. Retroviral infection allows to sparsely target developing neurons by either expression of the reporter only (e.g., in a wild-type or mutant environment) or using a viral vector that encodes

(Continued)

FIGURE 2 | Continued
a wild-type or mutant version of the gene of interest in combination with a reporter. This facilitates the inactivation or rescue of the gene of interest in either wild-type or mutant environments, allowing for the distinction of cell-autonomous gene function and non-cell-autonomous effects. Appropriately diluted retrovirus encoding the reporter and gene of interest allows for the discrimination of individual neurons and one can adjust the viral titer to generate more or less sparsely targeted neuronal populations. **(C)** *In utero* electroporation. Timed *in utero* electroporation for inactivation of a gene allows the sparse targeting of nascent migrating neurons in an otherwise wild-type environment. The inactivation of a specific gene can either be achieved by gene knockdown in combination with a reporter in a wild-type mouse or by electroporation of an expression vector which drives expression of CRE and a reporter into a mouse carrying a conditional floxed allele. In this paradigm one can mainly dissect the cell-autonomous gene function in the targeted neurons, although the presence of non-cell-autonomous effects provided by the wild-type environment will be present (mutant cells in wild-type environment). To investigate non-cell-autonomous effects, it is necessary to electroporate of a separate set of tissue only with the fluorescent reporter in an otherwise mutant environment (mutant cells in mutant environment). Thus, neurons mutant for the same gene in two different environments allows for the distinction of non-cell-autonomous effects, provided that a different phenotype is observed between the mutant cells in each specific environment. Wild-type neurons in an otherwise mutant background by (over)expression of a rescue construct would further allow determination of non-cell-autonomous effects originating from the mutant environment (wild-type cells in mutant environment). The comparison of these three distinct paradigms will facilitate detailed description of cell-autonomous gene function and non-cell-autonomous effects. **(D)** Consecutive electroporation. Consecutive electroporation enables labeling, genetic manipulation and the monitoring of two or more distinct neuronal populations in the developing embryonic brain. The first neuronal population is electroporated for gene knockdown and the consecutive population with control fluorescent markers or vice versa (first mutant, then wild-type). In such assay, the phenotype of the first cohort of electroporated cells can reflect cell-autonomous gene function whereas the phenotype of the second cohort of cells could reflect a combination of directed non-cell-autonomous effects originating from the first cohort and more global community effects. **(E)** MADM. Mosaic analysis with double markers (MADM) allows for the analysis of sparse genetic mosaic (sparse mosaic) versus global/whole tissue (full-KO) ablation of a candidate gene with single cell resolution. This allows to quantitatively analyze non-cell-autonomous effects by subtracting the phenotype present in the sparse mosaic from the full-KO (cell-autonomous + non-cell-autonomous) versus cell-autonomous (sparse mosaic). It is important to note that the background cells in a MADM sparse mosaic are heterozygous and may need adjustment of the paradigm in the case of investigation of a dosage-sensitive gene (haploinsufficiency). In that case, the MADM experiment can also provide a solution by comparing all genotypes/colors, e.g., green –/–, red + / + and yellow ±. For details of such application the reader is referred to Hippenmeyer et al., 2010. **(F)** Optogenetics. Optogenetics facilitates the use of genetically encoded tools to temporally control gene expression or protein function with light. Viral infection approaches and transgenic mice expressing optogenetic effector proteins in a Cre-dependent manner can be utilized to generate photoactivatable tissue. These approaches can create experimental paradigms which enable investigation of mutant neurons in an otherwise wild-type environment vs. wild-type neurons in a mutant environment in a spatiotemporal manner **(G)** *In toto* imaging. *In toto* live-imaging can visualize the movement of individual cells and their interactions with the surrounding cells within the whole developing tissue. This would allow for a direct assessment of non-cell-autonomous effects exerted by the neighboring cells on an individual cell or *vice versa*. *In toto* imaging mostly involves labeling of all cell membranes so each cell in the organism/microenvironment can be tracked and segmented. Here, a two-color combination of a membrane-localized fluorescent protein and a histone-fused fluorescent protein labeling chromatin which allows for tracking the cell membrane morphologies and nuclei movement has been displayed. Tracking the exact cell boundaries of the neurons spatiotemporally would enable the mapping of the physical interactions and forces which are exerted by the individual cell and that of the surrounding cells.

challenging. Yet, a few studies have very successfully applied chimeras to study radial neuronal migration in the cerebral cortex and have described the presence of non-cell-autonomous effects (Hammond et al., 2001; Yang et al., 2002; Hammond, 2004).

Viral Infection

In utero injection of virus encoding a reporter, e.g., green fluorescent protein (GFP) has widely been used to investigate neuronal migration, lineage tracing and clonal analysis *in vivo* (Gaiano et al., 1999; Malatesta et al., 2000; Kaspar et al., 2002; Gupta et al., 2003; Sanada et al., 2004; Stott and Kirik, 2006; He et al., 2015). Retroviral encoding allows to sparsely target developing neurons by either expression of the reporter only (e.g., in a wild-type or mutant environment) or using a virus vector that encodes a wild-type or mutant gene of interest in combination with a reporter. This facilitates the inactivation or rescue of the gene of interest in either wild-type or mutant environments, allowing for the distinction of cell-autonomous gene function and non-cell-autonomous effects (**Figure 2B**). Appropriate dilution of the retrovirus titer and thus lowering infection rate allows for the discrimination of individual neurons and one can generate more or less sparsely targeted neuronal populations (Noctor et al., 2001; Sanada et al., 2004). In addition, delivery of an adeno-associated virus (AAV) encoding a fluorescent protein and Cre recombinase in combination with a reporter mouse carrying a conditional floxed allele of a candidate gene of interest, can be also be used to target a specific population of neurons (Kaspar et al., 2002). Generally, any approach using a virus which can infect the cell population of interest, achieve specific stable gene expression and reporter labeling can be used to create paradigms for studying cell-autonomous gene function and non-cell-autonomous effects in radial projection neuron migration.

In utero Injection and Electroporation

Timed *in utero* electroporation for inactivation of a gene allows for the sparse targeting of developing neurons in an otherwise wild-type environment (**Figure 2C**). The inactivation of a specific gene can either be achieved by electroporation of shRNA or miRNA for gene knockdown, in combination with a reporter in a wild-type animal. Alternatively, electroporation of an expression vector which drives expression of CRE and a reporter in a mouse carrying a conditional floxed allele of a candidate gene of interest, can be used (Franco et al., 2011). These paradigms permit the dissection and analysis of cell-autonomous gene function in the targeted neurons. However, the presence of non-cell-autonomous effects originating from the wild-type environment may be present but not easily visualized (**Figure 2C**). Most studies so far have used this paradigm to study cell-autonomous gene function (Franco et al., 2011; Jossin and Cooper, 2011; Szczurkowska et al., 2018; Kon et al., 2019). To investigate non-cell-autonomous effects and mechanisms one would also need a separate set of tissue only electroporated with the fluorescent reporter to sparsely label the already mutant neurons in an otherwise non-labeled mutant environment (**Figure 2C**). Having neurons mutant for the same gene in two different environments would

allow for the distinction of non-cell-autonomous mechanisms, provided that a different phenotype is observed between the mutant cells in each specific environment. In addition, having wild-type neurons in an otherwise mutant background by (over)expression of a rescue construct would further permit the determination of non-cell-autonomous effects originating from the mutant environment (**Figure 2C**). Only a few studies have applied this paradigm of rescuing a few cells sparsely in a mutant environment (Gupta et al., 2003; Sanada et al., 2004). Similar to chimeras (**Figure 2A**), it is important to consider the ratio of the mutant versus wild-type cells. For instance, sparse electroporation allows for the investigation of the direct interaction of cells of distinct genotypes. However, generating a high amount of mutant cells within the wild-type environment might create a local mutant microenvironment where specific interactions between mutant cells could dominate. As a consequence, the presence of a local mutant microenvironment would make it difficult to distinguish cell-autonomous from non-cell-autonomous responses. Therefore, the amount of the electroporated cells should be considered carefully. While sparse single cell deletion of a candidate gene may truly report cell-autonomy of gene function, progressive local increase in the number of mutant cells may lead to a sweet spot from which onward non-cell-autonomous community effects emerge (Nakagawa et al., 2019). Another way of generating very sparse populations of cells using this method, is to transplant micro dissected mutant cells from electroporated corticies to either another wild-type or mutant brain by intraventricular injection (Elias et al., 2007).

Consecutive electroporation enables cellular labeling, genetic manipulation and the monitoring of two or more distinct neuronal populations in the developing embryonic brain (**Figure 2D**). The first neuronal population could be electroporated for gene knockdown and the consecutive population with control fluorescent markers or vice versa. In such assay, the phenotype of the first cohort of electroporated cells can reflect cell-autonomous gene function whereas the phenotype of the second cohort of cells could reflect a combination of directed non-cell-autonomous cues originating from the first cohort and more global community effects (Jossin and Cooper, 2011; Gil-Sanz et al., 2013; Baek et al., 2015; Greenman et al., 2015).

In summary, sparse *in utero* electroporation for gene knockdown or CRE-dependent conditional gene inactivation in combination with fluorescent reporters facilitates the comparison of mutant phenotypes in distinct cellular environments. Such comparative studies, in principle, enable the systematic dissection of cell-autonomous gene function and/or phenotypes in response to gene inactivation and non-cell-autonomous mechanisms in radial neuronal migration.

Mosaic Analysis With Double Markers (MADM)

Mosaic analysis with double markers (MADM) technology allows for the analysis of sparse genetic mosaic (sparse mosaic) versus global/whole tissue (full-KO) ablation of a candidate gene, and with single cell resolution (**Figure 2E**) (Zong et al., 2005; Hippenmeyer et al., 2010; Beattie et al., 2017; Laukoter et al., 2020). Therefore MADM provides a unique genetic tool to investigate cell-autonomous gene functions and the relative contribution of non-cell-autonomous effects. By using MADM one can quantitatively analyze these effects (**Figure 2E**) (Youn et al., 2009; Hippenmeyer et al., 2010; Hippenmeyer, 2014). In the sparse mosaic animals, mutant neurons are surrounded by 'normal' neurons and therefore mainly provide information about cell-autonomous gene function. In addition, the presence of non-cell-autonomous effects originating from the 'normal' environment may be present but not easily measured. In the full-knockout of a particular candidate gene, mutant neurons are surrounded by other mutant neurons, and it is not straightforward to distinguish between cell-autonomous gene function and non-cell-autonomous effects. However, one could quantitatively deduct non-cell-autonomous effects by subtracting the phenotype present in the sparse mosaic from the full/cKO (cell-autonomous + non-cell-autonomous versus cell-autonomous (sparse mosaic) (**Figure 2E**). The sparse mosaic versus full/cKO paradigm thus offers a promising experimental platform to investigate non-cell-autonomous effects because any phenotypic differences observed when the two paradigms are compared can be quantitatively assessed at single cell resolution (Beattie et al., 2017; Laukoter et al., 2020). Nevertheless, generating a full-knockout where all cells are mutant for a particular candidate gene can be problematic since many migration genes are lethal when knocked out completely (Hirotsune et al., 1998; Sasaki et al., 2005). Conditional-knockout mice could be analyzed, provided that floxed alleles are available. In the future, systematic assay of almost any candidate gene will be in principle enabled by the whole-genome MADM library resource (Contreras et al., 2020).

Optogenetics

Optogenetics facilitates the use of genetically encoded tools to temporally control gene expression or protein function with light. It can facilitate localized modifications spatiotemporally within living cells and animals, targeting a wide array of proteins, e.g., involved in cell-migration, cell–cell adhesion, and force transduction (Guglielmi et al., 2016; Mühlhäuser et al., 2017). Using this method one can investigate how changes in individual cells influence neighboring cells and global tissue remodeling. So far, most experiments applying optogenetics for studying cell-migration have mainly been applied to *in vitro* cell culture systems and small *in vivo* systems, e.g., during gastrulation (Wang et al., 2010; Kim et al., 2014; Weitzman and Hahn, 2014; Valon et al., 2017; Wang and Cooper, 2017). In the mouse brain, optogenetics have mainly been used to activate, inhibit, or detect neuronal activity (Montagni et al., 2019). However, spatiotemporal control of the expression of a candidate gene or the activity of a specific signaling pathway could provide valuable insights into the dissection of non-cell-autonomous mechanisms in projection neuron migration. Currently, various viral infection approaches and transgenic mice expressing optogenetic effector proteins in a Cre-dependent

manner can be utilized to generate photoactivatable cells and tissue (Madisen et al., 2012; Guglielmi et al., 2016). These approaches can create experimental paradigms similar to the ones described above (mutant neuron in an otherwise wild-type environment vs. wild-type neurons in a mutant environment) (**Figure 2F**), however, with spatiotemporal control of gene expression or protein function. This would allow exact targeting of specific neurons at specific sequential steps along the migratory path. In addition, one would be able to perturb cells of a specific cohort to see the exact non-autonomous effects on the surrounding non-stimulated neurons (**Figure 2F**). An interesting aspect for which an optogenetic approach could also provide information is to what extent the ratio of mutant and wild-type cells in the same tissue is needed to see non-cell-autonomous effects. Starting from targeting only one cell and then increasing the area which is activated by light stimulation could reveal the threshold for when non-cell-autonomous mechanisms emerge dependent on the cell ratio of mutant vs. wild-type present. However, for *in vivo* and *in situ* experiments of mouse tissue, such an optogenetic approach might prove technically difficult. Activating one specific moving cell or a certain area of the tissue with a beam of light can be quite difficult *in vivo* and in three-dimensional intact tissues.

In toto Live-Imaging

In toto live-imaging can visualize the movement of individual cells and their interactions with the surrounding cells within the whole developing tissue (Megason and Fraser, 2007; Veeman and Reeves, 2015; McDole et al., 2018). This would enable a direct assessment of non-cell-autonomous effects exerted by the neighboring cells on an individual cell or *vice versa*. So far, this method has mostly been used to visualize cell and collective migration behaviors in smaller *in vivo* systems such as, e.g., *Drosophila* (Krzic et al., 2012; Tomer et al., 2012), zebrafish (Nogare et al., 2017; Hiscock et al., 2018; Shah et al., 2019) and larger systems such as mouse gastrulation and heart tissue (Megason and Fraser, 2007; Stewart et al., 2009; McDole et al., 2018; Yue et al., 2020). *In toto* imaging mostly involves labeling of all cell membranes so each cell in the organism/microenvironment can be tracked and segmented (Nogare et al., 2017). In addition, both the cell membranes and the cell nuclei can be labeled in two individual colors for a more precise segmentation which does not rely on estimation. For *in vivo* studies of embryogenesis, a two-color combination of a membrane-localized fluorescent protein and a histone-fused fluorescent protein labeling chromatin enables the tracking of the cell membrane morphologies and nuclei movements (Megason, 2009; Stewart et al., 2009). Tracking all cells in an area of interest and their physical interactions would allow for a much more detailed analysis of the cellular dynamics which are ongoing during neuronal migration (**Figure 2G**). Achieving a resolution in which the exact cell boundaries of the neurons could be tracked spatiotemporally would enable the mapping of the physical interactions and forces which are exerted by the individual cell and that of the surrounding cells. Such mapping could help understand where and when certain cell dynamics are being subjected to non-cell-autonomous forces that evoke a response in the individual cell from the surrounding environment or *vice versa*. Future development of imaging approaches like, e.g., light-sheet microscopy could facilitate the spatiotemporal resolution needed to visualize the migration of interacting neighboring cells in bigger tissues like the mouse cerebral cortex.

OUTLOOK

Non-cell-autonomous mechanisms play an important role during brain development. However, little is known about the exact nature and physiological function of these non-autonomous mechanisms in radial neuronal migration. Thus, a number of open key aspects and questions require attention in future investigations. First, how can non-cell-autonomous mechanisms be distinguished from cell-autonomous cues and intrinsic gene function? Second, how can non-cell-autonomous effects be quantified and the underlying mechanisms determined? Third, what role do non-cell-autonomous mechanisms play in disease? Focal malformations of cortical development (FMCD) represent one example of a disorder where a localized cortical lesion, i.e., mutations in a small fraction of cells, disrupts the entire cortical architecture. In the most severe cases, devastating pediatric hemimegalencephaly may emerge, which is characterized by enlargement of one entire cerebral cortex hemisphere (Lee et al., 2012; Poduri et al., 2012, 2013; Rivière et al., 2012). Hence, it is also important from a clinical perspective to precisely dissect the contribution of non-cell-autonomous, tissue-wide and systemic mechanisms in cortical development in general and neuronal migration in particular. The better understanding of the interplay of cell intrinsic gene function and non-cell-autonomous effects will enable further comprehension of the underlying etiology of neurodevelopmental disorders due to genetic mutations (Guerrini et al., 2008; Guerrini and Parrini, 2010).

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Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Radial Migration Dynamics Is Modulated in a Laminar and Area-Specific Manner During Primate Corticogenesis

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The orderly radial migration of cortical neurons from their birthplace in the germinal zones to their final destination in the cortical plate is a prerequisite for the functional assembly of microcircuits in the neocortex. Rodent and primate corticogenesis differ both quantitatively and qualitatively, particularly with respect to the generation of neurons of the supragranular layers. Marked area differences in the outer subventricular zone progenitor cell density impact the radial glia scaffold compactness which is likely to induce area differences in radial migration strategy. Here, we describe specific features of radial migration in the non-human primate, including the absence of the premigratory multipolar stage found in rodents. *Ex vivo* approaches in the embryonic macaque monkey visual cortex, show that migrating neurons destined for supragranular and infragranular layers exhibit significant differences in morphology and velocity. Migrating neurons destined for the supragranular layers show a more complex bipolar morphology and higher motility rates than do infragranular neurons. There are area differences in the gross morphology and membrane growth behavior of the tip of the leading process. In the subplate compartment migrating neurons destined for the supragranular layers of presumptive area 17 exhibit radial constrained trajectories and leading processes with filopodia, which contrast with the meandering trajectories and leading processes capped by lamellipodia observed in the migrating neurons destined for presumptive area 18. Together these results present evidence that migrating neurons may exhibit autonomy and in addition show marked area-specific differences. We hypothesize that the low motility and high radial trajectory of area 17 migrating neurons contribute to the unique structural features of this area.

Keywords: cerebral cortex, macaque, area-specific, supragranular neurons, migration

INTRODUCTION

Radial migration of glutamatergic neurons from their birthplace in the germinal zones (GZ) to their final destination in the cortical plate (CP) is a complex process requiring a series of highly coordinated cellular events. Pasko Rakic established the crucial role of radial migration- where pyramidal glutamatergic neurons follow a trajectory that is perpendicular to the ventricular surface, and parallel to radial glial fibers (Rakic, 1972; Misson et al., 1991)-thereby translating the topography of the proliferative fate map in the ventricular zone to the CP (Rakic, 1988; Dehay et al., 1993; Polleux et al., 1997). Birthdating studies showed that layers II–VI of the cerebral cortex are generated in an "inside-out" sequence (Rakic, 1974). Neurons generated early reside in deeper infragranular (IG) layers (layers 5, 6), whereas late born neurons after completing their migration form the superficial, supragranular (SG) layers (layers 2–4).

Several studies have shown that electrical coupling between sister excitatory neurons ensures an important early step in the functional development of the cortex (Yu et al., 2009, 2012; Li Y. et al., 2012). During their radial migration sister excitatory neurons progressively and selectively form gap junctions with each other (He et al., 2015). These observations suggest that the spatial precision of radial migration is a key determinant of highly specific neuronal connectivity as has been shown in the spinal cord (Surmeli et al., 2011). These observations suggest that the area differences in progenitor cell and radial glia scaffold densities could require different migration strategies (Lukaszewicz et al., 2005; Betizeau et al., 2013).

In primates there are two features which could lead to differences in the migrational strategies with respect to those observed in rodents. Firstly, there is a massive increase in the thickness of the cortical subplate (SP), a partially transient compartment of the embryonic neuroepithelium (Kostovic and Rakic, 1990; Smart et al., 2002). The cortical SP increases throughout corticogenesis reaching its maximum extent during the final stages of corticogenesis when the SG layers are being generated. This results in the migratory trajectory of SG layers neurons in primates compared to rodents being multiplied by at least a factor of 10, which could be expected to lead to adaptive mechanisms to ensure accurate and timely arrival of postmitotic neurons to their final location in the brain. Secondly, in the primate, the temporal sequence of rates of neuronal production departs largely from that of the rodents. While in the rodent cortical progenitors show declining proliferative capacities overtime (Smart, 1972; Takahashi et al., 1995a,b), primate cortical neuron production is characterized by a late upsurge of proliferation of the outer subventricular zone (OSVZ) progenitors generating the SG layers (Lukaszewicz et al., 2005; Dehay and Kennedy, 2007; Hansen et al., 2010; Betizeau et al., 2013). This increased SG neuron production is responsible for the selective enlargement of SG layers in the human and non-human primate (Hutsler et al., 2005; Betizeau et al., 2013), which in turn could play a significant role in the increased computational and cognitive abilities in this order (Harris and Mrsic-Flogel, 2013).

Because rates of neuron production and migration have to be coupled to ensure a coherent process, the spatiotemporal variations in neuron production rates combined with changing migratory distances for different populations of neurons might put important constraints on neuron migration. Hence, we hypothesize that differences in the tempo of neuron production will require regulation of radial migration dynamics, that can be optimally explored by comparing neuron migration across layers and areas. Given the marked differences in rates of neurogenesis in visual areas 17 and 18 we have explored the morphodynamic properties of IG and SG migrating neurons traversing the subplate (SP) of these two areas (Lukaszewicz et al., 2005, 2006).

Based on *ex vivo* observations of organotypic slices of embryonic macaque cortex, we provide evidence of primate-specific features of migration. In particular, we show that primate early postmitotic neurons exhibit a bipolar morphology at the pre-migratory stage, in sharp contrast with the multipolar shape described in rodents (Tabata et al., 2009). We describe distinct morphodynamic features of IG and SG neurons during radial migration on organotypic slices of embryonic cortex. Migrating SG neurons exhibit a more complex gross morphology and significantly higher motility rates than do IG neurons in both areas. Remarkably, A17 and A18 SG neurons exhibit different radial migration strategies: whereas A17 neurons migrate according to a radial axis, A18 SG neurons follow curvilinear, ab-radial trajectories. Using an *in vitro* assay, which recapitulates the area-specific differences in migration trajectories, we further characterize the morphology of radial migrating neurons in both areas. Specifically, we identified distinctive features of the growing tip of the leading process in SG migrating neurons from A17 and A18 that we hypothesize could be related to the radial and ab-radial modes of migration. The fact that the area differences in gross morphology along with differences in area migration strategy were preserved *in vitro*, point to cell autonomous properties.

In sum, the comparative analysis of migrating neuronal populations to different layers (IG and SG) and areas (A17 and A18) highlights laminar and area-specific characteristics of radial migratory rates and trajectories in the primate cortex.

MATERIALS AND METHODS

Primates

The cynomolgus monkey (*Macaca fascicularis*) facility in this study and all experimental protocols were approved by the Animal Care and Use Committee CELYNE (C2EA#42). The animals were housed in a controlled environment (temperature: 22 ± 1°C) with 12 h light/12 h dark cycle (lights on at 08:00 a.m.). All animals were given commercial monkey diet twice a day with tap water *ad libitum* and were fed fruits and vegetables once daily. During and after experiments, monkeys have been under careful veterinary oversight to ensure good health. Fetuses from timed-pregnant cynomolgus monkeys (*M. fascicularis*) were delivered by cesarean section according to protocols

described in Lukaszewicz et al. (2005). Surgical procedures and animal experimentation were in accordance with European requirements 2010/63/UE. Protocols C2EA42-12-11-0402-003 and APAFIS#3183 have been approved by the Animal Care and Use Committee CELYNE (C2EA #42).

Plasmids

pCMV-EGFP retrovirus (Betizeau et al., 2013) were produced by M. Afanassieff (SBRI, INSERM U1208 Bron, France) via pTG5349, pTG13077 (Transgene SA, Illkirch-Graffenstaden, France), and phCMV-G [Gift from D. Nègre ENS Lyon (Yee et al., 1994)].

mCherry construct is as follows. NheI-mcherry-XhoI PCR of mCherry cDNA, from pmCherry-N1 plasmid from Clontech (PT3974-5), was cloned into modified pEGFPC1 plasmid from Clontech (ref 6084-1) where EGFP-C1 was previously switched for MCS NheI-SmaI-EcoRV-ClaI-XhoI.

Organotypic Slice Culture

Lethally anesthetized E63-E65 and E77-E80 fetuses were perfused through the heart with cold supplemented HBSS (Gibco, 14180046) (HBSS with glucose 18%, MgSO4 and CaCl2).

Occipital poles of embryonic hemispheres were isolated and embedded in 3% low-gelling agarose (Sigma, A9045) in supplemented HBSS at 37°C. 300 μm-thick parasagittal slices were cut in 4°C supplemented HBSS using a vibrating blade microtome (Leica VT1000 S). Slices were mounted on Laminin (10 μg/ml)/Poly-L-lysine (100 μg/ml) (Sigma, L2020 and P1399) coated 0.4 μm Millicell Culture Insert (Millipore, PICM0RG50) on a drop of type I collagen (BD Biosciences, 354236). Slices were cultured at 37°C and 7.5% CO_2, in 6-well plates in 1.2 mL of GMEM/10% FCS: Glasgow minimum essential medium (GMEM, Gibco, 21710-025) supplemented with 1% sodium pyruvate (Gibco, 11360-039), 100 μM beta-mercapto-ethanol (Gibco, 31350-010), 1% non-essential amino acids (Gibco, 11140-035), 2 mM glutamine, 1% penicillin/streptomycin (Gibco, 10378-016), and 10% fetal calf serum (FCS, Pan Biotech, P30-2600). Culture medium was renewed twice a day.

Retroviral Infection in Embryonic Primate Cortex

Cycling progenitors in the germinal zones were infected with a pCMV-EGFP retrovirus. Floating E63-E65 and E77-E80 cortical slices (300 μm thick) were incubated in GMEM (Gibco, 21710-025) culture medium containing pCMV-EGFP retrovirus (1–5.10^5 pi/mL), for 2–3 h at 37°C. The slices were then mounted on a Millicell Culture Insert system on a drop of type I collagen (see above for detailed procedure).

Dissociated Subplate (SP) Cell Culture and Lipofection

The SP was isolated from parasagittal slices via manual microdissection. SP were dissociated with trypsin 1X (Gibco, 1540054) for 3 min at 37°C, manually triturated, washed in GMEM/10% FCS and centrifuged for 5 min at 1000 rpm. Individual cells were plated at 5.10^4 cells per well on poly-L-Lysine (Sigma, P1399,100 μg/ml)/Laminin (Sigma, L2020, 10 μg/ml) coated lab-tek (Thermo Scientific, 155409) or at 1.10^5 cells on 14 mm diameter poly-L-Lysine (100 μg/ml) /Laminin (10 μg/ml) coated glass cover slips. Cells were maintained for 1 DIV in GMEM/10% FCS medium, before being transfered into Neurobasal A medium (Gibco, 10888-022) supplemented with B27 (1:50^e, Gibco, 17504-044), N2 (1:100^e, Gibco, 17502-048) and PSG (1X, Gibco, 10378-016) and maintained at 37°C in 7.5% of CO2.

Dissociated neurons were lipofected with the mCherry plasmid using Lipofectamine 2000 (Invitrogen, 11668019) according to the manufacturer procedures.

Neurosphere Assay

A17 and A18 GZ were isolated from E77 to E80 parasagittal organotypic slices via manual microdissection and cells dissociated using TrypLE Express 1X (Thermo Fisher, 12604013). For each area, 1–2.10^6 cells were diluted in 4 ml of NS medium, DMEM:F12, (Gibco, 31331-028), N2 supplement, (1:100^e, Gibco, 17502-048), 1% Non-essential amino acids (Gibco, 11140-035), 2 mM glutamine, 1% penicillin/streptomycin (Gibco, 10378-016), 100 μM beta-mercapto-ethanol (Gibco, 31350-010), 20 ng/ml bFGF, (Millipore, GF003AF), 20 ng/ml EGF, (Millipore, 01-107), 1000 U/ml human recombinant LIF, (homemade, Gift from P. Savatier) and grown as neurospheres for 2–10 days in a 50 mm Petri Dish. Neurospheres were then plated on 6 or 24 glass well plates or on 14 diameter glass coverslips coated on Laminin (10 μg/ml)/Poly-L-lysine (100 μg/ml) (Sigma, L2020 and P1399), and allowed to differentiate in Neurobasal A medium supplemented with B27 (1:50^e, Gibco, 17504-044), N2 (1:100^e, Gibco, 17502-048) and 2 mM glutamine, 1% penicillin/streptomycin (Gibco, 10378-016).

For live recording: Two to five days after plating, neurospheres on glass well plates were imaged under the time lapse for 5–7 days. Neurospheres were fixed at the end of recording session with 2% PFA and immunostained for Ki67 (Neomarker, clone sp6, RM9106S1) and NeuN (Millipore, MAB377). For A17 and A18 experiments, $NeuN^+$ /$Ki67^-$ post-mitotic neurons were tracked.

Brain and Organotypic Slices Cryosections

For organotypic cortical slices (300 μm thickness), cultured slices were fixed 1h by immersion in cold buffered 2% paraformaldehyde and then cryoprotected in 10 and 20% sucrose. For whole brain cryosections, lethally anesthetized primate fetuses (via intraperitoneal injection of Sodium Pentobarbital 60 mg/kg) were perfused through the heart with buffered 4% Paraformaldehyde (PFA) during 30 min. After sequential cryoprotection in 10% and 20% sucrose (in phosphate buffer), brains were embedded in Tissue-Tek. Immunolabelling against Vimentin was performed on either 80 μm thick organotypic slices (**Figure 3I**) or on 20 μm thick parasagittal sections performed with a cryostat (Microm, HM550) then mounted on superfrost glass slides (Superfrost Plus, Thermo Scientific) and stored à −20°C (**Figure 3J**).

Immunofluorescence, Antibodies and Confocal Imaging

Cryosections were air-dried for 30 min and hydrated in Tris-buffered saline (TBS) for 30 min. Glass slides or coverslips were rinsed three times in TBS Triton (0.5%) and incubated in Normal Goat Serum 10%, (Gibco, 16210-064) diluted in Dako Diluent (Dako, S3022) for 30 min. Primary antibodies were incubated overnight in Dako Diluent at 4°C. Chicken anti-EGFP (Invitrogen, A10262, 1:1000), mouse anti-Vimentin (Sigma, V6630, 1:400), mouse anti-NeuN (Millipore, MAB377, 1:100), Rabbit anti Ki67 (Neomarker, clone sp6, RM9106S1, 1:400). After 3 TBS wash, relevant secondary antibodies were incubated in Dako Diluent (Dako, S3022) 1 h at RT, at the following concentrations: Alexa Fluor 488 goat anti-chicken IgY (Invitrogen, A11039, 1:1000), Alexa Fluor 555 goat anti-mouse IgG (Invitrogen, A21422, 1:800), Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, A11001, 1:1000), Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, A21428, 1:800). Nuclear staining was performed using DAPI (Invitrogen, D1306, 3 μM in TBS), for 10 min at RT. Mounting was realized in Fluoromount-G Medium (Southern Biotech, 0100-01).

Confocal examination of the fluorescent labeling was carried out on a LEICA DM 6000 CS SP5 equipped with an Argon laser tuned to 488 nm, a HeNe laser 543 nm, a HeNe laser 633 nm, and a diode 405 nm. Acquisition were performed using oil objectives (×40), thanks to the LAS AF software (Leica).

Two-Photon Time-Lapse Video Recordings on Organotypic Slices

Real time video recordings were performed on an inverted Axio-Observer Z1 (Zeiss) two-photon microscope, equipped with Zeiss optics and a Chameleon system Ultra (I) Titanium Sapphire 80 MHz laser. The recording system is equipped with a Microscope Cage Incubation System (Okolab) maintaining temperature at 37°C and CO_2 at 7.5%. Millicell inserts were imaged in a 6-well glass bottom plate (Iwaki, #5816-006). The medium was renewed twice a day. Laser was tuned to 910 nm for EGFP imaging (power range 14–20%). Observations were performed using a plan apochromatic dry objective 10×/0.45 with a digital zoom of 1.5. Video-analysis was initiated 72 h after EGFP retroviral infection (E65) and 96 h post infection (E78). Using the Multi Time Series macro of Zeiss Zen software, 4D stacks were acquired over 80 μm thickness (14 optical sections spaced at 6 μm intervals), which allows following of the 4D migration pattern of progenitors and postmitotic neurons.

Recording was performed using a single scanning run at 1024 × 1024 pixels resolution with a scanning speed of 6 μsec/pix. Images were acquired every 1.5 h for up to 15 days.

Time-Lapse Recording on Dissociated SP Neurons

Video recordings were performed using a Nikon Eclipse Ti S inverted fluorescence microscope with an Andor Clara camera using a Nikon S ELWD Plan-fluor 40×/0.60 objective. The microscope was equipped with a humidified chamber (Nikon, the Box) and recordings were done at 37°C (Nikon, The Cube) under 7.5% CO2 (Nikon, the Brick). Recordings were made on a single focal plane at a rate of 0.1–2 s for each acquisition over 1 min.

Time-Lapse Recording on Neurospheres

Video recordings were performed using a Nikon Eclipse Ti S inverted fluorescence microscope with an Andor Clara camera using a 10×/0.3 Nikon plan fluor objective or a Plan fluor 20×/0.45. The microscope is equipped with a humidified chamber (Nikon, the Box) and recordings were done at 37°C (Nikon, The Cube) under 7.5% CO2 (Nikon, the Brick). Alternatively, a Leica DMIRBE inverted microscope was used. Recordings were done at 37°C under 7.5% CO2 (PECON). Phase contrast recordings were made every 10 min for up to 7 days. Half of the culture medium was renewed every day.

Manual Tracking of Radial Migration on Video Recordings in Organotypic Slices and Neurospheres

Organotypic Slices

Tracking of $EGFP^+$ migrating neuron soma movements was done manually by the experimenter by using the plugin MTrackJ, from GZ to the SP (a java program developed by Erik Meijering at the Biomedical Imaging Group Rotterdam). A neuron is considered to pause if it covers a distance less than 3.5 μm over a 6 h period. The analysis has been performed in lower two thirds of the SP (up to 850 microns). Typical SP neurons trajectories (**Figure 3**) are reconstructed using raw coordinates (x,y) obtained by MTrackJ tracking and modeled with a common origin ($x = 0$; $y = 0$) using the R Studio® software or the Excel software.

The radiality index was calculated for each displacement (i.e., movement between two recorded positions) within each track. The radial vector is defined as the axis perpendicular to the horizontal upper limit of the OFL. The radial index corresponds to the ratio between the radial distance (rd) and the distance corresponding to the shortest path (sp) measured between the start of migration and the recorded positions. The radiality index is an indicator of neuron dispersion with respect to strict radial migration.

Neurospheres

Cell tracking was performed for the whole migration trajectory: from the time the neurons exit the neurosphere until migration cessation, using the plugin MTrackJ. A neuron was considered to pause if it moves less than 3.5 μm over a 2 h period. Morphological analysis was performed for each track and for each position within a single track. Two main migrating neurons morphotypes were distinguished: (i) neurons exhibiting an elongated bipolar shape, classified as "Bipolar" and neurons exhibiting a multibranched morphology with neurites growing at the rear and on the sides of the soma, classified as "multibranched." Scarce migrating neurons with a split leading process were also observed in both A17 and A18 neurospheres.

Frequencies of each morphotype are monitored by computing the time spent by the neuron in each morphology with respect to the total migration time. Typical neuronal trajectories are reconstructed using raw coordinates (x,y) obtained by MTrackJ

tracking and modeled with a common origin ($x = 0$; $y = 0$) using the R Studio® software or the Excel software.

The radial vector used to calculate the radiality index (cf above) is defined by the radial glia processes orientation.

The straightness index is computed over the entire trajectory and quantifies directional persistence. It corresponds to the ratio of the straight distance between the origin and the endpoint of migration (tsd) divided by the total distance covered by the neuron (td).

Nuclear Orientation, Process Orientation

The nuclear and process orientation analysis was performed on MTrackJ individual trajectories data. The angles of the nucleus and the process orientation with respect to the radial vector were computed for each movement. In slices, the radial vector is defined by the axis perpendicular to the horizontal upper limit of the OFL (i.e., parallel to the ventricular border). In neurospheres, the radial vector is defined by the radial glia processes orientation. Radially orientated nucleus and processes present a displacement angle close to the radial vector (i.e., 90°).

IG and SG Morphological Analysis in the Germinal Zones and in the OFL

E64–E65 and E77–E78 organotypic cortical slices (300 μm thick) infected with a pCMV-EGFP retrovirus were fixed 3–7 days after infection and immunostained for GFP. Confocal acquisitions of $EGFP^+$ cells were performed using a Leica HC PL Apo immersion oil 20×/0.70 objective with a digital zoom of 3. 40 μm stacks were taken 1 μm apart. The number of processes starting from the soma were determined based on EGFP visualization. Cells were classified as: no process (np), radial (1–2 processes) and multipolar (≥3 processes).

IG and SG Subplate Neurons Morphological Complexity Assessment Using Sholl Analysis

The Sholl analysis was performed using the Sholl Analysis plugin for ImageJ (Anirvan Ghosh Laboratory, UCSD), on either fixed or live imaged neurons expressing mCherry (*in vitro*) or cytoplasmic EGFP (*ex vivo*). The following parameters were used: starting radius: 2 μm; ending radius: 100 μm, radius step size: 2 μ m.

Protrusions Analysis

Protrusions on growth cones and neurites of the leading process were analyzed using time lapse video recording of mCherry (pZou-mCherry) lipofected dissociated neurons. Cellular protrusions were scored manually and classified as filopodia (spike-like long protrusions) or lamellipodia (broader sheet-like protrusions). Results were expressed as a percentage of the total protrusions observed in a given neuron population.

Statistical Analysis of Nucleus and Processes Orientation

Statistical analyses were performed in the R statistical environment (R Core Team, 2017). Generalized Linear Models (GLM) were fit to each experiment with a Poisson family and the canonical log link function. This is equivalent to fitting a log-linear model with a multinomial distribution (Bishop et al., 1975). For the nucleus and process orientation, the linear predictor corresponded to a 6 × 2 (Angle × Experimental Factor) design in which the Experimental Factor was nested within Angle and the model contained no intercept term. These model designs generated pre-planned contrasts in which the differences of the two levels of the Experimental Factor were estimated and tested for significance for each orientation or angle. The possibility of overdispersion was excluded by examining the ratio of the residual deviance to the residual degrees of freedom and by comparing the fits with a negative binomial model, which includes an additional parameter to account for overdispersion. These measures along with examination of model diagnostic plots of the residuals did not reveal any systematic evidence for overdispersion. Statistical significance from the GLM analyses (referred to as GLM test) was evaluated by comparing nested models with and without an interaction of Orientation and the Experimental Factor by likelihood ratio tests and by the Wald statistics for the individual coefficients of the models. $p < 0.05$ was considered statistically significant. Sample number (n) corresponds to the total number of independent biological samples for all the experiments. Data are presented as the mean ± 95% confidence interval (CI).

Sholl Models and Statistical Analysis

The data for IG and SG neurons and for both conditions (*ex vivo* and *in vitro*) were modeled with the *glm.nb* function from the MASS package (Venables and Ripley, 2002) in the R programming environment (R Core Team, 2017). This fits a GLM with a negative binomial family and a log link function by maximum likelihood. The log volume of the sampling region was used as an offset variable to model estimated densities of the intersections. A segmented linear predictor was used to model the log of the density as a function of distance (Muggeo, 2008). The curves were obtained from the back-transformation of the estimated densities by multiplying them by the volume of the sampling region. The error bars are standard errors of the mean. The correspondence between the *ex-vivo* and the *in vitro* model was evaluated by comparing the log ratio of SG to IG densities among the two conditions.

RESULTS

The present study focuses on two developmental stages: E65 and E78 (**Figure 1A**) that correspond, respectively to the generation of the bulk of IG *(E55–E71)* and SG layer neurons *(E72–E90)* in the occipital cortex of the macaque monkey (Rakic, 1972; Dehay et al., 1993; Lukaszewicz et al., 2005; Betizeau et al., 2013). Both stages are prior to initiation of gyrification of the occipital lobe (Smart et al., 2002). Embryonic organotypic cortical slices provide an unrivaled *ex vivo* non-human primate model of early corticogenesis, where morphology, proliferation, differentiation and migration can be explored in an intact cytoarchitecture over a one to 2 week period (Betizeau et al., 2013; **Figures 1B–E** and

Supplementary Figure S1). IG and SG neurons were labeled via EGFP retroviral infection of cycling progenitors [as in Betizeau et al. (2013)] on organotypic slices from the most posterior pole of the occipital lobe cut in the parasagittal plane, encompassing the primary visual area (area 17 -A17) and its neighbor, area18 (A18) at E65 and E78 (**Supplementary Figure S2**).

Early Radially Migrating Neurons Do Not Go Through a Multipolar Stage in the Primate Cortex

We first analyzed the behavior and morphology of IG and SG postmitotic neurons during the pre-migratory stage in the GZ of A17 and A18. Morphological attributes of early postmitotic neurons have been extensively described in the mouse (Nadarajah and Parnavelas, 2002; Tabata et al., 2012). Immediately after cell-cycle exit, mouse postmitotic neurons enter a static stage when they pause for 48 h and assume a multipolar morphology in the SVZ, prior to resuming a bipolar morphology and reinitiating radial migration. Real time observations in the NHP embryonic slices showed that early postmitotic EGFP+ neurons pause for 24–40 h before reinitiating radial migration within the GZ. Upon exit from the OSVZ, newborn radially migrating neurons are characterized by a bipolar, elongated morphology of the nucleus and the soma. The vast majority (>85%) of pre-migratory neurons exhibit a bipolar morphology, and multipolar neurons account only for 12% of IG and 4% of SG premigratory pausing neurons (**Figures 1F,G**), indicating that the multipolar shape is rare in the germinal zones. This contrasts with the high (88%) proportion of multipolar premigratory postmitotic neurons observed in the SVZ/IZ compartment at mid-corticogenesis in the mouse (Tabata and Nakajima, 2003).

IG and SG Radial Migrating Neurons Show Distinct Morphodynamic Features

In a first instance, we analyzed the gross morphology of EGFP+ radially migrating neurons in the SP at E65 and E78 in A17 and A18 organotypic slices (**Figure 2**), 4–6 days following retroviral infection. At E65, IG migrating neurons in the SP present a bipolar morphology in both areas (**Figures 2A,B**). By contrast SG migrating neuron morphology is more complex, as quantified by the Sholl analysis (**Figure 2C**), exhibiting a bipolar, elongated soma with a single leading process extending in the direction of migration and a variable number of trailing neurites (**Figures 2A,B**). In A17, SG migrating neurons possess a single trailing neurite opposing the leading process. By contrast in A18 migrating neurons exhibit a multibranched morphology, with several trailing neurites, resulting in a higher complexity as quantified by a Sholl analysis (**Figure 2D**). No difference in morphological complexity between A17 and A18 IG migrating neurons has been detected (data not shown).

Using 2-photon real time imaging, we monitored the migratory behavior of EGFP+ newborn IG and SG neurons on parasagittal organotypic slices. Importantly, the parasagittal plane of section of the occipital lobe cuts parallel to the radial glia scaffold. Cell movements were recorded every 1.5 h (**Supplementary Movie S1**) and migration trajectories tracked manually from the GZ to the SP (**Figure 2E** and **Supplementary Movie S1**). At both stages, migrating neurons display a saltatory locomotion with pauses following a period of displacement (**Supplementary Movie S2**) as reported in mouse (Nadarajah and Parnavelas, 2002; Nadarajah et al., 2003; Kriegstein and Noctor, 2004). We focused on the early stages of radial migration and conducted our analysis in the OFL and the lower two thirds of the SP. Both IG and SG neurons follow a radial trajectory in the upper part of the OSVZ as well as in the OFL and the SP (**Figure 2E**).

Migration kinetics were characterized by measuring (i) migration velocity, defined in terms of units of distance with respect to time; and (ii) motility index defined by units of distance with respect to time minus cumulative pause duration. A neuron was considered to pause when its movement amplitude is less than 3.5 μm in any 6h period. In both areas, SG neurons show significantly increased migratory kinetics compared to IG neurons in both the OFL and the SP (**Figures 2F,G**). The higher migration velocity of SG compared to IG neurons (**Figures 2F,G**) is not due to pausing in either the OFL or SP as indicated by increased values of motility (**Figure 2H**), reflecting intrinsic differences in motility rates of these two populations. We did not observe differences in IG neurons velocity and motility rates between A17 and A18. By contrast SG neurons show significantly higher velocity and motility rates in A18 compared to A17.

These results point to laminar differences in morphodynamic properties of cortical migrating neurons that are conserved across A17 and A18.

SG Neurons Show Area-Specific Differences in Radial Migration Mode

We next focused on the analysis of trajectories of individual IG and SG migrating neurons in the SP. Global trajectories were observed to be strictly radial in the IG population in both areas (**Figures 3A,B**). SG neurons show distinctive behaviors in the two areas. SG migrating neurons in A17 follow relatively linear, radially constrained trajectories. By contrast SG migrating neurons in A18 exhibit meandering, ab-radial trajectories (**Figures 3C,D**). In order to quantify these differences in SG neurons, we have computed the radiality index (**Figure 3E**), defined as the ratio between the radial distance with respect to the shortest path between two time points. Deviation values from 1 of the radiality index indicate deviation with respect to the radial axis (perpendicular to the ventricular border). SG migrating neurons in A18 have significantly lower radiality index values compared to SG migrating neurons in A17, indicating that A18 SG neurons significantly deviate from radial routes (**Figure 3F**).

Migrating neurons in the SP are highly polarized in the direction of migration and exhibit a single leading process exhibiting forward motion. To investigate if the radial and ab-radial trajectories in the SP are associated with differences in cell body morphology and leading process dynamic, we monitored the cell body and the leading process orientations with respect to the radial axis. For each recorded movement in the SP, the cell bodies and leading process of SG migrating neurons in A17 show a high proportion of radial orientations (**Figures 3G,H**). By contrast, SG migrating neurons in A18 exhibited a significantly

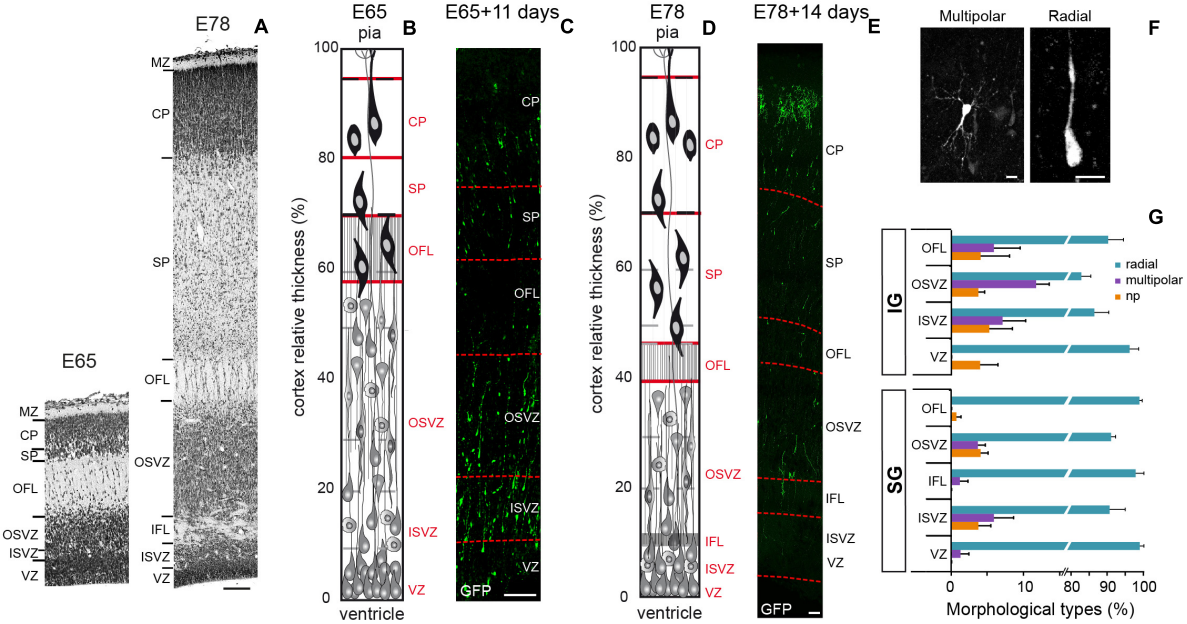

FIGURE 1 | (A) Transects of Area 17 at E65 (left) and E78 (Right). **(B)** Area 17 cellular organization at E65. **(C)** Transect of a A17 parasagittal organotypic slice 11 days after EGFP retroviral infection of cycling precursors at E65. A fraction of EGFP$^+$ neurons have migrated and reached the CP. **(D)** Area 17 cellular organization at E78. **(E)** Transect of a A17 parasagittal organotypic slice 14 days after retroviral infection at E78. EGFP$^+$ neurons have reached the CP. **(F)** Microphotographs of EGFP$^+$ neurons at the OFL/SP border showing multipolar (left panel) and radial morphology (right panel). **(G)** Frequency of the three morphological classes of EGFP$^+$ early postmitotic migrating neurons in the occipital cortex subsequently to retroviral infection at E65 and E78, respectively: no process (np), radial (1–2 processes), and multipolar (≥3 processes). VZ, ventricular zone; ISVZ, inner subventricular zone; IFL, inner fiber layer; OSVZ, outer subventricular zone; OFL, outer fiber layer; SP, subplate; CP, cortical plate; MZ, marginal zone. Scale bars: **A,C,E:** 100 μm; **F:** 10 μm.

lower proportion of radial orientations for both the soma and the leading process (**Figures 3G,H**).

Our observations were made at stages E65 and E78, which is prior to gyrification, in linear portions of A17 and A18 of the developing visual cortex (**Supplementary Figure S2**). The radial glia scaffold plays a key role in guiding migrating neurons (Rakic, 1972; Elias et al., 2007; Evsyukova et al., 2013). The tortuous trajectories of migrating A18 neurons at E78 in the SP do not result from an alteration or a disruption of the radial glia scaffold which is preserved over time in organotypic slices (**Figure 3I**). To further explore whether these area differences in radial and ab-radial trajectories could be due to differences in the radial scaffold density, we quantified the numbers of radial glia processes per surface area in the SP of A17 and A18 (**Figure 3J**). This shows a higher density of radial glia processes in A17 than in A18, in agreement with the higher numbers of bRGS in A17 OSVZ than in A18 (Lukaszewicz et al., 2005; Betizeau et al., 2013).

The Leading Process of SG Migrating Neurons Exhibit Area-Specific Features

The differences in the radial glial density in A17 and A18 could mean that the observed area differences in morphology and migration radiality reflect environmental factors. We designed *in vitro* assays in order to achieve high resolution analysis of neuron morphology and membrane behavior at the growing tip of the leading process of migrating neurons derived from cortical progenitors. First, we prepared neurospheres derived from A17 and A18 OSVZ primary GZ progenitors at E78. Four days following plating on a polylysine/laminin coated glass substrate, radial processes were observed to grow evenly out of the neurosphere and postmitotic neurons (Ki67$^-$/ NeuN$^+$) to migrate out of the sphere (**Figures 4A,B**). The sparse growth of radial processes emanating from the neurospheres only partially recapitulates cues encountered by the *in vivo* migrating neurons in the SP. Reconstruction of the trajectories of postmitotic neurons migrating out of the neurospheres showed significantly straighter radial paths in migrating neurons derived from A17, compared to tortuous, ab-radial routes in migrating neurons derived from A18 (**Figures 4C,D**). In addition to the radiality index (**Figure 4E**), we measured the straightness index, which allows to estimate the tortuosity of migration trajectories (**Figure 4J**). Both indices are significantly higher in A17 than in A18 neurospheres (**Figures 4E,J**). In A18, we observed a distinctive pattern of motility where migrating neurons make frequent changes in directionality. Similarly to what we observed during radial migration on organotypic slices, the soma orientation show greater deviation with respect to the radial axis in migrating neurons derived from A18 compared to those derived from A17, a feature that is accentuated in the leading process (**Figures 4F,G**), on par with what we observed in the organotypic slices (**Figures 3G,H**). The neurosphere migration assay recapitulates the area-specific distinctive features of SG migrating neurons, in absence of the dense radial scaffold

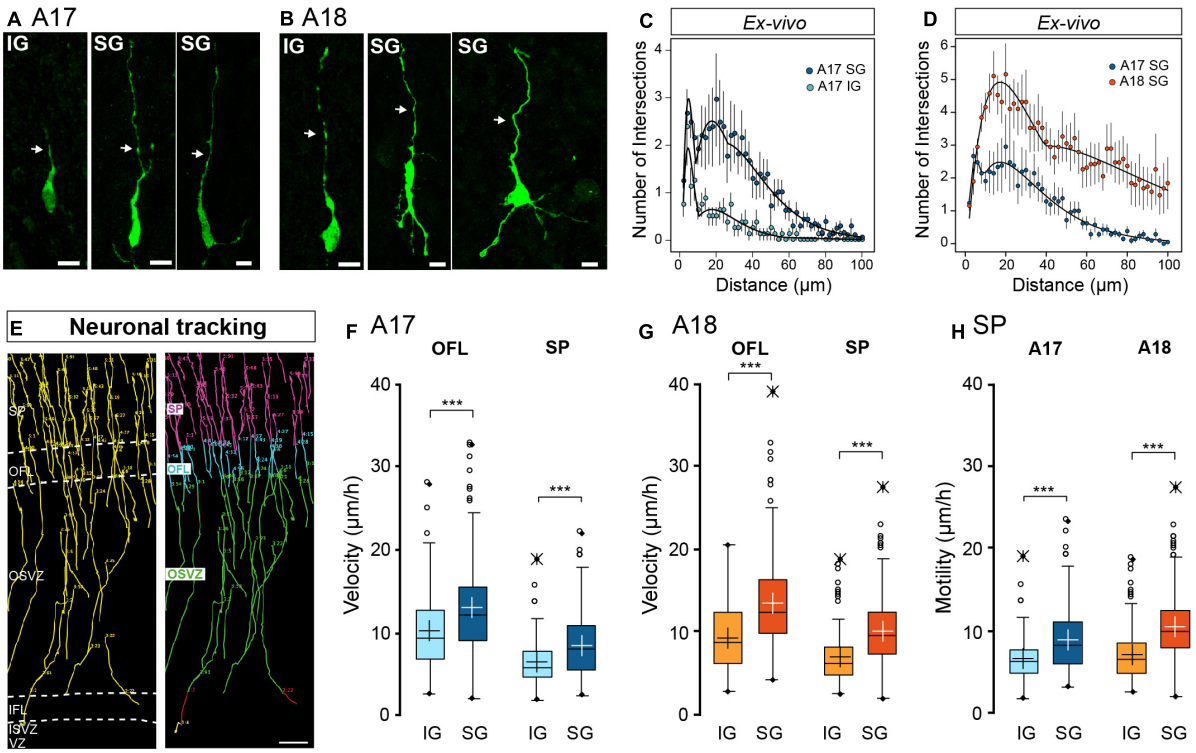

FIGURE 2 | (A,B) Characteristic neuronal morphology of A17 **(A)** and A18 **(B)** IG and SG in the SP revealed by GFP immunolabelling. Leading process is highlighted by a white arrow. **(C)** Sholl analysis comparison between A17 IG (light blue) and A17 SG (dark blue) **(D)** Sholl analysis comparison between A17 SG neurons (blue dots) and A18 SG neurons (orange dots) *ex vivo* using the Sholl analysis. **(E)** Typical trajectories of migrating SG neurons reconstructed via manual tracking from A17. 2-photon TLV observations of organotypic slices over a 10 days period (see **Supplementary Movie S1**), right panel: color-coded tracks with respect to cortical compartments Green: OSVZ, Blue: OFL, Pink: SP. **(F)** IG and SG migration velocity in the OFL and SP of A17. **(G)** IG and SG migration velocity in the OFL and SP of A18, **(H)** Motility index in A17 and A18 SP. Black and white crosses indicate the mean. Average values ± sem. Statistical analysis: Two-tailed and unequal variance Student test, *p* values <0.005***. Scale bars: **A,B** = 10 µm, **E** = 100 µm.

observed *in vivo* and *ex vivo* in the SP, suggesting that these differences are in part expression of cell autonomy.

Having shown that area specificity in migration trajectories are maintained *in vitro*, we checked that differences in gross morphology were also maintained in dissociated neurons, as measured by a Sholl analysis (**Supplementary Figure S3**). We then took advantage of the higher spatiotemporal resolution provided by *in vitro* dissociated cells, to implement an in-depth analysis of morphology dynamics and leading process behavior. High frequency TLV analysis revealed dynamic changes in the morphology of migrating neurons. We observed two main migrating neurons morphotypes: neurons exhibiting an elongated bipolar shape, classified as "bipolar" and neurons exhibiting a multibranched morphology with trailing and lateral neurites, classified as "multibranched" (**Figure 4H**). During their migration, migrating neurons derived from A18 show a higher frequency of multibranched morphology than do migrating SG neurons derived from A17, meaning that compared to A17, A18 derived neurons spend a larger fraction of their trajectory in the multibranched state (**Figures 4H,I**). In addition to these two main morphotypes, we observed scarce migrating neurons with a split leading process emanating from both A17 and A18 neurospheres (**Figure 4H**), a morphology also seldomly encountered *ex vivo* on the organotypic slices.

Subsequently, we proceeded to characterize the membrane growth behavior of the leading process on dissociated SP migrating neurons. *In vitro*, the leading process of dissociated migrating neurons was identified as the thickest primary process. Membrane protrusions were observed at the growing tip and along the leading process. These protrusions were classified as filopodia (long spike-like protrusions) or lamellipodia (broad sheet-like protrusions) (**Figure 4K**). Migrating IG neurons in both areas show a predominant filopodia phenotype (**Figure 4L**). In A17 SG migrating neurons, the vast majority (>95%) of protrusions of the leading process were filopodia (**Figures 4M,N**). By contrast, A18 SG migrating neurons exhibit up to 50% of lamellipodia which showed curvilinear extension and retraction movements (**Figures 4M,O**). These area differences observed *in vitro* point to cell autonomous characteristics in membrane behavior at the growing tip of the leading process. In sum, the filopodia membrane growth of the leading process is associated with radial trajectories (IG migrating neurons in A17/A18 and SG migrating neurones in A17), while the lamellipodia protrusion behavior in A18 SG migrating neurons is associated with ab-radial migration trajectory.

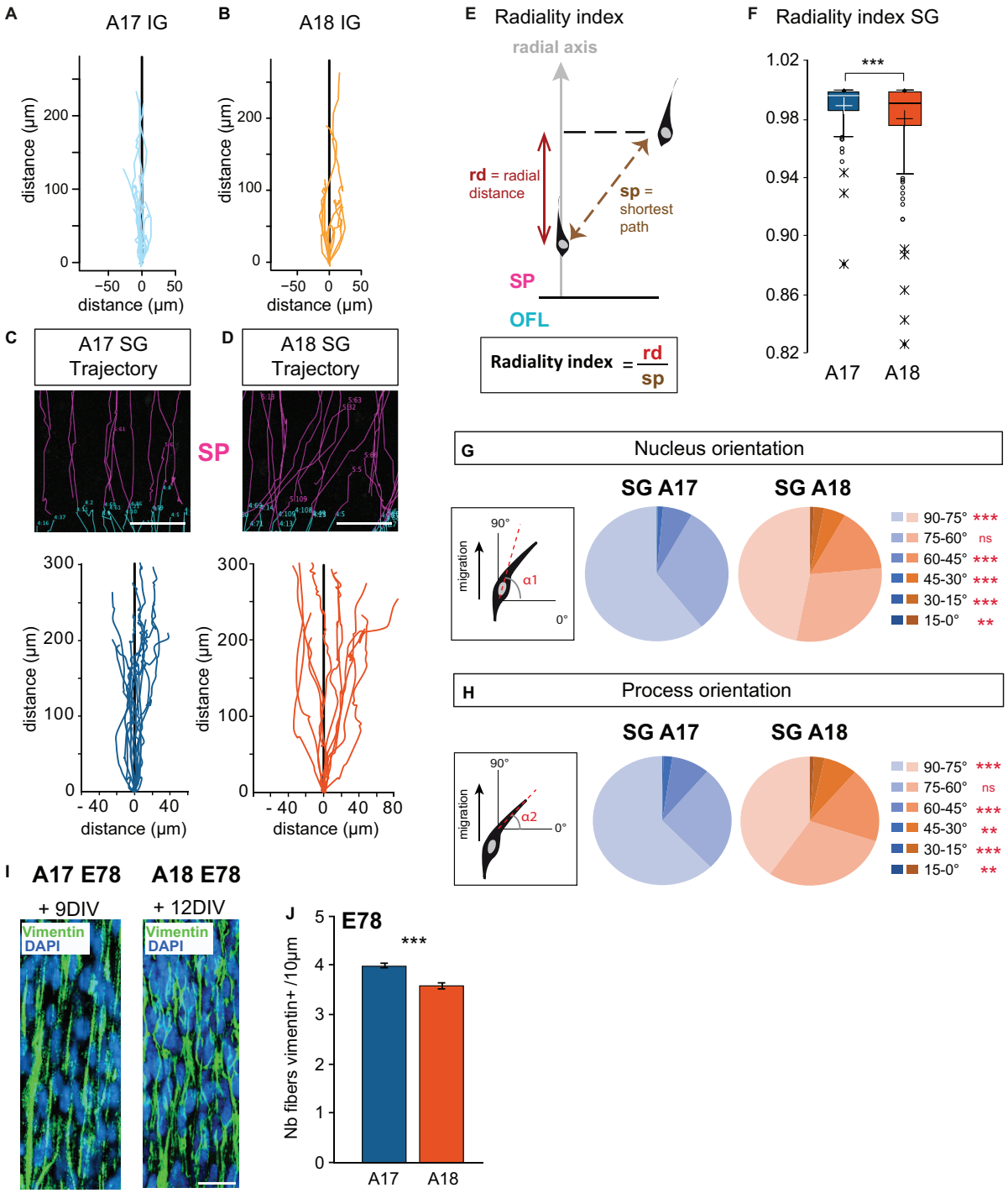

FIGURE 3 | (A,B) Trajectories of migrating IG neurons observed in Area 17 and A18 SP using 2-photon TLV on organotypic slices. **(C,D)** Typical radial Area 17 and ab-radial Area18 SG trajectories observed in the SP up to 12 days after retroviral infection. Examples of original SP trackings are presented above the graphs. **(E)** Radiality index. The radiality index corresponds to the ratio between the radial distance (rd) and the distance corresponding to the shortest path (sp) measured between two positions. **(F)** A17 and A18 SG neurons radiality index. Black and white crosses indicate the mean. **(G,H)** Nucleus and leading process changes in orientation during A17 and A18 SG trajectories. Nucleus **(G)** and leading process **(H)** orientations are defined with respect to the surface of the OFL (= 0°) and measured for each recorded position. **(I)** Vimentin expression on A17 and A18 in the lower SP during SG migration, as shown after immunofluorescent labeling on 80 microns thick section from E78 organotypic slices at, respectively, 9–12 days after infection. Nuclei are stained by DAPI. Note the well-preserved radial scaffold. **(J)** Graph showing the density of Vimentin+ fibers in the SP of area 17 and Area 18 per surface area quantified on 20 micron thick sections from E78 cortex. Average values ± sem. Statistical analysis: **(F,J)** Two-tailed and unequal variance Student test, *p* values <0.005***. **(G,H)** GLM test, *p* values <0.01**, <0.001***. SP: subplate. Scale bars: **C,D** = 100 µm, **I** = 20 µm.

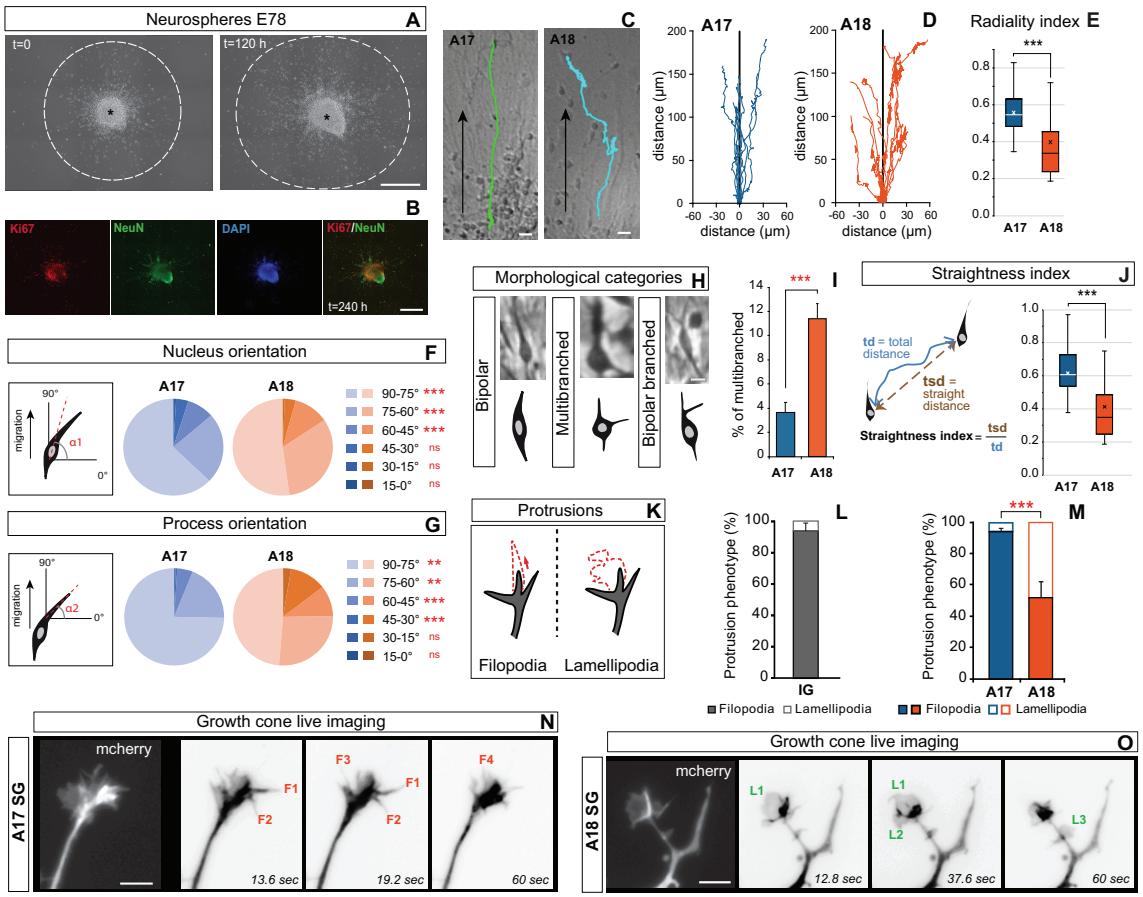

FIGURE 4 | (A) Photographs of A18 SG derived neurospheres 4 days after plating, (t = 0) (left panel), and 10 days after plating (t = 120 h) (right panel) showing neuronal migration extent (dotted line) out of the neurosphere. **(B)** At the end of the live recording, neurons are identified using NeuN and Ki67 immunostaining. NeuN^{+}/Ki67^{-} cells, are considered post-mitotic neurons. **(C)** Typical trajectory of A17 and A18 SG neurons migrating out of the neurosphere. **(D)** Typical trajectories of migrating SG neurons reconstructed from time lapse observations. **(E)** Area 17 and Area18 SG neuron trajectories radiality index. **(F,G)** Nucleus **(F)** and process **(G)** change in orientation during A17 and A18 SG neuronal migration out of neurospheres. **(H)** Morphology of SG neurons migrating out of the A17 and A18 neurospheres along their trajectory. Three main categories were identified: bipolar, with two processes located at the opposite poles of the soma; multibranched: with multiple processes growing out of the cell body; bipolar branched: with a branched leading process. **(I)** Percentage of time spent as multibranched for A17 and A18 SG neurons during their migrating trajectory **(J)** The straightness index is computed over the entire trajectory and corresponds to the ratio of the straight distance between the origin and the endpoint of migration (tsd) divided by the total distance covered by the neuron (td). **(J)** Area 17 and Area18 SG neuron trajectories straightness index. Black and white crosses indicate the mean. **(K)** Two types of growing membrane protrusions are observed on the growth cone and/or along the leading process of migrating SP neurons: filopodia (spike-like long protrusions, Left) or lamellipodia (broader sheet-like protrusions, Right). **(L)** Protrusions phenotype frequencies in occipital IG neurons. **(M)** Frequency of filopodia and lamellipodia observed on A17 and A18 SG neurons. **(N)** High magnification protrusions observed on a typical growth cone of an A17 SG SP neuron leading process labeled with mCherry. Imaging took place over 1 min. Four growing protrusions, classified as filopodia **(F1** to **F4)**, are highlighted. **(O)** High magnification of a typical growth cone of A18 SG SP neurons labeled with mCherry Three growing protrusions, classified as Lamellipodia **(L1** to **L3)** are highlighted. **(E,I,J,M)** Two-tailed and unequal variance Student test, p values < 0.005***. **(F,G)** GLM test, p values < 0.01**, < 0.001***. Scale bars: **A,B:** 500 µm, **C:** 20 µm, **H** = 10 µm, **N,O** = 5 µm.

DISCUSSION

Multipolar Stage Is Dispensable for Primate Cortex Radial Migration

Numerous observations in the rodent cortex (Tabata and Nakajima, 2003; Kriegstein and Noctor, 2004; Noctor et al., 2004; Ohtaka-Maruyama et al., 2018) have reported that before initiating their radial journey toward the CP, around 80% of migrating neurons undergo a transient multipolar phase in the upper SVZ/lower intermediate zone. The present observations in the NHP cortex reveal that multipolar postmitotic neurons represent only a minute fraction of the migrating neurons (4% for SG neurons) and that an overwhelming majority of young migrating neurons exhibit a bipolar morphology. Compared to rodent, the pause duration in macaque between cell-cycle exit and initiation of radial migration corresponds to a considerably reduced window of time. Of note, 90% of the VZ and OSVZ cycling progenitors in the macaque correspond to four morphotypes showing a polarized morphology, which have significantly higher neurogenic potential than the 10% of unpolarized progenitors (Betizeau et al., 2013). This suggests

that inheritance of this bipolar morphology establishes the optimal conditions for effective initial steps of radial migration in primate, by contrast with the rodent situation where a large majority of late-born pyramidal precursors are generated from unpolarized intermediate Tbr2 expressing progenitors with multipolar morphologies and highly dynamic processes (Kowalczyk et al., 2009; Kriegstein and Alvarez-Buylla, 2009; Nelson et al., 2013).

Species-Specific Temporal Regulation of Migration and Proliferation Rates

Despite drastic interspecies variation in the distance to be covered from the GZ to the CP, radial migration rates of cortical neurons appear to be conserved between species. Using real-time imaging on organotypic cortical slices, we observed average radial migration velocities for IG and SG neurons in primate Area 17 and 18 ranging from 6 to 12 μm/h - depending on the developmental stage, compartment and area. These values are in the range reported for bipolar neuron radial migration on organotypic slices of the gyrencephalic cortex of the ferret (Gertz and Kriegstein, 2015) and of the mouse cortex during mid-neurogenesis (Britto et al., 2011; Adnani et al., 2015). Note that the relative invariance of migration speeds between species stands in sharp contrast with the significant interspecies differences in cell-cycle duration (Tc is five times longer in monkey than in mouse-) (Kornack and Rakic, 1998; Lukaszewicz et al., 2005; Betizeau et al., 2013). This suggests that migration speed regulatory mechanisms might be less of an evolutionary target than cell-cycle control mechanisms. The lengthening in migration phase duration between mouse, ferret and primate (Jackson et al., 1989; Noctor et al., 2004; Borrell et al., 2006; Lukaszewicz et al., 2006) is on par with the enlargement of the cortex in these species corresponds to a major evolutionary adaptation offsetting similar rates of migration. This evolutionary constraint in migration rates is likely to result from an exquisitely tuned balance between intrinsic properties and extrinsic factors. Indeed, during their migration journey, IG and SG neurons have to cross different species-specific embryonic compartments, each characterized by distinctive ECM components (Fietz et al., 2010; Ayoub et al., 2011; Zeng et al., 2012).

Coordinated Regulation of Migration and Proliferation Rates

Given the exquisite correlation between the temporal sequence of cortical neuron birthdates and their laminar distribution in the CP, migration speed might need to be tightly adjusted to rates of neuron production in order to prevent crowding of newborn neurons and to achieve correct neocortical layering. Detailed lineage analysis showed that IG and SG progenitors differ with respect to their proliferative behavior (Betizeau et al., 2013). Specifically SG progenitors have increased proliferative capacities that allow the enlargement of SG layers that characterizes cortical areas in primates (Dehay et al., 2015). Here we find that radial migration velocity of SG neurons is significantly higher than that of IG neuron, on par with the higher production rates of SG neurons. This suggests coordination between proliferation and migration rates. Coordination mechanisms have been described in molecular investigations of neurogenesis and radial migration (Heng et al., 2010; Pacary et al., 2011). In addition, recent data suggest that radially propagative Ca++ activity in radial glial fibers could mediate such a coordination (Rash et al., 2016).

Major rodent-primate differences in corticogenesis may impact radial migration. Throughout rodent corticogenesis, the radial migration scaffold is provided by the basal processes of the apical progenitors (APs) of the VZ that extend both an apical and a basal process anchored to the ventricle and the basal membrane, respectively. During primate corticogenesis, the predominant progenitor pool is the bRGs of the OSVZ (Smart et al., 2002; Lukaszewicz et al., 2005; Lui et al., 2011; Florio and Huttner, 2014). While the vast majority of bRGS extend a long basal process directed to the CP, only a fraction (40%) exhibit an apical process, which is not anchored in the ventricular border (Betizeau et al., 2013). Therefore, one might posit that the scaffolding cues for radial migrating neurons differ between primates and rodents (Nowakowski et al., 2016). The sharp arealization that characterizes the primate cortex is supported by differences in the density of the OSVZ progenitor pool and proliferative programs (Dehay et al., 1993; Smart et al., 2002; Lukaszewicz et al., 2005). The area differences in density of bRGs observed between A17 and A18 (Lukaszewicz et al., 2005), translate into different SP microenvironments for migrating neurons, possibly requiring area-specific regulatory mechanisms.

Area-Specific Features of Radial Migration

In our experimental design, care has been taken to monitor radial migration in rectilinear regions of cortex in both A17 and A18, prior to folding and away from presumptive sulci and gyri. Hence the ab-radial meandering migration that we observe in A18 SG is not related to a fanning array of the radial glial fibers scaffold associated with gyrification as has been reported in ferret where migration has been studied in presumptive gyri (Borrell et al., 2006; Reillo et al., 2011) or in the GZ during folding (Gertz and Kriegstein, 2015). This is further supported by the observations within the neurosphere migration assay that recapitulates the radial and ab-radial trajectories.

Membrane lamellipodia have been hypothesized to serve as a sensor of the local microenvironment permitting cells to optimize their functional adhesion (Myat et al., 1997; Skalski et al., 2010). The higher occurrence of lamellipodia observed in A18 SG migrating neurons could confer them the ability to probe the environment more efficiently than A17 SG migrating neurons, thereby accommodating the sparser scaffold of radial glial fibers in A18. Compared to A18, the A17 OSVZ expresses higher levels of the primate-specific microRNA miR550-3P at E78 (Arcila et al., 2014). miR550-3p targets srGAP2 (Dennis et al., 2012) which, when overexpressed in mouse cortical progenitors, induces filopodia and highly dynamic membranes with large transient protrusions (Guerrier et al., 2009). Assuming that miR550-3p negatively regulates the expression of srGAP2 leads to

the prediction that A17 SG migrating neurons should exhibit less protrusions and lamellipodia than do A18 migrating SG neurons.

We observed that area differences in radial rates of migration as well as the differences in morphology are preserved in dissociated cultures, pointing to intrinsically determined properties of radially migrating SG neurons. How the cell-intrinsic control of the cytoskeleton interfaces with extracellular signal-regulated pathways that control the migration of neurons remains elusive (Pacary et al., 2011). The morphology of the leading process varies in different migrating neuronal types, which is considered to reflect an adaptation to the local migratory requirements (Marin et al., 2010; Valiente and Marin, 2010). While the majority of radially migrating pyramidal neurons exhibit a simple single leading process (Martínez-Martínez et al., 2019), fast tangentially migrating neurons display a more complex leading process with branched morphologies (Bellion et al., 2005). One cannot exclude the possibility that the differences in the morphodynamics of radial migration is partly correlated to changes in geometry and /or adhesive properties of the extracellular environment in the SP. This would reflect an adaptability of migratory strategies in order to maintain high motility as has been reported in other cell models (Tozluoglu et al., 2013, 2015).

Our data point to area-specific features in the trade-off between speed and distance to optimize radial migration efficiency. The tortuous trajectories in A18 SG neurons, indicative of less directional persistence, result in increased path length. This is reminiscent of the migrating behavior of early postmitotic neurons in the GZ in reelin mutants. Compared to control neurons showing a strict radial path, the loss of reelin results in an increased speed and deviation from the rectilinear radial path at the earliest stages of their trajectories (Britto et al., 2011) which is thought to be related to the greater extent of cell dispersion observed in the reeler cortex (Tissir et al., 2002).

Speculation on the Functional Consequences of Area-Specific Distinct Migratory Strategies

In the present study, our *in vitro* findings indicate that the area differences in migratory characteristics between A17 and A18 postmitotic neurons rely at least in part on cell autonomous mechanisms. This is reminiscent of the area differences in A17 and A18 OSVZ progenitor cell cycle durations that are maintained *in vitro* (Lukaszewicz et al., 2005). Together, these cell autonomous properties suggest that there might be a tight coupling between the proliferative behavior of progenitors and their post mitotic migratory behavior.

In addition to constraining the organization of the ontogenic cortical columns (Rakic, 1988; Jones and Rakic, 2010), the radiality of migration has been shown to influence cortical circuitry assembly in the neocortex. Clonally related neurons have been shown to be preferentially inter-connected and to share functional properties (Yu et al., 2009; Li H. et al., 2012; Ohtsuki et al., 2012; Gao et al., 2014). Induced lateral dispersion of migratory sister excitatory neurons disrupts preferential electrical coupling in the early developing mouse cortex (Yu et al., 2012; He et al., 2015), a crucial early step to ensure its proper functional development (Yuste and Katz, 1991; Elias et al., 2007). The relatively high radial organization of A17 cortical neurons (Rockland and Ichinohe, 2004), which is likely to result from the migration trajectories in the early stages of migration, could therefore have functional consequences on A17 connectivity. In many ways area 17 shows unique properties in terms of adult structure and function that could perhaps require strict radial migration and one could speculate that the slower velocities observed in migrating A17 neurons is the price that has to be paid to achieve maximum radiality. It remains to be determined whether the observed area differences in radial migration modes parallel differences in the local circuitry of primary versus associative areas (Douglas and Martin, 2007). Neuron densities vary across cortical areas (Collins et al., 2010) and SG neurons densities are particularly high in A17 (Rockel et al., 1980). The radial characteristics of migration observed in A17 SG migrating neurons could be linked to the high adult SG neuron density compared to other cortical areas. The spatial modulation of migration so as to accommodate area variation in SG neuron density could be an important adaptive feature given that high densities of SG neurons would favor the sparse coding strategy characteristic of these neurons that is thought to allow enhanced computational capacities (Harris and Mrsic-Flogel, 2013).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Comité d'Ethique Lyonnais pour les Neurosciences – CELYNE (C2EA #42).

AUTHOR CONTRIBUTIONS

DD, HK, and CD: conceptualization and writing – original draft. VC, DP, and ND: methodology. VC and PG: validation and data curation. KK and VC: formal analysis. VC, DD, DP, EG, and CH: investigation. CH and ND: resources. CD and HK: writing – review and editing. DP, PG, and VC: visualization. CD: supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.588814/full#supplementary-material

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frontiers in Cell and Developmental Biology

REVIEW
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Extracellular Control of Radial Glia Proliferation and Scaffolding During Cortical Development and Pathology

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During the development of the cortex, newly generated neurons migrate long-distances in the expanding tissue to reach their final positions. Pyramidal neurons are produced from dorsal progenitors, e.g., radial glia (RGs) in the ventricular zone, and then migrate along RG processes basally toward the cortex. These neurons are hence dependent upon RG extensions to support their migration from apical to basal regions. Several studies have investigated how intracellular determinants are required for RG polarity and subsequent formation and maintenance of their processes. Fewer studies have identified the influence of the extracellular environment on this architecture. This review will focus on extracellular factors which influence RG morphology and pyramidal neuronal migration during normal development and their perturbations in pathology. During cortical development, RGs are present in different strategic positions: apical RGs (aRGs) have their cell bodies located in the ventricular zone with an apical process contacting the ventricle, while they also have a basal process extending radially to reach the pial surface of the cortex. This particular conformation allows aRGs to be exposed to long range and short range signaling cues, whereas basal RGs (bRGs, also known as outer RGs, oRGs) have their cell bodies located throughout the cortical wall, limiting their access to ventricular factors. Long range signals impacting aRGs include secreted molecules present in the embryonic cerebrospinal fluid (e.g., Neuregulin, EGF, FGF, Wnt, BMP). Secreted molecules also contribute to the extracellular matrix (fibronectin, laminin, reelin). Classical short range factors include cell to cell signaling, adhesion molecules and mechano-transduction mechanisms (e.g., TAG1, Notch, cadherins, mechanical tension). Changes in one or several of these components influencing the RG extracellular environment can disrupt the development or maintenance of RG architecture on which neuronal migration relies, leading to a range of cortical malformations. First, we will detail the known long range signaling cues impacting RG. Then, we will review how short range cell contacts are also important to instruct the RG framework. Understanding how RG processes are structured by their environment to maintain and support radial migration is a critical part of the investigation of neurodevelopmental disorders.

Keywords: apical radial glia, cortical development, neuronal migration, scaffold, cell-cell interaction, cell signaling, extracellular matrix

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INTRODUCTION

The cerebral cortex is an intricate brain structure responsible for many precise functions such as thinking, decision making and long term memory, and is required for the final processing of sensory inputs and motor control. These functions rely on the way the neuronal network is precisely organized. The structure of the cortex is composed of different layers of neuronal subtypes (Taverna et al., 2014; De Juan Romero and Borrell, 2015). In the mouse, these layers are established during embryonic development in an inside-out manner via the successive migration of young neurons generated directly or indirectly from apical radial glial cells (aRGs) in the ventricular zone (VZ) to their final location in distinct superficial regions (Rakic, 1972; Kriegstein and Gotz, 2003). aRGs have a particular morphology as they grow processes that extend from the apical to the basal side of the cortex. In both rodent and primate, aRGs generate further basal intermediate neurogenic progenitors (IPs) residing in the subventricular zone (SVZ). In gyrencephalic species such as humans and other primates, neurons can also be generated from basal radial glia (bRGs), also called outer radial glia (oRG), which are distributed in an outer SVZ (Penisson et al., 2019; Matsumoto et al., 2020). bRGs can extend processes to the apical, the basal or both surfaces of the cortex (Betizeau et al., 2013). In all situations their structure provides a linear support for neuronal migration. Therefore, RGs are not only the source of neurons during embryonic cortical development but also the scaffold necessary for their proper distribution throughout the expanding cortex. The formation and maintenance of the RG scaffold is essential for the correct positioning of neurons and thus, the organization of the neuronal network.

Several cellular processes are important to consider for proper RG morphology. As they are dividing and self-renewing progenitors, RGs have been widely studied in the context of the mechanisms underlying their proliferative features (Taverna et al., 2014; Uzquiano et al., 2018). This will have an impact on the density of fibers available for supporting migration. RGs (e.g., expressing factors such as Pax6, Sox2, Hes5) can self-renew via symmetric divisions but can also carry out asymmetric divisions giving rise to different progeny including Tbr2 + IPs (**Figure 1**; Taverna et al., 2014; De Juan Romero and Borrell, 2015). RGs are also able to directly produce neurons. In particular, cell intrinsic mechanisms acting on mitotic spindle orientation or nucleokinesis via cytoskeletal or polarity proteins are tightly linked to daughter cell production and fate. At the structural level, how the radial processes critical to RG function are created, modulated or maintained relies on additional molecular mechanisms, which is the topic of this review.

Since aRGs are structured in a very defined way, with their soma and apical processes at the border of the ventricle, they are exposed to many secreted factors from the embryonic cerebrospinal fluid (eCSF). In particular, the primary cilium extends inside the ventricle and this is a crucial signaling center for the activation of numerous molecular cascades (Sarkisian and Guadiana, 2015). At the level of their cell bodies, aRGs and bRGs are both exposed to cell−cell and cell−environment interactions. They interact with each other as well as with additional cell types such as IPs or neurons. Importantly they interact with the surrounding extracellular matrix (ECM). For example, human bRGs have been shown to produce specific proteins which interact with the ECM in their basal position (Pollen et al., 2015). Finally, their basal processes are also exposed to external signals throughout the intermediate zone (IZ), cortical plate (CP), marginal zone (MZ), and at the pial surface (**Figure 2**). First, we will review the different secreted molecules involved in the establishment or maintenance of RG morphology from the eCSF or in the ECM. Then, we will describe how short range interactions between cells are essential for these processes. Finally, we will detail the impact of relevant molecular players on the origin and evolution of several human neurodevelopmental diseases.

ROLE OF SECRETED PROTEINS DERIVED FROM THE CSF IN THE FORMATION AND MAINTENANCE OF THE RG SCAFFOLD

Secreted Factors From the Embryonic Cerebrospinal Fluid (eCSF)

The cortex develops primarily from the neuroepithelium during embryonic development. Between E8.5 and E9.5 in mice, the neural tube closes, forming the ventricular cavity in which the amniotic fluid is sequestered and forms the basis for the eCSF (Lowery and Sive, 2009). Later during mouse brain development, the choroid plexus arises and secretes many factors, modifying the composition of the ventricular fluid (Chau et al., 2015). The deepest apical region of the developing brain is composed of neuroepithelial-derived aRG progenitors from E10.5. These aRGs are exposed to a variety of secreted factors from the ventricle during development. Proteomics analyses of the CSF indicates that the precise composition of secreted molecules varies during development. For instance, the concentration of Bone Morphogenic Proteins (BMPs) is higher in the amniotic fluid than in the eCSF, Sonic Hedgehog (Shh) concentration is higher in the eCSF at the beginning of aRG development (E10.5) and decreases thereafter, whereas the concentration of retinoic acid (RA) is higher at later stages (E14.5) (Chau et al., 2015). These variations in composition are required to induce the production of RGs (Sox2+) at the right time during the formation of the cortex (Chau et al., 2015). These data also suggest that certain secreted proteins or combinations of proteins in the eCSF during murine corticogenesis are required for evolving aspects of RG production and maintenance.

The composition of secreted factors in the eCSF not only changes with developmental stages but is also specific to different ventricles. Indeed, the different choroid plexus tissues present in each ventricle develop in a sequential manner (Lehtinen et al., 2011). Firstly, the choroid plexus from the fourth ventricle appears (E11 in the mouse), then the choroid plexus develops in the lateral ventricles (E12) and lastly, it develops in the third ventricle by E14. Each type of choroid plexus will express a different panel of secreted factors. For example, Shh is mainly

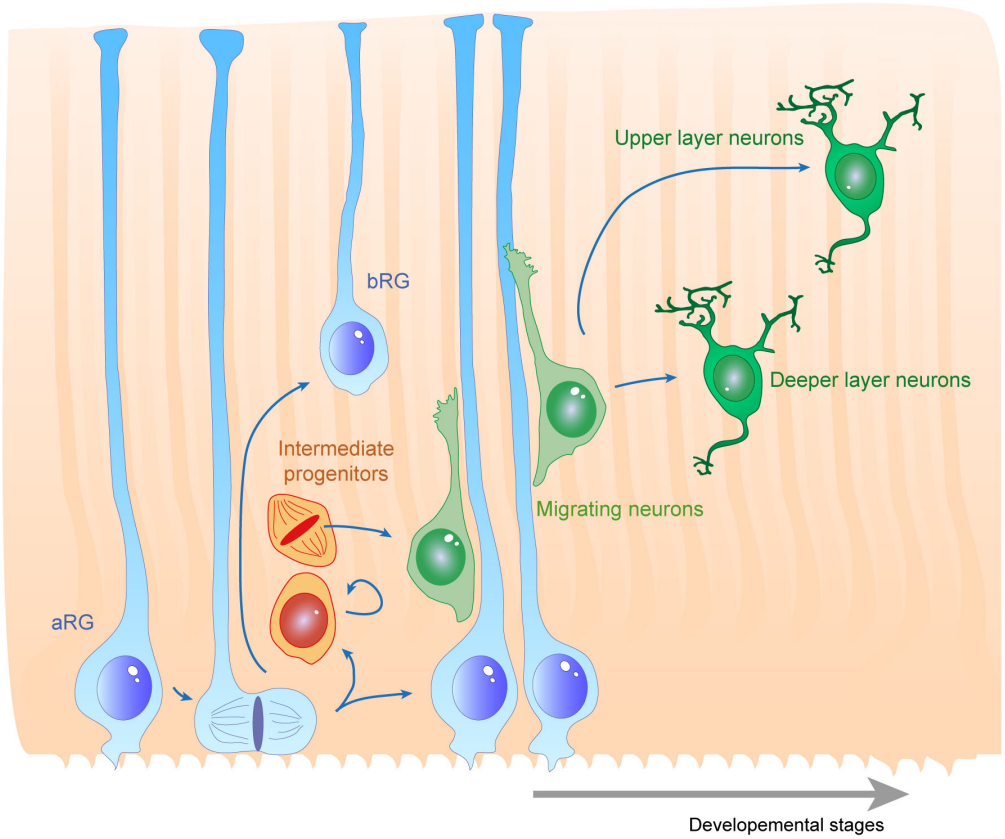

FIGURE 1 | Radial glias function as both the source and the support of newborn neurons in the developing cortex. Apical radial glia (aRG) extend an apical process reaching the ventricular surface, where they expose their primary cilia, as well as a basal process reaching the cortical surface. Basal radial glia (bRG) have their cell bodies located in more basal areas of the cortical wall. Apical and basal processes from these cells (blue) establish the scaffold across the whole cortical wall. RGs undergo cell division, giving birth to a daughter cell which can be either another RG (apical or basal – symmetric division) or a basal progenitor (asymmetric division, intermediate progenitors are represented in orange). These cells give rise to migrating neuroblasts (green) which move along RG basal processes to reach their final position within the cortical layers. First deep layer neurons are generated, then upper layer neurons are born.

produced in the fourth ventricle by the choroid plexus close to the hindbrain (Huang et al., 2010), whereas many other proteins are found only in the lateral ventricles (Zappaterra et al., 2007). More recently, proteomics data were integrated with RNA sequencing datasets, comparing telencephalic and hindbrain choroid plexuses (Lun et al., 2015). This spatial heterogeneity of their secretomes argue in favor of a precise and specific regulation of different brain areas. Overall, the eCSF plays multiple important roles in the formation of the nervous system (for review, Fame and Lehtinen, 2020). In this part of our review, we will describe the functional role of the main secreted factors present in the eCSF for the maintenance of RGs and therefore the formation of the RG scaffold.

Growth Factors

As mentioned in the previous section, several cytokines are found in the eCSF. Different types of molecules can be found within this family such as growth factors like Transforming Growth Factors (TGF, developed later on in this review). But not all of these cytokines have a direct effect on radial scaffolding. For example, chemokines are best known for their action on neurons (Zhu and Murakami, 2012). On the other hand growth factors are diffusible cytokines widely known to activate RG proliferation and/or sustain cell survival. Therefore, we first provide a non-exhaustive list of eCSF-derived growth factors (**Figures 3**, **4** and **Table 1**) necessary for cortical development and in particular for the integrity of the RG scaffold.

Multiple **Fibroblast growth factor** (FGF) ligands are expressed in the developing telencephalon. At early stages (E10–E12), FGFs 8, 17, and 18 are expressed in the frontal midline area where they act as morphogens (see section "Morphogens" below). In the ventral telencephalon, FGF15 is expressed (Rash and Grove, 2006; Cholfin and Rubenstein, 2007; Hebert and Fishell, 2008), whereas in dorsal regions, FGFs 2, 9, and 10 are expressed (Vaccarino et al., 1999; Raballo et al., 2000; Sahara and O'Leary, 2009). Here, we focus on FGF2, which increases the total number of neurons in the mouse cerebral cortex and promotes self-renewal of cortical progenitor cells (Vaccarino et al., 1999; Raballo et al., 2000).

Fibroblast growth factor 2 is one of the most important growth factors for the production and maintenance of RGs during cortical development. Initially, FGF2 proteins were

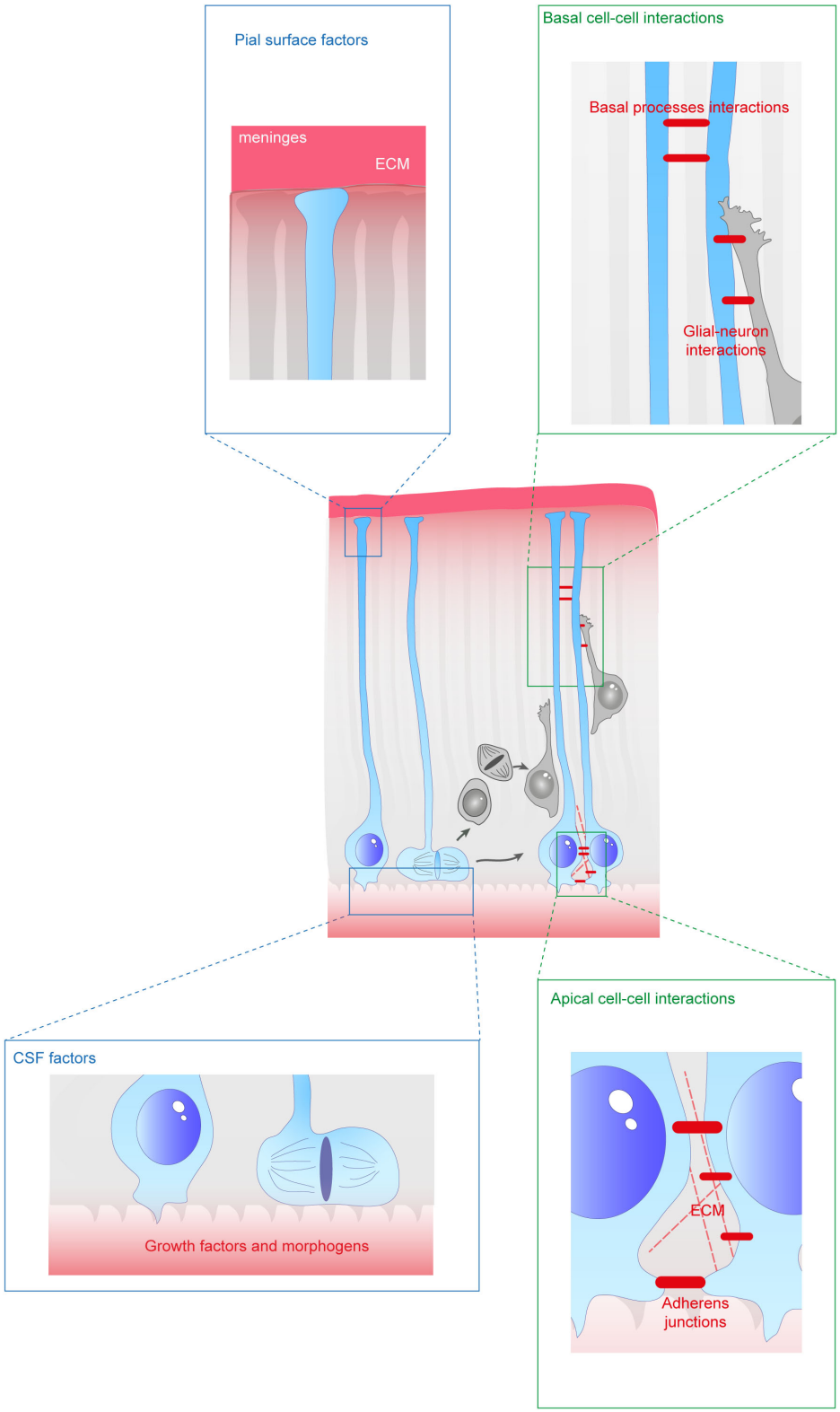

FIGURE 2 | Extracellular factors controling the scaffolding of RGs. RGs are exposed to a variety of extracellular cues. These signals can be secreted molecules (blue boxes) or received directly from other cells (green boxes). In apical regions aRGs receive signals from the eCSF as their cell bodies and primary cilia are in contact with the ventricles. They also establish contacts between themselves and with the extracellular matrix (ECM). In basal regions, RG basal processes are exposed to secreted cues from the meninges and from already differentiated neurons. These interactions can occur while neurons are migrating along them. Basal processes also exhibit interactions between themselves.

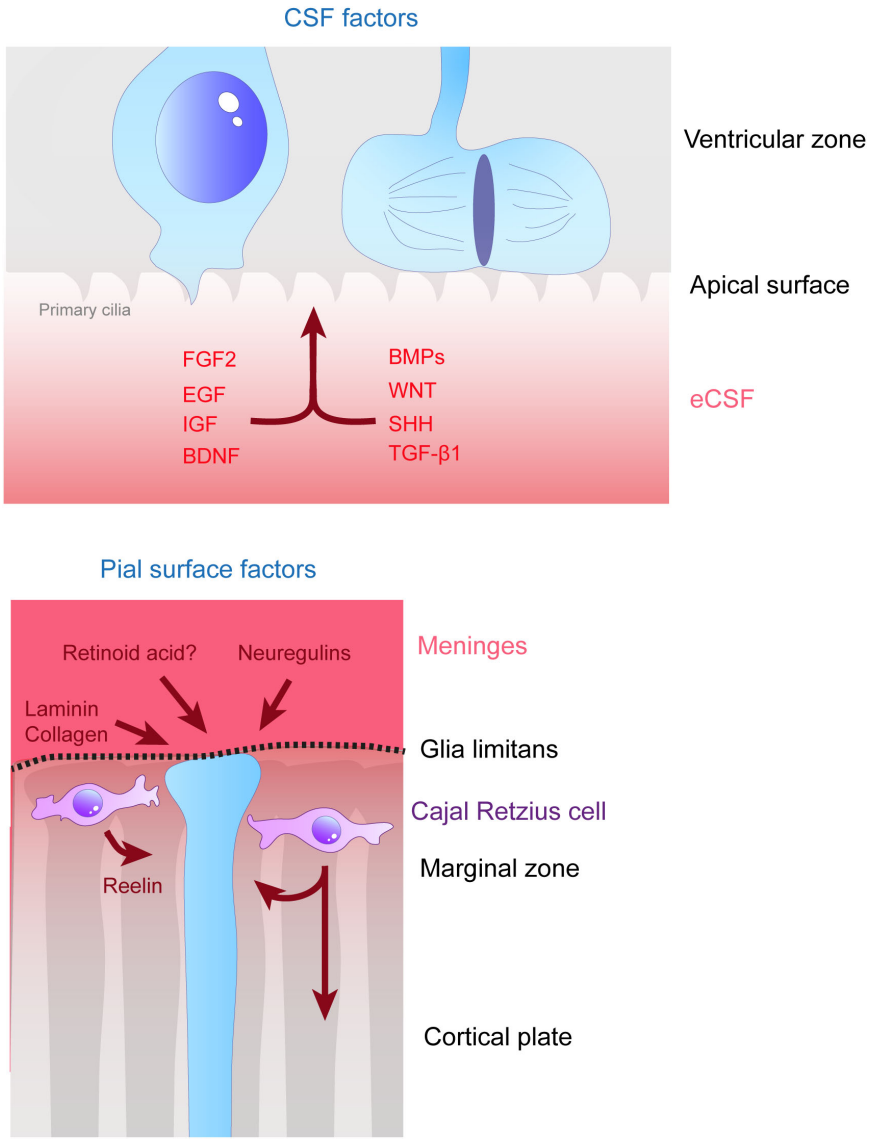

FIGURE 3 | Remote extracellular factors controling the scaffolding of RGs. Some of the extracellular cues controlling RG development are produced and secreted from relatively remote locations. Here are represented the factors present in the CSF (upper schema) which are detailed in this review, namely FGF2, EGF, IGF, BDNF, BMPs, WNT, SHH, and TGF- β1. On the bottom schema, extracellular cues derived from the meninges and acting on the extremities of basal processes are depicted, namely, laminin, collagen, neuregulins and retinoid acid. Cajal Retzius cells (in purple) are migrating cells which in early stages of development tangentially move in the MZ of the developing cortex. These cells are a source of Reelin amongst other molecules which influence RG scaffolding.

found present in the VZ of the murine developing cortex by immunohistochemistry (Dono et al., 1998). The source of the protein was not clearly defined but later the protein was detected in chicken eCSF (HH25) by western blot experiments (Martin and Groves, 2006), suggesting that it might be produced remotely and captured at the ventricular surface. In this study, authors show that the actual origin for FGF2 production in the chick embryo is the notochord, the mesonephros, the hepatic primordia and the brain neuroectoderm. The receptor for FGF2 (FGF2R) is expressed in the mouse VZ (E14.5) as shown by *in situ* hybridization (Dono et al., 1998). More recently single cell RNA-seq data shows that the *Fgf2r* gene is expressed in mouse RGs (Telley et al., 2019), suggesting that these cells can receive the FGF2 signal from the eCSF. The cortex of *Fgf2* mutant mice is thinner and there is abnormal distribution of neurons in the cortical wall. Indeed, pulse chase analyses indicate an increase of neurons generated at E14.5 in the deep layers of the cortex (Dono et al., 1998). This suggests a defect in the ability of these cells to colonize their final target place in more superficial layers. Defects in proliferation were also identified in *Fgf2* KO embryonic cortices in a separate study explaining the decrease in the size of the cortex (Raballo et al., 2000). This is in agreement with the fact that FGF2 is one of the major factors necessary for the renewal of RGs *in vitro* (Sun et al., 2011). The knockout

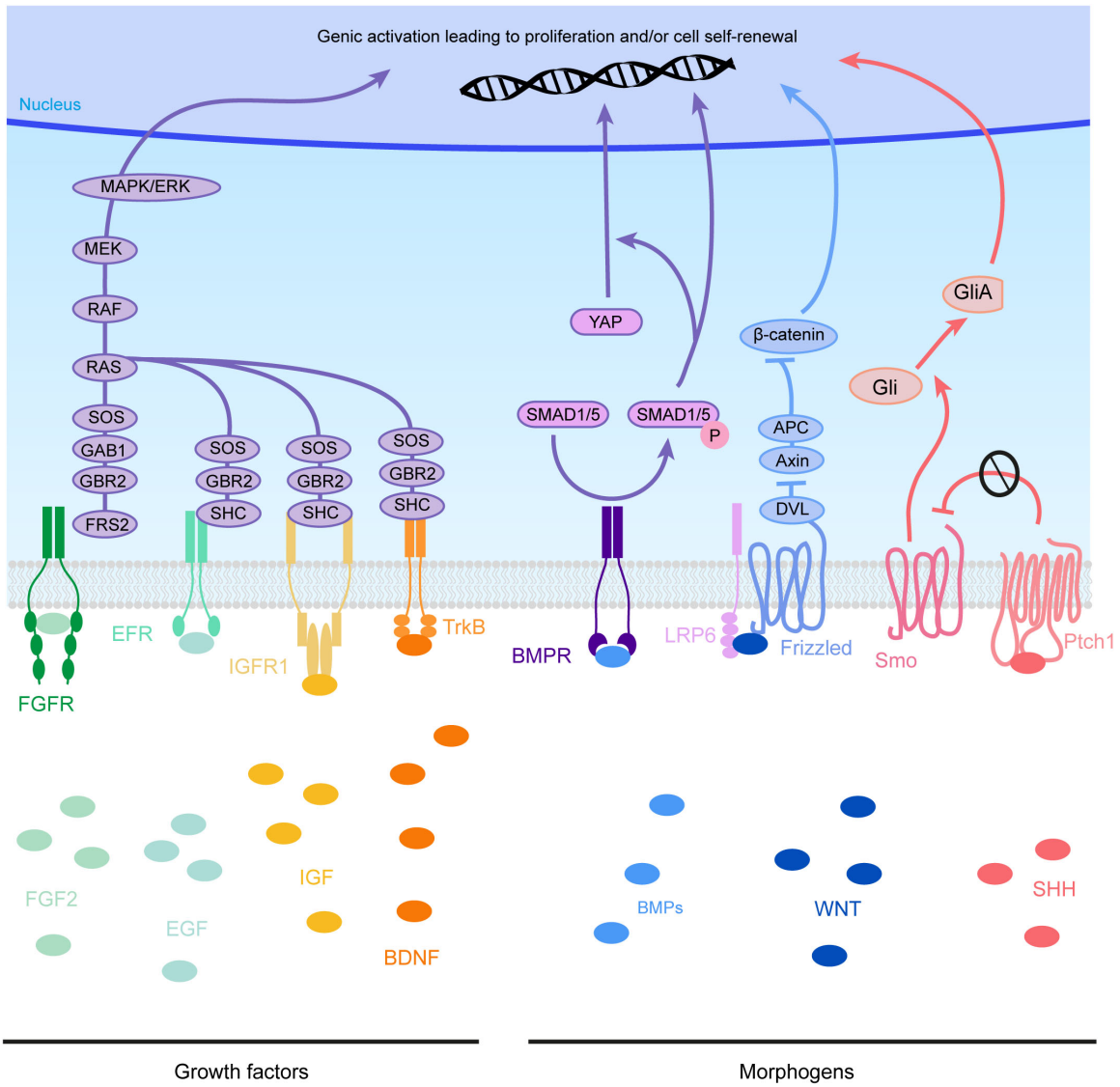

FIGURE 4 | Molecular pathways triggered by eCSF-derived factors. The growth factors found in the eCSF are mainly known to trigger the mitogen-activated protein kinases (MAPK) pathway (also known as the RAS-RAF-MEK-ERK pathway). This molecular signaling pathway is involved in the regulation of several essential cellular processes such as proliferation, differentiation, survival and death. BMP receptors (BMPR) activate the phosphorylation of SMAD1/5, which can activate directly transcription of target genes or act via the translocation of YAP into the nucleus. WNT molecules activate the Frizzled receptors and LRP6 co-receptors which will allow Disheveled (DVL) to inhibit the Axin-APC complex. This complex is a major inhibitor of β-catenin. Therefore, upon WNT activation, β-catenin is free to be directed into the nucleus to activate its target genes. Finally SHH binds to its receptor Patched1 (Ptch1), which then releases the 7 transmembrane protein Smoothened (Smo) from its inhibition. Smo activation triggers the cleavage of Gli transcription factors into their active form (GliA). GliA is then enriched in the nucleus to allow transcription of target genes (such as Cyclin D1 or Gli itself).

(KO) of *Fgfr* genes in the anterior neural plate using Foxg1-Cre, inhibits the formation of the telencephalon, leaving just the midline (Paek et al., 2009). When Fgfrs are removed only from RG, their development is impaired resulting in lower numbers of *Pax6* and *Hes5* + cells (Kang et al., 2009). These combined data show how crucial FGF2 is for the maintenance of RGs and therefore, the formation of the cortex. Moreover, gain of function experiments performed by *in utero* injection of FGF2 first in the telencephalic ventricles of rat embryos at E15 (Vaccarino et al., 1999), then in mouse embryos at E11.5 (Rash et al., 2013), induces an increase in proliferation. When FGF2 signaling is overactivated locally by these manipulations, this induces the formation of gyri in the mouse cortex (Rash et al., 2013). Although gyrification can be associated with the appearance of bRGs during evolution (Penisson et al., 2019), in this case FGF2 injections did not appear to increase the proportion of bRGs in the cortical wall. This suggests that FGF2 modified the development of the architecture of the cortex via other unknown mechanisms. At the molecular level, FGF2 triggers the mitogen-activated protein kinase (MAPK) pathway to induce cell cycle

and proliferation (for review, Iwata and Hevner, 2009, **Figure 4**). FGF2, as well as Notch signaling, can also induce calcium (Ca2+) bursting which can support communication along the RG fiber (Rash et al., 2016). Indeed, both along the RG fiber and the communication with neurons can be mediated by calcium waves, in a bidirectional manner.

Fibroblast growth factor 2 can be used in culture in combination with **Epidermal growth factor** (EGF). Indeed, the action of EGF on cortical progenitors has been studied for many years (Burrows et al., 1997; Lillien and Raphael, 2000). Recently, FGF2 and EGF were shown to regulate self-renewal of rat cortical progenitors in organotypic cultures *in vitro* (Lamus et al., 2020). These two growth factors can activate the same molecular pathways to initiate proliferation. The action of FGF2 and EGF is not a simple synergy since FGF2 can modulate the responsiveness of RGs to EGF (Lillien and Raphael, 2000). RGs are first responsive to FGF2 alone and later during cortical development, start to be also responsive to EGF (Ciccolini and Svendsen, 2001). Moreover, the effect of EGF on the proliferation of cortical progenitors is dose-dependent (Nelson et al., 2008). The combined action of EGF and FGF2 is therefore essential for the development of RGs and their maintenance during the development of the cortex. At the beginning of mouse cortical expansion (e.g., E13), the EGF receptor (EGFR) is present in the VZ and SVZ (Sun et al., 2005). At the cellular level, EGFR has been found to be localized asymmetrically in dividing RGs, controlling the fate of daughter cells. The cell inheriting EGFR is the daughter cell retaining proliferative capacity and glial markers (Sun et al., 2005). This indicates that RGs need to keep their ability to respond to EGF in order to maintain their progenitor identity. EGF signaling is therefore required for the maintenance of the RG scaffold during cortical development. Certain studies have investigated the mechanisms controlling the expression of EGFR. First the ganglioside GD3 was identified as an EGFR partner, responsible for its sustained expression in cortical progenitors *in vitro* (Wang and Yu, 2013). More recently, the expression of miR-129-5p, modulated by choline availability in the microenvironment, was shown to inhibit the expression of EGFR, thus impacting RG maintenance and cortical development (Trujillo-Gonzalez et al., 2019). All these data underline the essential role for EGF in the maintenance of RGs necessary for cortical development.

The **Insulin-like Growth factors** (IGF1 and IGF2) are a group of hormones which are present in the eCSF (Salehi et al., 2009; Zappaterra and Lehtinen, 2012; Bueno et al., 2020). IGF2 concentration in rat eCSF increases from E16 to E19 (Lehtinen et al., 2011). Gain of function experiments such as IGF1 overexpression in mouse embryo showed that this hormone induces a shortening of the cell cycle, acting in particular on S-phase. This increase in the speed of proliferation is linked to cortical hyperplasia, which is an increase in global cortical size via an increase in cell number (Hodge et al., 2004; Popken et al., 2004; Mairet-Coello et al., 2009). Both *in vivo* and *in vitro* data show that IGF2 can induce cortical growth by stimulating RG proliferation (Lehtinen et al., 2011). The reverse result is observed in *Igf2* KO mice which present a neurogenesis decrease affecting the production of neurons destined for the upper cortical layers. At the apical membrane level, CSF-derived IGF2 binds to primary cilia of RGs, where IGF receptors are localized. IGF1 receptor (IGF1R) is the main receptor allowing IGFs to trigger proliferation (Zappaterra and Lehtinen, 2012). Like FGF2 and EGF, IGF can trigger the MAPK pathway but can also activate a non-canonical pathway via Gβγ signaling, which regulates the timing of the cell cycle (Yeh et al., 2013). All of these data explain why the *Igf1r* conditional knockout (cKO) in neural precursors leads to microcephaly (Lehtinen et al., 2011).

Brain Derived Neurotrophic Factor (BDNF) has the particularity of being expressed directly by RGs and also by Cajal-Retzius cells (Fukumitsu et al., 1998). The role of BDNF on RGs has been investigated by both injection of BDNF itself directly in ventricles at mouse E13.5 (Fukumitsu et al., 2006) and also by overexpression of *Bdnf* in cortical precursors *in vivo* via *in utero* electroporation (Bartkowska et al., 2007). Both strategies led to an increase in proliferation. BDNF is one of the ligands which can activate the tropomyosin receptor kinase B (TrkB) receptor. The loss of function of its gene (*Ntrk2*) in the mouse was achieved by different approaches such as by short hairpin RNAs (shRNAs) or by expression of a dominant-negative variant of TrkB. Blockade of TrkB signaling elicited a decrease in RG self-renewal (Bartkowska et al., 2007). TrkB receptors can be phosphorylated, which will activate the MAPK or phosphoinositide 3-kinase (PI3K) pathways (Numakawa et al., 2018). Both pathways are implicated in different functions of RG behavior during cortical development. PI3K is important for RG survival whereas MAPK activation is required for the production of neurons (Barnabe-Heider and Miller, 2003). Therefore, the pathway linking BDNF, TrkB and PI3K is essential for the maintenance of the RG scaffold, indeed activation of the BDNF-TrkB-MAPK axis can lead to premature RG differentiation into neurons via the activation of BMP7 (Ortega and Alcantara, 2010 and see below). BDNF can also activate Anoctamin 1 (ANO1), a Ca2+-activated chloride channel which is expressed in RGs (Hong et al., 2019). The growth of RG basal processes is dependent on the activity of this channel as its loss of function disrupts the extension of RG protrusions. ANO1 overexpression inversely increases this process (Hong et al., 2019). The lack of basal process growth in *Ano*1-deficient mice leads to disorganized cortical layers and microcephaly (Hong et al., 2019).

Transforming growth factor β 1 (TGF-β1) is a cytokine which is involved at many levels of neuronal development (Meyers and Kessler, 2017). TGF-β1 is present in the VZ of the developing cortex (Mecha et al., 2008) and its receptor, TGFRII, is highly expressed by RGs (Stipursky et al., 2014). Although its role is mainly associated with the differentiation of RGs into either neurons or glia (Stipursky et al., 2012, 2014), injection of TGF-β1 directly into the embryonic ventricles at E14 induces drastic changes in RG scaffold morphology. Basal processes seem shorter and disorganized. In fact, TGF-β1 triggers early transition of RGs into astrocytes which alters their morphology from radial to multipolar. Interestingly, these effects are similar to the action of a morphogen, as described in the next paragraph, more than a growth factor.

TABLE 1 | Non-exhaustive list of proteins influencing RG scaffolds during cortical development.

Protein	Localization	Implication in RG scaffold	Phenotype	References
Afadin	Apical endfeet	Apical process arrangement AJ maintenance	Apical process irregularly arranged Loss of AJ markers	Yamamoto et al., 2015
aPKCλ (Atypical protein kinase C λ)	Apical endfeet	RG polarity Apical process maintenance	Apical process retraction RG detachment	Fumiyasu et al., 2006
APC (adenomatous poli C)	RG tips Soma	Maintenance and extension of RG processes Scaffold polarity	Mis-oriented scaffold (basal process not directed at pial surface) Shorter processes	Yokota et al., 2009
Arp2/3 (Actin Related Protein 2/3)	Basal/apical endfeet Soma Nucleus	Formation and maintenance of AJs	Shorter RG processes and misoriented Lower speed of basal process formation Ventricular surface is altered	Wang P.S. et al., 2016
Bone morphogenic proteins/SMADs	eCSF Meninges Hem	Control of neurogenesis	Premature differentiation Thinner cortex/microcephaly	Najas et al., 2020
Brain Derived Neurotrophic Factor	eCSF RG Cajal-Retzius cells	RG self-renewal	Decrease of RG proliferation	Bartkowska et al., 2007
Cdc42 (Cell division control protein 42)	Leading process (basal fiber)	Basal process growth Inter−radial fiber interactions	Shorter basal process Decreased contacts between RG fibers	Yokota et al., 2010
Ece2 (endothelin converting enzyme-2)	RG apical compartment Cortical plate	Apical process maintenance RG morphology Ventricular surface integrity	Loss of apical process Ventricular surface alteration Loss of radial morphology	Buchsbaum et al., 2020
ECM components and receptors	Pial surface VZ SVZ	Apical process integrity Basal process integrity RG morphology		Milev et al., 1996; Li et al., 2008; Loulier et al., 2009; Sittaramane et al., 2009; Okamoto et al., 2013; Buchsbaum et al., 2020
Epidermal growth factor	eCSF	Maintenance of RG identity and self-renewal		Burrows et al., 1997; Lillien and Raphael, 2000; Sun et al., 2005
Fibroblast growth factor	eCSF	Production and maintenance of RG	Decrease in cortical size	Dono et al., 1998; Raballo et al., 2000
FSTL1 (Follistatin like-1)	Pial basement membrane	RG basal process orientation Basal endfeet branching	RG basal process not parallel Less endfeet branched	Liu et al., 2015
Glial growth factor	Neuronal secretion	Basal process elongation	Loss of endfeet formation and disrupted morphology	Anton et al., 1997
GSK3 (Glycogen synthase kinase 3)	Leading process (basal fiber)	Basal process growth and orientation Whole scaffold morphology	Shorter basal process Basal process mis-oriented Scaffold morphology altered	Yokota et al., 2010
Insulin-like Growth factors	eCSF	RG proliferation	Neurogenesis decrease	Lehtinen et al., 2011
N-cadherin	AJs	AJ maintenance Apical process maintenance	RG detachment Apical process retraction Premature differentiation	Rousso et al., 2012; Wong et al., 2012; Das and Storey, 2014

(Continued)

TABLE 1 | Continued

Protein	Localization	Implication in RG scaffold	Phenotype	References
Neuregulins	RG	Maintenance of RG proliferation and radial morphology	Reduced number of RG	Schmid et al., 2003; Nakagawa et al., 2019
Notch	RG	RG identity Promotion of radial morphology Increase expression of adhesion proteins	Premature differentiation Overexpression: Radial morphology increased Adhesion protein expression increased	Li et al., 2008; Yoon et al., 2008
Numb/Numbl	Apical endfeet	Radial polarity Apical process maintenance AJ maintenance	Altered ventricular surface Loss of radial polarity Loss of apical process	Rasin et al., 2007
Plekha7	Apical endfeet	Apical process maintenance Apical contact integrity	Loss of apical contact Apical process retraction	Tavano et al., 2018
Reelin	Cajal-Retzius cells	Maintenance of RG morphology	RG process branching defects	Hartfuss et al., 2003; Schaefer et al., 2008; Chai et al., 2015
Sonic Hedgehog	eCSF and interneurons	Radial glia proliferation	Reduction in RG number	Komada et al., 2008; Dave et al., 2011; Wang L. et al., 2016
TAG-1 (Transient axonal glycoprotein-1)	Basal region	Basal process maintenance	Basal process loss Basal process retractation	Okamoto et al., 2013
Transforming growth factor β 1	eCSF	Control of RG morphology and processes	ND	Stipursky et al., 2014
Wnt	eCSF	RG self-renewal RG radial morphology	Basal process disruption Premature differentiation	Woodhead et al., 2006; Nakagawa et al., 2017

The column phenotype takes into account results of experiments done when the protein is lacking (cKO, KO, pharmacological inhibition etc.) Proteins are presented in alphabetical order.

Thus, different growth factors play apparently critical roles influencing the formation and maintenance of RG scaffolds.

Morphogens

Contrary to growth factors which are known classically to act at the proliferation level, morphogens are instead also associated with an action at the differentiation level and to control cell fate decisions (Briscoe and Small, 2015). We review here the known roles of morphogens in the maintenance of RGs and therefore the RG scaffold (**Figures 3**, **4** and **Table 1**).

Certain members of the TGF family, e.g., the **bone morphogenic proteins** (BMP), have important roles in the maintenance of RG scaffolding. *In vitro* experiments on cultures of RGs indicated that BMP signaling is involved in the control of neurogenesis (Li et al., 1998; Mabie et al., 1999). Bmp7 has been detected in the meninges, hem and also in the eCSF (Segklia et al., 2012). When Bmp7 is removed from the mouse brain, this leads to reduced cortical thickness and number of neurons at E14.5. On the other hand, when Bmp signaling is activated by expressing a constitutively active form of its receptors (Bmpr1a or Bmpr1b), over proliferation and defects in global morphology are observed in the developing cortex (Panchision et al., 2001). In particular, folds can be seen at the brain surface, suggesting differences in RG scaffolding. More recently, the implication of Smad1/5 (canonical BMP transcription factors) was revealed by loss of function experiments in both mouse and chick (Najas et al., 2020). In these models, RG maintenance was disrupted, and premature differentiation occurred, which leads to a microcephaly phenotype. The consequences of KO were assessed on neurogenesis but not the RG scaffold *per se*. However, SMADs are likely to regulate neurogenesis by modulating YAP (Yes-associated protein) activity (Najas et al., 2020), since decreasing SMAD1/5 leads to a decrease in YAP translocation into RG nuclei. This is crucial for cortical development as Hippo signaling has been linked to apical RG surface integrity and adhesion (Roy et al., 2019). Moreover, KO of YAP and TAZ, a transcriptional coactivator with PDZ-binding motif, can rescue genetically driven (via a *Pard3* deletion in the mouse) cortical heterotopia associated with detached RGs and higher YAP levels (Liu et al., 2018, see also section "Further Factors Identified via Human Pathology" *Human pathology*). Overall, these data indicate an important role of BMPs via their activation of SMADs in the control of RG behavior during cortical development.

The **Wnt** morphogen is implicated at many levels of neural system development and in particular in the cortex (Harrison-Uy and Pleasure, 2012). Wnt proteins are present and active in the eCSF where they are transported by lipoprotein particles (Johansson et al., 2013; Kaiser et al., 2019). Many different studies point to the role of Wnt as an essential factor in maintaining RG identity and self-renewal. A scaffolding disruption phenotype as well as proliferative defects are described in the developing hippocampus in the *Lrp6* gene mouse KO, one of the most important Wnt co-receptors (Pinson et al., 2000; Zhou et al., 2004; Wang Y. et al., 2016). Concerning the intracellular signaling triggered by Wnt, the canonical pathway relying on β-catenin inhibits neurogenesis by keeping RG undifferentiated (Woodhead et al., 2006; Wrobel et al., 2007; Mutch et al., 2010; Munji et al., 2011). β-catenin can be involved in different cellular processes such as cell-cell adhesion in addition to its transcriptional role. In one study, the authors specifically abrogated β-catenin's transcriptional role by expression of a truncated form of this molecule in the telencephalon (Draganova et al., 2015). This study showed that Wnt/β-catenin signaling regulates a network of transcription factors involved in specific stages of cortical development including Dach1, Eya2, Etv5, and also Nfix (Draganova et al., 2015). In the Wnt/β-catenin pathway, Adenomatous polyposis coli (APC) is a regulator of β-catenin driving its degradation in the absence of Wnt binding at the membrane (Nelson et al., 2015). In an APC conditional KO in mouse RGs, the scaffold of basal processes is disturbed (Nakagawa et al., 2017). It is also interesting to note that Wnt signaling has been implicated in the maintenance of basal progenitors via the regulation of N-myc (Kuwahara et al., 2010). Therefore, since several Wnt molecules are expressed at different levels of the developing cortex (i.e., Wnt7a at the apical surface and Wnt7b in the basal parenchyma), it is possible that this morphogen can regulate the RG scaffold throughout the cortex and even in superficial regions.

The presence of the **Sonic Hedgehog** morphogen (Shh) ligand in the developing cortex has been known for several years (Komada et al., 2008). Shh is a well-known morphogen which can control a lot of different aspects of neurodevelopment at different locations of the nervous system (for review see Ferent and Traiffort, 2015). Shh is present in the eCSF, providing a source for the VZ, as identified by the ELISA method (Huang et al., 2010; Chau et al., 2015; Lun et al., 2015). Shh production occurs in cells of the choroid plexus of the fourth ventricle of the hindbrain (Huang et al., 2010) but not from the choroid plexus from the telencephalon (Lun et al., 2015). This would suggest that ventricular derived-Shh derived from the hChP would have to travel long distances to reach the ventricular wall of the developing cortex. Very recently, Shh secretion in the eCSF was linked to the ESCRT-III system (Endosomal sorting complex required for transport). Indeed, the *Chmp1a* (a gene coding for the charged multivesicular body protein *1a*, a subunit of the ESCRT complex) null mice present a decrease in the amount of Shh in the eCSF, correlated with a reduction in RG proliferation and the development of microcephaly (Coulter et al., 2018). This phenotype can be rescued when Shh signaling is genetically activated, showing that the ESCRT system is indeed upstream of Shh secretion. Migrating interneurons and Cajal-Retzius cells also produce Shh locally within the cortex (Dahmane et al., 2001; Flandin et al., 2011).

Several studies focused on the role of receptors or downstream signaling components of the Shh pathway during cortical development. Loss of function of the Smoothened Shh signaling activator in RG using GFAP-Cre or Nestin-Cre mice showed a decrease in proliferation, whereas activating the pathway via Patched1 receptor KO showed an increase (Dave et al., 2011; Wang L. et al., 2016). Overexpression of a constitutive form of Smo (SmoM2) increases the proportion of bRG in the developing cortex, suggesting a potential role for Shh signaling in the formation of bRGs (Wang L. et al., 2016). The role of the Patched1 co-receptor *Cdon* in cortical development has been highlighted

by a loss-of-function study showing that deletion of Cdon leads to cortical microcephaly and reduction in RG proliferation (Zhang et al., 2006). At the molecular level, Shh controls the activity of Gli transcription factors to favor Gli2 activating forms over Gli3 repressor forms (**Figure 4**). Therefore, *Gli2* mutant mice present a decrease in RG proliferation (Palma et al., 2004) whereas Gli3 repressor form invalidation leads to an increase in cell cycle speed (Wilson et al., 2012). Suppressor of Fused (Sufu) is an important inhibitor of Shh signaling activity. Some ectopic progenitor clusters are detected in the cortical wall, showing over proliferation, when Sufu is conditionally knocked-out in the murine cortex (using Emx1-Cre) (Yabut et al., 2015). Ultimately, this leads to major defects such as a thinner cortex and strong differentiation disruption. Very recently, Yabut et al. showed that Sufu regulation of the Shh pathway controls the expression of *Fgf15* which is responsible for lineage progression of RGs (Yabut et al., 2020). This is a good example of how different extracellular cues can influence one another to modulate RG behavior.

Thus morphogens can have multiple effects but they are notable in their impact on RG structure, maintenance sand behavior.

SECRETED FACTORS FROM CLOSE RANGE CELLS

The eCSF is not the only source of secreted factors controlling the RG scaffold. Extracellular cues can be also sent from neighboring cells throughout the tissue. For example, the formation and maintenance of the basal process is dynamic (Yokota et al., 2010), with important information received from the meninges (Radakovits et al., 2009; Siegenthaler et al., 2009). This basal communication is not well known, including the mechanisms by which the meninges provide information to basal processes for their maintenance. Here, we provide examples of proteins involved in RG scaffold maintenance in response to extracellular cues produced locally within the developing cortex or from the meninges (**Figure 3**).

Neuregulins (NRG) play a major role in neuronal migration and RG integrity (Anton et al., 1997; Lopez-Bendito et al., 2006). In particular, mouse KO of *Nrg-1* leads to reduced cell numbers in primary cultures of embryonic progenitors (Schmid et al., 2003). NRG activates the v-Erb-a erythroblastic leukemia viral oncogene homolog (ErbB) family of tyrosine kinase receptors. ErbB2, 3, and 4 are expressed by RGs and are present along their basal processes (Schmid et al., 2003). Importantly, ErbB2 expression is specific to RGs and its loss of function in the mouse unbalances the astrocyte/RG population ratio by reducing the number of elongated RGs in the developing cortex (Schmid et al., 2003). ErbB2 interacts specifically with a redox active protein, Memo1 (Newkirk et al., 2018). Although Memo1 has been known for some time to be important for cell migration (Marone et al., 2004), its role in the branching and the maintenance of the RG scaffold was identified relatively recently (Nakagawa et al., 2019). A link has also been established between Nrg signaling and mGluR5 receptors. Indeed mGluR5 is coupled to the non-selective cation channel, canonical transient receptor potential 3 (Trpc3) (Louhivuori et al., 2015) and its loss of function in the mouse disrupts the formation of RG processes. This RG growth defect mediated by the mGluR5/Trpc3 signaling blockade can be rescued by Nrg/ErbB4 signaling showing that Nrg/ErbB4 is downstream of mGluR5/Trpc3 (Louhivuori et al., 2018).

Retinoic acid (RA) is a very well-known neurogenesis modulator. The particularity of this factor is that it is produced by different sources which could each impact cortical development. Although RA is secreted in the eCSF as described in chick (Alonso et al., 2011) and in zebrafish (Chang et al., 2016), its role on RG behavior has mainly been attributed to the meninges source (Siegenthaler et al., 2009). Indeed, when meninges are disrupted, limiting the supply of RA, or when a hypomorphic allele for the RA synthesizing enzyme Rdh10 is generated in the mouse, production of IPs is decreased (Siegenthaler et al., 2009). Nevertheless, this phenotype was not observed in *Rdh10* -/- mouse embryos (Chatzi et al., 2013; Haushalter et al., 2017), nor in conditional KO embryos for the other enzyme responsible for RA synthesis, Raldh2 (Haushalter et al., 2017). Therefore, it seems that although meninges-derived RA is important, its role with respect to RGs stills needs clarifying. The role of eCSF RA has also not yet been clearly identified.

Cajal-Retzius cells, present in basal regions in the MZ of the developing cortex, secrete, amongst other factors, **Reelin**, a glycoprotein which interacts extracellularly with receptors on migrating neurons (Sekine et al., 2014, see also section "Further Factors Identified via Human Pathology" *Human pathology*). When RG basal processes reach the MZ, they branch, however this branching is impaired in the *reeler* mutant mouse (deficient for Reelin, Chai et al., 2015). This indicates that besides its classical role influencing migrating neurons, Reelin may also control some aspects of RG morphology and influence the scaffolding (see also Hartfuss et al., 2003; Schaefer et al., 2008). Also, Reelin was linked to maintaining hippocampal RG integrity, since *reeler* tissue also showed precocious conversion of RGs to astrocytes, rescued by exogenous sources of Reelin (Zhao et al., 2004). Amongst the signals secreted by the meninges, **CXCL12** (chemokine (C-X-C motif) ligand 12) also called SDF1 (stromal cell-derived factor 1) can directly act on Cajal-Retzius cells and therefore indirectly modify the formation of RG scaffolding (Borrell and Marin, 2006). Briefly, CXCL12 controls tangential migration of Cajal-Retzius cells and disruption of its receptor CXCR4 leads to their displacement in deeper layers of the cortex, resulting in a dysplastic cortex (Paredes et al., 2006). Similarly in the hippocampus, CXCR4 invalidation also leads to severe phenotypes, including dentate gyrus granule neuron migration defects but also reduced proliferation of RG-like progenitors (Lu et al., 2002; Berger et al., 2007).

Bidirectional interactions between migrating neurons and RGs are essential for RG fiber growth. The **glial growth factor** (GGF), a soluble form of neuregulin, is expressed by migrating neurons along RG fibers and influences positively the growth of the RG fiber. In a pioneering study, Anton et al. (1997) provided evidence suggesting that the effect of GGF signaling on fiber elongation via ErbB2 is mediated through BLBP (brain lipid binding protein), an RG-expressed molecule (see also Hartfuss et al., 2003; Poluch and Juliano, 2010). It is thought that the rate of

migratory neurons influences the lengthening of RG fiber, which also influences the rate of migratory neurons (Anton et al., 1997).

In the pial basement membrane (BM), a novel role for the secreted glycoprotein, **Follistatin like-1** (FSTL1) was identified in RG scaffolding (Liu et al., 2015). Indeed, authors showed that in embryonic mouse cortices, RG basal processes were not parallel and their endfeet less branched. Thus, they provide data suggesting that this protein is important for the basal but not the apical process and plays its role through a unique mechanism that does not include Cdc42 and GSk3β (Liu et al., 2015). This emphasizes the fact that multiple mechanisms are involved in the formation of the RG scaffold.

ROLE OF CELL TO CELL AND CELL TO ECM CONTACTS IN THE FORMATION AND MAINTENANCE OF THE RG SCAFFOLD AND PROLIFERATION

Because RGs extend across the whole cortical wall, they make numerous and various contacts. This section will focus on the impact of different contacts on their scaffold and their proliferative capacity.

Adherens Junctions (AJs)

At the onset of neurogenesis, neuroepithelial cells become RGs and lose tight junctions, but AJs are maintained (Aaku-Saraste et al., 1996). These are composed of junctional complexes including N-cadherin, β catenin, α catenin and the cytoskeleton, which connect the apical regions of RGs to each other at the apical ventricular surface facing the eCSF (**Figure 5**).

The extracellular domain of **N-cadherin** enables the anchoring of the cells to each other, while the intracellular domain is connected to β and α catenins to link the AJ to the cytoskeleton. Therefore, this complex links the actin cytoskeleton to the plasma membrane to form cadherin mediated cell-cell adhesion sites (Drees et al., 2005; Nelson, 2008; Pokutta et al., 2008; Benjamin et al., 2010; Maiden and Hardin, 2011). Many studies (Miyamoto et al., 2015; Veeraval et al., 2020) emphasize the fact that N-cadherin based AJs are key elements for the development of cortical architecture. Several proteins involved in the maintenance of AJs, including afadin, as well as N-cadherin, αE catenin, β catenin, are essential for the formation and maintenance of the RG scaffold (**Table 1**).

First, RG N-cadherin based AJs are Numb-dependent (Kadowaki et al., 2007; Rasin et al., 2007). Numb acts as an inhibitor of Notch signaling and is localized in apical endfeet of RGs. Numb/Numbl (homolog of Numb) interact with cadherin-catenin complexes during cortical development and are essential to maintain N-cadherin AJs. When Numb and Numbl are lacking in mouse cortices, the ventricular surface is altered, and RGs lose their radial polarity and their apical process suggesting an essential role of Numb/Numbl in apical process maintenance (Rasin et al., 2007). Moreover, N-cadherin has been shown to be required to prevent RG delamination, apical process retraction and premature differentiation (Rousso et al., 2012; Das and Storey, 2014; Wang P.S. et al., 2016). The maintenance of cadherin based AJs enables the activation of the β catenin phosphodegradation complex (Gsk3β, APC, Axin) and so reduces its level in the cytoplasm (Maher et al., 2009). As mentioned in section "Secreted Factors From Close Range Cells," β catenin is an effector of Wnt signaling, and is stabilized in the cell, influenced by the N-cadherin and Akt pathways. Interestingly, the presence of N-cadherin also allows Akt activity, and the phosphorylation of β catenin by Akt increases its translocation to the nucleus (Zhang et al., 2010; Zhang et al., 2013). These data further suggest a role of N-cadherin in the regulation of β catenin level and distribution. N-cadherin function is also impacted by the conditional KO of afadin in the cortex, and mice exhibit a double cortex (a normotopic cortex as well as a heterotopic cortex) due to detached RGs. In these mutants Yamamoto et al. (2015) showed that major proteins (including N-cadherin) of RG AJs are not maintained at E14.5, suggesting that afadin plays an essential role in the maintenance of the AJs in the apical processes. In this case, ectopic detached Sox2 + progenitors result. RG basal processes were not altered but the apical processes were irregularly arranged in the deficient embryonic mouse cortices. Interestingly, no defects in RG proliferation and differentiation were found in these mutants (Yamamoto et al., 2015), further suggesting a crucial role of basal attachment on RG proliferation (Uzquiano et al., 2018).

It is important to mention the **Arp2/3 complex** that is involved in the formation and maintenance of AJs. When Arp2/3 is conditionally deleted in the mouse, RG processes are shorter and mis-oriented (Wang P.S. et al., 2016). More precisely, the ventricular surface is altered with the presence of ectopic progenitors and the speed of formation of the basal process is also reduced. The Arp2/3 complex is an effector of β catenin, and establishes a link between the formation of the RG scaffold and the cytoskeleton (Wang P.S. et al., 2016). Cdc42 and RhoA are known to be upstream regulators of the Arp2/3 complex, and control both basal process extension and apical process adhesion (Cappello et al., 2006; Yokota et al., 2010).

Also, **Atypical protein kinase C** λ (aPKC λ) is a protein kinase present at the level of AJs during mammalian corticogenesis, and this protein forms complexes with polarity proteins Par6 and Par3. When aPKC is conditionally deleted in mouse cortex, apical processes of RGs retract more often and RGs detach from the ventricular surface. aPKC is indispensable for neuroepithelial cells to form AJs and maintain cell polarity in the neuroepithelium (Imai et al., 2006). Also, classical polarity proteins (Par3, Llg1) are phosphorylated by aPKC during RG polarity establishment (leading to the specific bipolar RG morphology) (Yamanaka et al., 2003) emphasizing a role of classical polarity proteins in this scaffolding. As mentioned above, *Pard3* conditional KO leads to detached RGs (Liu et al., 2018).

Plekha7 is another protein associated with apical AJs, and is involved in the maintenance of the RG scaffold, preventing RG delamination. Tavano et al. (2018) showed the importance of this AJ-associated protein by forcing the expression of *Insulinoma-associated* 1 (*Insm*1) in RGs. Insm1 is

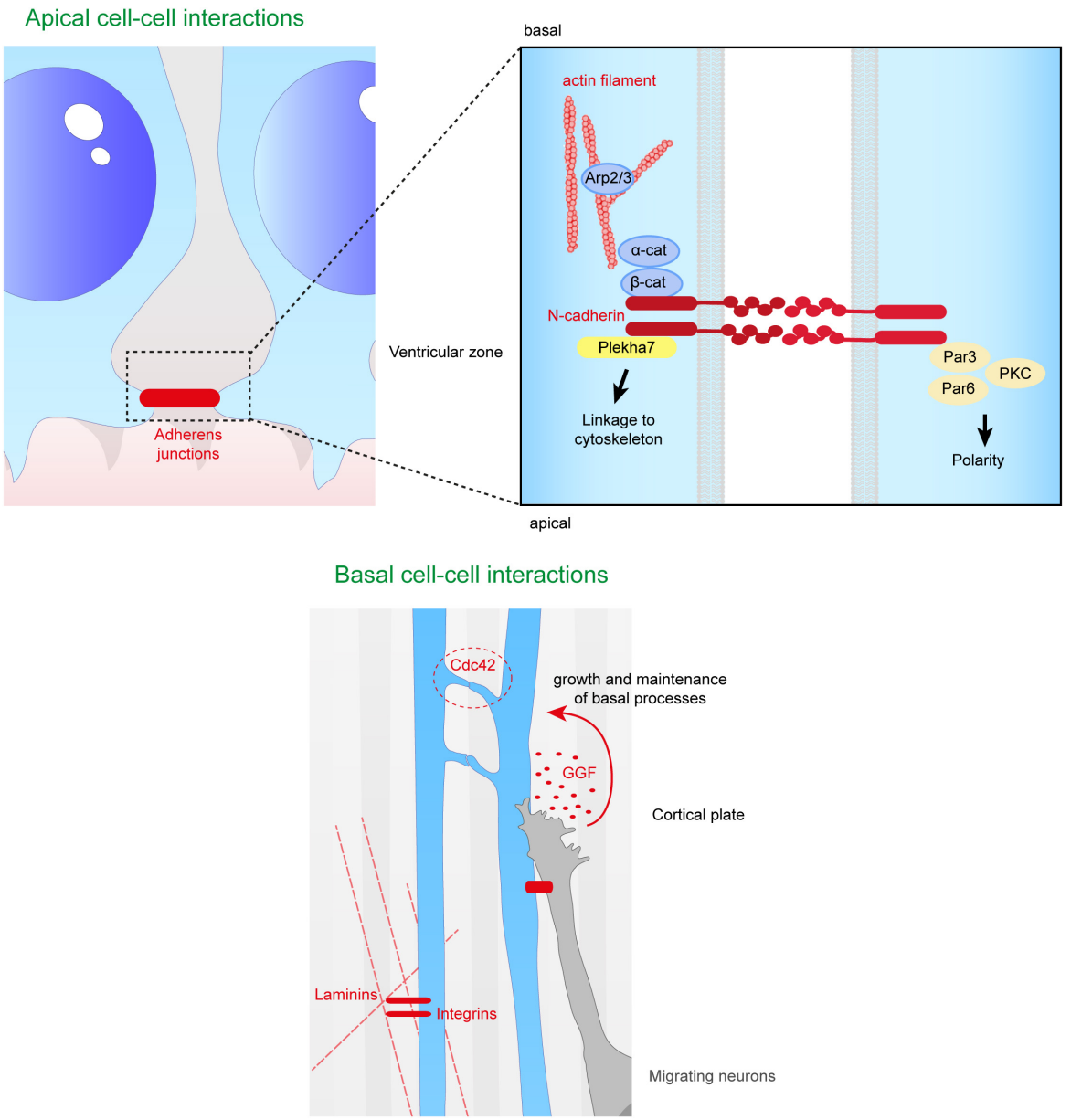

FIGURE 5 | Close range contacts controling the scaffolding of RGs. RGs directly receive signals from neighboring cells such as other RGs or migrating neurons. On the top panel are depicted the cell–cell interactions occurring at the apical side of aRGs. Adherens junctions between aRGs are crucial for the maintenance of the scaffold. In the enlarged box is represented the binding of N-cadherins which can link extracellular contacts with the cytoskeleton (via Plekha7 or β-catenin) or with polarity proteins such as Par3, Par6, and PKC. On the bottom panel is illustrated basal cell–cell interactions. Basal processes of RGs can interact with each other inducing a Cdc42 response intracellularly. Neurons can also directly act on the glial scaffold by secreting factors such as GGF which controls growth and maintenance of basal processes. Finally, basal processes receive information from the extracellular matrix, especially via the interaction between intergins and laminins.

a transcription factor that represses *Plekha7* transcription. By forced expression of Insm1, there was an increased proportion of bIPs (multipolar cells) and bRGs, suggesting an alteration of the RG scaffold across the brain. Thus, when the level of Plekha7 is reduced by Insm1 forced expression, RGs lose their apical contact and their apical processe retracts (Tavano et al., 2018). This study confirms that AJ components are crucial for the RG scaffold and more specifically for apical process integrity.

Thus, perturbing or changing AJs influences RG attachment and this is one method by which apically detached RGs can arise (Penisson et al., 2019; Kalebic and Huttner, 2020). The fate of the detached cell can be variable (e.g., aberrant RG, bRG, IP or neuron) depending often on mutant conditions.

Also, as described later in this review (see section "Further Factors Identified via Human Pathology" *Human pathology*), certain human cortical malformation gene mutations have been identified, related to further apical adhesion complexes. These data also emphasize the importance of RG apical contacts for the intact RG scaffold and correct neuronal migration.

Extracellular Matrix (ECM) Components

The ECM is essential for corticogenesis and neural development (for review see Long and Huttner, 2019). In the embryonic cortex the ECM, composed of various proteins such as laminins, proteoglycans, dystroglycans and collagens, surrounds the cells (including RGs). Transcriptome analyses of human and mouse germinal zones in developing cortex (identified from laser microdissected material) showed that the variation of expression of ECM protein interactions and cell adhesion is likely to regulate the ability of neuronal progenitors to proliferate. Also, the expression profile of ECM proteins emphasizes differences between mouse SVZ (containing IPs and few bRGs) and human SVZ (oSVZ, iSVZ containing numerous bRGs and IPs) (Arai et al., 2011; Fietz et al., 2012). As already mentioned, further transcriptome analyses showed that an increase in the production of bRGs which no longer have contact with the eCSF, seems likely to require a modified ECM compared to ventricular aRGs (Pollen et al., 2015; Kalebic and Huttner, 2020). In a gyrencephalic model (ferret), it has been shown that inhibiting integrin (a major ECM receptor, Yamada and Sekiguchi, 2015) in the developing neocortex leads to a reduction in the number of bRGs (Fietz et al., 2010). Conversely, in the mouse increased expression of integrin increases the proliferation of basal progenitors (Stenzel et al., 2014). Recently, Kalebic et al. (2019) showed that the increased ability of bRGs to proliferate was associated with an increased number of RG processes. Indeed, increased processes allows the bRGs to multiply the reception of proliferative signals *via* integrin.

Radial glia basal processes extend across the cortical wall from the VZ to reach the pial surface (Rakic, 1972). These basal fibers are attached at the pial surface in part by **integrin−laminin-interactions**, allowing the migration of newborn neurons to reach their correct place in the CP (Graus-Porta et al., 2001; Belvindrah et al., 2007). Integrin−laminin interactions help anchor RG basal processes to the pial BM. Interestingly, laminin induces intracellular signaling via several receptors (e.g., as well as integrin, also dystroglycan, see section "Further Factors Identified via Human Pathology" *Human pathology*).

Related to this, **TAG-1**, for transient axonal glycoprotein-1, is a cell surface molecule expressed in the basal region of the cortical wall during embryonic development. This molecule also known as contactin-2 is essential for the maintenance of RG basal processes. Indeed, knockdown of TAG-1 in the mouse leads to basal process retraction and ectopic progenitors (Okamoto et al., 2013). The role of TAG-1 in basal RG process maintenance is cell autonomous, and knockdown does not affect apical surface integrity even if it increases mechanical stress in the ventricular zone. Indeed, the role of TAG-1 in the apical process is not well established. The mechanisms underlying its role in the basal RG fiber are not well defined but one hypothesis is that it is through the interaction of TAG-1 and basal lamina components such as Anoxin-1 and Laminin (Milev et al., 1996; Sittaramane et al., 2009; Okamoto et al., 2013).

Furthermore, *via* isolated stabilized RG clones, Li et al. (2008) showed that activated **Notch** promotes radial morphology, increases expression of BLBP (mentioned at the end of the section "Role of Secreted Proteins Derived From the CSF in the Formation and Maintenance of the RG Scaffold" as an actor in RG process elongation) and promotes RG adhesion on a laminin/nidogen complex. In this *in vitro* model, the authors observed increased expression of other adhesion proteins such as proteoglycans contributing to the brain ECM. Regarding these findings we can hypothesize that Notch action on radial morphology is likely to involve these ECM elements (Li et al., 2008). Also indirectly, Notch has been shown to be important in RG scaffolding. Indeed, the first step of the establishment of the RG scaffold is the maintenance of RG identity itself. Yoon et al. described data involving Notch signaling in the maintenance of the RG pool. By a specific deletion of *mind bomb 1* in mouse embryonic neuronal progenitors, Notch activation was inhibited and premature differentiation of RGs to IPs and neurons was observed (Yoon et al., 2008).

Loulier et al. (2009) demonstrated that the integrin-laminin interaction may act also in apical processes at the ventricular surface. Using blocking antibodies delivered into the cerebral ventricle *in utero*, preventing the fixation of laminin to its ligand β1 integrin, they observed detachment of RG apical processes, suggesting an apical role of the integrin-laminin interaction (Loulier et al., 2009). These findings provided evidence of the ECM's role in RG bipolar shape and proliferative ability. More recent transcriptome and proteome analyses continue to contribute information concerning the ECM and different progenitor types including RGs (Fietz et al., 2012; Pollen et al., 2015; Buchsbaum et al., 2020; Kalebic and Huttner, 2020). For example, recently, interested in periventricular heterotopia (see section "Further Factors Identified via Human Pathology" *Human pathology*), Buchsbaum et al. (2020) provide novel information concerning the endothelin converting enzyme-2 (ECE2) gene and RG morphology. In cerebral organoids and the developing mouse cortex, they show that knockdown of *ECE2*/*Ece2* changes aRG morphology since these cells are less radial and bipolar. The ventricular surface was also altered, and aRG lose their apical process suggesting a role of this protein in apical RG processes. Interestingly, this ECE2-deficient phenotype is associated with ECM protein and receptor dowregulation.

Extracellular matrix components thus clearly have crucial roles in RG morphology and proliferative ability. This is a current exciting area of research which will further clarify the precise mechanisms involved.

Interactions via Basal Processes (RG−RG, RG-Neuron)

As RGs are a physical support for post-mitotic neuron migration, it is clear that neurons and RG interact. This interaction provides to neurons a mechanical support for migration but also a way to communicate with RGs that can influence the migration

process. It is thus not only the integrity of the RG scaffold that is essential to allow this post-mitotic neuron migration, but also the communication between the different cell types. Coherent with this, it has been shown that **connexin 43** (Cx43) and **26** (Cx26) connect migrating neurons and RG fibers via gap junction dynamic adhesive contacts (Elias et al., 2007). Importantly, when either *Cx43* or *Cx26* is downregulated via shRNAs injected in mouse embryonic cortex, the neuron's ability to migrate is reduced, without however, affecting the RG scaffold and expression of other cell-cell adhesion proteins. Also, as explained in section "Role of Secreted Proteins Derived From the CSF in the Formation and Maintenance of the RG Scaffold" (see BLBP section), neuron attachments to RG fibers are important for RG process elongation. In this context, it is important to mention that N-cadherin also plays a role in the attachment of the migrating neuron to the RG fiber, with its knockdown diminishing this interaction. The correct level of N-cadherin at the neuronal cell surface is mediated via endocytic pathways dependent on Rab GTPases (Kawauchi et al., 2010; Shikanai et al., 2011).

Cdc42 is a small GTPase localized at the leading edge of basal radial fibers, where it allows the recruitment of protein complexes such as Par6-aPKC (Etienne-Manneville and Hall, 2003; Heasman and Ridley, 2008). Cdc42 plays a role in RG−RG interactions via inter-radial fibers, during the dynamic extension of the basal process (**Figure 5**). Indeed, *Cdc42* KO in the mouse leads to shorter basal processes which do not reach the pial surface during cortical development. The number of contacts between RG fibers is also reduced. Very little is known about the role of the inter-radial fiber on the scaffold, but there is a correlation between less inter-radial fibers and shorter RG basal processes (Yokota et al., 2010). Cdc42 signaling is known to be regulated via GSK3β phosphorylation, but the phenotype of the RG scaffold when GSK3 is pharmacologically inhibited is not the same as the *Cdc42* cKO phenotype. Indeed, although basal processes are shorter as in the *Cdc42* cKO, they are not well oriented at the pial surface after inhibition of GSK3 and the whole scaffold shows a wavy morphology. This suggests distinct roles for these two proteins influencing RG scaffolding and basal processes (Yokota et al., 2010).

As mentioned previously, in the Wnt signaling pathway **APC** has a role in the maintenance and extension of the RG scaffold. APC is localized in RG tips and in the soma. When it is specifically deleted in RGs *in vivo*, the scaffold is mis-oriented with basal processes not directed to the pial surface (Yokota et al., 2009). Over corticogenesis, the processes appear also shorter, suggesting a role for APC in maintenance of scaffold polarity but also fiber extension. APC is involved in the response of basal process extension via neuregulin 1 signaling (see section "Role of Secreted Proteins Derived From the CSF in the Formation and Maintenance of the RG Scaffold"), but also in the stability of microtubules at cell contacts (apical AJs and in basal RG endfeet at BM sites, Yokota et al., 2009). Indeed, APC is known to interact with microtubule proteins such as EB1 and microtubules themselves, and to allow the correct localization of polarity proteins (Numb, Cdc42) in subcellular compartments. Without APC, the integrity of apical and basal cell-cell contacts may hence be altered. This may explain why in its absence, basal fibers do not respond to neuregulin 1 since the interactions are not made correctly. However, its intrinsic role in microtubule stability may also play a role in this mechanism (Yokota et al., 2009).

FURTHER FACTORS IDENTIFIED VIA HUMAN PATHOLOGY

As previously mentioned, depletion of long range and short range factors can disrupt RG proliferation leading to microcephaly. Disruption of RG architecture on which neuronal migration relies, can also lead to other cortical malformations mentioned here, including human lissencephaly, polymicrogyria, and heterotopia. We describe the key features and genes involved in these disorders, shedding further light on external influences of neuronal migration.

Apically Disrupted RGs

When RG architecture is perturbed apically it can lead to heterotopias associated with epilepsy and sometimes intellectual disability (Bizzotto and Francis, 2015). Firstly, perturbation of RGs and neuron migration can lead to **periventricular nodular heterotopia** (PH) where clusters of neurons are identified close to the ventricles. In PH models, during development abnormal clusters of progenitors and neurons are found trapped at the ventricular surface (Bizzotto and Francis, 2015; **Table 2**). Secondly, although **subcortical band heterotopia** (abnormal neuron clusters found within the white matter) is usually associated with an intrinsic problem in migrating neurons, other subcortical heterotopias (SH) can arise due to perturbed and apically detached RG, which subsequently perturb migration (Kielar et al., 2014; Stouffer et al., 2016).

A number of PH genes highlighted here code for proteins regulating apical RG functions, with often as well evidence for a role in migrating neurons. PH is classically associated with mutations in *Filamin A*, coding for an actin cross-linking protein interacting with cell adhesion molecules such as integrins as well as other membrane proteins, enabling their anchoring to the cytoskeleton (Fox et al., 1998; Lian and Sheen, 2015; **Table 2**). Other PH proteins have been implicated in vesicle trafficking, e.g., ARFGEF2 required for trafficking from the Golgi apparatus (Sheen et al., 2004); and ERMARD and TMTC3, endoplasmic reticulum (ER) proteins (Conti et al., 2013; Larsen et al., 2017). *ARFGEF2* mutations can perturb proliferation and have been shown to affect the localization of cadherins and β catenin at the cell surface (Sheen et al., 2004), thus disrupting AJs. Mutations in α *N-catenin* (*CTNNA2*) also give rise to severe brain malformations (complex cortical dysplasia, Schaffer et al., 2018). The apical protocadherin receptor-ligand pair *DCHS1* and *FAT4* also show mutations in Van Maldergem syndrome which includes PH (Cappello et al., 2013). Acute knockdown in the mouse of these genes showed accumulation of cells in the VZ, as well as migration defects. Klaus et al. (2019) went on to show defective RG morphologies and transcriptional signatures, a discontinuous apical surface and slowed migration in human *in vitro* organoid models (Klaus et al., 2019). RG delamination most probably due to perturbed apical adhesion or signaling was

TABLE 2 | Genes mutated in human pathology associated with apical defects.

Gene	Pathology	OMIM_ number/acronym	OMIM neurological	LOF or GOF Model	Brain phenotype	Gene function	References
ARFGEF2/ Arfgef2	Peri ventricular heterotopia	608097 PERI VENTRICULAR HETEROTOPIA WITH MICROCEPHALY; ARPHM; AUTOSOMAL RECESSIVE	Microcephaly, progressive Delayed psychomotor development Mental retardation, severe Seizures Hypsarrhythmia Quadriparesis Periventricular nodular heterotopia seen on MRI Thin corpus callosum	LOF Mouse models: gene-trap; early postnatal intraventricular injections of 40 μm brefeldin-A (BFA).	Gene-trap: Early embryonic lethality. BFA: heterotopic nodules below the ventricular surface; discontinuous N-cadherin staining	ADP-ribosylation factor guanine nucleotide-exchange factor-2; brefeldin A (BFA)-inhibited GEF2 protein (BIG2), which is required for vesicle and membrane trafficking from the *trans*-Golgi network (TGN)	Sheen et al., 2004; Grzmil et al., 2010
CTNNA2/ Ctnna2	Pachygyria; Cerebellar hypoplasia	618174 CORTICAL DYSPLASIA, COMPLEX, WITH OTHER BRAIN MALFORMATION 9; CDCBM9, AUTOSOMAL RECESSIVE	Microcephaly, acquired Global developmental delay, Intellectual disability, severe Absent speech Inability to walk Ataxia Spastic tetraplegia Hyperreflexia Seizures, myoclonic, atonic, intractable Abnormal EEG Pachygyria Thickened cortex Thin CC Absent anterior commissure	LOF Cerebellar-deficient folia' (cdf) mice	Cerebellar ataxia and hypoplasia. Cerebellar and hippocampal lamination defects	Alpha-N-catenin, cadherin-associated protein related; cytoskeleton protein anchoring cadherins	Cook et al., 1997; Schaffer et al., 2018
DCHS1/ Dchs1	Periventricular heterotopia, van Maldergem	601390 VAN MALDERGEM SYNDROME 1; VMLDS1; AUTOSOMAL RECESSIVE	Mental retardation Intellectual disability Periventricular nodular heterotopia Subcortical band heterotopia Pachygyria Simplified gyral pattern Thin corpus callosum	LOF Dchs1-null embryonic mice; mouse IUE ShRNA; human *in vitro* organoid model	Early lethality; IUE: cells accumulated in the proliferative zones of the developing cortex. Changed proliferation, differentiation balance. Human: changed morphology of progenitor cells, defective migration of a subset of neurons, PH	Transmembrane cell adhesion molecule that belongs to the protocadherin superfamily. Apically located adhesive complex.	Cappello et al., 2013; Klaus et al., 2019
ECE2/ Ece2	Periventricular heterotopia	None	None	LOF Mouse IUE and human cerebral organoid models	Ectopic localization of neural progenitors and neurons (including non-cell autonomous). Rosettes of progenitors and neurons in cortex. Perturbed ventricular surface, progenitor detachment.	Endothelin-converting enzyme 2; type II metalloprotease; Links cytoskeleton and adhesion. Regulates secretion of extracellular matrix molecules	Buchsbaum et al., 2020

(Continued)

TABLE 2 | Continued

Gene	Pathology	OMIM_ number/acronym	OMIM neurological	LOF or GOF Model	Brain phenotype	Gene function	References
EML1/ Eml1	MEG, heterotopia	600348 BAND HETEROTOPIA; BH; AUTOSOMAL RECESSIVE	Macrocephaly Hydrocephalus Delayed development Intellectual disability Spasticity Seizures Sleep problems Subcortical band heterotopia Polymicrogyria Agenesis CC Dilated ventricles Behavioral problems	LOF *HeCo* heterotopic cortiex mice	Subcortical heterotopia; abnormal primary cilia	Microtubule-associated protein playing a role in trafficking from the Golgi apparatus.	Kielar et al., 2014; Uzquiano et al., 2019
ERMARD/ Ermard/ C6orf70	Periventricular heterotopia	615544 PERIVENTRICULAR NODULAR HETEROTOPIA 6; PVNH6; AUTOSOMAL DOMINANT	Delayed psychomotor development Seizures Delayed speech Hypsarrhythmia Hypoplastic corpus callosum, hippocampus and cerebellum Periventricular nodular heterotopia Polymicrogyria	LOF (haplo insuffi-ciency) IUE rat brain	Massive neuronal migration defect, significant arrest of cells within the ventricular zone, and development of heterotopic nodules along the walls of the lateral ventricles	Endoplasmic reticulum membrane-associated RNA degradation protein	Conti et al., 2013
FAT4/ Fat4	Periventricular heterotopia, van Maldergem	615546 VAN MALDERGEM SYNDROME 2; VMLDS2; AUTOSOMAL RECESSIVE	Mental retardation Intellectual disability Periventricular nodular heterotopia Subcortical band heterotopia Thin corpus callosum	LOF Fat4-null mouse mutants; IUE mouse. Human *in vitro* organoid model.	Mouse mutants early lethality. IUE: cells accumulated in the proliferative zones of the developing cortex, heterotopia. Human organoid: disorganized germinal layer, premature delamination of progentors, abnormal neuronal migration, nodules	Member of a large family of protocadherins; role in vertebrate planar cell polarity	Cappello et al., 2013; Klaus et al., 2019
FLNA/ FlnA	Periventricular heterotopia	300049 PERI VENTRICULAR NODULAR HETEROTOPIA 1; PVNH1; X-LINKED	Seizures, refractory to treatment Imaging shows non-calcified subependymal periventricular heterotopic nodules Mental retardation, mild Strokes due to coagulopathy Neuronal migration disorder	LOF FlnA knockout mice. Conditional mice (neural progenitors)	Knockout mice die at E14.5. Conditional mice have disrupted ventricular surface, perturbed intermediate progenitors. Exuberant angiogenesis.	Actin-binding protein making a link with plasma membrane proteins	Fox et al., 1998; Feng et al., 2006; Houlihan et al., 2016
GNAI2/ Gnai2	Periventricular heterotopia	No obvious OMIM number Periventricular Nodular Heterotopia and Intellectual Disability, *de novo*	Intellectual disability Periventricular nodular heterotopia	LOF IUE knockdown mice	Delayed radial migration of excitatory neurons during corticogenesis, perhaps because of impaired morphology. No effects on proliferation or position of progenitors.	Guanine nucleotide binding protein, alpha inhibiting activity polypeptide 2. G-proteins transduce signals from seven−transmembrane−type receptors (G−protein−coupled receptors) to various downstream effectors	Hamada et al., 2017

(Continued)

TABLE 2 | Continued

Gene	Pathology	OMIM_ number/acronym	OMIM neurological	LOF or GOF Model	Brain phenotype	Gene function	References
GPSM2/ Gpsm2/ LGN	Periventricular heterotopia, PMG, Chudley-McCollough	604213 CHUDLEY-MCCULLOUGH SYNDROME; CMCS; AUTOSOMAL RECESSIVE	Hydrocephalus Ventricomegaly Intellectual disability rare Seizures rare CC abnormality Cerebellar hypo or dysplasia Obstruction of the foramen of Monro (variable) Subcortical nodular heterotopia Polymicrogyria Arachnoid cysts	LOF Drosophila mutant. Mouse knockout mutant.	Drosophila: mutant neuroblasts rapidly fail to self-renew. Randomized orientation of normally planar neuroepithelial divisions. Abnormally localized progenitors.	G-protein signaling modulator 2, Leu-Gly Asn repeat enriched protein. Modulates activation of G proteins which transduce extracellular signals received by cell surface receptors into integrated cellular responses. Involved in orientation of divisions	Lee et al., 2006; Konno et al., 2008; Doherty et al., 2012
HNRNPK/ Hnrnpk	Au-Kline syndrome, Periventricular heterotopia	616580 AU-KLINE SYNDROME; AUKS; KABUKI-LIKE SYNDROME, AUTOSOMAL DOMINANT	Delayed psychomotor development Intellectual disability Poor speech High pain tolerance Nodular heterotopia (in 1 patient)	LOF (haplo insuffi-ciency) Mouse mutant	Down- regulation of hnRNPK in cultured hippocampal neurons by RNAi results in an enlarged dendritic tree and a significant increase in filopodia formation. Link to actin cytoskeleton.	Heterogeneous nuclear ribonucleoprotein K. Involved in chromatin remodeling, transcription, and mRNA splicing, translation, and stability. Pre-mRNA metabolism of transcripts containing cytidine-rich sequences.	Proepper et al., 2011; Lange et al., 2016
INTS8/ Ints8	Periventricular heterotopia	618572 NEURODEVELOPMENTAL DISORDER WITH CEREBELLAR HYPOPLASIA AND SPASTICITY; NEDCHS; AUTOSOMAL RECESSIVE	Microcephaly, borderline Dysmorphic facial features Optic atrophy Hypertelorism Developmental delay Intellectual disability severe Inability to walk, talk Spastic paraplegia Seizures Cerebellar hypoplasia Pontine hypoplasia Brainstem hypoplasia Periventricular nodular heterotopia	LOF Drosophila mutant	Ectopic type II neuroblasts. Normally prevents de-differentiation of intermediate neural progenitors back into neural stem cells. IntS8 genetically interacts with ERM to suppress the formation of ectopic neuroblasts.	Integrator complex subunit. Associates with the C-terminal domain of RNA polymerase II large subunit. Mediates 3-prime end processing of small nuclear RNAs U1	Oegema et al., 2017; Zhang et al., 2019
KAT6B/ Kat6b	Periventricular heterotopia	606170; 603736 GENITOPATELLAR SYNDROME; GTPTS; OHDO SYNDROME, SBBYS VARIANT; SBBYSS; AUTOSOMAL DOMINANT	Microcephaly Agenesis of corpus callosum Psychomotor retardation, severe Hypotonia Colpocephaly Periventricular neuronal heterotopia	LOF? Mouse gene-trap mutant. *Querkopf* mutant.	Homozygous die before weaning. Brain developmental defects. Less cells in cortical plate especially layer 5. Fewer interneurons.	Histone (lysine) acetyltransferase. Activated by the chromatin regulator Brpf1	Thomas et al., 2000; Clayton-Smith et al., 2011
MED12/ med12	Heterotopia	305450; 309520 OPITZ-KAVEGGIA SYNDROME; OKS; LUJAN-FRYNS SYNDROME; X-LINKED	Macrocephaly Developmental delay Intellectual disability Neonatal hypotonia Seizures Hydrocephalus Agenesis CC Heterotopia Attention deficit disorder Hyperactivity Friendly, sociable personality (some) Aggressive behavior (some) Autistic-like behavior (some) Poor	LOF Zebrafish mutant and over-expression of *med12* RNA.	Embryos showed defects in brain, neural crest, and kidney development and do not survive beyond 1 week after fertilization. Re-expression of *med*12 RNA leads to premature neuronal differentiation.	Mediator of RNA polymerase II transcription. Coactivator of Sox9. Regulates the expression of distinct neuronal determination genes.	Hong et al., 2005; Wang et al., 2006; Caro-Llopis et al., 2016

(Continued)

TABLE 2 | Continued

Gene	Pathology	OMIM_ number/acronym	OMIM neurological	LOF or GOF Model	Brain phenotype	Gene function	References
		social interactions Emotional instability (some) Obsessive compulsive disorder (some) Poor impulse control (some) Low frustration tolerance (some) Psychosis (some)					
NEDD4L/ Nedd4l	Periventricular heterotopia, PMG	617201 PERIVENTRICULAR NODULAR HETEROTOPIA 7; PVNH7; AUTOSOMAL DOMINANT	Delayed psychomotor development Intellectual disability Poor or absent speech Delayed or absent walking Seizures (in some patients) Periventricular nodular heterotopia Cortical dysplasia (in some patients) Thin corpus callosum (in some patients)	GOF IUE mouse to express mutant proteins. Knockdown.	Mutants: increased mitotic index, and arrest of neuronal cells within the ventricular and periventricular zone, depletion of neurons in the cortical plate. Terminal translocation disrupted? Knockdown - no differences.	E3 ubiqutin ligase. One target is the epithelial sodium channel (ENaC). Influences different signaling pathways. Player in regulation of the crosstalk between PI3K–mTORC2 and TGF-β–activin–Smad2–Smad3 (Smad2/3) signaling pathways	Broix et al., 2016
RPGRIP1L/ Rpgrip1l/ FTM/ Ftm	Subcortical heterotopia	None	None	LOF Mouse IUE	Ectopic localization of neural progenitors Rosettes of progenitors in cortex. Perturbed ventricular surface, progenitor detachment.	Can associate with base of the primary cilia; Involved in proteasome degradation and autophagy	Uzquiano et al., 2019
TMTC3/ Tmtc3/ Smile	Periventricular heterotopia, cobblestone brain malformation;	617255 LISSENCEPHALY 8; LIS8; AUTOSOMAL RECESSIVE; PERIVENTRICULAR NODULAR HETEROTOPIA	Microcephaly Delayed psychomotor development Intellectual disability Poor or absent speech Seizures Appendicular spasticity Lissencephaly, cobblestone Polymicrogyria Ventricomegaly Abnormal myelination Nocturnal seizures Hypoplasia CC Hypo and dysplasia of the brainstem Hypo and dysplasia of the cerebellum Occipital encephalocele Autistic features	LOF *Smile* mouse mutant; Crispr/Cas9 *in vitro*. Fly model; post-mitotic neuron-specific knockdown	Mouse, early neonatal death; Fly, seizures, presynaptic function?	Transmembrane and tetratricopeptide repeat containing 3 gene. Positive regulator of the endoplasmic reticulum (ER) stress response. Also co-localization of TMTC3 in the rat brain with vesicular GABA transporter at pre-synaptic terminals. CDH and PCDH O-Man glycosylation.	Farhan et al., 2017; Larsen et al., 2017

LOF, loss of function; GOF, gain of function; CC, corpus callosum; IUE, in utero electroporation; ShRNA, short hairpin RNA; PH, periventricular heterotopia. Human clinical information obtained from https://omim.org/.

also observed in these and other models (e.g., also with *EML1* mutations giving rise to SH, Kielar et al., 2014) and this can lead to subtle or severe disruption of the ventricular surface, sometimes resulting in heterotopia.

Several PH proteins (e.g., GNAI2, GSPM2 involved in G-protein signaling) are likely to be involved in the transduction of extracellular signals to intracellular effectors (Lee et al., 2006; Hamada et al., 2017). Further proteins impact intracellular signaling (e.g., NEDD4L, Broix et al., 2016). Extracellular signaling was also revealed as important for RG function in the case of *ECE2* mutations (Buchsbaum et al., 2020). This enzyme localizes to the plasma membrane and its mutation in the developing mouse cortex led to RG delamination and the formation of rosettes (progenitors clustered in a circle within the tissue). Surrounding non-mutant cells also appeared to be affected (non-cell autonomous phenomenon). The ventricular surface showed morphological alterations suggesting a weakening of cell junctions and indeed proteomic analyses revealed deregulated ECM molecules, as well as cytoskeletal and cell adhesion proteins. Thus, transduction of extracellular signals as well as cell adhesion regulation are clearly important in RGs and migrating neurons, their disruption leading to PH (Lian and Sheen, 2015; Buchsbaum et al., 2020).

Breakages in the BM and Cajal-Retzius Cells

Cobblestone lissencephaly is associated with disorganized cerebral and cerebellar cortices and multiple coarse gyri, with agyric regions (**Table 3**, OMIM; Guerrini and Dobyns, 2014). It is often included in a broader spectrum of disorders including muscular dystrophy and eye defects, as well as sometimes agenesis of the corpus callosum, cerebellar hypoplasia and hydrocephalus (OMIM; Guerrini and Dobyns, 2014). The dysfunctional mechanisms involve an over-migration of neurons at the pial surface, due to breaks in the cortical BM (Nickolls and Bonnemann, 2018). This has been associated with RG basal process end-feet that are not well attached to the ECM (e.g., laminin, **Figure 3**), leading to subsequent disintegration of the RG scaffold. In mouse models, mis-localization of Cajal-Retzius cells is also observed, in some models correlated with rostro-caudal and medio-lateral gradients of the lesions and the severity of the brain phenotype (Booler et al., 2016).

As mentioned previously, receptors and glycoproteins present on RG basal membranes normally make interactions with the ECM, helping to form and maintain the BM (Nickolls and Bonnemann, 2018). Mutations in some of these molecules (see below) leads to a cobblestone lissencephaly phenotype (**Table 3**). In other cases, a **polymicrogyria** phenotype in human patients is associated with a cobblestone-like phenotype revealed in mouse models. Indeed, polymicrogyria, associated with multiple small folds at the surface of the brain (Francis et al., 2006) also often involves over-migration and BM breakages. In this disorder, regions of fused gyri within the brain parenchyma can also contain BM components (Squier and Jansen, 2014). Thus, cobblestone lissencephaly is associated with polymicrogyria and indeed, often the two are found together (Squier and Jansen, 2014). These disorders can also often be associated with other neuronal migration defects such as periventricular or subcortical heterotopia (**Table 3**). We examine here causes of these linked disorders, from human genetics and animal model data.

Central to cobblestone lissencephaly hypotheses is post-translational regulation of dystroglycan (coded by DAG1, dystrophin-associated glycoprotein 1), a glycoprotein present in RG basal processes, that acts as an anchor point with the ECM (Booler et al., 2016), being also important for BM structure (Henry and Campbell, 1998). It normally gets cleaved giving rise to the peripheral membrane protein α-DG and the transmembrane protein β-DG. α-DG undergoes O-linked mannosylation allowing its binding to ECM proteins such as laminin (which has α, β and γ subunits, Burgeson et al., 1994), agrin, neurexin, pikachurin, and perlecan. The ER is involved in trafficking and glycosylating secretory pathway cargo. β-DG interacts with the actin cytoskeleton via dystrophin, utrophin and plectin, and thus α-DG and β-DG link the cytoskeleton to the ECM (Booler et al., 2016).

Muscle eye brain disease (MEB), Fukuyama congenital muscular dystrophy (FCMD), and Walker Warburg syndrome (WWS) have each been associated with aberrant glycosylation of α-DG (e.g., Kobayashi et al., 1998; Yoshida et al., 2001; Michele et al., 2002). Abnormal dystroglycan—ligand interactions underlie the pathogenic mechanism of muscular dystrophy as well as brain abnormalities. Rare mutations in *DAG1* (coding for dystroglycan, Hara et al., 2011; Geis et al., 2013), laminin subunit genes (e.g., *LAMA2*, *LAMB1*, and *LAMC3*, Barak et al., 2011; Radmanesh et al., 2013; Nelson et al., 2015; Zambonin et al., 2018) and genes coding for proteins likely to influence glycosylation and maturation of α-DG as well as potentially other proteins (ATP6V0A2, B4GAT1, B3GALNT2, FKTN, FKRP, ISPD, LARGE, POMK, POMT1, POMT2, POMGNT1, POMGNT2, SRD5A3, TMEM5, Kobayashi et al., 1998; Brockington et al., 2001; Yoshida et al., 2001; Beltran-Valero De Bernabe et al., 2002; Willer et al., 2004; Van Reeuwijk et al., 2005, 2007; Cantagrel et al., 2010; Manzini et al., 2012; Roscioli et al., 2012; Vuillaumier-Barrot et al., 2012; Buysse et al., 2013; Jae et al., 2013; Stevens et al., 2013 and see **Table 3**) can give rise cobblestone lissencephaly and/or polymicrogyria.

Several other genes involved in cobblestone phenotypes (*TBC1D10, TMTC3,* Liegel et al., 2013; Jerber et al., 2016) code for proteins which are likely to be involved in membrane trafficking and/or the ER stress response. Interestingly, TMTC3 has also been associated with 0-mannosyl glycosylation in the ER of the cell adhesion cadherins and proto-cadherins, but not α-DG (Larsen et al., 2017). As mentioned previously, cadherins are involved in RG apical cell-cell contacts, which may hence be disrupted if glycosylation does not occur correctly. Several patients with PH, already associated with abnormal RG contacts and neuronal migration (Cappello et al., 2013), exhibit mutations in *TMTC3* (Larsen et al., 2017). Thus, most likely apical (periventricular) as well as basal interactions require correct glycosylation.

TABLE 3 | Genes mutated in human pathology associated with basal defects.

Gene	Pathology	OMIM_ number/acronym	OMIM (neurological)	LOF or GOF Model	Brain phenotype	Gene function	References
ADGRG1 (GPR56), Gpr56	Polymicrogyria	606854; 615752 POLYMICROGYRIABI LATERAL FRONTOPARIE TALBFPP; POLYMICROGYRIABI LATERAL PERISYLVIAN; BPPR; AUTOSOMAL RECESSIVE	Developmental delay Psychomotor delay Intellectual disability, moderate to severe Seizures Cerebellar signs Pyramidal signs Polymicrogyria, most severe in the frontoparietal regions Polymicrogyria, anterior to posterior gradient Areas of dysmyelination on MRI Brainstem hypoplasia Cerebellar hypoplasia	LOF Mouse knockout	Neuronal ectopia in the cerebral cortex, a cobblestone-like cortical malformation.	7 transmembrane domains, as well as a mucin-like domain. Autoproteolytic cleavage to produce N-terminal adhesion ectodomain and transmembrane domain, which associate on cell surface. Receptor for collagens	Piao et al., 2004; Li et al., 2008; Paavola et al., 2011
ATP6V0A2/ Atp6V0A2	Cobblestone brain malformation	219200 CUTIS LAXA, AUTOSOMAL RECESSIVE, TYPE IIA; ARCL2A	Microcephaly Delayed motor development Intellectual disability Seizures Hypotonia Partial pachygyria Cobblestone lissencephaly, posterior frontal and parietal regions Board and poorly defined gyri Polymicrogyria Dandy-Walker malformation	LOF Studies in the mouse (e.g., monoclonal antibody, anti-a2V)	Spontaneous abortions due to placental expression; role also in sperm	Integral membrane subunit of a vacuolar-type proton pump (H (+)-ATPase or V-ATPase) for acidification of diverse organelles and vesicles. Involved in N-glycosylation at the level of processing in the Golgi apparatus	Kornak et al., 2008; Van Maldergem et al., 2008; Jaiswal et al., 2015
B3GALNT2/ B3Galnt2	Cobblestone brain malformation	615181 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 11; MDDGA11; AUTOSOMAL RECESSIVE	Delayed psychomotor development, severe Lack of acquisition of motor milestones Severe cognitive impairment Hydrocephalus Polymicrogyria Cobblestone lissencephaly Frontotemporal leukoencephalopathy Cerebellar dysplasia Pontocerebellar hypoplasia Cerebellar cysts	LOF Zebrafish knockdown	Retinal degeneration, hydrocephalusand severely impaired motility.	Transmembrane protein, beta-1,3-N-Acetyl Galactosaminyltransferase 2; Adds galactose residues, to synthesize poly-N-acetyllactosamine.	Stevens et al., 2013
B4GAT1/ B3GNT1/ B4Gat1/ B3Gnt1	Cobblestone brain malformation	615287 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 13; MDDGA13; AUTOSOMAL RECESSIVE	Lack of psychomotor development Hydrocephalus Anencephaly Occipital encephalocele Enlarged ventricles Seizures Spasticity Agenesis of the corpus callosum Brainstem hypoplasia Cerebellar hypoplasia Cortical dysplasia Cobblestone lissencephaly Nodular heterotopia Dandy-Walker malformation	LOF Mouse ENU, null mutation (B3gnt1 LacZ/LacZ) and hypomorphs B3gnt1LacZ/M155T	Null: E9.5 lethality. Hypomorphs: Defective glycosylation of alpha-dystroglycan. Congenital muscular dystrophy. Radial glial endfoot detachment and cobblestone-like phenotype.	Transmembrane protein i-beta-1,3-N-acetylglucosaminyl transferase. N-acetylglucosamine residues added to synthesize poly-N-acetyllactosamine, a linear carbohydrate that can be incorporated into either N- or O-linked glycans.	Wright et al., 2012; Buysse et al., 2013; Shaheen et al., 2013
COL3A1/ Col3A1	Cobblestone brain malformation	618343 POLYMICROGYRIA WITH OR WITHOUT VASCULAR-TYPE EHLERS-DANLOS SYNDROME; (PMGEDSV); AUTOSOMAL RECESSIVE	Delayed motor development Impaired intellectual development Seizures Speech delay Polymicrogyria Cobblestone-like malformation of the cortex Anterior to posterior gradient Enlarged ventricles Cerebellar	LOF Mouse knockout	Early postnatal death. At E18.5, cobblestone like cortical malformation with pial breakdown in the basement membrane, neuronal overmigration, RG detachment,	ECM molecule present in basement membranes	Liu et al., 1997; Jeong et al., 2012; Horn et al., 2017; Vandervore et al., 2017

(Continued)

TABLE 3 | Continued

Gene	Pathology	OMIM_ number/acronym	OMIM (neurological)	LOF or GOF Model	Brain phenotype	Gene function	References
			hypoplasia Cerebellar cysts Brainstem hypoplasia Abnormal corpus callosum White matter abnormalities		and formation of marginal zone heterotopias		
COL4A1/ Col4A1	Cobblestone-like brain malformation	POLYMICROGYRIA SCHIZENCEPHALYPOREN CEPHALY, WWS or MEB, AUTOSOMAL RECESSIVE	Variable	LOF Mouse mutant Col4a1 + /Δex40 (splice acceptor mutation)	Homozygous lethal. Heterozygote mice develop porencephaly secondary to focal disruptions of vascular basement membranes, Col4a1 + /Δex40 mice also show pial basement membrane disruptions and cerebral cortical lamination defects	ECM molecule, ubiquitously present in basement membranes. Interacts with COL4A2	Gould et al., 2005; Labelle-Dumais et al., 2011; Cavallin et al., 2018; Zagaglia et al., 2018
DAG1/ Dag1	Cobblestone brain malformation	616538 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 9; MDDGA9; AUTOSOMAL RECESSIVE	Macrocephaly Delayed psychomotor development, severe Lack of speech Poor head control Hydrocephalus Thin cortical layer Polymicrogyria Frontal agyria Migration defects Dilated ventricles Thin corpus callosum Kinking of the pons and brainstem Hypoplastic cerebellar vermis Cerebellar cysts White matter abnormalities Leukodystrophy Cystic lesions Intracranial calcifications	LOF Mouse KO and cKO; KI	KO, embryonic lethal. Brain-specific deletion: discontinuous *glia limitans*, perturbed cortical layering, fusion of cerebral hemispheres and cerebellar folia, aberrant migration of granule cells. KI: muscular dystrophy and neurologic motor impairment, glycosylation by LARGE is decreased.	Glycoprotein, membrane associated. Interacts with ECM molecules	Williamson et al., 1997; Henry and Campbell, 1998; Moore et al., 2002; Hara et al., 2011; Geis et al., 2013
FKRP/ Fkrp	Cobblestone brain malformation (WWS, MEB or less severe form)	613153 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 5; MDDGA5; AUTOSOMAL RECESSIVE	Intellectual disability, profound Delayed motor development, severe Hydrocephalus Cobblestone lissencephaly Agyria Cerebellar cyst Absence of the cerebellar vermis Pontine hypoplasia Cerebellar hypoplasia Cerebellar dysplasia Pachygyria Hypoplastic brainstem Ventricular dilatation Absence of the corpus callosum White matter abnormalities Dandy-Walker malformation Hyporeflexia	LOF Mouse hypomorphic knockin. Zebrafish morpholino	KI mice die around birth. decreased muscle mass, perturbation of the limiting membrane of the eye, and a disturbance in neuronal migration. Zebrafish: muscle and eye phenotype	Golgi-resident glycosyltransferase. Could impact dystroglycan maturation.	Brockington et al., 2001; Ackroyd et al., 2009; Kawahara et al., 2010
FKTN/ Fktn	Cobblestone brain malformation (Fukuyama congenital muscular dystrophy, WWS, MEB or less severe form)	253800 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 4; MDDGA4; AUTOSOMAL RECESSIVE	Intellectual disability Poor motor development Polymicrogyria Leptomeningeal thickening Focal interhemispheric fusion Low density white matter Cobblestone lissencephaly Pachygyria Agyria Agenesis of the corpus callosum Encephalocele (rare) Hydrocephalus Cerebellar cysts Seizures Hyperekplexia (rare) Pyramidal tract hypoplasia Brainstem hypoplasia Cerebellar hypoplasia Hypo- or areflexia	LOF Mouse	Laminar disorganization of the cortical structures in the brain with impaired laminin assembly, focal interhemispheric fusion, and hippocampal and cerebellar dysgenesis. Loss of laminar structure in the retina,	Golgi-resident glycosyltransferase. Secreted protein. Expressed in Cajal Retzius cells and cortical neurons. In ECM, modifies glycosylation of DAG1	Kobayashi et al., 1998; Sasaki et al., 2000; Hayashi et al., 2001; Beltran-Valero De Bernabe et al., 2002; Takeda et al., 2003

(Continued)

TABLE 3 | Continued

Gene	Pathology	OMIM_ number/acronym	OMIM (neurological)	LOF or GOF Model	Brain phenotype	Gene function	References
ISPD/ Ispd	Cobblestone brain malformation	614643 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 7; MDDGA7; AUTOSOMAL RECESSIVE	Macrocephaly Intellectual disability, profound Hydrocephalus Ventriculomegaly Encephalocele Dandy-Walker malformation Cobblestone lissencephaly Agyria Pachygyria Polymicrogyria Hypoplasia of the corpus callosum Partial agenesis of the corpus callosum Cortical thinning Subcortical heterotopia Cerebellar hypoplasia Brainstem hypoplasia Brain vascular anomalies (rare) Areflexia	LOF Zebrafish morpholino-based knockdown; Mouse ENU mutant (stop mutation)	Zebrafish: Hydrocephalus and incomplete brain folding, with significantly reduced eye size. Mouse: lethal P1, defective axon guidance. Cobblestone-like phenotype. Reduced glycosylation of dystroglycan	ISPD has an isoprenoid synthase domain characteristic of nucleotide diP-sugar transferases	Roscioli et al., 2012; Vuillaumier-Barrot et al., 2012; Wright et al., 2012
LAMA2/ Lama2	Polymicrogyria, Cobblestone brain malformation (some patients)	607855 MUSCULAR DYSTROPHY, CONGENITAL MEROSIN-DEFICIENT, 1A; MDC1A; AUTOSOMAL RECESSIVE	White matter hypodensities seen on MRI Abnormal cortical gyration (rare) Seizures (rare) Intellectual disability (rare) Lissencephaly (rare)	LOF Mouse KO or transgenic	Lethlality. Symptoms of congenital muscular dystrophy. Full or partial laminin deficiency. Brain phenotype may require other gene mutations	ECM molecule, alpha-2 laminin subunit	Guo et al., 2003; Dominov et al., 2005; Nelson et al., 2015; Oliveira et al., 2018
LAMB1/ Lamb1	Cobblestone brain malformation; no eye and muscle phenotypes.	615191 LISSENCEPHALY 5; LIS5; AUTOSOMAL RECESSIVE	Macrocephaly due to hydrocephalus Psychomotor retardation Intellectual deficiency progressive Hypotonia Seizures Spastic paraplegia Cobblestone lissencephaly (posterior brain regions more affected than anterior regions) Subcortical band heterotopia Occipital encephalocele Cerebellar hypoplasia Brainstem hypoplasia Leukoencephalopathy White matter cysts Porencephaly White matter atrophy, progressive	LOF? Mouse - spontaneous mutant (stop codon) leads to modest truncation	Homozygous lethal. Dystonia-like phenotype in heterozygote state.	ECM molecule, beta-1 laminin subunit, regulates axon guidance	Radmanesh et al., 2013; Liu et al., 2015; Tonduti et al., 2015
LAMC3/ Lamc3	Polymicrogy riaoccipital pachygyria	614115 CORTICAL MALFORMATIONS, OCCIPITAL; OCCM; AUTOSOMAL RECESSIVE	Seizures, absence Seizures, tonic-clonic (1 patient) Delayed psychomotor development (1 patient) Autonomic symptoms Pachygyria, occipital Polymicrogyria, occipital EEG abnormalities	LOF Mouse	Retinal phenotype. Gene expressed in vessels and meninges	ECM molecule, gamma-3 laminin subunit	Denes et al., 2007; Barak et al., 2011; Radner et al., 2013; Zambonin et al., 2018
LARGE/ Large	Cobblestone brain malformation (WWS, MEB or milder)	613154 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 6; MDDGA6; AUTOSOMAL RECESSIVE	Intellectual disability Areflexia Cobblestone lissencephaly Ventricular dilatation Absence of the cerebellar vermis Hypoplasia and dysplasia of the cerebellum Hydrocephalus White matter changes Pontine hypoplasia Dandy-Walker malformation (rare)	LOF Mouse myd mutation deletion in Large gene	myd mice have abnormal neuronal migration in the cerebral cortex, cerebellum, and hippocampus, and show disruption of the basal lamina.	Transmembrane protein N-acetylglucosaminyl transferase. Adds a glycan repeat to dystroglycan	Michele et al., 2002; Longman et al., 2003; Van Reeuwijk et al., 2007

(Continued)

TABLE 3 | Continued

Gene	Pathology	OMIM_ number/acronym	OMIM (neurological)	LOF or GOF Model	Brain phenotype	Gene function	References
POMGNT1/ Pomgnt1	Cobblestone brain malformation (WWS, MEB or milder)	253280 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 3; MDDGA3; AUTOSOMAL RECESSIVE	Microcephaly Intellectual disability, severe Hypotonia, severe Seizures Hydrocephalus Lack of motor development (WWS) Disorganized brain cytoarchitecture Ventricular dilatation White matter changes Cerebellar hypoplasia Cerebellar dysplasia Brainstem hypoplasia Brainstem concavity Flattening of the pons Complete or partial absence of the corpus callosum Cobblestone lissencephaly, type II Pachygyria Polymicrogyria Cerebellar cysts	LOF Mouse knockout (gene-trap)	Abnormal cortex, disappearance of molecular layer I (overmigration); cerebral hemispheres fused. Hippocampal dysplasia and scalloped DG. Enlarged lateral ventricles	Type II transmembrane protein. O-mannose beta-1,2-N-acetyl glucosaminyl transferase, participates in O-mannosyl glycan synthesis	Yoshida et al., 2001; Liu et al., 2006; Bouchet et al., 2007
POMGNT2/ Pomgnt2, GTCD2/ Gtcd2 (AGO61)	Cobblestone brain malformation (WWS)	614830 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 8; MDDGA8; AUTOSOMAL RECESSIVE	Lack of psychomotor development Hydrocephalus Enlarged ventricles Cobblestone lissencephaly Cerebellar hypoplasia	LOF Zebrafish morpholino-based knockdown; Mouse knockout	Zebrafish: hydrocephalus, ocular defects, and muscular dystrophy. Mouse: lethal P1, abnormal basal lamina formation and a neuronal migration defect. RG endfoot detachment.	Endoplasmic reticulum (ER)-resident protein with N terminal signal peptide, that catalyzes the second step of the O-mannosyl glycosylation in the mucin-like domain of alpha-dystroglycan. Glycosyltransferase-like domain-containing protein-2, O-mannose β-1,4-N-acetyl glucosaminyltransferase.	Manzini et al., 2012; Yagi et al., 2013
POMK/ Pomk, SGK196	Cobblestone brain malformation	615249 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 12; MDDGA12; AUTOSOMAL RECESSIVE	Microcephaly, progressive (1 patient) Delayed psychomotor development, severe Psychomotor retardation, severe Loss of ambulation Poor speech Seizures (1 patient) Hydrocephalus Cerebellar hypoplasia Brainstem hypoplasia (1 patient) Cobblestone lissencephaly (1 patient) Agenesis of the corpus callosum (1 patient) Agyria (1 patient) Brain hypomyelination	LOF Zebrafish morpholine knockdown, Mouse knockout	Zebrafish: small head, delayed ocular development, shortened thicker tail; Mouse, neuronal migration defects, cerebellar dysplasia, hydrocephlaus	Protein-O-mannose kinase. Transmembrane protein with extracellular kinase-like domain, phosphorylates the 6-position of O-mannose.	Vogel et al., 2012; Jae et al., 2013; Yoshida-Moriguchi et al., 2013; Di Costanzo et al., 2014; Von Renesse et al., 2014

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TABLE 3 | Continued

Gene	Pathology	OMIM_ number/acronym	OMIM (neurological)	LOF or GOF Model	Brain phenotype	Gene function	References
POMT1/ Pomt1	Cobblestone brain malformation (WWS, MEB and a less severe form)	236670 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 1; MDDGA1; AUTOSOMAL RECESSIVE	Microcephaly Hypotonia, severe Seizures Hydrocephalus Ventricular dilatation White matter changes Cerebellar hypoplasia Cerebellar dysplasia Brainstem hypoplasia Flattening of the pons Agenesis of the CC Occipital encephalocele Meningoencephalocele Thin cortical mantle Cobblestone lissencephaly Agyria Pachygyria Fused hemispheres Posterior fossa cysts Virtual absence of pyramidal tracts Polymicrogyria (MEB) Cerebellar cysts (MEB)	LOF Mouse	Embryonic lethal.	Integral membrane protein. O-mannosyl transferase that catalyzes the first step in the synthesis of the O-mannosyl glycan found on alpha-dystroglycan (see also POMT2)	Beltran-Valero De Bernabe et al., 2002; Willer et al., 2004; Bouchet et al., 2007
POMT2/ Pomt2	Cobblestone brain malformation (WWS, MEB and a less severe form)	613150 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 2; MDDGA2; AUTOSOMAL RECESSIVE	Microcephaly Intellectual disability, Hypotonia, severe Hydrocephalus Ventricular dilatation Cerebellar hypoplasia Cerebellar dysplasia Brainstem hypoplasia Flattening of the pons Cobblestone lissencephaly, type II Smooth, thin mantle Aplasia of the CC Encephalocele (1 patient, MEB) Cerebellar cysts (MEB) Pachygyria with frontoparietal involvement (MEB) Polymicrogyria (MEB) Periventricular white matter changes (MEB) Diffuse white matter changes (MEB) Seizures (MEB)	LOF Mouse knockout (constitutive and conditional)	KO embryonic lethal. cKO Emx1-Cre: neocortical dysplasia (over-migration), migration failure in cerebellum, hippocampal dysplasia, displaced Cajal–Retzius cells, disruption of the BM. Hypo glycosylation of alpha-DG.	Integral membrane protein. Sequence similarity with a family of protein O-mannosyl transferases, that catalyze the first step in the synthesis of the O-mannosyl glycan found on alpha-dystroglycan	Willer et al., 2002; Van Reeuwijk et al., 2005; Bouchet et al., 2007; Hu et al., 2011
RELN/reln	Lissencephaly, Pachygyria, cerebellar hypoplasia	257320 LISSENCEPHALY SYNDROME, NORMAN-ROBERTS TYPE NORMAN-ROBERTS SYNDROME; AUTOSOMAL RECESSIVE	Microcephaly Lissencephaly, type I Thick cerebral cortex Cerebellar hypoplasia	LOF Mouse spontaneous "reeler"	Impaired motor coordination, tremors, and ataxia. Neurons fail to reach their correct locations in the developing brain, disrupting the organization of the cerebellar and cerebral cortices and other laminated regions.	Secreted glycoprotein expressed in Cajal-Retzius cells	D'Arcangelo et al., 1995; Hong et al., 2000
SRD5A3/ Srd5A3	Cobblestone brain malformation	612379 CONGENITAL DISORDER OF GLYCOSYLATION, TYPE Iq; CDG1Q; AUTOSOMAL RECESSIVE	Brachycephaly Intellectual disability Hypotonia Delayed motor development Pituitary gland hypoplasia Polymicrogyria, frontal Cerebellar vermis hypoplasia	LOF Mouse knockout	Embryonic lethal at day E12.5; upregulation of genes involved in regulation of the unfolded protein response (ER - related to role of N-glycan?)	Polyprenol reductase. Reduction of polyprenol is the major pathway for dolichol biosynthesis during N-glycosylation	Cantagrel et al., 2010

(Continued)

TABLE 3 | Continued

Gene	Pathology	OMIM_ number/acronym	OMIM (neurological)	LOF or GOF Model	Brain phenotype	Gene function	References
TBC1D20/ Tbc1D20	Cobblestone brain malformation	615663 WARBURG MICRO SYNDROME 4; WARBM4; AUTOSOMAL RECESSIVE	Postnatal microcephaly Brachycephaly Congenital hypotonia, axial or generalized Postnatal development of hypertonic extremities Spastic quadriplegia Speech severely limited Seizures Cortical atrophy Hypoplastic CC Bilateral frontoparietal polymicrogyria Widened lateral ventricles Progressive cerebellar atrophy Mega cisterna magna Autistic features	LOF Mouse spontaneous *"blind-sterile"*	Nuclear cataracts and male infertility; no obvious brain abnormalities	TBC1 domain family member; TBC (Tre2, Bub2, and Cdc16) domains found in most Rab-GTPase-activating proteins (GAPs), which are important in vesicular membrane transport. Associates with the ER.	Sklan et al., 2007; Liegel et al., 2013
TMTC3/ Tmtc3/ Smile	Cobblestone brain malformation; no eye and muscle phenotypes.	617255 LISSENCEPHALY 8; LIS8; AUTOSOMAL RECESSIVE; PERIVENTRICULAR NODULAR HETEROTOPIA	Microcephaly (in some patients) Delayed psychomotor development Delayed walking Intellectual disability Poor or absent speech Seizures Appendicular spasticity Lissencephaly, cobblestone Polymicrogyria Ventriculomegaly Abnormal myelination (in some patients) Hypoplasia of the CC Hypoplasia of the brainstem Hypoplasia of the cerebellum Dysplasia of the brainstem Dysplasia of the cerebellum Occipital encephalocele (in some patients) Autistic features (in some patients)	LOF *Smile* mouse mutant; Crispr/Cas9 *in vitro*. Fly model; post-mitotic neuron-specific knockdown	Mouse, early neonatal death; Fly, presynaptic function?	Transmembrane and tetratricopeptide repeat containing 3 gene. Positive regulator of the endoplasmic reticulum (ER) stress response. Also co-localization of TMTC3 in the rat brain with vesicular GABA transporter at pre-synaptic terminals. CDH and PCDH O-Man glycosylation.	Racape et al., 2011; Yun and Vu, 2012; Jerber et al., 2016; Farhan et al., 2017; Larsen et al., 2017
TMEM5/ Tmem5	Cobblestone brain malformation	615041 MUSCULAR DYSTROPHY-DYSTROGLYCANOPATHY (CONGENITAL WITH BRAIN AND EYE ANOMALIES), TYPE A, 10; MDDGA10; AUTOSOMAL RECESSIVE	Cobblestone lissencephaly Occipital neural tube defects Cerebellar dysplasia Macrocephaly (in some patients)	LOF None		Transmembrane protein believed to have glycosyltransferase function	Vuillaumier-Barrot et al., 2012; Jae et al., 2013
VLDLR/ Vldlr	Lissencephaly; abnormal neuron migration	224050 CEREBELLAR ATAXIA, MENTAL RETARDATION, AND DYSEQUILIBRIUM SYNDROME 1; CAMRQ1; AUTOSOMAL RECESSIVE	Psychomotor retardation Mental retardation Poor speech development Gait ataxia Truncal ataxia Disturbed equilibrium Quadrupedal gait (in some) Intention tremor Dysarthria Dysmetria Dysdiadochokinesis Hypotonia Hyperreflexia Broad-based gait Seizures (rare) Cortical gyral simplification Pachygyria Cerebellar hypoplasia Cerebellar ataxia Small brainstem	LOF Vldlr knockout mouse (or double knockout with ApoeR2)	Invasion of migrating neurons in the MZ. Double knockout with ApoeR2 leads to inverted and disorganized cortical layers	Receptor for reelin expressed on migrating neurons	Trommsdorff et al., 1999; Hack et al., 2007; Valence et al., 2016

WWS, Walker-Warburg syndrome; MEB, Muscle eye brain; KO, knockout; cKO, conditional knockout; KI, knockin; LOF, loss of function; GOF, gain of function; ECM, extracellular matrix; BM, basement membrane; CC, corpus callosum. Human clinical information obtained from https://omim.org/.

GPR56 mutations were identified in bilateral frontoparietal or perisylvian polymicrogyria (Piao et al., 2004; Bae et al., 2014). GPR56 is a member of an adhesion family of G protein-coupled receptors (GPRs), expressed on the basal membrane of RGs. Mouse mutants show a cobblestone-like phenotype, with abnormal basal RG processes, a disrupted BM, with neuronal over-migration and Cajal Retzius cell disruption (Li et al., 2008). Type III collagen, as well as being important in connective tissue including blood vessels, is one of the major constituents of the pial BM and a ligand for GPR56 (Luo et al., 2011). Although several disorders are associated with altered levels of the collagen COL3A1 (Kuivaniemi and Tromp, 2019), some mutations give rise to frontal predominant bilateral polymicrogyria or a cobblestone−like cortical malformation (Horn et al., 2017; Vandervore et al., 2017). COL3A1 is the α1 chain of type III collagen, an ECM protein. COL4A1, the α1 chain of type IV collagen, is also a crucial component of the BM. Cobblestone lissencephaly, polymicrogyria, schizencephaly or porencephaly were observed in certain patients with *COL4A1* mutations (Labelle-Dumais et al., 2011; Cavallin et al., 2018; Zagaglia et al., 2018). Cavallin et al. (2018) discuss prenatal stroke and suggest that hydranencephaly, schizencephaly, porencephaly and polymicrogyria represent a continuum of brain injury depending on the timing and the severity of the insult. Both genetic as well as environmental factors may hence contribute to these disorders. Interestingly, *COL4A2* mutations were also identified in a patient presenting heterotopia in addition to bilateral polymicrogyria suggesting perturbed neuronal migration (Cavallin et al., 2018).

By activating signaling cascades, Reelin acts at the terminal steps of neuronal migration, notably terminal translocation, without however, influencing RG-guided migration (Sekine et al., 2014). As mentioned previously however (section "Role of Secreted Proteins Derived From the CSF in the Formation and Maintenance of the RG Scaffold"), Reelin may also influence RG basal processes at least in some species. Lower concentrations of Reelin are also present in the lower IZ, in this case influencing the neuronal multipolar-bipolar transition (Sekine et al., 2014). Mutations have been identified in *Reelin* in autosomal recessive pachygyria associated with cerebellar hypoplasia (Hong et al., 2000). *Reeler* mutant mice show a similar combination of defects, including disorganized and inverted cortical lamination (D'Arcangelo et al., 1995). There are also patients which exhibit mutations in the Reelin receptor gene, *very low density lipoprotein receptor*, *VLDLR* (Valence et al., 2016). Mutations give rise to the CARMQ1 syndrome (cerebellar ataxia and mental retardation with or without quadrupedal locomotion), which can include mild simplification or thickening of cortical gyri (Valence et al., 2016). *Vldlr* mouse mutants show an invasion of neurons in the MZ (Hack et al., 2007) and double KO with a second Reelin receptor gene, *ApoER2*, phenocopies the cortical disorganization observed in *reeler* mutants (Trommsdorff et al., 1999). Thus, extracellular Reelin via the above-mentioned receptors, is critical for neuron migration and notably translocation in the most superficial regions of the developing cortex, as well as potentially maintenance of the RG scaffold (Weiss et al., 2003).

CONCLUSION AND PERSPECTIVES

Overall, in this review we summarize the variety of different factors which together regulate the formation and the maintenance of RG scaffolding. The need for this variety, ranging from secreted factors in the eCSF to very local cell to cell contacts, is probably due to the unique morphology of RGs. Indeed, they spread from one side of the developing cortex to the other, and are therefore exposed to very different environments. RGs have basal endfeet in contact with secreted factors derived from the meninges and from cells migrating from remote places, such as Cajal Retzius cells or neurons, and at the same time aRGs have their cell bodies and primary cilia in contact with other RG and the eCSF (**Figure 1**). For the stability of the whole cortical architecture, which is based on RGs, it therefore seems to be essential that there are multiple spatial environments which signal to the different RG compartments. aRGs will also differ from bRGs in this respect and it is also clear that environments change over time further regulating these essential cell types.

It appears that there is not a single mechanism that governs the RG scaffold but instead elaborated mechanisms involving numerous actors. However, although this review is not exhaustive, it is striking that unlike what it is already known for neuronal growth and guidance, there is little research which has up till now identified specific modulators of RG growth and maintenance. We attempted here to identify and group such factors. RGs have to grow a long process to reach a remote location, crossing a relatively thick tissue, however, they are often seen as a constant scaffold acting as a support for neuronal migration. The RG scaffold is not though as static as we might imagine. Indeed, evidence provided by the work of Yokota et al. (2010), showed using videomicroscopy that RG processes, endfeet and radial fiber interactions are constantly dynamic. The function of this dynamic behavior could impact neuronal migration at the local level to finely regulate neuron positioning in the CP and hence the structure of the brain. How and why the RG scaffold is plastic still remains to be investigated. Experiments taking into account this dynamic behavior could help acquire further information to distinguish factors involved in RG growth aspects from those involved in maintenance.

Radial glia are not only defined by their morphology. They are also able to adapt their gene expression to match the changing cues in their environment and to adjust their cellular responses (Telley et al., 2019). The timing of these mechanisms is crucial as cues can be produced in waves during development (Chau et al., 2015). How these genic modulations occur still needs to be studied. However, factors influencing chromatin and RNA processing are likely to play a role (e.g., it is notable that there are several PH genes involved in these processes, including HNRNPK, INTS8, KAT6B, MED12, Clayton-Smith et al., 2011; Caro-Llopis et al., 2016; Lange et al., 2016; Oegema et al., 2017, see **Table 2**), although for the moment in relatively unknown ways. Further studies across corticogenesis could shed further light on such mechanisms. Transcriptomic or proteomic experiments related to these genes could further identify key targets (acting on adhesion and/or signaling) impacting progenitor and/or neuron migration function at different timepoints.

When we think of the role and formation of the RG scaffold, another aspect to consider is the evolution from lissencephalic species to gyrencephalic species, especially with respect to the appearance of bRGs. Indeed, related to this relatively new progenitor type, whose somata lie in more basal areas of the developing cortex of gyrencephalic species, the RG network is not the same as in lissencephalic species. Some questions are: what are the relationships between aRG basal processes and bRG basal processes? Do they interact? Do they respond in the same manner to extracellular cues? How is bRG maintenance initially put in place, since these cells do not receive the same extracellular cues as their apical counterparts? Are there specific extracellular cues regulating bRGs, forming a special niche in the center of the developing cortex? For instance, a study identified the specific role of PDGFD signaling in the maintenance of bRG in human cortical development compared to mouse progenitors (Lui et al., 2014). Pollen et al. found several genes specific to bRGs which are highly related to ECM interactions. For instance tenascin C (TNC) is specific to bRGs and has the ability to bind to PTPRZ1, syndecans or integrins (Pollen et al., 2015). Moreover as mentioned earlier Notch is important for RG maintenance in mouse but recently a study showed that three paralogs of human-specific NOTCH2NL are essential to control the proliferation/differentiation balance of human RGs, which can be directly linked with the expansion of the human cortex (Fiddes et al., 2018). The function of the bRG scaffold is indeed still mysterious although it is widely assumed to contribute to gyrification during evolution. These ideas and relevant mechanisms are discussed in a review on the role of bRG morphology in cortical development and evolution (Kalebic and Huttner, 2020).

Radial glia are very unique and fascinating cells: the correct orchestration of their formation, organization, and localization is crucial for correct cortical development. Since the discovery of these cells, much work has been done to understand better how these progenitors communicate and interact with their environment but still a lot remains to be investigated. This review highlights a level of complexity inherent to these cells with respect to the impact of extracellular cues on their development and maintenance. Further understanding this aspect will help clarify mechanisms involved in health and neurodevelopmental disorders.

AUTHOR CONTRIBUTIONS

JF wrote the sections "Role of Secreted Proteins Derived From the CSF in the Formation and Maintenance of the RG Scaffold" and "Secreted Factors From Close Range Cells" and designed all the figures and parts of **Table 1**. DZ wrote the section "Further Factors Identified via Human Pathology" and generated **Table 1**. FF wrote the section "Role of Cell to Cell and Cell to ECM Contacts in the Formation and Maintenance of the RG Scaffold and Proliferation" and generated **Tables 2**, **3**. All authors contributed to the article and approved the submitted version.

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REVIEW
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How Do Cortical Excitatory Neurons Terminate Their Migration at the Right Place? Critical Roles of Environmental Elements

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Interactions between neurons and their environment are crucial for proper termination of neuronal migration during brain development. In this review, we first introduce the migration behavior of cortical excitatory neurons from neurogenesis to migration termination, focusing on morphological and behavioral changes. We then describe possible requirements for environmental elements, including extracellular matrix proteins and Cajal–Retzius cells in the marginal zone, radial glial cells, and neighboring neurons, to ensure proper migration termination of these neurons at their final destinations. The requirements appear to be highly linked to sequential and/or concurrent changes in adhesiveness of migrating neurons and their surroundings, which allow the neurons to reach their final positions, detach from substrates, and establish stable laminar structures.

Keywords: cell adhesion, layer formation in the neocortex, marginal zone, radial glial cell, radial migration

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INTRODUCTION

The cerebral cortex is critical for memory formation, language, perception, attention, and other intellectual activities. These functions are supported by six layered neuronal structures, which are composed of excitatory and inhibitory neurons. The former account for about 80% of neurons in the cerebral cortex and transmit signals over long distances, projecting to multiple cortical areas as well as subcortical regions.

Neuronal migration is one of the most fundamental processes for constructing functional brain circuits in development. In the cerebral cortex, excitatory neurons are born in the ventricular zone (VZ) facing the ventricle and migrate toward their final positions, where they form a specific layered structure. Their aberrant migration and consequent mispositioning result in structural and functional abnormality, which underlies neuronal disorders such as epilepsy and intellectual disability (Romero et al., 2018).

Among the several stages in neuronal migration, termination of migration is the final important step and is directly associated with the establishment of the cortical cytoarchitecture. However, our knowledge about how neurons terminate their migration is still limited. Although this event must ultimately be analyzed *in situ*, most studies so far have been carried out in organotypic brain slice cultures. In such preparations, it is not easy to preserve intact radial glial (RG) cells that maintain the contact between their fibers and meninges, which is required for recapitulating proper

termination of migration. The analysis also involves technical limitations in gene manipulation: *in utero* electroporation or viral infection to introduce a gene of interest usually targets neural stem cells, which can sometimes prevent us from examining the gene's role in migration or migration termination when the transgene severely impairs neuronal differentiation and/or neuronal migration in the early phase. Nevertheless, recent studies using conditional knockout mice, or temporally and/or spatially controlled gene manipulation, are increasingly uncovering the process of migration termination, with particular attention being directed to sequential changes in adhesiveness between a migrating neuron and the extracellular components, including neighboring neurons, in its environment (Franco et al., 2011; Sekine et al., 2011, 2012; Gil-Sanz et al., 2013; Kohno et al., 2015; Ha et al., 2017; Matsunaga et al., 2017; Hirota et al., 2018; Hatanaka et al., 2019; Hirota and Nakajima, 2020).

In this review, we focus on the terminal phase of neuronal migration and discuss the role of these environmental components including extracellular matrix proteins and Cajal–Retzius (CR) cells in the marginal zone (MZ), RG cells, and neighboring neurons. Their cooperation is indispensable for proper migration termination, and thus for the construction of the proper cortical laminar structure.

MIGRATION BEHAVIOR OF CORTICAL EXCITATORY NEURONS

The development of cortical excitatory neurons from their progenitors is well documented. They are derived from neural stem cells in the cortical VZ through interkinetic nuclear migration, a cell cycle-dependent periodic movement of the nuclei. Initially, they divide symmetrically to amplify self-renewing stem cells. These cells have a bipolar morphology, extending apical and basal processes that are attached to the ventricular surface and the pia matter, respectively. They then further elongate their basal process and are called RG cells from around this stage, based on their molecular and morphological features (Malatesta et al., 2000).

In the neurogenic period, an RG cell divides asymmetrically, producing two daughter cells: one of them remains an RG cell, while the other differentiates into either a neuron or an intermediate neuronal progenitor (IP) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; **Figure 1**). Both neurons and IPs migrate toward the sub-VZ (SVZ), retracting their apical and basal processes (Tabata and Nakajima, 2003). They lose apicobasal polarity and execute "multipolar migration," alternately extending and retracting thin and short processes, and gradually move into the intermediate zone (IZ). IPs further divide to produce two or more daughter neurons during this period.

These daughter neurons in the IZ suddenly begin to elongate a dynamically moving short process, which often eventually becomes an axon (Hatanaka and Yamauchi, 2013; Namba et al., 2014). After forming pia-directed thick leading processes, they initiate radial migration toward the pial surface. Migrating neurons, which are closely apposed to RG fibers (Rakic, 1971, 1972), show a bipolar shape, extending a leading process in front and a long trailing process, a nascent axon, at the rear (Hatanaka and Yamauchi, 2013). They show repeated saltatory movements, termed "locomotion" (Nadarajah et al., 2001): typically, they first extend a leading process forward up to a certain length away from the soma, and then the leading process becomes anchored, followed by a forward movement of the soma. Although the dynamic movement of the leading process does not always appear to be strictly coupled with that of the soma (Schaar and McConnell, 2005), their overall combined behavior results in saltatory movement. When these neurons approach the top of the cortical plate (CP), they appear to change their migration mode. After the leading process reaches the MZ, their somas pause transiently and then move quickly along the shortening leading process, which is called "terminal translocation" (Nadarajah et al., 2001; Sekine et al., 2011). Finally, the neurons settle at the top of the CP. Since later-born neurons migrate past the neurons in existing layers before terminating their own migration, these sequential neuronal migratory behaviors lead to the establishment of a cortical laminar structure that exhibits an "inside-out" organization (Angevine and Sidman, 1961; Rakic, 1974).

ROLES OF MATRIX PROTEINS AND CR CELLS IN THE MZ DURING THE TERMINAL PHASE OF MIGRATION

As noted above, when migrating neurons approach the MZ, they change their mode of migration from locomotion to terminal translocation (Sekine et al., 2011). The first step of this change is to anchor their leading process to the MZ (Nadarajah et al., 2001; Sekine et al., 2011; **Figure 1**), which is a critical step toward migration termination (Sekine et al., 2011). Morphologically, when cortical neurons migrate radially in a deep part of the CP, they show the saltatory nuclear movement typical of locomotion. They then transiently pause when they approach the top of the CP, followed by rapid somal movement accompanied by shortening of the leading process, whose tip remains attached to the MZ (Sekine et al., 2011).

Several studies have indicated that Disabled homolog 1 (Dab1) plays a critical role in terminal translocation (Olson et al., 2006; Franco et al., 2011; Sekine et al., 2011), molecularly accounting for this mode change (**Figure 2A**). Dab1 is an intracellular adaptor protein that transduces signaling of Reelin (Rice et al., 1998; Howell et al., 1999), an extracellular matrix protein synthesized by CR cells. Two receptors of Reelin, apolipoprotein E receptor 2 (ApoER2, also known as LRP8) and very low-density lipoprotein receptor (VLDLR) (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999), are expressed on the leading process of migrating neurons that extend into the MZ (Hirota et al., 2015). When Dab1 in cortical neurons destined for layers 2/3 is suppressed by RNA interference (Olson et al., 2006; Sekine et al., 2011) or knocked out (Franco et al., 2011), the neurons approach the top of the CP but fail to reach their final positions. These findings support the notion that Dab1 is required for the terminal translocation of cortical neurons.

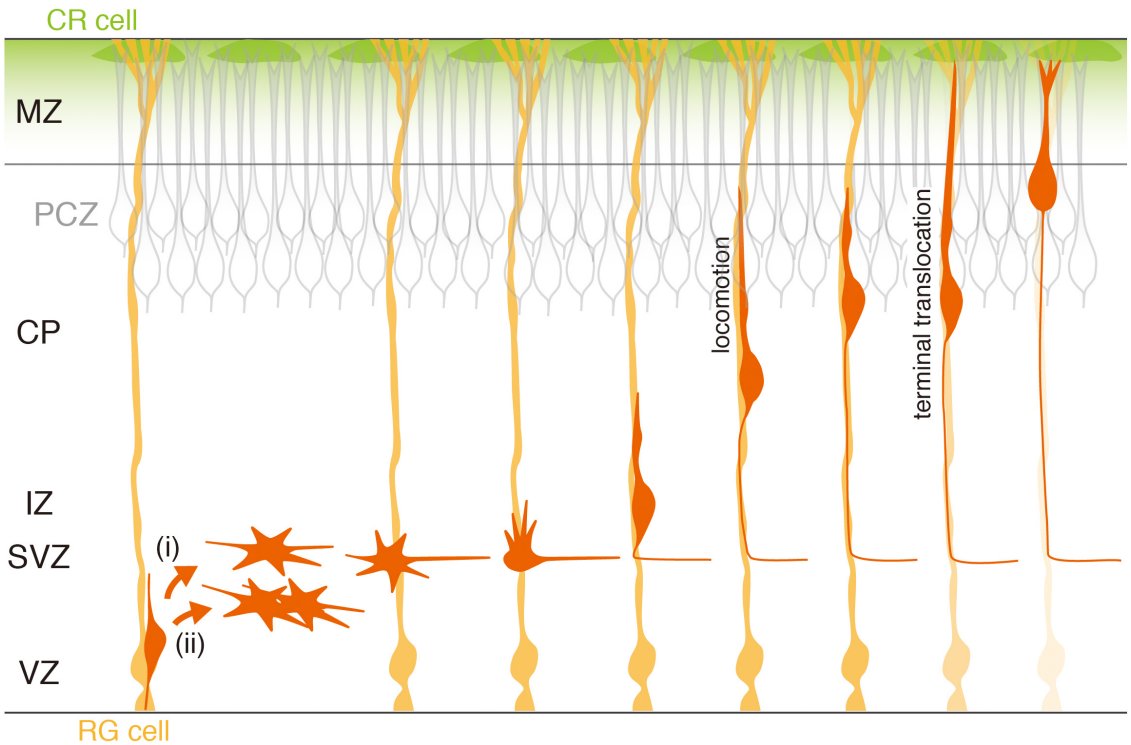

FIGURE 1 | Neurogenesis, migration, and migration termination of excitatory cortical neurons. RG cells (orange) are neural progenitors as well as substrates for radially migrating neurons. They produce neurons (red) directly (i) or indirectly via intermediate neuronal progenitors (ii). Newly generated neurons reside transiently in the SVZ/IZ as multipolar neurons, and initiate axon formation. After forming a leading process, they start radial migration along RG fibers, leaving the elongating axon in the rear. They initially migrate in locomotion mode but finally change to terminal translocation mode by anchoring the leading process to the marginal zone (MZ). Underneath the MZ, postmigratory neurons are densely packed, forming the primitive cortical zone (PCZ) (Sekine et al., 2011). The mode change likely allows newly arrived neurons to integrate into the PCZ. Neuron–RG cell adhesion disappears during the terminal phase of migration (as indicated by the fading RG color).

Stable attachment of the leading process to the MZ may be a key for terminal translocation. Indeed, Dab1-deficient neurons extend the leading process into the MZ but fail to maintain contact with the MZ. They often retract the process and rarely undergo terminal translocation (Franco et al., 2011). Consistent with this, although upper-layer neurons whose Dab1 is knocked down extend the tip of the leading process into the MZ, the process is underdeveloped and less likely to contact the MZ (Olson et al., 2006). The attachment is likely mediated by a Reelin–Dab1–Crk/CrkL–C3G–Rap1 pathway that activates $\alpha_5ß_1$ integrin on neurons, which promotes neuronal adhesion to fibronectin in the MZ (Sekine et al., 2012). However, deletion of $ß_1$ integrin from neurons does not cause major defects in brain lamination (Belvindrah et al., 2007; see also next section), suggesting that fibronectin binding via $\alpha_5ß_1$ integrin activation is dispensable for terminal translocation. Homophilic N-cadherin adhesion between leading processes and CR cells via the Rap1 pathway also seems to function (Franco et al., 2011) (see also below).

The cell-dense outermost part of the CP is named the primitive cortical zone (PCZ), which is occupied by newly settled immature neurons (Sekine et al., 2011; **Figure 1**). The terminal translocation step brings somas of migrating neurons into the PCZ, where inside-out placement of neurons occurs (Sekine et al., 2011). Indeed, sequential labeling of VZ cells at embryonic day (E)14.5 and 15.5 with different fluorescent proteins shows clear birthdate-dependent inside-out alignment of neurons in the wild-type cortex. However, when Dab1 in VZ cells at E15.5 is suppressed, these labeled neurons are not segregated at the top of the CP, failing to form the inside-out layer pattern. Thus, terminal translocation appears to be critical for migrating neurons to properly position their somas within the PCZ to establish the inside-out alignment.

Adhesion molecules expressed by CR cells also appear to play a critical role in terminal translocation. Gil-Sanz et al. (2013) showed that CR cells express an immunoglobulin-like cell adhesion molecule, Nectin1, while the leading processes of migrating cortical neurons express its preferred binding partner, Nectin3 (**Figure 2A**). Knockdown of either of these genes causes a failure of terminal translocation. The binding of Nectin1 to Nectin3 in migrating neurons stabilizes homophilic N-cadherin interactions between neurons and CR cells. This is mediated by the recruitment of an Afadin/Rap1 complex, an essential regulator of cadherin function via p120 Catenin, to the Nectin1–Nectin3 contact site (Gil-Sanz et al., 2013). Disturbance of N-cadherin function in CR cells or migrating cells leads to impairment of the leading process, which then displays reduced arborization in the MZ, and failure of

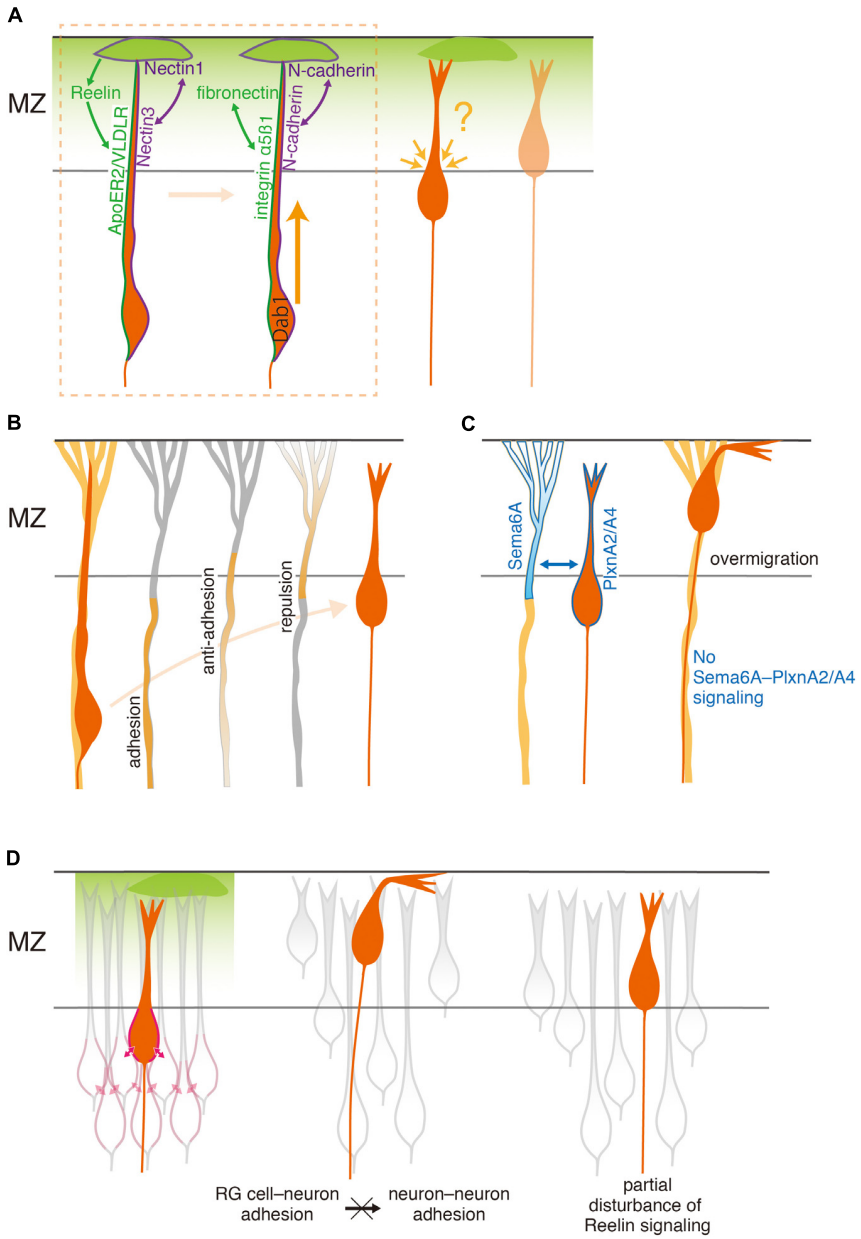

FIGURE 2 | Cellular and structural elements required for proper migration termination. **(A)** Roles of extracellular matrix and CR cells in the MZ. Radially migrating neurons anchor their leading process to the MZ or to CR cells in the MZ, through contact-independent Reelin–receptor interaction as well as contact-dependent Nectin1–Nectin3 interaction (left cell in the area surrounded by a broken line). These interactions promote adhesion between fibronectin in the MZ and integrin $\alpha_5\beta_1$ on the neuron, and by homophilic N-cadherin adhesion between CR cells and neurons, respectively (right cell). Dab1 is essential for executing terminal translocation. Although the MZ is important for the terminal translocation, it remains unsolved whether it also contributes to determining the position of the soma, which never invades the MZ. **(B)** Roles of RG cells. Radially migrating neurons detach from the RG cell fiber at the distal part. This detachment likely occurs by a decrease of adhesion, increase of anti-adhesion, and/or increase of repulsion between RG cells and neurons. **(C)** Sema6A on RG cells and PlxnA2/A4 on migrating SLNs appear to work as a repulsion signal that detaches neurons from RG cells. The absence of a Sema6A–PlxnA2/A4 signaling results in ectopic SLNs positioned beyond their proper final destination, likely due to the lack of detachment. **(D)** Roles of neighboring neurons. Radially migrating neurons are stably settled at the final position. This may be achieved by increased neuron–neuron adhesion (left). Over-adhesion of neurons to RG cells may reduce neuron–neuron adhesions, and lead to loosely packed neurons in the CP and ectopically located neurons in the MZ (middle). Direct inhibition of neuron–neuron adhesion, which may be induced by reduction of Reelin signaling, also causes loosely packed neurons and neurons ectopically located in the MZ (right).

somal translocation (Gil-Sanz et al., 2013). Interestingly, Reelin promotes recruitment of p120 Catenin and N-cadherin to Nectin/Afadin complexes, thereby stabilizing N-cadherin so that it can mediate homophilic interactions at the cell surface. Thus, cooperation between secreted and contact-dependent signals from CR cells may be essential for terminal translocation.

While leading processes are anchored to the MZ, migrating neurons arrest somal movement just beneath the MZ, forming a sharp boundary between the CP and the MZ. Therefore, there must be mechanisms that regulate the somal movement. So far, however, there is no evidence for direct regulation of somal movement by the MZ. Reelin signaling possibly contributes indirectly to this process through enhancement of neuron–neuron interactions at the top of the CP, but we need further studies to test this hypothesis (see also section "Roles of neighboring neurons during the terminal phase of migration").

ROLES OF RG CELLS DURING THE TERMINAL PHASE OF MIGRATION

Radially migrating neurons migrate along RG fibers. During this mode of migration, they maintain specific adhesive interactions with RG cells, indicating the importance of these interactions for the migration. A special junction termed "interstitial density" is observed between actively migrating, but not stationary, neurons apposed to glial fibers (Gregory et al., 1988). When these neurons enter the terminal phase of migration, their specific adhesive interactions with RG cells are presumed to be dissolved. This could occur before the terminal translocation (Nadarajah et al., 2001) or concurrently with the neurons settling in their final destinations. Studies including previous *in vitro* culture analyses as well as recent gene-manipulated mouse experiments have uncovered several molecules that are involved in these processes (Anton et al., 1996, 1999; Gongidi et al., 2004; Hatanaka et al., 2019).

Anton et al. (1996, 1999) first reported molecules that might mediate adhesive interactions between neurons and RG cells. These include RG cell membrane proteins, recognized by specific antibodies (Anton et al., 1996), and $\alpha_3ß_1$ integrins (Anton et al., 1999). Gap junction subunits connexin 26 and connexin 43 may also mediate adhesion, since they are expressed at the contact sites between migrating neurons and RG fibers (Elias et al., 2007). These molecules contribute to the continuation of migration. Importantly, the membrane proteins, localized at the plasmalemmal junction between migrating neurons and RG fibers, are distributed along the RG fibers but are virtually absent in their distal part that resides within the MZ (Anton et al., 1996). Moreover, functionally blocking the molecules perturbs migration, sometimes leading to detachment of neurons from their RG fiber substrates *in vitro* (Anton et al., 1996, 1999) and *in vivo* (Elias et al., 2007). Although the distribution of these molecules has not been fully reported, these findings suggest that proper migration termination depends on the spatial distribution of RG adhesive molecules, and that a reduction of such molecules on RG fibers causes premature termination of neuronal migration (**Figure 2B**). However, the identity of the membrane antigen remains unknown. Furthermore, later studies raised a question regarding the direct contribution of integrins to neuron–RG cell adhesion, because removal of ß1 integrin from RG cells, but not from neurons, perturbs layer formation, accompanied by disruption of endfeet anchorage on the pial basement membrane (Graus-Porta et al., 2001; Belvindrah et al., 2007). Thus, it is possible that integrin functions indirectly in neuronal migration *in vivo* by maintaining the integrity of the pial basement membrane (Graus-Porta et al., 2001; Halfter et al., 2002; Belvindrah et al., 2007).

There is another type of RG surface protein, SPARC (secreted protein acidic and rich in cysteine)-like 1, which is contrastingly expressed in the distal segment of the RG fibers spanning the upper CP. Its spatial expression profile and anti-adhesive activity between neurons and RG cells in culture suggest that it functions as a trigger for migrating neurons to detach from RG cells at their final positions (Gongidi et al., 2004; **Figure 2B**). Consistently, mutant mice lacking this molecule exhibit diffuse laminar organization. However, their gross cortical organization is normal, suggesting that other molecules are also involved in terminating migration. Molecules that interact with SPARC-like 1 remain unknown, and a better understanding of the active detachment process is awaited.

As a novel molecular cue, we recently found that a Semaphorin (Sema) 6A–Plexin (Plxn)A2/A4 interaction is responsible for the detachment of migrating neurons from RG fibers (Hatanaka et al., 2019; **Figures 2B,C**). Sema–Plxn interactions were originally determined as repulsive signals in axonal guidance (Tamagnone and Comoglio, 2000). Either Sema6A single mutants or PlxnA2/A4 double mutants show mislocalization of superficial layer neurons (SLNs) in the MZ, as the result of overmigration of SLNs beyond their final destinations. Sema6A is expressed in RG cells, while PlxnA2 and A4 are predominantly expressed in SLNs at the time when they terminate migration. Conditional knockout of Sema6A in RG cells recapitulates the overmigration phenotype, while forced expression of PlxnA2 in SLNs rescues the phenotype of PlxnA2/A4 double mutants, indicating that Sema6A and PlxnA2/A4 function in RG cells and neurons, respectively. Since Sema6A–PlxnA2/A4 trans-interaction typically elicits a repulsive effect, it is very likely that interaction between Sema6A on RG cells and PlxnA2/A4 on SLNs terminates neuronal migration by detaching SLNs from their RG substrates at their final destinations. Consistent with this interpretation, the extracellular domain of PlxnA2 most strongly binds to the MZ, highlighting their potential interacting site (Hatanaka et al., 2019). These results support the idea that active changes in adhesiveness, or a repulsive interaction, between neurons and RG cells function for proper termination of radial migration.

Terminal translocation is often referred to as an RG cell-independent process (Nadarajah et al., 2001), which implies that the detachment from RG cells itself is somewhat coupled with the terminal translocation. Supporting this notion, for example, Dab1 signaling is implicated in regulating the de-adhesion from RG cells; radially migrating neurons in *Dab1* deficient mice remain closely attached to the process of parental RG cells, but they detach from the process when they are forced to express wild-type Dab1, but not Dab1 mutants that lack potential phosphorylation sites (Sanada et al., 2004). The Nectin3–Nectin1 interaction also seems to contribute to the detachment process; their interaction switches N-cadherin-mediated neuronal adhesion from RG cells (Kawauchi et al., 2010) to CR cells (Gil-Sanz et al., 2013). In addition, the

detachment from RG cells can play a role in stopping somal movement at the top of the CP by removing the migration substrate. Therefore, in addition to identifying the molecules involved in the detachment, determining the precise timing of neurons' detachment from the RG substrate, in relation to the terminal translocation, is an essential piece of information to understand the mechanisms of migration termination.

ROLES OF NEIGHBORING NEURONS DURING THE TERMINAL PHASE OF MIGRATION

It is conceivable that cortical neurons arriving at their final destinations preferentially adhere to each other, allowing them to make laminar structures (Goffinet, 1984; **Figure 2D**). Indeed, the formation of the PCZ, where neurons are tightly packed underneath the MZ, suggests an increase of postmigratory neuron–neuron adhesiveness (Sekine et al., 2011). A PCZ-like structure is observed not only at late stages but also at earlier stages when numerous cortical neurons are migrating (Catalano et al., 1991). Therefore, all radially migrating neurons, when they reach the top of the CP, encounter a wall of cells compacted by neuron–neuron homophilic interactions. It is also hypothesized that the increase of adhesiveness among neurons helps detachment of neurons from the RG substrate by counteracting neuron–RG interaction (Goffinet, 1984).

Theoretically, the switch in adhesiveness of migrating neurons from RG cells to neighboring neurons can be achieved by either weakening of the neuron–RG cell interaction or strengthening of the neuron–neuron interaction. Several studies suggest that both occur, not independently but cooperatively or sequentially. As described above, the molecules that are responsible for migrating neuron–RG cell adhesion include integrins (Anton et al., 1999). Interestingly, while α_V integrin is important to maintain optimal neuron–RG cell adhesive strength, α_3 integrin appears to modulate neuron–RG recognition cues; impairment of α_3 integrin function switches adhesive preference of neurons from gliophilic to neurophilic in dissociated cell culture (Anton et al., 1999). Thus, it is possible that a decrease of α_3 integrin expression in the upper part of the CP weakens neuron–RG cell interactions and conversely strengthens neuron–neuron interactions.

Another example is observed in Sema6A–PlxnA2/A4 signaling-deficient mice, in which SLNs are less densely packed in the PCZ compared with those in wild-type mice at the stage when they reach their final position (Hatanaka et al., 2019). When the overmigration of SLNs in PlxnA2/A4 double mutant mice was rescued by forced expression of PlxnA2, these neurons were also clustered densely, possibly because of weakened neuron–RG cell interactions. These observations suggest that weakened neuron–RG cell interactions lead to an increase of neuron–neuron interaction, implying that they are interconnected events.

Interestingly, Reelin appears to play a direct role in the increase of neuron–neuron interaction. Matsunaga et al. (2017) have found that application of Reelin to dissociated cortical neurons transiently enhances neuronal adhesion. This is consistent with the finding that forced Reelin expression in migrating neurons induces neuronal aggregation (Kubo et al., 2010). Although a low level of Reelin expressed in the lower IZ (Uchida et al., 2009; Hirota et al., 2015) is reported to have a different role for migrating multipolar neurons in the IZ (initiation of the multipolar–bipolar transition/control of neuron–RG cell interaction, Jossin, 2011; Jossin and Cooper, 2011; Kon et al., 2019), accumulation of these neurons in the lower IZ (Tabata et al., 2009) may also support this view. The increase of neuron–neuron interaction seems to be N-cadherin-dependent (Matsunaga et al., 2017). In this context, a Reelin–receptor–Dab1 pathway likely functions, because the aggregation fails to occur when binding of Reelin to the receptor is prevented by 2A-Reelin, or when Dab1 is removed from the system. *In vivo*, neurons are mislocalized in the MZ of mice that partially lack Reelin signaling, such as the single-gene deletion of Reelin receptor components (VLDLR, Hack et al., 2007; Hirota and Nakajima, 2020, and ApoER2, Hirota et al., 2018), and of Reelin mutants that lack the C-terminal region (Kohno et al., 2015; Ha et al., 2017). Moreover, forced Reelin expression in migrating neurons in these mutant mice indicates a requirement of these receptors for the formation of properly packed PCZ-like aggregates (Hirota et al., 2018; Hirota and Nakajima, 2020). Collectively, these observations reveal that it is highly likely that Reelin–ApoER2/VLDLR receptor signaling controls neuron–neuron adhesions. Thus, a likely scenario is that Reelin secreted from CR cells controls the neuron–neuron adhesions during the terminal phase of migration, thereby indirectly suppressing ectopic neuronal invasion of the MZ, and eventually assures the stable settlement of newly arriving neurons at the top of the CP.

Dab1 stability appears to be important as a cell-autonomous determinant of neuronal positioning. Knockdown of Cullin-5 (Cul5), a key component of the E3 ubiquitin ligase complex, prevents the Reelin-dependent degradation of phosphorylated Dab1, causing activated Dab1 to accumulate in migrating neurons (Feng et al., 2007; Simo et al., 2010). These neurons are positioned more superficially, suggesting their overmigration. If activated Dab1 induces neuron–neuron adhesion to terminate migration, overmigration caused by Dab1 activation would appear to be contradictory. This seeming discrepancy may not arise, however, given that Dab1 is normally degraded upon Reelin stimulation (Arnaud et al., 2003). Moreover, the Cul5-knocked down neurons show an increase in migration speed as well as persistence at the top of the CP (Simo et al., 2010). These observations suggest that temporally regulated activation and degradation of Dab1 normally occur *in vivo*, thereby effecting transient strengthening and weakening in neuron–neuron adhesion at the top of the CP to terminate migration.

Finally, we would like to discuss the phenotypic similarity between mutant mice that have primary defects in different cellular contexts. As described above, a decrease of Reelin signaling leads to mislocation of SLNs in the MZ (Hack et al., 2007; Kohno et al., 2015; Ha et al., 2017; Hirota et al., 2018; Hirota and Nakajima, 2020), which is reminiscent of the phenotype observed in mice lacking Sema6A–PlxnA2/A4 signaling (Hatanaka et al., 2019). However, unlike Sema6A–PlxnA2/A4

knockout mice that display impaired neuron–RG cell interactions, the mutation in Reelin signaling appears to cause changes in neuron–neuron interactions, because none of VLDLR, ApoER2, or Reelin appear to be expressed by RG cells at the stage when SLNs reach the top of the CP (Alcantara et al., 1998; Hirota et al., 2015). Although it is still possible that Reelin signaling affects neuronal migration through control of the RG scaffold (Hartfuss et al., 2003; Chai et al., 2015), the above observation raises the possibility that neuron–RG cell and neuron–neuron interactions are interrelated processes in the proper location of neurons in cortical layers.

CONCLUDING REMARKS

Recent studies illuminate the roles of environmental elements in migration termination and proper positioning of cortical excitatory neurons. These include extracellular matrix proteins and CR cells in the MZ, RG cells, and neighboring neurons. Although their roles are not completely separable, each element appears to directly control the contiguous processes of terminal translocation, neuronal positioning, and proper alignment of newly arrived neurons at the top of the CP. Migrating neurons dynamically change their adhesiveness to these elements during the terminal phase of migration. Importantly, changes in adhesiveness are cooperatively regulated by these different elements, so that migrating neurons can sequentially switch their adhesion during the terminal phase of migration. This is achieved by multiple signaling molecules, such as Reelin and N-cadherin, that control the strength of cell adhesion, as well as adhesion-related molecules that regulate adhesion specificity between a neuron and each element.

Because migration termination is a highly dynamic process, understanding such a process will require dynamic analyses of adhesion-related molecules within cells as well as between cells in live-cell imaging, utilizing techniques such as SLENDR that clarifies the localization of intrinsic proteins (Mikuni et al., 2016), pHluorins to monitor protein surface expression (Miesenbock et al., 1998; Ashby et al., 2004), and FRET (Förster resonance energy transfer) biosensors to detect downstream intracellular signaling (Nakamura et al., 2006; Pertz et al., 2006) in a spatiotemporal context. Also, we will need to identify molecules that are directly involved in adhesion, as well as those that serve to switch adhesiveness during migration termination, and to analyze them in a temporally and spatially controlled manner.

AUTHOR CONTRIBUTIONS

YH wrote the manuscript in consultation with TH. Both authors contributed to the article and approved the submitted version.

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REVIEW
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The Extracellular Matrix in the Evolution of Cortical Development and Folding

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The evolution of the mammalian cerebral cortex leading to humans involved a remarkable sophistication of developmental mechanisms. Specific adaptations of progenitor cell proliferation and neuronal migration mechanisms have been proposed to play major roles in this evolution of neocortical development. One of the central elements influencing neocortex development is the extracellular matrix (ECM). The ECM provides both a structural framework during tissue formation and to present signaling molecules to cells, which directly influences cell behavior and movement. Here we review recent advances in the understanding of the role of ECM molecules on progenitor cell proliferation and neuronal migration, and how these contribute to cerebral cortex expansion and folding. We discuss how transcriptomic studies in human, ferret and mouse identify components of ECM as being candidate key players in cortex expansion during development and evolution. Then we focus on recent functional studies showing that ECM components regulate cortical progenitor cell proliferation, neuron migration and the mechanical properties of the developing cortex. Finally, we discuss how these features differ between lissencephalic and gyrencephalic species, and how the molecular evolution of ECM components and their expression profiles may have been fundamental in the emergence and evolution of cortex folding across mammalian phylogeny.

Keywords: radial glia, gene expression, microenvironment, folding, evolutionary conservation, extracellular matrix

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INTRODUCTION

The largest part of our brain is the cerebral cortex, or neocortex, which is considered the seat for our higher cognitive abilities and complex reasoning. The extraordinary size and complexity of the human cerebral cortex are the result of a sophisticated and exquisitely orchestrated developmental program, which emerged during mammalian evolution. This stemmed from an increase in the number of neuronal and glial cells, followed by a dramatic expansion in cortical size and folding. The selective pressure on these traits was the basis for the evolution of the mammalian cortex towards human (Florio and Huttner, 2014; De Juan Romero and Borrell, 2015). Recent efforts in understanding this remarkable process of mammalian cortex evolution have begun to shed light on key cellular and molecular mechanisms involved.

The neocortex is a large sheet of neural tissue characteristically organized in six main layers of neurons. This sheet may be smooth, typical of mammals with small brains like mice, or three-dimensionally arranged in folds and fissures, typical of mammals with a large brain like primates and carnivores, including human (De Juan Romero et al., 2015; Fernandez et al., 2016). The cerebral cortex originally develops from the early telencephalic primordium, a pseudostratified epithelium with apical-basal polarity composed by neuroepithelial cells (NECs; Götz and Huttner, 2005; Taverna et al., 2014). Cortical neurogenesis begins with the transformation of NECs into apical Radial Glia Cells (aRGCs), the lineage of which gives rise to all excitatory neurons of the neocortex. aRGCs are highly polarized and elongated cells, with an apical process contacting the ventricular surface, a basal process contacting the pial surface, and the cell body in the vicinity of the telencephalic ventricle, which altogether constitute the ventricular zone (VZ; Boulder_Committee, 1970). Similar to NECs, the cell body of aRGCs migrates apico-basally during the distinct phases of the cell cycle, in a movement known as interkinetic nuclear migration (INM). After mitosis at the apical surface, the cell nucleus moves basally during G1, undergoes DNA replication (S phase) at the basal side of the VZ, and moves apically during G2 to undergo mitosis again at the apical surface (Takahashi et al., 1993). aRGCs typically express the paired-box transcription factor Pax6, and may produce neurons either directly upon mitosis, or indirectly via producing Basal Progenitors (BPs; Noctor et al., 2001, 2004; Haubensak et al., 2004; Miyata et al., 2004). BPs generated by aRGCs migrate to the basal border of the VZ, where they coalesce forming the subventricular zone (SVZ) and divide to eventually produce neurons. There are two main types of BPs: intermediate progenitor cells (IPCs), which lack obvious polarity and characteristically express the T-box transcription factor Tbr2; basal radial glia cells (bRGCs), similar to aRGCs with a basal process contacting the pial surface, but without an apical process contacting the ventricle (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011; Shitamukai et al., 2011). In species with a smooth cortex (lissencephalic) like mouse, the SVZ is relatively thin and contains few BPs, with IPCs being the predominant type. These BPs largely undergo self-consuming neurogenic divisions, producing two neurons each. In contrast, in species with a folded cortex (gyrencephalic), the SVZ contains much larger numbers of BPs and is much thicker, displaying two cytoarchitectonically distinct sublayers: inner (ISVZ) and outer subventricular zone (OSVZ; Smart et al., 2002; Reillo et al., 2011). The high abundance of BPs in gyrencephalic species is largely due to their high potential for self-amplification (Fietz et al., 2010; Hansen et al., 2010; Betizeau et al., 2013). Both ISVZ and OSVZ are rich in bRGCs and IPCs, which after several rounds of self-amplification start producing massive numbers of neurons (Reillo et al., 2011; Betizeau et al., 2013; Martínez-Martínez et al., 2016). Neurogenesis from BPs occurs either by asymmetric self-renewing divisions (producing one neuron and one progenitor), or by terminal symmetric self-consuming divisions (producing two neurons). Thus, the abundance of BPs is ultimately proportional to the final number of cortical neurons and to cortical folding, these parameters being low in lissencephalic and high in gyrencephalic species (Borrell and Reillo, 2012; Betizeau et al., 2013; Pilz et al., 2013; Dehay et al., 2015; Llinares-Benadero and Borrell, 2019).

The extracellular matrix (ECM) is a key part of the cellular microenvironment during cortical development, contributing to define the local niche of the different cell populations. The ECM is formed by a complex combination of structural proteins and proteoglycans that act as a cell-supporting scaffold. However, in addition to this classical concept, recent studies show that the ECM plays fundamental roles in the polarity, survival, proliferation, migration and differentiation of cells (Hynes, 2009). Recent major breakthroughs in transcriptomic and functional analysis of cortical development in both lissencephalic and gyrencephalic species have identified ECM components as key factors regulating the proliferation of specific types of cortical progenitors, with a direct impact on the expansion and folding of the cerebral cortex (Fietz et al., 2012; Florio and Huttner, 2014; Florio et al., 2017; Long et al., 2018; Long and Huttner, 2019).

Here, we review how the expression of ECM components is regulated and patterned during cortical development, across cortical layers and progenitor cell populations, in lissencephalic and gyrencephalic species. Then we elaborate on the impact of the ECM on cortical progenitor cell proliferation and neuronal migration across mammalian phylogeny, and discuss its influence on the mechanical properties of cortical tissue, altogether affecting cortex folding. Finally, we hypothesize that the modification of ECM components and their expression patterns may have been critical to the remarkable expansion and folding of the mammalian neocortex during evolution.

EXPRESSION OF ECM COMPONENTS DURING CORTICAL DEVELOPMENT

Transcriptomic analyses of the developing human, mouse and ferret neocortex have been key to our understanding of the relevance of ECM in cortical development (Fietz et al., 2010, 2012; Camp et al., 2015; De Juan Romero et al., 2015; Florio et al., 2015; Pollen et al., 2015; Martínez-Martínez et al., 2016; Telley et al., 2019). High-throughput bulk RNA sequencing (RNAseq) analyses of isolated cortical germinal layers in mouse and human at mid-neurogenesis highlight that specific sets of ECM components are differentially expressed (Fietz et al., 2012). In human embryos, cortical germinal zones including VZ, ISVZ and OSVZ exhibit higher mRNA expression levels of ECM components and cytoskeletal proteins than the neuronal layer Cortical Plate (CP; **Table 1**). The mouse VZ also has a distinct signature of ECM gene expression, such that these genes are downregulated when progenitor cells are undergoing neurogenesis (Arai et al., 2011). Transcriptomic microarray data from the ferret neocortical VZ also revealed differential expression of ECM components, in this case along cortical developmental stages (**Table 1**; Martínez-Martínez et al., 2016).

Extracellular matrix components are extraordinarily diverse, and many of those expressed in the developing cerebral cortex

TABLE 1 | Differentially expressed extracellular matrix (ECM) components, Integrins, growth factors, and transferases, across lissencephalic, and gyrencephalic species.

ECM Genes		Human NCBI Gene ID	A Human (Fietz et al., 2012)			Mouse (Fietz et al., 2012)		B Ferret (Martínez-Martínez et al., 2016)			C Human cell populations (Florio et al., 2015)	
			hVZ	hISVZ = hOSVZ	hCP	mVZ	mCP	E34VZ-E30VZ	P1VZ-E34VZ	P1VZ-E30VZ	aRG > bRG > N	bRG ≥ aRG > N
Proteoglycans	ACAN	176	–	–	–	–	–	nr	nr	nr		ACAN
	BCAN	63827	–	–	–	–	–	BCAN	BCAN	BCAN	–	–
	BGN	633		BGN				nr	nr	nr	–	–
	DCN	1634					DCN	–	–	DCN	–	–
	HAPLN1	1404	–	–	–	–	–	nr	nr	nr	HAPLN1	
	HAPLN4	404037	HAPLN4					nr	nr	nr	–	–
	NCAN	1463	NCAN					–	NCAN	NCAN	–	–
	LUM	4060	–	–	–	–	–	nr	nr	nr	LUM	
	RELN	5649	–	–	–	–	–	–	–	RELN	–	–
	SCUBE3	222663					SCUBE3	–	–	SCUBE3	–	–
	SPARC	6678	–	–	–	–	–	–	SPARC	–	–	–
	SPARCL1	8404	–	–	–	–	–	–	SPARCL1	–	–	–
	SPOCK1	6695	–	–	–	–	–	–	SPOCK1	SPOCK1	–	–
	SPOCK2	9806	–	–	–	–	–	–	SPOCK2	SPOCK2	–	–
	SUSD1	64420	–	–	–	–	–	–	–	SUSD1	–	–
	VCAN	1462					VCAN	–	VCAN	VCAN	–	–
ECM proteins	ATRN	8455	–	–	–	–	–	–	ATRN	ATRN	–	–
	BMPER	168667				BMPER		nr	nr	nr		BMPER
	CD248	57124		CD248				nr	nr	nr	–	–
	CNTN4	152330			CNTN4			nr	nr	nr	–	–
	COCH	1690			COCH			nr	nr	nr	–	–
	ECM1	1893				ECM1		nr	nr	nr	–	–
	FBLN2	2199				FBLN2		–	FBLN2	FBLN2	–	–
	FBLN5	10516	FBLN5					nr	nr	nr	–	–
	LGALS3	3958	–	–	–	–	–	–	–	LGALS3	–	–
	LGALS8	3964			LGALS8			nr	nr	nr	–	–
	LGALSL	29094	–	–	–	–	–	–	LGALSL	LGALSL	–	–
	LTBP1	4052	–	–	–	–	–	–	LTBP1	–	–	–

(Continued)

TABLE 1 | Continued

ECM Genes		Human NCBI Gene ID	A Human (Fietz et al., 2012)			Mouse (Fietz et al., 2012)		B Ferret (Martínez-Martínez et al., 2016)			C Human cell populations (Florio et al., 2015)	
			hVZ	hISVZ = hOSVZ	hCP	mVZ	mCP	E34VZ-E30VZ	P1VZ-E34VZ	P1VZ-E30VZ	aRG > bRG > N	bRG ≥ aRG > N
	LTBP4	8425	–	–	–	–	–	–	LTBP4	LTBP4	–	–
	MATN2	4147	MATN2					–	MATN2	MATN2	–	–
	MFAP1	4236	–	–	–	–	–	–	MFAP1	MFAP1	–	–
	NTN1	9423		NTN1				nr	nr	nr	–	–
	NTN3	4917					NTN3	nr	nr	nr	–	–
	NTN4	59277				NTN4		nr	nr	nr	–	–
	NTNG1	22854	–	–	–	–	–	–	–	NTNG1	–	–
	PRELP	5549	–	–	–	–	–	nr	nr	nr	PRELP	
	RELN	5649	–	–	–	–	–	–	–	RELN	–	–
	TMEFF2	23671			TMEFF2			nr	nr	nr	–	–
	VIT	5212	–	–		–	–	–	–	VIT	–	–
	VWF	7450			VWF			nr	nr	nr	–	–
Collagens	COL1A1	1277	–	–	–	–	–	–	–	COL1A1	–	–
	COL2A1	1280	COL2A1					–	–	COL2A1	–	–
	COL1A2	1278	–	–	–	–	–	nr	nr	nr	COL1A2	
	COL3A1	1281	–	–	–	–	–	–	–	COL3A1	–	–
	COL4A1	1282		COL4A1				–	COL4A1	COL4A1		COL4A1
	COL4A2	1284		COL4A2				nr	nr	nr	–	–
	COL4A6	1288	–	–	–	–	–	–	–	COL4A6	–	–
	COL5A2	1290	–	–	–	–	–	–	–	COL5A2	–	–
	COL5A3	50509	COL5A3					nr	nr	nr	–	–
	COL8A1	1295	–	–	–	–	–	nr	nr	nr		COL8A1
	COL9A3	1299		COL9A3				nr	nr	nr	–	–
	COL11A1	1301	–	–	–	–	–	–	–	COL11A1	–	–
	COL11A2	1302	COL11A2					nr	nr	nr	–	–
	COL12A1	1303				COL12A1		nr	nr	nr	–	–
	COL15A1	1306				COL15A1		–	–	COL15A1	–	–
	COL16A1	1307	–	–	–	–	–	–	COL16A1	COL16A1	–	–
	COL17A1	1308	–	–	–	–	–	–	COL17A1	COL17A1	–	–
	COL18A1	80781				COL18A1		–	COL18A1	COL18A1	–	–

(Continued)

TABLE 1 | Continued

ECM Genes		Human NCBI Gene ID	A Human (Fietz et al., 2012)			Mouse (Fietz et al., 2012)		B Ferret (Martínez-Martínez et al., 2016)			C Human cell populations (Florio et al., 2015)	
			hVZ	hISVZ = hOSVZ	hCP	mVZ	mCP	E34VZ-E30VZ	P1VZ-E34VZ	P1VZ-E30VZ	aRG > bRG > N	bRG ≥ aRG > N
	COL21A1	81578	–	–	–	–	–	COL21A1	COL21A1	COL21A1	–	–
	COL22A1	169044	COL22A1					nr	nr	nr	–	–
	COL24A1	255631	–	–	–	–	–	–	COL24A1	COL24A1	–	–
	COL28A1	340267	–	–	–	–	–	nr	nr	nr	COL28A1	
	COLQ	8292	COLQ					nr	nr	nr	–	–
Laminins	LAMA1	284217	–	–	–	–	–	–	–	LAMA1	–	–
	LAMA3	3909	LAMA3					nr	nr	nr	–	–
	LAMA5	3911				LAMA5		nr	nr	nr	–	–
	LAMB1	3912	–	–	–	–	–	–	LAMB1	LAMB1	–	–
	LAMB2	3913	–	–	–	–	–	–	–	LAMB2	–	–
	LAMB4	22798	–	–	–	–	–	nr	nr	nr		LAMB4
	LAMC2	3918	–	–	–	–	–	nr	nr	nr		LAMC2
Integrins	ITGA1	3672		ITGA1				nr	nr	nr	–	–
	ITGA3	3675			ITGA3			nr	nr	nr	–	–
	ITGA5	3678				ITGA5		nr	nr	nr	–	–
	ITGA10	8515				ITGA10		nr	nr	nr	–	–
	ITGB5	3693	–	–	–	–	–	ITGB5	–	–	–	–
Growth Factors	BMP3	651					BMP3	nr	nr	nr	–	–
	CRELD1	78987	–	–	–	–	–	–	CRELD1	–	–	–
	EREG	2069	–	–	–	–	–	nr	nr	nr		EREG
	FGF5	2250	–	–	–	–	–	nr	nr	nr		FGF5
	FGF9	2254	–	–	–	–	–	FGF9	–	–	–	–
	FGF12	2257			FGF12			nr	nr	nr	–	–
	FGF18	8817					FGF18	nr	nr	nr	–	–
	GDF1	2657					GDF1	nr	nr	nr	–	–
	GDF5	8200					GDF5	nr	nr	nr	–	–

(Continued)

TABLE 1 | Continued

ECM Genes		Human NCBI Gene ID	A Human (Fietz et al., 2012)			Mouse (Fietz et al., 2012)		B Ferret (Martínez-Martínez et al., 2016)			C Human cell populations (Florio et al., 2015)	
			hVZ	hISVZ = hOSVZ	hCP	mVZ	mCP	E34VZ-E30VZ	P1VZ-E34VZ	P1VZ-E30VZ	aRG > bRG > N	bRG ≥ aRG > N
	IGF2	3481	IGF2					nr	nr	nr	–	–
	INHA	3623					INHA	nr	nr	nr	–	–
	INHBA	3624			INHBA			nr	nr	nr	–	–
	MEGF6	1953		MEGF6				nr	nr	nr	–	–
	MEGF8	1954	–	–	–	–	–	–	–	MEGF8	–	–
	MEGF10	84466	–	–	–	–	–	–	MEGF10	–	–	–
	MSTN	2660	MSTN					nr	nr	nr	–	–
	PDGFA	5154			PDGFA			nr	nr	nr	–	–
	PDGFB	5155			PDGFB			nr	nr	nr	–	–
	PDGFC	56034				PDGFC		nr	nr	nr	–	–
	PDGFRA	5156			PDGFRA			nr	nr	nr	–	–
	TGFA	7039				TGFA		nr	nr	nr	–	–
	TGFB3	7043				TGFB3		nr	nr	nr	–	–
	TMEFF2	23671			TMEFF2			–	–	TMEFF2	–	–
	VEGFC	7424				VEGFC		nr	nr	nr	–	–
Transferase	CHPF	79586	CHPF					nr	nr	nr	–	–
	CHSY3	337876			CHSY3			nr	nr	nr	–	–
	HS2ST1	9653				HS2ST1		nr	nr	nr	–	–
	HS6ST1	9394					HS6ST1	nr	nr	nr	–	–
	NDST1	3340				NDST1		nr	nr	nr	–	–
	NDST2	8509				NDST2		nr	nr	nr	–	–
	ST3GAL2	6483			ST3GAL2			nr	nr	nr	–	–
	SULF1	23213	SULF1					nr	nr	nr	–	–
	SULT1B1	27284	–	–	–	–	–	nr	nr	nr		SULT1B1
	SULT1C2	6819	–	–	–	–	–	nr	nr	nr		SULT1C2
	SULT1C4	27233	–	–	–	–	–	nr	nr	nr	SULT1C4	

(A) *Genes differentially expressed between cortical layers in human and mouse (Fietz et al., 2012). The gene name is indicated where it is expressed at significantly higher levels compared to the other layers; (–) means no significant difference.* ***(B)*** *Genes differentially expressed between embryonic (E) and postnatal (P) cortical Ventricular Zone (VZ) in ferret (Martínez-Martínez et al., 2016). The gene name is indicated where it is differentially expressed; (–), no significant difference; (nr), not reported.* ***(C)*** *Genes differentially expressed between specific cell populations of the developing human cortex (Florio et al., 2015). The gene name is indicated in the comparison where it is differentially expressed; (–), no significant difference.*

are polyvalent in regulating stem cell proliferation and niche maintenance (Fietz et al., 2010; Marthiens et al., 2010; Stenzel et al., 2014; Güven et al., 2020). Each mammalian species expresses in cortical germinal zones a unique combination of ECM components at unique relative levels, which suggests that their precise abundance and overall combined composition may be important in fine-tuning cortical progenitor proliferation, self-renewal and expansion, which are also unique among species. In the human OSVZ, very rich in highly proliferative BPs, specific ECM components are expressed at high levels (**Table 1**). A landmark study by Florio et al. (2015) compared the transcriptomic profile of isolated aRGCs, bRGCs and neurons in the developing human and mouse cerebral cortex. This analysis revealed that ECM components and cell surface receptors were more highly expressed in human aRGCs and bRGCs than in mouse, pointing to the notion that these components may influence the proliferation of aRGCs and bRGCs in human versus mouse (Florio et al., 2015, 2016, 2017). Hence, a notion emerges that each species, either lissencephalic or gyrencephalic, elaborates its own ECM niche in germinal zones to implement the particular proliferative and neurogenic program for their unique set of progenitor cell composition, thus contributing to species differences in cortical development. Accordingly, changes in the expression of ECM components strongly regulate cortical progenitor proliferation and may have been central in the evolutionary expansion of the human neocortex (Fietz et al., 2012). Importantly, germinal zones appear to be a reservoir of ECM components. For example, HAPLN1 and collagen I mRNAs are expressed at high levels in human germinal zones (**Table 1**), but at the protein level these are concentrated in the CP and cortical wall. This shows that germinal zones are the site of transcription of these genes, but the proteins they encode are only active at the CP and cortical wall (Long et al., 2018).

One of the most salient features of mammalian cortex evolution is its folding. Transcriptomic studies in ferret have shed light on the genetic basis of cortex folding, which also appears to be strongly influenced by the ECM. By comparing the transcriptomic profile of the cortical germinal zones prospectively forming the Splenial Gyrus and the Lateral Sulcus in the ferret visual cortex, we discovered a large number of genes differentially expressed between these two regions, including genes that encode for cell adhesion molecules and ECM components (De Juan Romero et al., 2015). This analysis also showed that the largest amount of differentially expressed genes, and the greatest differences in expression levels between prospective gyrus and sulcus, occur at the OSVZ, further supporting the central importance of this germinal layer in the differential expansion and folding of the cerebral cortex. This pioneer notion has been substantiated experimentally by, for example, the disruption of Integrin receptor function in the OSVZ of ferret organotypic cortical slices (Fietz et al., 2010). The loss of function of Integrin αvβ3 caused a significant reduction in the abundance of bRGCs, but not IPCs. This indicates that ECM components specifically enhance the amplification of bRGCs and, consequently, promote the expansion of the OSVZ and cortex folding (Fietz et al., 2010; De Juan Romero et al., 2015; Dehay et al., 2015).

Single cell RNA sequencing (scRNAseq) revolutionized the field of transcriptomic analysis by providing a snapshot of cell diversity. scRNAseq has been extensively used to characterize the developing cerebral cortex in a variety of mammals, from mouse to human, and newly emerged *in vitro* experimental models such as cerebral organoids (Camp et al., 2015; Pollen et al., 2015; Arlotta and Pasca, 2019; Kanton et al., 2019; Telley et al., 2019; Bhaduri et al., 2020). Aiming to identify the transcriptomic changes that caused the evolutionary expansion of the neocortex, studies have compared aRGCs and bRGCs in human and mouse. Findings highlight ECM genes as a correlate with the high proliferative activity of RGCs in human and ferret as compared to mouse (Lui et al., 2014; Johnson et al., 2015; Pollen et al., 2015). For example, human bRGCs have higher expression levels of ECM genes than mouse, including Laminin, Tenascins, and Integrins, along with HOPX, PTPRZ1, and other genes that modulate the interaction between ECM components, self-renewal of progenitor cells and migration of neurons (Pollen et al., 2015). ScRNAseq analyses have also revealed that RGCs possess unique typological and temporal transcriptomic profiles, distinguishing lineages between the dorsoventral and the rostrocaudal telencephalon. Accordingly, the well-known topographic differences and gradients of development in the telencephalon have been proposed to result from the existence of spatially patterned transcriptomic programs (Nowakowski et al., 2017). Similarly, during development of the mouse somatosensory cortex aRGCs gradually switch from proliferation to neurogenesis, and this appears to be evolutionarily conserved, as it is largely recapitulated in embryonic human aRGCs (Telley et al., 2019). This temporal and spatial change in the transcriptomic profile of progenitor cells during cortical development is linked to ECM components and microenvironmental cues, suggesting that they may have a relevant impact on neurogenesis and cortical patterning.

Recently, cerebral organoids have emerged as a valid *in vitro* model to study cortical development in diverse species (Lancaster et al., 2013; Lancaster and Knoblich, 2014; Camp et al., 2015; Qian et al., 2019; Velasco et al., 2019; Bhaduri et al., 2020). Accordingly, scRNAseq studies comparing progenitor cell populations in human fetal tissue and cerebral organoids have shown that aRGC populations express similar ECM components in both systems (Camp et al., 2015). Interestingly, scRNAseq in human and chimpanzee organoids uncovered subtle differences in the expression levels of genes encoding ECM components and cell adhesion molecules. Given the relevance of differences between human and chimpanzee to understand human evolution, even these small variations in the transcriptomic profiles and signaling pathways of cortical progenitor cells may be key in understanding the evolution and expansion of the human brain (Pollen et al., 2015, 2019; Mora-Bermudez et al., 2016).

ECM AND PROLIFERATION OF NEURAL PROGENITOR CELLS

The ECM plays many roles during neural development, from the formation of a meshwork for structural support, to the activation

of signaling pathways that stimulate progenitor proliferation, either directly or indirectly (Barros et al., 2011). Prior to the onset of neurogenesis, NECs in the cortical primordium augment their number by self-amplification via symmetric divisions (Miyata et al., 2010; Fernandez et al., 2016). Already at that early stage, the ECM provides the microenvironment necessary to modulate the behavior of NECs (Perris and Perissinotto, 2000; Zimmermann and Dours-Zimmermann, 2008). The developing cortex exhibits high concentration of extracellular matrix molecules, including chondroitin sulfate (CS) and heparan sulfate (HS) proteoglycans, hyaluronic acid (HA), Laminins, and glycoproteins like Tenascins (Maeda, 2015). Proteoglycans have an influential role on the proliferation of NECs. These are complex macromolecules composed of a central core with sulphated glycosaminoglycan (GAG) and O- or N-oligosaccharides covalently linked. There are four types of GAGs: CS, dermatan sulfate (DS), Heparin and HS; Schwartz and Domowicz, 2018). Heparan sulfate proteoglycans (HSPGs) include Syndecans, Glypicans, Agrin, and Perlecan (Sarrazin et al., 2011). Glypican is abundant in the cortical VZ during neurogenesis. Mouse embryos mutant for Glypican 1 have an imbalance between proliferation and differentiation of NECs during one day of embryonic development (E8.5-9.5), which is sufficient to cause a significant reduction in brain size (**Figure 1**). At the signaling level, this reduction is due to the suppression of fibroblast growth factor signaling (FGF; Jen et al., 2009). The evolutionary conservation of the role of Glypican on NECs, and its relationship with FGF signaling, is evident in *Drosophila*, where it has been linked to organ development (Crickmore and Mann, 2007), and in *Xenopus* embryos, where Glypican 4 regulates dorsal forebrain development via FGF signaling activation (Galli et al., 2003).

Perlecan is an ECM component of the basement membrane important for both structural support and NEC proliferation (**Figure 1**). Mouse embryos mutant for Perlecan exhibit either exencephaly or microcephaly, the latter caused by a reduction in progenitor cell proliferation and impaired cell cycle progression. This phenotype results from a reduced dispersion of growth factors in the extracellular space mediated by Perlecan, such as FGF or SHH (Girós et al., 2007). Perlecan is also highly conserved, where the mutation of its *Drosophila* homolog *trol* leads to G1 cell cycle arrest, mediated by FGF and hedgehog (Hh) signaling (Park et al., 2003).

Syndecan-1 (Sdc1) is a transmembrane HSPG highly enriched in the cortical VZ. Knockdown of *Sdc1* in the developing mouse cortex led to a reduction in NEC proliferation and premature differentiation, accompanied by a reduction in ß-catenin. This suggests a possible implication of Sdc1 in regulating Wnt signaling (Wang et al., 2012; **Figure 1**). Another subclass of proteoglycan that plays a prominent role in NEC proliferation is chondroitin sulfate proteoglycans (CSPGs), which include the Lectican family (Brevican, Neurocan, Versican, and Aggrecan), Phosphacan, CD44 and the transmembrane component NG2 (Maeda, 2015). Previous studies have shown that depletion of CSPGs in mouse neurospheres *in vitro*, by means of the CSPG degrading enzyme Chondroitinase ABC, leads to a decrease in proliferation of NECs (Sirko et al., 2007). Intriguingly, a similar treatment with Chondroitinase ABC of rat neurospheres increased NEC proliferation and differentiation, indicating some functional divergence in this respect across species (Gu et al., 2009).

Laminins are a major class of ECM components with a role in cortical progenitor proliferation. Laminins are trimeric proteins composed of alpha, beta, and gamma subunits. They are expressed at high levels in stem cell niches like the VZ and SVZ, and are a major component of the VZ's apical surface (Lathia et al., 2007; Hall et al., 2008; Nirwane and Yao, 2019). Laminins exert their function by binding to Integrin and non-Integrin receptors, which transduce the Laminin signal in and out of the cell (Nirwane and Yao, 2019). *In vitro* studies illustrate that Laminin has an effect on expansion, maintenance and differentiation of mouse and human cortical progenitor cells (Drago et al., 1991; Kearns et al., 2003; Flanagan et al., 2007). Interestingly, enhanced expression of *Integrin-β1* in NECs of chick embryos led to two very distinct phenomena (Long et al., 2016). On the one hand, the generation of a population of cells that resemble subapical progenitors (SAPs) described in mouse (Pilz et al., 2013), dividing in the VZ away from the apical surface and producing IPCs. On the other hand, a non-cell autonomous effect where non-Integrin expressing cells undergo greater levels of neurogenesis driven by Wnt signaling and an increase in *Decorin* expression (Long et al., 2016). Because *Decorin* is only expressed in the OSVZ of the Human cortex (Fietz et al., 2012), this result further supports the notion that the ECM was key in the evolution of the mammalian cortex by enhancing the proliferation of progenitor cells and promoting cortical expansion and folding. So the next question regarding Laminins is: ¿how is their expression controlled during cortical development? A recent study reports that knock out of *Sox9* in the developing ferret cortex leads to a reduction in the proliferation of IPCs and bRGCs in the OSVZ. Conversely, conditional overexpression of *Sox9* in the embryonic mouse cortex leads to an increase in the proliferation of BPs, increased cell cycle re-entry and premature gliogenesis (**Figure 1**). In the long term, *Sox9* overexpression in mouse leads to an increase in the production of upper layer neurons, a hallmark of evolutionary cortical expansion. Importantly, *Sox9* overexpression in mouse cortex was accompanied by increased expression of ECM components, where Laminin 211 was the key in promoting BP proliferation (Güven et al., 2020).

Extracellular matrix components also influence the INM of NECs and aRGCs. Zebrafish *tab* mutants (analogue of *Laminin γ1*) exhibit abnormal INM in the neural tube, with nuclei entering mitosis prior to reaching the apical domain (Tsuda et al., 2010). Similarly, blockade of the β1-Integrin receptor in the VZ leads to detachment of aRGCs and affects INM and the cleavage plane of VZ progenitor cells (**Figure 1**; Lathia et al., 2007; Loulier et al., 2009). These studies confirm the key and evolutionarily conserved influence of Laminins and their receptors on progenitor proliferation and cortical development.

The basement membrane, produced by the meningeal membranes, is crucial for the survival of RGCs. Loss of *Integrin-β1* in aRGCs of the developing mouse cortex leads to the detachment of their end feet, followed by apoptosis. This detachment is recapitulated by surgical removal of the meninges,

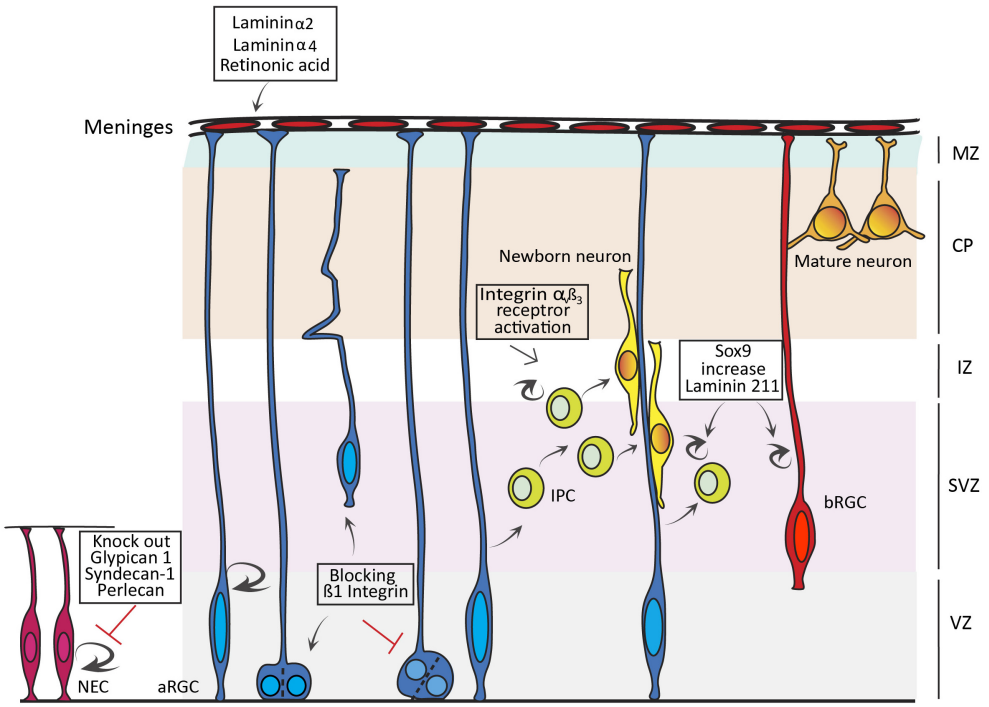

FIGURE 1 | Influence of extracellular matrix (ECM) on cortical progenitor cells. Schema summarizing the effects of ECM components on the proliferation and lineage of neuroepithelial cells (NEC), apical Radial Glia Cells (aRGC) and basal Progenitor Cells (bPC), including Intermediate Progenitor Cells (IPC) and basal RGCs (bRGC). Loss of Glypican 1, Syndecan-1, and Perlecan leads to a decrease in proliferation of NECs, while blocking β1 Integrin leads to apical detachment of aRGCs and loss of asymmetric divisions in the VZ. Knocking out Laminin α2, Laminin α4, and Retinoic acid secreted from the external meninges affects aRGC attachment to the basement membrane. Activation of Integrin αvβ3 increases IPC proliferation and cell cycle re-entry, while Sox9 activation increases bPC proliferation via Laminin 211.

and in mice lacking *Laminin α2* and *4* in their basement membrane (**Figure 1**; Radakovits et al., 2009). Furthermore, mutant mice with disrupted meningeal development exhibit an expansion of NECs in detriment of IPC production and neurogenesis (Siegenthaler et al., 2009). This phenotype was rescued with retinoic acid (RA) treatment, showing the importance of the factors secreted from the meninges for propagating a normal neurogenesis (Siegenthaler et al., 2009).

The concept that the self-renewal capacity of cortical progenitors is the driving force for cortical expansion during evolution, where gyrencephalic species have a larger capital of NECs underlying the generation of more aRGCs, IPs and bRGCs, and subsequently more neurons, has been supported experimentally (Florio and Huttner, 2014; Fernandez et al., 2016). Integrin αvβ3 is expressed at particularly high levels in human OSVZ, where highly proliferative bRGCs are abundant. Inhibition of Integrin αvβ3 signaling in species endowed with abundant bRGCs, including human and ferret, decreases proliferation of bRGCs in OSVZ (Fietz et al., 2010; Reillo et al., 2011). Concomitantly, activation of the Integrin αvβ3 receptor in mouse cortex leads to increased proliferation and cell cycle re-entry of IPs (Stenzel et al., 2014). Altogether, this strongly supports the notion that Integrin modulation of BPs plays an important role in cortical expansion, and that changes in ECM composition during mammalian evolution contributed critically to define the size and complexity of the cerebral cortex, including progenitor cell proliferation, neurogenesis and gliogenesis (Rash et al., 2019).

ECM IN CELL MIGRATION

Extracellular matrix molecules are also involved in regulating neuronal migration during cortical development (Franco and Müller, 2011; Franco et al., 2011). Excitatory cortical neurons travel radially from their place of birth in the germinal layers to their final destination in the CP, in a process known as radial migration (Rakic, 1972; Sidman and Rakic, 1973). In this process, neurons interact intimately with the basal process of aRGCs, known as radial glial fiber, which serves as guide and physical substrate for neuronal migration (Rakic, 1972; Sidman and Rakic, 1973). Thus, radial neuron migration depends on the integrity of RGCs, the actual movement of neurons, and the interaction between the two. Defects in neuron radial migration usually involve delayed or excessive migration, and lead to neuronal miss positioning and disorganization of cortical layers, direct causes of malformation of cortical development (Fernandez et al., 2016). Classically, studies of neuron radial migration have focused on intrinsic or cell-autonomous functions of candidate genes. However, radial neuron migration is also influenced by multiple non-cell autonomous signals, ranging from diffusible molecules to ECM proteins, and cell-cell interactions. This section mainly

focuses on the role of ECM components as primary non-cell autonomous factors that affect radial neuron migration.

Preservation of RGCs and the Basement Membrane

Radial neuron migration in the cerebral cortex depends on the integrity of RGCs, including the attachment of their basal process to the basement membrane, where ECM components are highly expressed. Laminins are critical for the structural integrity of the basement membrane, and patients with mutations in *Laminin beta-1* (*LAMB1*) develop cobblestone-lissencephaly. This is a neuronal migration disorder characterized by the breaching of the basement membrane, causing the detachment of the basal end-feet of aRGCs followed by the over migration of neurons, the loss of cortex folding and the acquisition of a bulgy appearance of the cortical surface (Timpl and Rohde, 1979; Radmanesh et al., 2013). Similarly, mutant mice deficient in *Laminin γ1III4* and *Perlecan* have severe defects on basement membrane integrity and neuron migration (Haubst et al., 2006), developing neuronal ectopias typical of cortical cobblestone (**Figure 2**).

Dystroglycan is another ECM component with an important role in neuron migration. This is a glycoprotein key in the dystrophin glycoprotein complex, which binds to α-Dystroglycan, a primary target for O-glycosylation. The Dystrophin glycoprotein complex is important for maintaining the integrity of the basement membrane by ensuring the attachment of the RGC end feet to the pial surface. Patients with genetic mutations resulting in hypoglycosylation of α-Dystroglycan display over-migration abnormalities and other malformations of cortical development (van Reeuwijk et al., 2005). This phenotype is mimicked in *Dag1* mutant mice, where RGCs fibers are truncated and the basement membrane is frequently breached, invaded by multiple cell types forming heterotopias (**Figure 2**; Myshrall et al., 2012).

The integrity of RGCs is also impaired upon the loss of the proteoglycan Syndecan-3 (Hienola et al., 2006) and of Endothelin Converting Enzyme 2 (ECE2; Buchsbaum et al., 2020). Both absence and overexpression of ECE2 in developing mouse embryos and human cerebral organoids lead to apical-basal detachment of RGCs and impaired radial neuron migration, resulting in the ectopic accumulation of neurons within the VZ. These features are typical of periventricular nodular heterotopia (PNH), a cortical malformation formed by clusters of cortical neurons that fail to undergo radial migration properly and accumulate next to the ventricular surface. Proteomic studies analyzing ECE2 mutant human cerebral organoids reveal a significant down regulation of ECM components such as *Laminin*, *Lumican* and six different collagens. These findings highlight the role of ECE2 in regulating the expression of ECM components that are important for normal neuron migration and cortical development (**Figure 2**; Buchsbaum et al., 2020).

Regulation of Neuron Movement

The role of ECM in cortical lamination also extends to a direct influence on migrating neurons. Reelin (Reln) is among the most studied, and yet most poorly understood, ECM molecules. Throughout cortical development, Reln is secreted by Cajal-Retzius (CR) cells in the marginal zone (D'Arcangelo et al., 1995; Alcantara et al., 1998). Reln binds to the VLDLR and/or ApoER2 lipoprotein receptors of target cells, driving the tyrosine phosphorylation of the adaptor protein Dab1 (Rice and Curran, 2001). Reln has been proposed to be a stop signal that instructs the end of radial migration to each new wave of cortical neurons, thus directly organizing the formation of cortical layers in an inside-out manner (older neurons occupy deep layers, newer neurons occupy superficial layers). Mutation of *RELN* leads to Norman-Roberts lissencephaly in humans (Hong et al., 2000) and to the *reeler* phenotype in mice (D'Arcangelo et al., 1995). Both human and mouse mutations disrupt cortical neuron migration, which in *reeler* mice is accentuated by the massive invasion of ectopic neurons into the marginal zone. This led to the suggestion that Reln acts as a "stop" signal to terminate neuronal migration at the cortical marginal zone (**Figure 2**; Curran and D'Arcangelo, 1998; Dulabon et al., 2000; Rice and Curran, 2001). CR cells and Reln have also been shown to be required for maintenance of the integrity of radial glia fibers in mouse (Super et al., 2000; Hartfuss et al., 2003), but this remains under debate as it seems not to be the case in ferret (Schaefer and Juliano, 2008). The sequence of Reln protein is conserved across more than 104 species (Manoharan et al., 2015), and the levels/patterns of expression of *Reln* and *Dab1* during cortical development in turtle, lizard, chicken and mouse are well corresponded with their respective laminar organization. In contrast to the subpial expression of *Reln* in mammals, in lizards it is expressed in a subcortical layer and cortical neurons are positioned in an inverted, outside-in manner. This suggests functional conservation of this extracellular protein in neuronal migration across amniotes. Its relevance in the well-defined laminar organization of the CP in mammals and lizards, as opposed to non-laminar in birds, is considered an example of homoplasy by convergent evolution (Bar et al., 2000).

Malformations of cortical development are also caused by delayed neuronal migration (Ross and Walsh, 2001). Targeted disruption of *Laminin γ1* expression in the cerebral cortex disrupts Integrin and Akt/Gsk-3β signaling, which impairs neuronal migration without affecting cell proliferation and neuronal cell death. The absence of Laminin γ1 – AKT signaling hinders the arrival of migrating neurons to the marginal zone and leads to defective cortical lamination (**Figure 2**; Chen et al., 2009). Neuroglycan C is a member of the family of CSPGs and a downstream interactor of PHF6, an X-linked protein mutated in the intellectual disability disorder Börjeson–Forssman–Lehmann. Loss of Neuroglycan C in mouse embryos leads to radial migration failure during cortical development (**Figure 2**; Zhang et al., 2013). The functional side chains of CSPGs possess a sulphated structure generated by a family of sulphotransferases, several of which are expressed during cortical development. Several sulphotransferases have been shown to play central roles in neuronal migration, by *in utero* electroporation of loss-of-function short hairpin RNAs. Following this manipulation, neuronal migration is

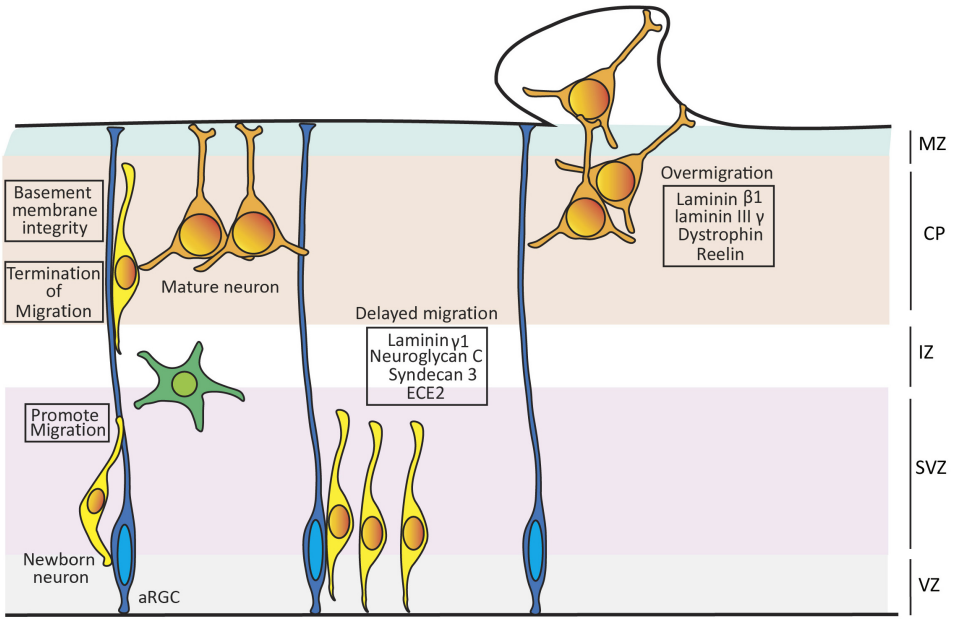

FIGURE 2 | Role of extracellular matrix (ECM) on neuronal migration. Schema showing the role of ECM components on promoting the migration, termination of migration and maintenance of the basement membrane integrity in mouse developing cortex. Loss of Laminin γ1, Neuroglycan C, Syndecan 3, and overexpressing ECE2 leads to delay in migration, while loss of Laminin β1, Laminin IIIγ, Dystrophin or Reelin leads to overmigration of neurons and breaching of the basement membrane.

blocked at the multipolar-to-bipolar transition but not at the level of RGCs, suggesting that the specific sulphated side chains play an important role during radial migration (Akita et al., 2008; Ishii and Maeda, 2008). Altogether, it is clear that the ECM is involved in controlling many aspects of cortical neuronal migration, and that this is largely conserved across phylogeny, further supporting the importance of the ECM on the expansion and folding of the cerebral cortex during evolution.

ECM IN CEREBRAL CORTEX FOLDING

As mentioned above, transcriptomic studies have demonstrated that expression of ECM components is very different between cortical layers and species, supporting a process of cortical expansion and folding via progenitor cell proliferation and neuron migration. The ECM also defines the stiffness and biomechanical properties of the developing cortex, thus additionally influencing its folding. Accordingly, changes in ECM composition during mammalian evolution may have dictated the occurrence, degree and pattern of cortex folding across phylogeny (Llinares-Benadero and Borrell, 2019).

ECM in Cortical Expansion

The mechanisms responsible for folding of the mammalian cerebral cortex have been under debate for many years. An early attractive hypothesis was that animals with large brains have folded cortices because they undergo a disproportionate expansion of the outer cortical surface (gray matter, composed of neuron) in comparison to the inner part (white matter, composed of axons and glial cells), and this leads to folding of the cortex. Notable exceptions to this trend are represented by the American beaver and the Florida manatee, which have a smooth cortex but brain size similar to other species with a highly folded cortex, such as the chimpanzee (Welker, 1990). A refined version of this hypothesis proposes that cortex folding results from the differential expansion of the upper neuronal layers in comparison to deep cortical layers (Armstrong et al., 1991). The relative expansion of upper layers has been proposed to result from increases in BP abundance and the formation of the OSVZ (Smart et al., 2002; Kriegstein et al., 2006; Reillo et al., 2011; Borrell and Reillo, 2012). In combination with differential neurogenesis, the tangential dispersion of radially migrating neurons in gyrencephalic species is thought to significantly contribute to the expansion of cortical surface and the formation of folds (Borrell, 2018; Llinares-Benadero and Borrell, 2019).

As discussed above, the ECM is a very important factor in the regulation of cortical progenitor cell proliferation, and recent studies support that it is also important in cortex folding. Patients with mutations in *RELN* (see above) display abnormal neuronal migration and axonal connectivity, and in the long term resulting in lissencephaly (loss of cortical folds; Hong et al., 2000). The importance of proper neuron migration for cortical gyrification has been recently highlighted with the analysis of mice mutant for Flrt proteins. Flrts are a family of cell adhesion transmembrane proteins rich in Fibronectin and Leucine repeats, which are involved in the radial migration of cortical neurons. The analysis of mice double mutant for

Flrt1/3 revealed the formation of *bona fide* cortical folds and fissures in the otherwise smooth mouse cortex (del Toro et al., 2017). This phenotype emerges from an imbalance in adhesion-repulsion forces in migrating neurons. Importantly, these experimental results are validated by observations in the normally folded cortex of ferrets, where *Flrt1* and *Flrt3* are expressed at much lower levels in migrating neurons of cortical fissures than folds (De Juan Romero et al., 2015; del Toro et al., 2017).

Influence of the ECM on the Mechanical Properties of Cortex During Folding

Folding of the cerebral cortex is ultimately a physical process of deformation of developing neural tissue (Kroenke and Bayly, 2018). Cortical folding has been described as a mechanism where the differential expansion rate between upper and lower cortical layers leads to elastic instability (Richman et al., 1975; Bayly et al., 2014). Experimental testing with hydrogel models has been fundamental to our understanding of this process beyond mathematical models. Hydrogel models are composed of an inner core hydrogel covered with an outer layer of second hydrogel with similar or different physical properties (elasticity, resistance, etc.). When subject to expansion, these compound gel models sustain significant and measurable elastic instability and compression. The use of these models has demonstrated that when the outer layer swells (grows) faster than the inner core, this results in material strain and compression, which is released by buckling and the formation of seeming folds and fissures (Tallinen et al., 2014). For greater realism, three-dimensional hydrogel models have been designed with the shape of a mid-gestational human embryo brain, and then the differential expansion of the bi-layered hydrogel results in the formation of folds and fissures mimicking the adult human brain (Tallinen et al., 2016).

The above studies and related transcriptomic analyses (Sheppard et al., 1991; Fietz et al., 2012) suggest that the ECM regulates cortical folding not only by affecting progenitor cell proliferation and neuron migration, but also by contributing to define the mechanical properties of the developing cortex. A seminal study by Long and colleagues used living slices of embryonic human cortex cultured *in vitro* to demonstrate the critical role of the ECM on cortex folding (Long et al., 2018). Slices of human fetal neocortex in culture were treated with a cocktail of ECM components (HAPLN1, Lumican, and Collagen I), which induced the ultra-rapid folding of the cortical surface, not occurring in untreated slices. Related to an increase in tissue stiffness, this folding was accompanied by an increase in expression of HA and its receptor (CD168) in the CP, followed by ERK signaling activation. Intriguingly, this ECM cocktail did not induce folding by promoting progenitor proliferation or neuronal migration, but by decreasing cell density at the CP. This was recapitulated in untreated slices from older fetuses, supporting that this combination of ECM components increases stiffness and induces folding by the same physiological mechanism as nascent folds that develop at later stages in the non-manipulated human embryo (Long et al., 2018).

The advent of cerebral organoids has become an additional alternative to study and understand cortical folding, by physical manipulation *in vitro*. An innovative organoid on-a-chip approach allows growing cerebral organoids that wrinkle and fold (Karzbrun et al., 2018). This enables to culture human cerebral organoids in millimeter-thick chambers and image them in whole mount, including the formation of folds. Under these conditions, organoids developed from hiPSCs from lissencephalic patients, mutant for *LIS1*, wrinkle significantly less than control organoids from healthy donors. Transcriptomic analyses of these mutant organoids has revealed a significant downregulation of ECM and cytoskeletal genes, suggesting that the underlying cause of this deficit in cortical folding is a pathological softening of the cytoskeleton. Unfortunately, cortical folding of on-chip organoids is due to contraction of the VZ and expansion of the progenitor cell nucleus (Karzbrun et al., 2018), which completely differs from the expanded basal germinal zones and increased neurogenesis observed in animal models (Reillo et al., 2011; Heide et al., 2018; Karlinski and Reiner, 2018; Karzbrun et al., 2018). Nonetheless, these results support the relevance of the ECM in maintaining the tissue contractility and stiffness that induce cortex folding (Karlinski and Reiner, 2018; Karzbrun et al., 2018).

The balance between softness and stiffness in the CNS microenvironment is also a key factor in fate determination. Mounting evidence demonstrates that the mechanical properties of tissue microenvironment exerted by ECM components, including stiffness or viscoelasticity, play a significant role in cell fate determination, dictating the output of cellular lineages from differentiation to proliferation or apoptosis (Holle et al., 2018). For example, microenvironments as soft as brain tissue promote mesenchymal stem cells to adopt a neuronal lineage, whereas stiffer microenvironments promote the same cells to enter myogenic differentiation (Engler et al., 2006). Analyses of the stiffness of the developing mouse cortex using atomic force microscopy (AFM) have shown that VZ and SVZ gradually increase in stiffness during development, while the neuron-rich CP increases in stiffness only until E16.5, decreasing by E18.5. Stiffness of the CP is due not only to neurons, which are stiffer than other cells in the cortex, but also to changes in the composition of the ECM (Iwashita et al., 2014). Indeed, differences in ECM composition along the human cortical surface, causing variations in tissue stiffness, have been proposed as a mechanism contributing to cortex folding (Long et al., 2018; Wianny et al., 2018).

EVOLUTION OF ECM COMPONENTS AND THE EVOLUTION OF CORTICAL FOLDING

Recent progress in neuroimaging techniques and neuroanatomy are providing major insights into fundamental differences in cortical organization across phylogeny. Using multiple

approaches to compare cortical folding, parcellation and neural connectivity in mouse, marmoset, macaque and human, David Van Essen and colleagues have revealed dramatic differences in the total number and arrangement of cortical areas (Van Essen et al., 2019). In this study, they also report that cortical folding patterns vary dramatically across species, and that individual variability in cortical folding increases with cortical surface area. In line with this evidence, recent hypotheses propose that the sophistication of cortical folding and expansion in development and evolution may be attributed to both cell autonomous mechanisms (i.e., increased progenitor cell proliferation) and non-cell autonomous mechanisms (i.e., ECM composition) known to impinge on the former (Fietz et al., 2010; Güven et al., 2020). The notion that the evolution of ECM components may have significantly contributed to the evolution of cortical folding is directly supported by the effects of ECM treatment on folding of cortical slices in culture (Long et al., 2018). Ectopic administration of ECM molecules (HAPLN1, Lumican and Collagen I) caused the folding of living cortical slices from human embryos, but not from ferrets or mice, although it did cause changes in tissue stiffness. This different response suggests that the ECM and signaling pathways that induce gyrification in humans are different from those with a similar role in ferret, as shown in **Table 1**. These findings highlight human specific ECM components as a game changer in mechanical and signaling processes during cortical folding (Wianny et al., 2018). Interestingly, Cromar et al. (2014) showed that ECM proteins underwent domain gain that occurs exclusively at the divergence of primates from other mammals. In agreement with this, primate-specific miRNAs regulating the expression of ECM genes are differentially expressed in CP and germinal zones in primates (Arcila et al., 2014). Taken together, this indicates the existence of evolutionary changes in the regulation of expression of ECM components, and supports the notion that the ECM contributes to regulate cortex size and folding (Fietz et al., 2012; Florio et al., 2017; Long et al., 2018).

A close inspection of the spatial and temporal patterns of expression of ECM components and cell adhesion molecules in the developing cerebral cortex highlights potential mechanisms evolved to induce cortical folding. As mentioned, *Flrt1/3* are expressed homogeneously and at high levels in the developing mouse cortex but not in ferret, where domains of medium and low expression alternate, correlating with the folding pattern. Interestingly, the loss of *Flrt1/3* in the mouse smooth cortex alters the adhesion-repulsion balance between migrating neurons thus promoting their tangential dispersion, leading to the formation of fissures and folds. This mimicks the native situation found in human and ferret, therefore emphasizing the importance of repression of *Flrt1/3* in the evolution of cortex folding (del Toro et al., 2017; Llinares-Benadero and Borrell, 2019).

The relevance of neuronal migration in the formation of cortical folds is further supported by comparative analyses in mouse and ferret (Gertz and Kriegstein, 2015; Martínez-Martínez et al., 2019). Whereas in mouse cortex radial neuron migration takes place in rather rectilinear trajectories, cortical neurons in ferret display much more tortuous and complex behaviors (Gertz and Kriegstein, 2015). Examination of the detailed cellular morphology and behavior demonstrates that, contrary to dogma, radially migrating cortical excitatory neurons extend a leading process that is frequently branched under normal physiological conditions, both in mouse and ferret (Martínez-Martínez et al., 2019). The frequency and degree of branching of this leading process are significantly greater in the gyrencephalic ferret than the lissencephalic mouse. We have proposed that this difference has a profound influence on the tangential dispersion of neurons migrating radially and, consequently, on cortical folding. Differences in branching between species may stem from differences in the expression profile of ECM and cell adhesion molecules (Fietz et al., 2012; Reillo et al., 2017).

In addition to the known and potential direct effects of ECM on cortex expansion and folding, a recent study in the developing ferret identified multiple cellular elements that may act as non-cell autonomous or "extrinsic" elements affecting cortical progenitor behavior and fate in different ways (Reillo et al., 2017). For example, axonal fiber tracts and tangentially migrating neurons with a marked laminar organization are proposed to be prominent sources of instructive signals onto cortical progenitor cells and radially migrating neurons. These extrinsic elements change quite dynamically during development, so their relevance on cortex development/folding are proposed to be also dynamic. This highlights the role that different combinations of ECM components and cell adhesion molecules may play in creating a complex laminar code of extrinsic influences, that modulate cortical development and folding in a selective manner (Nowakowski et al., 2017; Reillo et al., 2017).

CONCLUSION AND FUTURE PERSPECTIVES

The ECM is best known for providing structural support to cells and tissues. However, the burst of transcriptomic studies over the past few years has identified ECM components as prime candidates in controlling cerebral cortex development, expansion and folding, and the evolution of these features. A number of studies have shown the central importance of the ECM in regulating cortical progenitor proliferation and basal progenitor amplification, the basis for increased neurogenesis, expansion and folding. Other ECM molecules regulate neuron migration or define the stiffness of tissue, with profound implications in cell fate determination and cortex folding. Some of these functions are highly conserved across phylogeny, while others exert their function in a species-specific manner. Accordingly, functionally relevant interspecies differences in ECM composition suggest its co-evolution with the cortical phenotype.

New tools and technologies continuously provide unprecedented opportunities to increase our understanding of the ECM and its roles in brain development. Single cell

RNA sequencing now offers the unique opportunity to carefully examine differences in ECM expression profiles across progenitor cell populations and their lineages, and the impact of the ECM on transcriptional programs critical during cortical development. This may then allow identifying ECM signaling pathways implicated in the evolution and folding of the neocortex. A focus on the ECM is a promising strategy in the quest to reach a unified understanding of molecular mechanisms of cortical evolution and folding.

AUTHOR CONTRIBUTIONS

SA created the figures. SA and VB wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Beyond Axon Guidance: Roles of Slit-Robo Signaling in Neocortical Formation

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The formation of the neocortex relies on intracellular and extracellular signaling molecules that are involved in the sequential steps of corticogenesis, ranging from the proliferation and differentiation of neural progenitor cells to the migration and dendrite formation of neocortical neurons. Abnormalities in these steps lead to disruption of the cortical structure and circuit, and underly various neurodevelopmental diseases, including dyslexia and autism spectrum disorder (ASD). In this review, we focus on the axon guidance signaling Slit-Robo, and address the multifaceted roles of Slit-Robo signaling in neocortical development. Recent studies have clarified the roles of Slit-Robo signaling not only in axon guidance but also in progenitor cell proliferation and migration, and the maturation of neocortical neurons. We further discuss the etiology of neurodevelopmental diseases, which are caused by defects in Slit-Robo signaling during neocortical formation.

Keywords: Robo, Slit, neocortex, migration, proliferation, dendrite, spine, axon guidance

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INTRODUCTION

The neocortex is the six-layered outermost structure of the cerebrum, and is considered to be an evolutionarily new region of the brain that appeared soon after the emergence of mammals. Humans have the largest neocortex relative to their body size, which is thought to underlie their higher brain functions, such as cognition and emotion (Rakic, 2009).

The neocortex consists of two main types of neurons, i.e., excitatory projection neurons and inhibitory interneurons, which are generated from distinct germinal zones in the developing cerebrum, corresponding to the dorsal and ventral telencephalon, respectively. In both regions, the germinal zones are divided into two territories. The first is the ventricular zone (VZ), which lines the ventricles and occupies the apical-most region of the cerebral cortex. The second is the subventricular zone (SVZ), which is located adjacent to the VZ and basally toward the surface of the neocortex. The VZ comprises apical radial glial cells (aRGCs), which integrate into the apical junctional belt and extend long basal processes toward the pial surface (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Sun and Hevner, 2014, **Figure 1**). After the onset of neurogenesis, most aRGCs give rise to a secondary progenitor cell population in the SVZ, namely, basal intermediate progenitor cells (bIPs). bIPs demonstrate a multipolar morphology, delaminate from the apical junctional belt, and produce neurons after a limited number of cell divisions. In rodents, a subset of neocortical neurons are derived from the bIPs (Letinic et al., 2002;

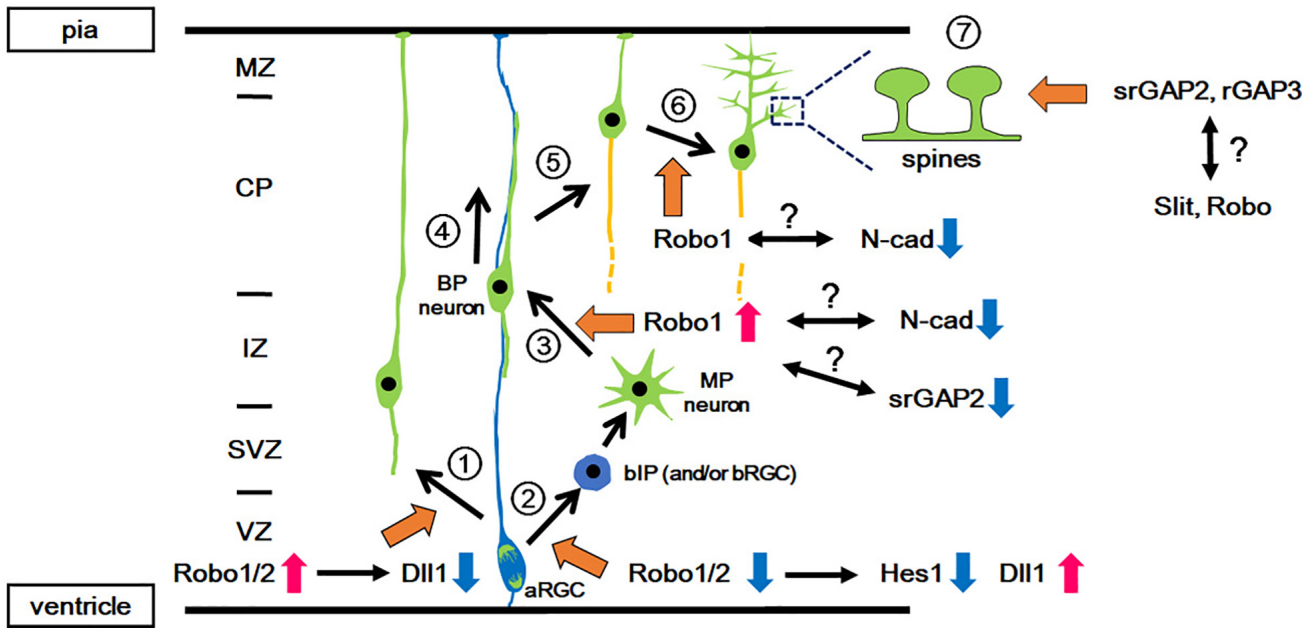

FIGURE 1 | Functions of Slit-Robo signaling in various events during neocortical development. During development, pyramidal projection neurons are directly generated from the aRGCs in the VZ (1: *Direct neurogenesis*) or via intermediate progenitors (2: *Indirect neurogenesis*). In indirect neurogenesis, bIPs give rise to neurons with multipolar processes (MP neurons) and then the MP neurons transform into bipolar (BP) neurons (3: *MP-BP transition*). The BP neurons migrate along the basal processes of the aRGCs toward the CP (4: *Locomotion*). When the BP neurons reach the upper part of the CP, they detach from the basal processes and migrate a short distance to the pial surface (5: *Terminal translocation*). Then, the neurons develop dendrites (6: *Dendrite formation*) followed by spine formation on the dendrites (7: *Spine formation*). Orange arrows indicate the involvement of Slit-Robo signaling and/or srGAP. Red and blue arrows indicate the activation and inactivation of molecules/signals, respectively. aRGC, apical radial glial cell; bIP, basal intermediate progenitor; BP, bipolar; CP, cortical plate; IZ, intermediate zone; MP, multipolar; MZ, marginal zone; N-cad, N-cadherin; SVZ, subventricular zone; VZ, ventricular zone.

Haubensak et al., 2004; Kowalczyk et al., 2009; Petros et al., 2015; Tyler et al., 2015; Vasistha et al., 2015). Notably, in gyrencephalic mammals, including humans, non-human primates, and ferrets, there is an additional type of basal progenitor cell that demonstrates a radial glia-like morphology, namely, the basal RGCs (bRGCs; also called outer radial glial cells) (Lui et al., 2011; Wang et al., 2011; Sun and Hevner, 2014; Namba and Huttner, 2017; Miller et al., 2019; Cárdenas and Borrell, 2019). bRGCs divide extensively and produce a large number of neurons, and therefore, expansion of the neocortex is thought to correlate with the presence of bRGCs.

In mice, after the onset of neurogenesis, neurons generated from aRGCs migrate radially toward the marginal zone (MZ) through direct somal translocation by processes that extend from the soma to the pial surface (**Figure 1** and Nadarajah et al., 2001). Subsequently, neurons arise from the bIPs, which demonstrate a multipolar morphology, and undergo repeated extension and retraction of their multiple thin processes in the intermediate zone (IZ) (Noctor et al., 2001; Tabata and Nakajima, 2003). The multipolar neurons first transform into bipolar neurons by extending a trailing process, followed by the formation of a leading process in the IZ and subplate (SP) (Hatanaka and Yamauchi, 2013; Namba et al., 2014). These bipolar neurons migrate radially toward the pial surface through the IZ and the cortical plate (CP), through a locomotion mode using radial glial fibers as a scaffold (Rakic, 1972; Nadarajah et al., 2001). Once the leading processes enter the MZ, the soma of migrating neurons translocate rapidly for a short distance toward the MZ (terminal translocation) (Nadarajah et al., 2001). Late-born neurons migrate past the earlier-born neurons that have settled in the CP, and therefore laminar formation proceeds in an inside-out manner.

Proper leading and trailing process formation and the subsequent migration of neurons are crucial for the establishment of neural networks. It has been shown that such neuronal morphogenesis and migration are regulated by environmental cues, including axon guidance molecules and cell adhesion molecules (Kawauchi, 2012; Inamura et al., 2012; Namba et al., 2015; Cadwell et al., 2019). Abnormalities in neuronal migration cause neuronal migration disorders, including lissencephaly, heterotopia, and focal cortical dysplasia (Guerrini and Parrini, 2010; Roberts, 2018). On the other hand, subtle alterations in neuronal migration cause mild changes in lamination and circuit formation, which lead to epilepsy and neuropsychiatric disorders, including autism, schizophrenia, and dyslexia (Cascella et al., 2009; Poelmans et al., 2011; Peterson and Pennington, 2012; Katsarou et al., 2017; Varghese et al., 2017; Keller et al., 2017).

In this review, we focus on Slit and Robo, which were originally identified as axon guidance molecules, and

discuss the novel roles of Slit-Robo signaling in neocortical development. We highlight the pleiotropic functions of Slit-Robo signaling beyond axon guidance, by focusing on their new roles in the proliferation, migration, and maturation of cortical neurons during development, and further discuss the involvement of Slit-Robo signaling in human neurodevelopmental disorders.

MOLECULAR PATHWAY OF SLIT-ROBO SIGNALING

Slit Ligands and Robo Receptors

Slit and Robo were first identified by screening of *Drosophila* mutants demonstrating abnormal projections of commissural axons in the central nervous system (Rothberg et al., 1988; Seeger et al., 1993). Slit is a protein that is secreted by midline glial cells, and Robo receptors are expressed in commissural axons (Rothberg et al., 1990; Kidd et al., 1998). Slit molecules act via binding to Robo receptors to regulate axonal guidance (Brose et al., 1999; Kidd et al., 1999; **Figure 2A**). Because Slit molecules act as a repulsive axon guidance cue, Slit-Robo signaling enables all commissural axons to cross the midline only once, and thus ensures them to project to the contralateral side (Brose et al., 1999; Kidd et al., 1999).

Slit ligands and Robo receptors are well conserved across species, from invertebrates to vertebrates. In mammals, three Slit subtypes (Slit1–Slit3) (Holmes et al., 1998; Itoh et al., 1998; Brose et al., 1999; Yuan W. et al., 1999) and four Robo subtypes (Robo1–Robo4) (Kidd et al., 1998; Sundaresan et al., 1998a,b; Yuan S.S.F. et al., 1999; Huminiecki et al., 2002) have been identified. Robo receptors are single-pass transmembrane proteins and are members of the immunoglobulin superfamily of cell adhesion molecules (IgCAMs), containing immunoglobulin-like (Ig) domains and fibronectin type III (FNIII) domains (**Figure 2A**). Upon binding to Slit through the Ig domains, the Robo receptor transduces intracellular signals (Brose et al., 1999; Liu et al., 2004). Whereas the role of Slit-Robo signaling in axon guidance is conserved from *Drosophila* to mammals (Bagri et al., 2002; Andrews et al., 2006; Fouquet et al., 2007; López-Bendito et al., 2007; Unni et al., 2012), several additional roles of Slit-Robo signaling have been identified in mammals. Studies have shown that Robo-mediated signaling is required for the proliferation of neural progenitor cells, as well as for the migration and morphological differentiation of cortical neurons (Andrews et al., 2006, 2008; Barber et al., 2009; Hernández-Miranda et al., 2011; Zheng et al., 2012; Borrell et al., 2012; Gonda et al., 2013; Yeh et al., 2014; Cárdenas et al., 2018; Blockus et al., 2019). These findings support the view that Robo signaling plays important roles in addition to axonal pathfinding in the developing neocortex.

To date, several downstream signals of the Robo receptor have been identified (Ypsilanti et al., 2010; Blockus and Chédotal, 2016; Dai et al., 2019; Jiang et al., 2019; Tong et al., 2019). Here, we focus on two Slit-Robo-mediated signal transduction systems that are involved in cerebral cortex formation.

Slit-Robo GTPase-Activating Protein (srGAP) in Slit-Robo Signaling

One of the downstream signal pathways of Slit-Robo is mediated by the Rho family of small GTPases (Wong et al., 2001; Hu et al., 2005, **Figure 2B**). Using yeast two-hybrid screening, Wong and colleagues identified Slit-Robo GTPase-activating protein (srGAP) as a molecule that interacts with the intracellular domain (CC3 domain) of Robo (Wong et al., 2001). In mammals, four srGAPs (srGAP1, srGAP2, srGAP3, and Arhgap4) have been identified. However, in addition to the ancestral copy of *srGAP2* (*srGAP2A*), the human genome has three human-specific paralogs of *srGAP2*, namely, *srGAP2B*, *srGAP2C*, and *srGAP2D*, which arose by gene duplications (Dennis et al., 2012; Sporny et al., 2017). All *srGAPs* contain three functional domains; i.e., from the N-terminus to C-terminus, the Fes-CIP4 homology BAR (F-BAR) domain, GTPase-activating protein (GAP) domain, and Src homology 3 (SH3) domain. Each srGAP has specific binding characteristics to the Rho family small GTPases; i.e., srGAP1 interacts with Cdc42 and RhoA upon Slit stimulation (Wong et al., 2001), srGAP2 has been reported to bind to Rac1, and srGAP3 binds to both Rac1 and Cdc42 (Wong et al., 2001; Endris et al., 2002; Guerrier et al., 2009).

In experiments using a human-derived cell line, the binding of Slit to Robo was demonstrated to promote the interaction between the intracellular CC3 domain of Robo1 and srGAP1, resulting in the inactivation of Cdc42. Cdc42 inactivation suppresses activation of the actin-related protein (Arp)2/3 complex and neuronal Wiskott-Aldrich syndrome protein (actin polymerization regulatory protein, N-WASP), resulting in actin depolymerization. This leads to the axon repulsion and the inhibition of cell migration (Wong et al., 2001).

Cell Adhesion Molecules and Slit-Robo

In addition to srGAPs, the crosstalk between Slit-Robo signaling and cell adhesion signals is mediated by cadherins (**Figure 2C**). Cadherins are trans-interacting calcium-dependent cell-cell adhesion molecules, and classical cadherins (such as N-cadherin) interact with adaptor proteins (such as catenin) to connect with the actin cytoskeleton (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; Reynolds et al., 1994). Interference of cadherin and catenin interactions leads to either increased or decreased adhesion depending on the context (Mège et al., 2006).

The binding of Slit to the Robo receptor induces an interaction between the Robo receptor and N-cadherin-Cable complex via Abelson (Abl) kinase, which binds to the intracellular domain (CC3) of Robo. This Robo and N-cadherin interaction leads to the phosphorylation of β-catenin by Abl, and thereby phosphorylated β-catenin is detached from N-cadherin. This, in turn, weakens N-cadherin-mediated cell adhesion. The phosphorylated β-catenin translocates to the nucleus and activates transcription (Rhee et al., 2002, 2007). The signal transduction of Robo receptors depends on its cytoplasmic interactors: the CC3 domain of Robo1 interacts with the SH3 domain of srGAPs in addition to the SH3 domain of Abl

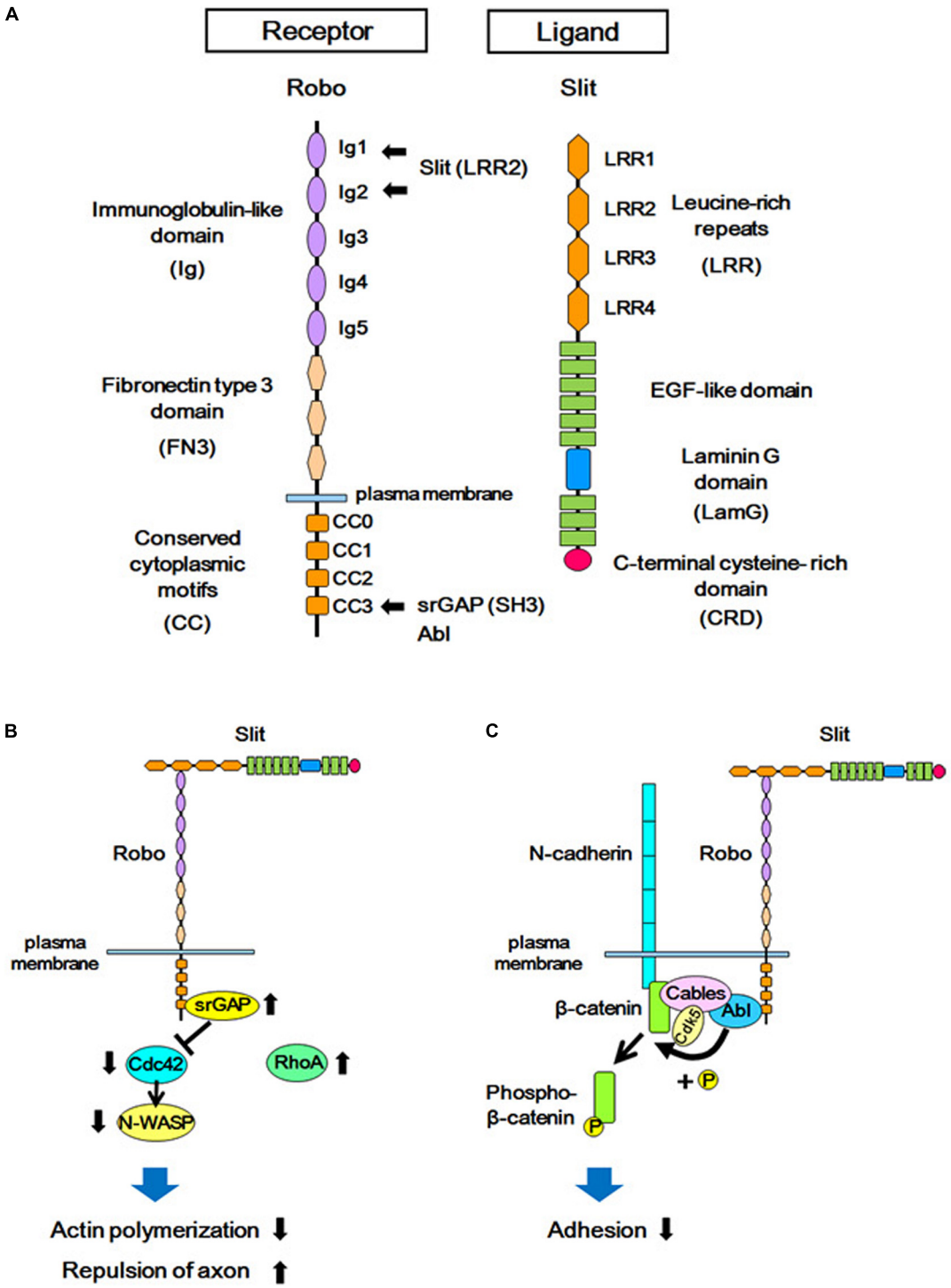

FIGURE 2 | Structures of Slit/Robo, and the Slit-Robo signaling pathway. **(A)** The Robo receptor contains five immunoglobulin-like domains (Ig), three fibronectin type III domains (FN3), and four conserved cytoplasmic domains (CC). Slit is a secreted glycoprotein and a major ligand of the Robo receptor. Slit contains four domains consisting of leucine-rich repeats (LRR), several EGF-like sequences, a laminin-G domain (LamG), and a C-terminal cysteine-rich domain (CRD). The LRR2 domain of Slit interacts with the Ig1 and Ig2 domains of Robo, and the SH3 domain of srGAPs and Abl kinase interacts with the CC3 domain of Robo. **(B)** The extracellular interaction between Slit and Robo increases the binding of srGAP with Robo, resulting in the activation of srGAP. Activated srGAP induces GTP hydrolysis of Cdc42, and therefore inactivates Cdc42. Inactivated Cdc42 is unable to stimulate actin polymerization via the downstream effector of Cdc42 (N-WASP). This in turn leads to actin depolymerization and repulsion of the axon. **(C)** Binding of Slit to Robo results in the interaction between Abelson (Abl) and Cable, which leads to tyrosine phosphorylation of β-catenin by Abl. This phosphorylation reduces the affinity between β-catenin and N-cadherin, and attenuates N-cadherin-mediated adhesion.

(Wong et al., 2001), suggesting that these two signal mediators act competitively via the same SH3 domain.

SLIT-ROBO SIGNALING IN NEURAL PROGENITOR CELLS

Effects of Slit-Robo Signaling on Neural Progenitor Cell Proliferation

During the early stages of cortical development, multiple signaling pathways regulate the proliferation and division modes of cortical progenitor cells. In this context, studies using *Robo* mutant mouse lines have implicated the roles of Slit-Robo signaling in controlling the balance between cell proliferation and differentiation.

Borrell's group reported that Slit-Robo signaling is involved in the proliferation-differentiation balance of neural progenitor cells (Borrell et al., 2012, **Figure 1**). *Robo1*/*Robo2* expression is detected in the VZ. Loss of Robo1/2 signaling leads to a decrease in the number of aRGCs and a concomitant increase in the number of basally located cells expressing the bIP marker Tbr2. However, these Tbr2-expressing cells retain apical processes and are integrated into the ventricular (apical) surface, suggesting that Robo signaling regulates two events, i.e., the production of intermediate progenitors (IPs) from aRGCs and their delamination from the apical junction belt.

The increased number of basally located progenitors in the neocortex of *Robo1*/*Robo2*-deficient mice is largely consistent with another report using distinct *Robo1/Robo2* mutant mice (Yeh et al., 2014). However, Yeh et al. reported that the number of aRGCs is increased in *Robo1/Robo2* mutant mice (Yeh et al., 2014). This discrepancy between the two studies may be due to the different mutant mouse lines used. The former used a hypomorphic *Robo1* mutant, in which Ig-domain 1 and half of Ig-domain 2, which are the domains responsible for Slit binding, are still expressed (Long et al., 2004; López-Bendito et al., 2007), whereas the latter study used a null mutant mouse line (Lu et al., 2007; Andrews et al., 2008). Interestingly, treatment of cortical progenitor cells with the extracellular domain of Robo1 resulted in a reduction in the number of progenitor cells expressing the aRGC marker Pax6 (Yeh et al., 2014). Thus, the remaining Ig domains in the hypomorphic *Robo1* mutant mice may affect the number of aRGCs.

The function of Robo in cortical progenitor cells has been mediated by a crosstalk between Robo signaling and Hes1, which is a transcription factor acting downstream of Notch (Borrell et al., 2012, **Figure 1**). Notch is a transmembrane protein that is known to promote neuroepithelial cell to aRGC transition, and inhibits the production of IPs from aRGCs (Gaiano et al., 2000; Mizutani et al., 2007; Ohata et al., 2011; Martynoga et al., 2012). Upon Notch activation, its intracellular domain is cleaved and translocates into the nucleus to induce transcriptional activation of its effector gene *Hes1* (Kageyama et al., 2019). As the neocortex of Robo mutant mice show reduced expression of Hes1, Robo signaling is thought to activate Hes1. Given that transcriptional activation of *Hes1* by Robo2 was observed in a cell line that lacks Notch expression, the activation of Hes1 is independent of Notch activation (Borrell et al., 2012). Furthermore, *Hes1* activation is also induced by Robo2 lacking the CC3 domain, which was previously identified to be the domain to which Robo-interacting proteins bind. These results suggest that other molecules may mediate Hes1 activation.

Altogether, Robo signaling does not affect Notch activity directly, but activates *Hes1* expression. This transcriptional activation of Hes1 by Robo signaling explains how the production of IPs from aRGCs is increased in *Robo* mutant mice (Borrell et al., 2012). In addition, *Slit1/Slit2* mutant mice show a phenotype similar to that seen in *Robo1/Robo2* mutant mice, which suggests that Slit1/Slit2 are candidate ligands for Robo signaling in regulating aRGC proliferation.

Role of Slit-Robo Signaling in Regulating Direct vs. Indirect Neurogenesis

The roles of Robo signaling in neuronal proliferation also implicates its roles in brain evolution (Cárdenas et al., 2018). The mammalian brain consists of distinct regions that developed at different times during evolution. The neocortex is the newest brain region that developed in mammals, whereas regions such as the olfactory bulb (OB) are conserved among vertebrates, and are thus considered to be older regions of the brain. The mode of neurogenesis differs among these regions; the neocortex undergoes indirect neurogenesis, in which aRGCs give rise to neurons via the production of bIPs, whereas OB neurons are produced by direct neurogenesis from the aRGCs (Díaz-Guerra et al., 2013; Luzzati, 2015; Cárdenas et al., 2018; **Figure 1**). Therefore, direct neurogenesis is assumed to be an evolutionarily older mode of neurogenesis, whereas indirect neurogenesis is an evolutionarily newer mode.

This difference in neurogenic modes appears to also be regulated by the level of Slit-Robo signaling. *Robo1/Robo2* are expressed at higher levels in the OB than in the neocortex during the early stages of neurogenesis (Cárdenas et al., 2018). High expression levels of *Robo1/Robo2* lead to direct neurogenesis, whereas low expression levels of *Robo1/Robo2* in the neocortex is required for maintaining indirect neurogenesis (Cárdenas et al., 2018). As Robo1/Robo2 induce the expression of the Notch ligands Jag1 and Jag2, but suppress the expression of another Notch ligand, Dll1 (Cárdenas et al., 2018), Robo regulates direct vs. indirect neurogenesis via the modulation of Notch ligand expression.

A comparative study of the reptile, bird, and mammalian telencephalon showed a negative correlation of Robo expression to the amount of indirect neurogenesis. That is, the highest level of Robo expression and the lowest amount of indirect neurogenesis were observed in reptiles, a moderate level of Robo expression and moderate amount of indirect neurogenesis were found in birds, and the lowest level of Robo expression

and the highest amount of indirect neurogenesis were detected in mammals (Cárdenas et al., 2018). Taken all together, Robo regulates the mode of neurogenesis and its low expression level enables neocortical progenitor cells to increase in number, which finally results in expansion of the telencephalon.

SLIT-ROBO SIGNALING IN NEURONAL MIGRATION

Excitatory projection neurons in the neocortex migrate radially toward the CP from the VZ by radial migration (Ohtaka-Maruyama and Okado, 2015; Hevner, 2019; Silva et al., 2019; **Figure 1**). By contrast, inhibitory interneurons are generated from the ganglionic eminence (GE) and migrate tangentially to the neocortex through two distinct zones, namely, the IZ/SVZ and MZ (Pleasure et al., 2000; Lim et al., 2018; Silva et al., 2019). The migration of interneurons from outside of the neocortex is another determinant of the number of neurons in the neocortex. Next, we describe the requirement of Slit-Robo signaling in these two migration modes.

Slit-Robo Signaling in Interneuron Migration

Several axon guidance molecules have been shown to regulate the tangential migration of inhibitory neurons (Zhu et al., 1999; Marín et al., 2001; Hirschberg et al., 2010). In the embryonic neocortex, Slit1 is expressed in the VZ and SVZ of the lateral and medial ganglionic eminences (Yuan W. et al., 1999; Bagri et al., 2002; Marillat et al., 2002), and has been suggested to regulate interneuron migration by repelling interneurons toward the neocortex (Zhu et al., 1999). However, Marín et al. (2003) show that the distribution of interneurons in the neocortex is unaffected in the absence of Slit1 and Slit2, suggesting that Slit is dispensable for the tangential migration of interneurons toward the neocortex.

Robo1 has been reported to regulate the migration of interneurons (Andrews et al., 2006). The Robo1 protein is detected in the SVZ of the GE and the MZ, and the lower IZ of the neocortex, where interneurons tangentially migrate to the neocortex (Andrews et al., 2006). Interneurons are aberrantly found in the striatum of *Robo1*-knockout mice (Andrews et al., 2006), which was not observed in *Slit1*- and *Slit2*-knockout mice (Marín et al., 2003). These data suggest that Robo signaling regulates interneuron migration through a Slit-independent mechanism. One possibility is a signal crosstalk between Robo signaling and Sema-Neuropilin (Nrp)/Plexin signaling. A previous study showed that Robo1 does not directly interact with Sema, but binds to Nrp1 in trans via the region including the first two Ig domains, which is known to bind to Slit molecules (Liu et al., 2004). Interestingly, interneurons in *Nrp1*-knockout mice demonstrate a phenotype similar to that of *Robo1*-knockout mice (Marín et al., 2001; Tamamaki et al., 2003). This phenotype may be due to the lack of a physical interaction between Robo1 and Nrp1, or the reduction in Nrp1 expression found in the interneurons of *Robo1*-knockout mice (Hernández-Miranda et al., 2011).

Slit-Robo Signaling in the Radial Migration of Projection Neurons

In addition to the role of Slit-Robo signaling in the migration of interneurons, the dynamics of Robo1 expression in cortical layer neurons during development indicated the roles of Slit-Robo signaling in the radial migration of neocortical projection neurons (Marillat et al., 2002; Whitford et al., 2002; Gonda et al., 2013).

Indeed, knockdown of *Robo1* in layer II/III neurons demonstrates a delay in their radial migration, particularly in their migration from the IZ to the CP (Gonda et al., 2013, **Figure 1**). This phenotype resembles that of N-cadherin overexpression and N-cadherin knockdown in migrating neurons, both of which caused a delay in neuronal migration (Kawauchi et al., 2010; Jossin and Cooper, 2011). In addition, the proper regulation of N-cadherin-mediated cell adhesion by controlling N-cadherin turnover in the plasma membrane of neurons was shown to be crucial for neuronal migration from the IZ to the CP (Kawauchi et al., 2010). As Robo1 inhibits the interaction between N-cadherin and β-catenin (Rhee et al., 2002, 2007), which may lead to N-cadherin endocytosis, Robo1 may regulate radial migration, possibly by attenuating N-cadherin-mediated cell adhesion. This possibility requires further investigation. Furthermore, there is still the open question of whether the delay in migration is dependent or independent of Slit.

Robo4 has also been reported to regulate the radial migration of layer II/III neurons (Zheng et al., 2012). Unlike *Robo1*-knockdown neurons, *Robo4*-knockdown neurons cannot migrate into the CP, and are retained in the white matter until at least postnatal day 20. *Robo4*-knockdown neurons do not show substantial changes in their transition from a multipolar to bipolar morphology, suggesting that Robo4 does not play a role in the polarization of neurons (La Fata et al., 2014; Barnat et al., 2017; Zhang et al., 2018). However, *Robo4*-knockdown neurons have leading processes with an aberrant orientation, suggesting that Robo4 regulates the interaction between the basal processes of aRGCs and migrating neurons. One possibility is that Robo4 acts as a cell adhesion molecule, similar to other IgCAMs.

In addition to the Robo1 and Robo4 receptors, srGAPs, which are the downstream effectors of Slit-Robo signaling, also function to regulate migration in the developing forebrain. Inhibition of srGAP1 activates Cdc42 in neurons migrating from the anterior SVZ of the neonatal forebrain and blocks Slit-mediated repulsion (Wong et al., 2001). srGAP2 expression becomes prominent in the CP of the neocortex from the late neurogenic period (embryonic day 16.5). Suppression of srGAP2 expression in neocortical neurons reduced the branching of leading processes, resulting in the promotion of radial migration (Guerrier et al., 2009, **Figure 1**). The expression of srGAP3, as well as Robo1, is decreased in the neocortex of *Ngn2*-knockout mice (Schuurmans et al., 2004; Mattar et al., 2004), which exhibits a delay in neuron migration (Hand et al., 2005), suggesting that Robo signaling

and srGAP3 regulate cell migration. This possible involvement of srGAP3 in cell migration is further supported by another study that showed the abnormal migration of progenitor cells in the postnatal *srGAP3*-knockout mouse brain (Kim et al., 2012).

Robo Signaling in the Terminal Positioning of Cortical Neurons

The terminal positioning of excitatory projection neurons takes place in the superficial region of the CP, designated as the primitive cortical zone (PCZ) (Sekine et al., 2011). Immature neurons undergo terminal translocation in the PCZ to complete their final positioning (Sekine et al., 2011). The terminal positioning process is known to be regulated by two distinct mechanisms (Sekine et al., 2012; Gonda et al., 2013). The first is terminal translocation, which is a mode of neuronal migration regulated by reelin, a classical secreted factor that is deposited in the MZ and is required for laminar formation (Kubo et al., 2010; Hirota et al., 2018). Terminal translocation has been shown to be independent of the radial glial scaffold (Nadarajah et al., 2001), and therefore the attenuation of N-cadherin-mediated cell adhesion between neurons and the radial glial scaffold may be important. Consistent with this view, N-cadherin protein expression is low in the PCZ (Kawauchi et al., 2010, **Figure 1**). As Robo1 attenuates N-cadherin-mediated cell adhesion by inducing the phosphorylation of β-catenin, which promotes the detachment of β-catenin from N-cadherin (Rhee et al., 2002, 2007), the internalization and subsequent proteolysis of N-cadherin might be increased in the PCZ where Robo1 is highly expressed.

The second mechanism is regulation through dendrite formation. In the PCZ, terminally translocated neurons stabilize the leading process, which eventually differentiates into an apical dendrite of a pyramidal neuron (O'Dell et al., 2015, **Figure 1**). Together with apical dendrite extension, the cell soma of the neuron moves down to the CP. In contrast, *Robo1*-knockdown neurons migrate through the CP and reach the MZ-CP border; however, these neurons accumulate there (Gonda et al., 2013). This phenotype indicates that terminal translocation is not affected; however, dendrite formation is impaired in *Robo1*-knockdown cells. The extension of apical dendrites toward the MZ creates a space for terminally translocated neurons to pass through the earlier-arriving resident neurons. In agreement with this notion, the inside-out layering pattern is disrupted in the cortex of *Robo1*-knockdown mice, suggesting that a defect in the terminal positioning of cortical layer neurons is due to abnormal dendrite formation.

ROLES OF SLIT-ROBO SIGNALING IN DENDRITE DEVELOPMENT

Dendritic patterning is a crucial developmental process in neocortical circuit formation and function. The dendritic development of neocortical projection neurons may be controlled by factors in the MZ (Polleux et al., 2000; O'Dell et al., 2012), because dendrites undergo dynamic changes after neurons reach the superficial part of the cortex and initiate differentiation (O'Dell et al., 2015, **Figure 1**).

During this process, Robo1 is required for proper apical dendrite formation (Gonda et al., 2013), however, the mechanisms by which Robo1 regulates the morphological development of differentiating cortical neurons remains unknown. One possible role of Robo1 is that it acts as a cell adhesion molecule similar to other IgCAMs, which are known to regulate dendrite formation during development (Moresco et al., 2005; Seong et al., 2015; Parcerisas et al., 2020). The other possibility is that Robo acts to attenuate N-cadherin-mediated cell adhesion, as described above (**Figures 1**, **2**C).

In vitro studies have demonstrated that Slit1 also promotes dendrite formation in both pyramidal and non-pyramidal neurons. Inhibition of the binding of Slit to Robo receptors by Robo1 and Robo2 ectodomains suppressed dendrite growth and branching in pyramidal and non-pyramidal neurons (Whitford et al., 2002). Furthermore, a dominant-negative form of Robo1 inhibited dendritic branching in cultured neurons (Whitford et al., 2002). In contrast, *Robo1* knockdown increased the number of apical dendrites of layer II/III neurons *in vivo* (Gonda et al., 2013). These differences may be due to the experimental conditions, as the former study was performed in cultured neurons (Whitford et al., 2002) lacking an *in vivo* microenvironment, whereas the latter study analyzed neocortical neurons *in vivo*, which maintains tissue polarity and a relevant microenvironment (Gonda et al., 2013). An alternative explanation is the difference of neuronal types between layer V neurons (Whitford et al., 2002) and layer II/III neurons (Gonda et al., 2013).

Slit-Robo signaling also affects the early neurite outgrowth of cortical interneurons *in vivo* (Andrews et al., 2008). Migrating interneurons in the SP and SVZ/IZ of *Robo1*-knockout mice have more processes and longer neurites compared with the interneurons of WT mice. As *Slit1/Slit2* double-knockout mice showed a marked increase in process length and neurite number, Slit1/2-Robo1 signaling acts as a negative regulator of neurite outgrowth in migrating interneurons. Taken together, Slit-Robo signaling inhibits the overgrowth of neurites, which in turn ensures the proper dendritic formation and migration of interneurons.

One of the downstream molecular mechanisms underlying Slit-Robo-mediated dendrite formation involves srGAPs. srGAPs are the downstream mediators of Robo, and have at least two distinct roles in neurite outgrowth. srGAPs, which are Rho family small GTPase inhibitors, regulate cytoskeletal dynamics, which is crucial for neurite outgrowth (**Figure 2B**). Because each srGAP demonstrates a specificity to particular Rho family small GTPases, they play distinct roles in neurite outgrowth, for example, srGAP3 inhibits neurite outgrowth via Rac1 inactivation (Soderling et al., 2002), whereas srGAP2 has been reported to promote neurite outgrowth (Guerrier et al., 2009). As srGAP2 also inactivates Rac1, the functional difference between srGAP2 and srGAP3 cannot be explained by their GAP specificities, and may be owing to another domain in srGAPs, namely, the F-BAR domain (**Figure 3A**).

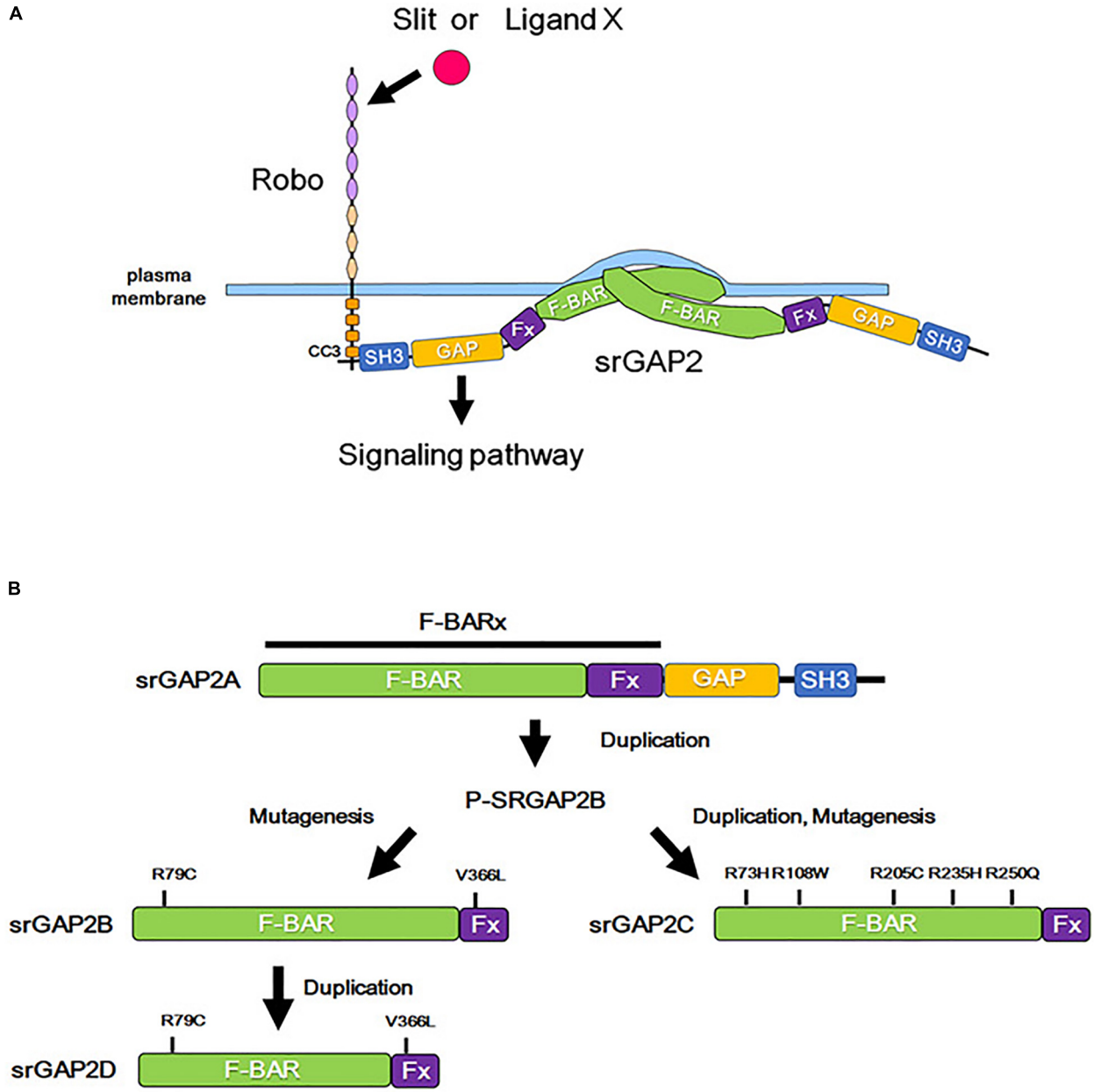

FIGURE 3 | Role of srGAP2 in membrane protrusion, and evolutionary history of srGAP2. **(A)** By the binding of Slit or some other stimulation, the CC3 domain of Robo interacts with the SH3 domain of srGAP2. srGAP2 forms a homodimer via its F-BAR domain and directly binds to the plasma membrane, inducing filopodia-like protrusions. **(B)** srGAP contains an amino-terminal extended F-BAR (F-BARx), GAP, and SH3 domains. srGAP2A is the ancestral form, and srGAP2B, srGAP2C, and srGAP2G evolved into their present forms after several evolutionary steps of duplication and/or mutagenesis.

The F-BAR domain normally induces invagination of the plasma membrane; however, the F-BAR domain of srGAP2 demonstrates a function of the I-BAR domain, that is, the induction of filopodia formation by outward bending of the plasma membrane (Guerrier et al., 2009). Consistent with this function, srGAP2 promotes filopodia formation and subsequent neurite outgrowth in cultured cortical neurons (Guerrier et al., 2009; Coutinho-Budd et al., 2012). In contrast, the filopodia-forming function of srGAP3 appears to be weaker than that of srGAP2. Furthermore, srGAP1 prevents filopodia formation (Coutinho-Budd et al., 2012). Therefore, a balance in the activities mediated by the GAP and the BAR domain may determine the effect of srGAPs on neurite outgrowth. As the activity of the GAP domain is regulated by Slit-Robo signaling (Wong et al., 2001), the presence of Slit-Robo signaling might enable the function of GAPs to dominate over the function of the BAR domain.

srGAP AND ROBO SIGNALING IN SPINE FORMATION

Dendritic filopodium is a structure found in the early stages of spine formation, which matures into a dendritic spine. Therefore, filopodium formation is thought to be crucial for the onset of spine formation. As mentioned above, srGAPs are known to regulate filopodium formation and thus control spine formation in neurons.

srGAP2 is detected in the spine head of excitatory synapses in neocortical projection neurons and promotes spine maturation (Charrier et al., 2012, **Figure 3A**). Interestingly, human-specific paralogs of srGAP2, namely, srGAP2B, srGAP2C, and srGAP2D arose by gene duplications during human evolution (Dennis et al., 2012, **Figure 3B**). Because of partial gene duplication, srGAP2C retains only a part of the F-BAR domain. srGAP2C binds to an ancestral paralog of srGAP2A, and inhibits the function of srGAP2A in spine formation (Charrier et al., 2012; Fossati et al., 2016; Sporny et al., 2017).

In addition, srGAP3 was initially reported as mental disorder-associated GAP protein, also known as WAVE-associated Rac GTPase-activating protein (WRP), through the analysis of a female patient with 3p deletion syndrome who had hypotonia and severe intellectual disability (Endris et al., 2002). srGAP3 interacts with a scaffold protein for actin remodeling, WAVE-1, and inhibits Rac1 activity (Soderling et al., 2002). Because either the inhibition of or activation of Rac1 leads to abnormal spine formation (Costa et al., 2020), precise regulation of Rac1 activity is crucial for normal spine formation. Consistent with this notion, both a reduced interaction between srGAP3 and WAVE-1 and knockout of srGAP3 have been shown to decrease the number of spines (Soderling et al., 2007; Carlson et al., 2011).

Recently, the association between Robo and spine formation has been reported. Robo2 is localized at the postsynaptic membrane of hippocampal CA1 pyramidal neurons, and directly binds to presynaptic neurexin irrespective of Slit (Blockus et al., 2019). This binding promotes spine formation and subsequent excitatory synapse formation.

In summary, srGAPs play a role in spine formation through its Rho GAP domain and/or F-BAR domain (**Figure 3A**). However, the involvement of Slit-Robo in the functions of srGAPs needs further investigation. One possibility is that Robo determines srGAP localization at the plasma membrane and therefore regulates the site of spine formation. Furthermore, it will be interesting to clarify the roles of Slit-Robo and srGAP signaling in the diversification of spine formation among different functional regions of the neocortex (Benavides-Piccione et al., 2002; Konur et al., 2003; Sasaki et al., 2010).

SLIT-ROBO SIGNALING AND NEUROPSYCHIATRIC DISORDERS

Abnormal development of the neocortex affects neural circuit formation and causes neuropsychiatric disorders. Here, we discuss two etiologies known to be caused by abnormalities in Slit-Robo signaling, i.e., dyslexia and autism spectrum disorder (ASD).

Robo and Dyslexia

ROBO1 and ROBO2 have been associated with dyslexia (Nopola-Hemmi et al., 2001; Stein et al., 2004). *ROBO1* and *ROBO2* genes are mapped at the dyslexia susceptibility loci *DYX5*, which is located on chromosome 3 (3p12-q13). Silent and 3′UTR SNPs of *ROBO1* and a translocation t(3; 8) (p12; q11) that causes reduced *ROBO1* transcription were found in individuals with dyslexia (Hannula-Jouppi et al., 2005). Furthermore, a study analyzing post-mortem brains of dyslexic subjects demonstrated the presence of abnormal microgyria in the left temporal speech region and ectopic neurons in the subcortical white matter (Galaburda and Kemper, 1979), which are thought to be caused by ectopic neuronal positioning.

However, recently, a magnetic resonance imaging study of children with dyslexia demonstrated the abnormal morphology of neurites in the language-associated regions of the neocortex (Caverzasi et al., 2018). In line with these observations, reduced expression of Robo1 in the embryonic mouse neocortex was shown to delay neuronal migration during development, followed by abnormal dendrite formation leading to subsequent impairment in the terminal positioning of neurons (Gonda et al., 2013).

Taken all together, the dyslexic phenotype in patients with *ROBO1* mutations may be caused by the abnormal formation of dendrites and terminal positioning of neurons. As dendrite formation and terminal positioning of neurons are potentially regulated by signals from the MZ and occur during the neonatal period, an interaction between ROBO1 and Slit or unknown molecules that reside in the MZ during the neonatal period might be important. Altogether, ROBO plays a crucial role in human neocortical development by regulating dendrite formation and neuron positioning, and such abnormalities occurring in language-associated regions can lead to dyslexia.

Slit-Robo Signaling and ASD

In addition to dyslexia, the downregulation of ROBO expression has also been associated with ASD, presumably through the modulation of serotonin levels in the neocortex.

Serotonin reuptake by serotonin transporters is crucial for maintaining normal levels of serotonin in the neocortex. Dysfunctions of serotonin transporters and resultant high serotonin levels are observed in ASD patients (Schain and Freedman, 1961; Muller et al., 2016). As Robo has been shown to promote serotonin transporter expression in *Drosophila* (Couch et al., 2004), and the expression of ROBO1, ROBO2, ROBO3, and ROBO4 was reduced in patients diagnosed as having ASD (Anitha et al., 2008), decreased ROBO expression might increase serotonin level, which is associated with ASD. As excess serotonin in the developing mouse neocortex is known to affect the migration of both pyramidal neurons and interneurons (Riccio et al., 2009, 2011), decreased ROBO expression might impair neuronal migration in a non-cell autonomous manner in addition to the cell-autonomous manner (see section "Slit-Robo Signaling in Neuronal Migration").

In addition, mutations in srGAPs are associated with intellectual and cognitive disabilities (Saitsu et al., 2012; Waltereit et al., 2012; Bertram et al., 2016). The disruption of SRGAP2 expression was found in patients diagnosed with West syndrome, who demonstrate intellectual disability (Saitsu et al., 2012). A microdeletion of 1q32.1, where the *SRGAP2* gene is localized, causes Van der Woude syndrome accompanied with intellectual disabilities (Rincic et al., 2016). In addition, rare copy number variations of *SRGAP2C*, a human-specific paralog of *srGAP2*, was identified in patients with ASD and intellectual disability (Dennis et al., 2012, **Figure 3B**). srGAP3-deficient mice demonstrate several behavioral abnormalities, including intellectual disability-associated behaviors and autism-associated behaviors (Kim et al., 2012; Waltereit et al., 2012; Koschützke et al., 2015; Bertram et al., 2016). srGAPs have been shown to play important roles in spine formation, and srGAP mutations are thought to cause intellectual disabilities, likely via abnormal spine formation.

CONCLUSION AND PERSPECTIVES

Whereas the roles of Slit-Robo signaling in the developing brain have been well studied regarding axon guidance, during the previous decade, new roles of Slit-Robo signaling in progenitor cell proliferation and dendritic formation have emerged. These studies have shed light on the fundamental roles of Slit-Robo signaling in multiple events of neocortical development, from the proliferation of progenitor cells to circuit formation (**Figure 1**).

Although the significance of Slit-Robo signaling in cortical development has been highlighted in this review, the detailed molecular mechanisms underlying Slit-Robo-mediated corticogenesis merits further investigation. As Slit molecules and Robo receptors have multiple binding partners in addition to their conventional ones, a comprehensive understanding of the Slit and Robo interactome in different cell types at different developmental stages is essential to understand the upstream and downstream signaling networks of Slit and Robo. This in turn will help us to understand the etiology of human diseases caused by abnormalities in Robo signaling.

The recent implication of Robo signaling in brain evolution (Cárdenas et al., 2018) has provided an important direction for future studies. The association of ROBO1 with literacy (Hannula-Jouppi et al., 2005), which is a unique characteristic of humans, suggests that Robo signaling is involved not only in the expansion of the brain during evolution, but also in the development of higher brain functions.

Taken together, now is the time to revise our classical view of Slit-Robo signaling as a regulator of axon guidance, and build a new perspective on these key molecules in orchestrating multiple steps of neocortical circuit assembly and function.

AUTHOR CONTRIBUTIONS

YG wrote and edited the manuscript. TN and CH edited the manuscript. All authors reviewed, discussed, and commented on the manuscript.

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REVIEW
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Involvement of Netrins and Their Receptors in Neuronal Migration in the Cerebral Cortex

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In mammals, excitatory cortical neurons develop from the proliferative epithelium and progenitor cells in the ventricular zone and subventricular zone, and migrate radially to the cortical plate, whereas inhibitory GABAergic interneurons are born in the ganglionic eminence and migrate tangentially. The migration of newly born cortical neurons is tightly regulated by both extracellular and intracellular signaling to ensure proper positioning and projections. Non-cell-autonomous extracellular molecules, such as growth factors, axon guidance molecules, extracellular matrix, and other ligands, play a role in cortical migration, either by acting as attractants or repellents. In this article, we review the guidance molecules that act as cell–cell recognition molecules for the regulation of neuronal migration, with a focus on netrin family proteins, their receptors, and related molecules, including neogenin, repulsive guidance molecules (RGMs), Down syndrome cell adhesion molecule (DSCAM), fibronectin leucine-rich repeat transmembrane proteins (FLRTs), and draxin. Netrin proteins induce attractive and repulsive signals depending on their receptors. For example, binding of netrin-1 to deleted in colorectal cancer (DCC), possibly together with Unc5, repels migrating GABAergic neurons from the ventricular zone of the ganglionic eminence, whereas binding to α3β1 integrin promotes cortical interneuron migration. Human genetic disorders associated with these and related guidance molecules, such as congenital mirror movements, schizophrenia, and bipolar disorder, are also discussed.

Keywords: axon guidance, netrin, DCC, Unc5, neogenin

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CORTICAL NEURON MIGRATION

The mammalian cerebral cortex is a highly organized laminar structure with six layers, each of which contains a characteristic distribution of different neurons with various connections to other cortical and subcortical regions. During development, excitatory neurons are generated from radial glia and progenitor cells in the ventricular zone (VZ) and subventricular zone (SVZ), and migrate radially toward the cortical plate in an inside-out pattern (Lui et al., 2011), whereas inhibitory GABAergic interneurons are born in the ganglionic eminence and migrate tangentially (Xu et al., 2004). Distinct subtypes of cortical GABAergic interneurons are generated in specific regions of the basal telencephalon. Parvalbumin- and somatostatin-expressing interneurons derive from the lateral and medial ganglionic eminence, while most calretinin-positive interneurons are born at

later stages in the caudal ganglionic eminence (Xu et al., 2004; Butt et al., 2005; Miyoshi and Fishell, 2011).

Radial glia can be classified as apical radial glia, which connect to the apical surface (lateral ventricle) with short processes and to the basal side (outer surface) with long processes, and basal radial glia, which have no apical processes and are located in the SVZ. Radial glia undergo asymmetric division, generating a progenitor cell or excitatory neuron. Intermediate progenitors are derived from radial glia disconnected from the ventricular surface and generate neurons after undergoing multiple rounds of symmetric cell division (Miyata et al., 2001; Haubensak et al., 2004; Noctor et al., 2004; Lui et al., 2011). Newly generated neurons have multipolar processes and migrate relatively slowly in the intermediate zone but transition to a bipolar morphology once they enter the cortical plate, where they migrate radially and rapidly along the basal processes of radial glia in an inside-out manner. Therefore, early born neurons (∼embryonic day 12 [E12] in rodents) form the deeper layers, and later born neurons migrate to the upper layers, stopping just below layer I by Reelin signaling (Hirota and Nakajima, 2017).

The migration of excitatory and inhibitory neurons is precisely organized by extracellular cues, including guidance molecules such as netrins, ephrins, semaphorins, and slits. These molecules are well-known for navigating axonal growth cones, but they can also regulate cell migration using similar ligand-receptor binding systems. In this review, we focus on netrin family proteins, their receptors, and related molecules, and describe the mechanisms by which migrating neurons in mammalian cerebral cortex utilize those molecules to navigate to their final destinations. Finally, we discuss the human genetic disorders of these guidance molecules, such as congenital mirror movements, schizophrenia, and bipolar disorder.

NETRIN FAMILY PROTEINS

Netrin was first identified as Unc-6 (uncoordinated), which regulates neural development in *Caenorhabditis elegans* (Ishii et al., 1992). It was named from the Sanskrit word *netr*, meaning "the person who guides." It is conserved among non-vertebrate and vertebrate species (Lai Wing Sun et al., 2011), including mammals, which express netrin family members netrin-1, -3, -4, -5, G1, and G2. Netrin-2 expression has only been identified in chickens and fish (Kennedy et al., 1994; Serafini et al., 1994; Park et al., 2005). Netrin-1, -3, -4, and -5 are secreted, whereas netrin G1 and G2 have a glycophosphatidylinositol anchor region that binds them to the membrane. Netrins typically contain an N-terminal laminin VI domain, laminin V-type EGF-like domains, and the NTR/C345C domain (**Figure 1A**). However, netrin-5 lacks portions of those domains depending on the splicing variant (Visser et al., 2015; Yamagishi et al., 2015). Netrin-1 was considered a typical guidance cue that attracts axons of commissural neurons growing dorsoventrally from the roof plate toward the floor plate in the spinal cord, where it is highly expressed. However, two independent groups recently showed that netrin-1 expression at the floor plate is dispensable for this wiring (Dominici et al., 2017; Varadarajan et al., 2017). Using conditional knockout mice, they showed that netrin-1 supplied from the VZ is required for growth of commissural axons, functioning as a local cue but not as a long-range attractant.

Netrin-1 is an important attractant and repellant for axon guidance depending on its receptors (DCC, neogenin, Unc5, DSCAM, and integrins; **Figure 1B**). DCC and neogenin both contain four Ig-like C2 domains and six fibronectin type-III domains, with 50% amino acid homology. However, their crystal structures revealed different architectures when bound to netrin-1 (**Figure 1C**). Whereas the netrin/DCC complex is constructed as a continuous netrin-DCC-netrin-DCC-repeating assembly, netrin/neogenin forms a 2:2 heterotetramer complex (Xu et al., 2014). Furthermore, neogenin is also a receptor for the repulsive guidance molecule a/b (RGM a/b), which does not bind DCC. Another well-known repulsive binding interaction, netrin/Unc5, has been characterized by crystallography (Grandin et al., 2016). The V2 domain of netrin-1 binds to the Ig1/Ig2 domain of Unc5B, which can bind and compete with FLRT proteins (other Unc5 ligands; **Figure 1B**) (Shirakawa et al., 2019). The different patterns of binding to receptors likely contribute to the variety of netrin functions, such as cell migration, axon branching, synaptogenesis, oligodendrocyte differentiation, angiogenesis, lymphangiogenesis, immune function, and tumor progression (Rajasekharan and Kennedy, 2009; Larrieu-Lahargue et al., 2011; Finci et al., 2015; Feinstein and Ramkhelawon, 2017; Bruikman et al., 2019; Meijers et al., 2020).

ROLES OF NETRIN FAMILY PROTEINS IN THE MIGRATION OF CORTICAL NEURONS

Netrin-1 is involved in the migration of GABAergic interneurons. In the developing mouse, netrin-1 is highly expressed in the VZ of the ganglionic eminence and expressed at a lower level in the marginal zone and intermediate zone of the cerebral cortex at the mid- to late-gestational stage (Hamasaki et al., 2001; Stanco et al., 2009). Hamasaki et al. (2001) showed that netrin-1 repels postmitotic GABAergic neurons from the ganglionic eminence. This repulsive effect is blocked by anti-DCC antibodies, indicating the involvement of DCC in this repulsion, possibly by complexing with the Unc5 receptor. By contrast, Marin et al. (2003) reported that netrin-1 does not contribute to the tangential cortical migration of GABAergic interneurons. Mice with genetic deletion of netrin-1, as well as triple-knockouts for Slit1 and Slit2 (expressed in the subpallium) in addition to netrin-1, exhibit a normal distribution of cortical interneurons at E18 (Marin et al., 2003). Nevertheless, Stanco et al. (2009) found that netrin-1 in the marginal zone and intermediate zone guides tangential migration of ganglionic eminence-derived interneurons, which is mediated by α3β1-integrin (**Figure 2A**). *In vivo* analysis of interneuron-specific α3β1-integrin- and netrin-1-deficient mice revealed abnormal interneuron migration along the top of the developing cortical plate, disrupting the distribution of interneurons throughout the cerebral cortex including the hippocampus. The interactions between the C terminus of netrin-1 and α6β4 and α3β1 integrins

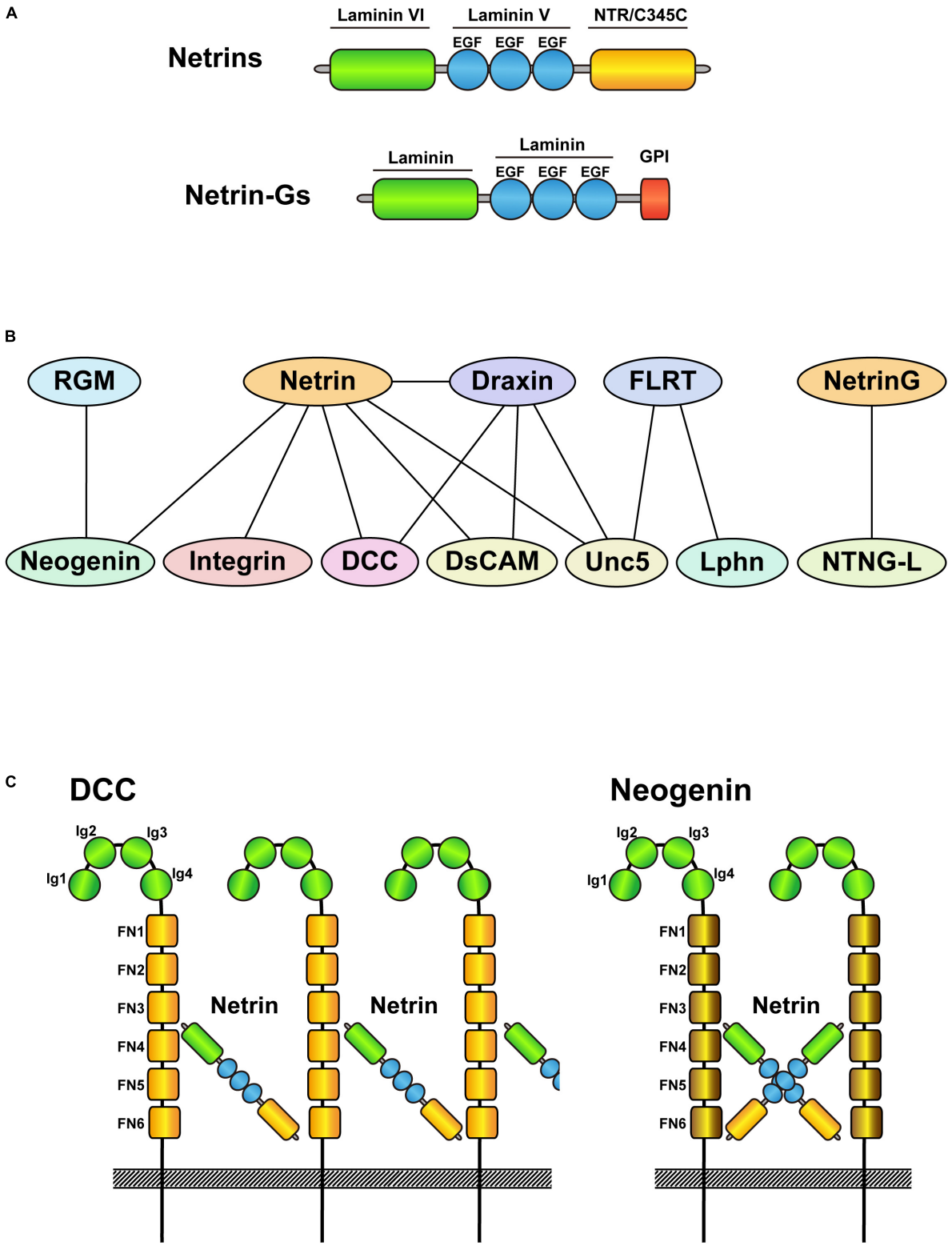

FIGURE 1 | Schematic drawings of netrin family proteins and their interacting proteins. **(A)** Schematic drawing of the domain structure of netrins and netrin-Gs. Netrins are secreted proteins, whereas netrin-Gs are membrane bound GPI-anchored proteins. **(B)** Protein interactions of netrin family proteins and their receptors. Upper side indicates ligands and lower side shows their receptors. Note that netrin-Gs bind to netrin-G ligands as receptors. **(C)** Schematic drawings of the interactions between netrin-DCC and netrin-neogenin. Although DCC and neogenin are structurally similar, netrin-DCC binds continuously and makes a large complex, whereas netrin-neogenin forms a 2:2 complex.

are also known to contribute to cell adhesion as well as to the migration of non-neuronal cells, such as pancreatic epithelial cells and mesenchymal stem cells (Yebra et al., 2003; Son et al., 2013; Lee et al., 2016). The binding of netrin-4 to α6β1 integrin, which makes a ternary complex with laminin γ1, promotes neurogenesis and migration in the rostral migratory stream (Staquicini et al., 2009). Furthermore, other combinations of netrin-4/integrin interactions are reported in non-neuronal systems. Namely, α2β1, α3β1, α6β1, and b4 integrins bind to netrin-4 on endothelial cells, epithelial cells, and glioblastomas (Larrieu-Lahargue et al., 2011; Yebra et al., 2011; Hu et al., 2012). Although these netrin–integrin interactions have not been extensively analyzed in neuronal cells, they might contribute to cortical migration.

Netrin-4 is known to influence the maturation of cortical neurons and is highly expressed in pyramidal cells of the neocortex and hippocampus, and Purkinje cells of the cerebellum (Zhang et al., 2004). Netrin-4 can bind to DCC and Unc5 receptors via its N-terminal domain, although an unidentified receptor can bind to its C-terminal domain. In layer 4 neurons in the visual cortex and somatosensory area, both netrin-4 and its receptor Unc5D are expressed (**Figures 2A,C**). As the Unc5D receptor has an intracellular death domain that triggers apoptosis without ligand binding as a dependent receptor, netrin-4 seems to serve to inhibit apoptotic cell death (Takemoto et al., 2011). Furthermore, netrin-4/Unc5B signaling regulates the branching of thalamocortical neuron axons in the somatosensory and visual cortices in an activity-dependent manner (Hayano et al., 2014). The contribution of netrin-4 to cortical migration has not yet been analyzed. However, according to Allen Developing Mouse Brain Atlas[1], netrin-4 expression does not occur at E15.5 and E18.5 in the cerebral cortex. Therefore, instead of functioning in cortical cell migration, netrin-4 may play a role in cell survival and maturation.

GPI-anchored netrin G1 and G2 show distinctive expression patterns. During the development of the cerebral cortex, netrin G1 is expressed in the marginal zone and subplate, whereas netrin G2 is expressed throughout the cortex (**Figure 2A**) (Nakashiba et al., 2002). Knockdown of either netrin G1 or G2 at E14.5 by IUE impairs radial migration at postnatal day 1 and 7 (Heimer et al., 2020). Netrin-G1 knockdown results in a major migration defect, with only ∼50% of cells entering the cortical plate at postnatal day 1 and ∼60% of transfected cells managing to migrate to layer 2/3. Netrin-G2 knockdown shows a similar migration deficit. Only 55% and 40% of transfected cells reached to the cortical plate at P1 and layer2/3 at P7, respectively (Heimer et al., 2020).

To the best of our knowledge, there is no other publication showing the function of netrin family proteins in either radial or tangential migration of cortical neurons. In 2015, we reported another member of netrin protein family, netrin-5, which lacks the N-terminal laminin VI domain (Yamagishi et al., 2015). It is not well characterized because netrin-5 mutant mice develop normally without any obvious phenotypes (Garrett et al., 2016). Garrett et al. (2016) also showed high expression of netrin-5 in the boundary cap cells (BCCs) in the spinal cord, which prevents migration into the ventral root. When netrin-5 is absent in BCCs, subsets of motor neurons migrate to the peripheral nervous system. Interestingly, this phenocopies $DCC^{-/-}$ mice, suggesting that the interaction between netrin-5 and DCC induces a repulsive signal, possibly together with the Unc5 receptor. Biochemical screens revealed that DSCAM is another receptor for netrin-5 (Visser et al., 2015); however, the function of this interaction is not known, as *in vitro* growth cone collapse and turning assays have not been performed. Recently, we reported that netrin-5 is involved in organizing the rostral migratory stream in the adult mouse brain (Ikegaya et al., 2020). However, the contribution of netrin-5 to cortical development remains to be determined.

ROLES OF DCC IN THE MIGRATION OF CORTICAL NEURONS

Deleted in colorectal cancer (DCC) regulates the radial migration of cortical neurons. Zhang et al. (2018) reported that DCC interacts with Dab1, an intracellular transducer of Reelin signaling, by binding to ApoER2 and VLDLR in multipolar migrating neurons. Netrin-1 induces Dab1 phosphorylation, and knockdown or truncation of the C-terminal P3 domain of DCC impairs the multipolar-to-bipolar transition of neurons, dramatically delaying their migration. These results indicate that Dab1 mediates netrin-1/DCC signaling. Myosin-10 (Myo10), a non-traditional myosin family member, interacts with DCC for radial migration (Ju et al., 2014). Full-length Myo10 is expressed in the VZ/SVZ, and headless Myo10 is expressed in the intermediate zone as well as in the VZ/SVZ. Knockdown of full-length Myo10 results in abnormally oriented bipolar neurons, whereas knockdown of the headless isoform impairs the multipolar–bipolar transition. Interestingly, overexpression of DCC rescues the full-length Myo10 knockdown phenotype but not the headless Myo10 knockdown phenotype, indicating that DCC is involved in full length Myo10-regulated migration, but not in headless Myo10-controlled morphological transition. Although the downstream signaling of DCC in migrating neurons is not fully understood, *in vitro* analyses of non-neuronal cells suggest that FAK, Nck1, Rac, cdc42, and RhoA may be involved in the netrin-DCC signaling pathway (Li et al., 2002a,b; Shekarabi and Kennedy, 2002).

NEOGENIN/RGM

Neogenin, which is expressed throughout the telencephalon, including in dividing neuroepithelial cells, at E12.5 (Fitzgerald et al., 2006), is involved in the migration of both excitatory and inhibitory neurons. At E14.5, neogenin is expressed on nestin- and GLAST-positive radial glia and within the VZ, SVZ, and intermediate zone of the cortex (**Figure 2B**). The expression of neogenin on neurons migrating through the intermediate zone is turned off once they reach the cortical plate, where they begin

[1]http://developingmouse.brain-map.org

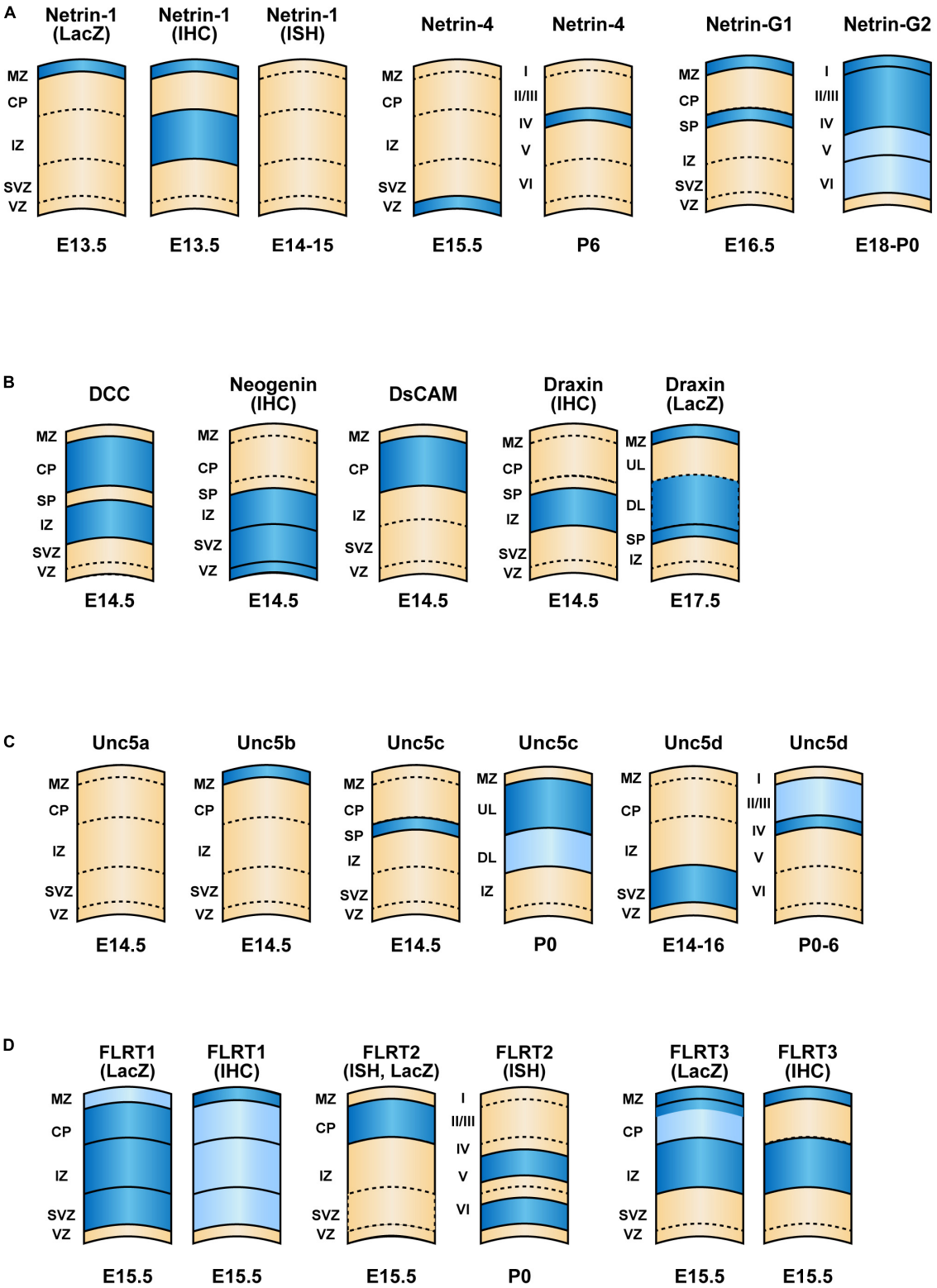

FIGURE 2 | Expression patterns of netrin family proteins and their interacting proteins in the cerebral cortex. Schematic of cortical layers depicting the laminar-specific expression of **(A)** netrins, **(B)** their interacting proteins (DCC, neogenin, DsCAM and Draxin, **(C)** Unc5 family receptors and **(D)** FLRT family proteins within the neocortex. Dark blue and light blue indicate higher and lower relative levels of expression, respectively. The expression levels are based on results of *in situ* hybridization unless mentioned otherwise. The development stages are indicated. References to publications on each of the genes are listed in **Table 1**. ISH, *in situ* hybridization; IHC, immunohistochemistry.

expressing DCC. Thus, neogenin expression is limited to the immature stage of excitatory neurons, but is also expressed by newborn cortical interneurons and the maturing calbindin- and parvalbumin-positive subpopulations (O'Leary et al., 2013). The ligand of neogenin is RGMa, which provides a repulsive cue for newborn interneurons migrating away from the VZ and medial ganglionic eminence. Interestingly, this repulsion is suppressed by netrin-1, suggesting that RGMa and netrin-1 compete for binding to neogenin to control migration. The expression of neogenin is regulated by Rb, a tumor suppressor (Andrusiak et al., 2011). In Rb mutant mice in which expression is driven by Foxg1-cre recombinase, neogenin is strongly upregulated in the telencephalon. This results in augmented interneuron adhesion and a defective migratory response to netrin-1 *in vitro* (Andrusiak et al., 2011). *In vivo*, overexpression of neogenin impairs migration of neuroblasts from the SVZ and medial ganglionic eminence.

Radial migration is regulated in a RGMa/neogenin dependent manner. RGMa is expressed in the cortical plate and VZ. Knockdown of neogenin in migrating neurons results in their abnormal distribution to the areas where RGMa is expressed (van Erp et al., 2015). A similar phenotype was observed with knockdown of Lrig2, a negative regulator of the proteolytic cleavage of neogenin by ADAM17, as this results in RGMa insensitivity. These results indicate that RGMa-neogenin-Lrig2 signaling propels migrating neurons out of the VZ/SVZ and prevents their premature entry into the cortical plate (van Erp et al., 2015). Interestingly, Unc5B can interact with neogenin as a coreceptor for RGMa (Hata et al., 2009), such that knockdown of the Unc5 receptor eliminates the repulsion mediated by RGMa, including growth cone collapse in cortical neurons *in vitro*. However, whether the Unc5 family protein is involved in the RGMa-mediated repulsion in tangential and radial migration *in vivo* remains unknown.

DOWN SYNDROME CELL ADHESION MOLECULE (DSCAM)

Down syndrome cell adhesion molecule, another factor regulating radial migration, is a large (>200 kDa) neural cell adhesion molecule that consists of 10 Ig C2-type domains, six FN type-III domains, a transmembrane domain, and a C-terminal intracellular domain. DSCAM and DSCAML1, a splice variant, are widely expressed in all layers of the cerebral cortex. More precisely, DSCAM is highly expressed in layer V, whereas DSCAML1 is more prominent in the superficial layer and layer V. Knockdown of either DSCAM or DSCAML1 impairs the radial migration of upper layer neurons at P0. The DSCAMs deficient neurons remained trapped in the deep layers and intermediate zone, which was rescued by overexpression of full length DSCAM. At P7, a large number of shDSCAM-transfected neurons failed to migrate to layers II–III, whereas most shDSCAML1-transfected neurons did (Zhang et al., 2015). These knockdowns disrupt the callosal projections of cortical neurons to the contralateral hemisphere, and increase the dendritic branching in cultured cortical neurons. However, it is not clear whether these phenotypes involve netrin family proteins.

DRAXIN

Draxin (dorsal repulsive axon guidance protein) binds to various netrin-related proteins. Islam et al. (2009) characterized draxin as a repulsive cue regulating midline crossings of axons in the corpus callosum, hippocampal commissure, anterior commissure, and commissure neurons of the spinal cord, regulation that is phenocopied in netrin-1$^{-/-}$ and DCC$^{-/-}$ models. Indeed, draxin directly interacts with netrin-1 and DCC, as well as with DSCAM and Unc5a-c (Ahmed et al., 2011; Gao et al., 2015; Meli et al., 2015; Liu et al., 2018). In draxin knockout mice, not only midline-crossing commissural axons but also thalamocortical and corticofugal projections are severely affected (Shinmyo et al., 2015). Interestingly, draxin promotes the growth of thalamic neuron axons *in vitro*, which is abolished by DCC deficiency, indicating that draxin acts as an attractant, similarly to netrin-1. Although it is highly expressed in the developing cortex,

TABLE 1 | List of references to publications describing the expression patterns of netrin family proteins and their interacting proteins in the cerebral cortex depicted in **Figure 2**.

Name	Age	Method	References
Netrin-1	E13.5	LacZ	Stanco et al. (2009)
Netrin-1	E13.5	IHC	Stanco et al. (2009)
Netrin-1	E14–15	ISH	Yamagishi et al. (2011); Miyoshi and Fishell (2012)
Netrin-4	E15.5	ISH	Yamagishi et al. (2011)
Netrin-4	P6	ISH	Takemoto et al. (2011)
Netrin-G1	E16.5	ISH	Nakashiba et al. (2002)
Netrin-G2	E18, P0	ISH	Allen Developmental Mouse Brain Atlas; Nakashiba et al. (2002)
DCC	E14.5	ISH	Miyoshi and Fishell (2012)
Neogenin	E14.5	IHC	Fitzgerald et al. (2006)
DsCAM	E14.5	ISH	Miyoshi and Fishell (2012)
Draxin	E14.5	IHC	Shinmyo et al. (2015)
Draxin	E17.5	LacZ	Shinmyo et al. (2015)
Unc5a	E14.5	ISH	Miyoshi and Fishell (2012)
Unc5b	E14.5	ISH	Miyoshi and Fishell (2012)
Unc5c	E14.5	ISH	Srivatsa et al. (2014)
Unc5c	P0	ISH	Srivatsa et al. (2014)
Unc5d	E14.5–16.5	ISH	Takemoto et al. (2011); Yamagishi et al. (2011); Miyoshi and Fishell (2012)
Unc5d	P6	ISH	Takemoto et al. (2011); Yamagishi et al. (2011)
FLRT1	E15.5	LacZ	Del Toro et al. (2017)
FLRT1	E15.5	IHC	Del Toro et al. (2017)
FLRT2	E15.5	ISH, LacZ	Yamagishi et al. (2011); Del Toro et al. (2017)
FLRT2	P0	ISH	Yamagishi et al. (2011)
FLRT3	E15.5	LacZ	Del Toro et al. (2017)
FLRT3	E15.5	IHC	Del Toro et al. (2017)

neither radial migration nor tangential migration of interneurons is affected by draxin deficiency (**Figure 2C**) (Shinmyo et al., 2015). These results suggest that draxin is not involved in neuronal migration, but rather is specifically involved in axon guidance, unlike netrin-1 and DCC. It is also possible that another molecule compensates for the absence of draxin to ensure proper cortical organization of neuronal migration.

Unc5/FLRT

Among four members of Unc5 protein family, Unc5b regulates interneuron migration and Unc5d regulates radial migration. During tangential migration to the cortex, GABAergic interneurons express transcription factor Sip1, also known as ZEB2 or Zfhx1b, which regulates Unc5b expression (van den Berghe et al., 2013). In Sip1 mutant mice, interneurons exhibit a migration defect, and Unc5b and netrin-1 are highly upregulated. Overexpression of Unc5b, but not netrin-1, contributes to the migration defect. Furthermore, Unc5b knockdown rescues the aberrant migration in Sip1 mutants, indicating that downregulation of Unc5b by Sip1 is necessary for normal interneuron migration (van den Berghe et al., 2013).

Unc5d is the most-characterized molecule among four Unc5 family proteins involved in radial migration. A portion of the *Unc5d* gene was first characterized as an *in situ* probe, *Svet1*, a specific marker of the embryonic SVZ and the upper layers of the mature cortex (**Figure 2C**) (Tarabykin et al., 2001). *Svet1* cDNA consists of 3,934 bp without an open reading frame and was later identified as part of a 324 kb intron between exon 1 and exon 2 of *Unc5d* (Sasaki et al., 2008). *Svet1*/*Unc5* is expressed in multipolar neurons in the SVZ, which migrate to the upper layers. Interestingly, when the neurons migrate through deep layers, where a high-affinity repulsive ligand to Unc5d, FLRT2, is expressed, Unc5d is temporarily shut down by the suppression of nuclear RNA splicing. Upon arrival to the upper layer, Unc5D is re-expressed (Yamagishi et al., 2011). Overexpression of Unc5d delays radial migration, whereas knockout of *Unc5d* results in broader distribution of $Tbr2^{+}$ intermediate progenitor cells, typically confined to the SVZ, toward the cortical plate (Yamagishi et al., 2011; Seiradake et al., 2014). Such dynamic expression of Unc5d is highly regulated by transcription factor FoxG1 (Miyoshi and Fishell, 2012). FoxG1 gain-of-function cells fail to express Unc5d and show a migration defect, which is rescued by Unc5d overexpression, whereas a loss of FoxG1 function arrests cells in an early multipolar phase. Upregulation of FoxG1 is required to exit the multipolar cell phase and to enter the cortical plate. Furthermore, *in situ* pattern analysis revealed that *Unc5d* and *Dcc* were among the genes with the highest expression induced by *Eomes* (Tbr2) (Cameron et al., 2012).

The zinc-finger transcriptional repressor, RP58, controls the multipolar-to-bipolar transition by suppressing the neurogenin2–Rnd2 pathway (Heng et al., 2008; Ohtaka-Maruyama et al., 2012, 2013). RP58 forms a transcriptional complex with FoxG1, and chromatin immunoprecipitation sequencing revealed associations with *Neurog2*, *NeuroD1*, *Rnd2*, and *Unc5D* (Cargnin et al., 2018). Another transcription factor, PRDM8, regulates multipolar-to-bipolar transition by modulating Unc5d levels (Inoue et al., 2014). Although the expression patterns of PRDM8 and Unc5d partially overlap, overexpression of PRDM8 inhibits Unc5d expression and *vice versa*.

FLRT family proteins are involved in the radial migration and gyrus formation of the cerebral cortex. High-affinity binding of FLRT2 to Unc5D (K_d = 0.31 μM) induces a repulsive signal and controls radial migration (Yamagishi et al., 2011; Seiradake et al., 2014). FLRT proteins also bind Latrophilin3 (Lphn3), which is involved in cell adhesion and synaptogenesis (K_d = 40 nM) (O'Sullivan et al., 2012; Jackson et al., 2015). In addition, FLRT2/Unc5D/Lphn3 forms a ternary complex in a stoichiometry of 1:1:2, which further dimerizes to make a larger supercomplex at 2:2:4 (Lu et al., 2015; Jackson et al., 2016). As FLRT and Lphn also form a ternary complex with teneurin (Sando et al., 2019; Del Toro et al., 2020), it would be interesting to know whether they form a large tetra-complex with Unc5. The multiple FLRT bindings with repulsive/adhesive functions play important roles in radial migration, tangential distribution, and synapse formation. Indeed, FLRT1/3 double-knockout mice show ectopic cortical gyrus formation (Del Toro et al., 2017). The expression pattern of FLRTs is summarized in **Figure 2D**. The functions of FLRTs in cortical migration and gyrus formation have been the subject of a previous review article (Peregrina and del Toro, 2020).

HUMAN DISEASES

Recent genetic analyses have revealed that mutations in the above mentioned guidance molecules are involved in congenital disorders. Mutations in genes encoding netrin-1 (*NTN1*) and DCC (*DCC*) in human result in abnormal targeting of corticospinal tracts and congenital mirror movements, a disorder characterized by involuntary movements of one hand that mirror the intentional movements of the opposite hand. Three mutations in exon 7 of *NTN1*, I518del, C601R, and C601S, encoding the C-terminal NTR domain, were identified in two unrelated families and one sporadic case. In the patients, only a portion of the corticospinal tracts crossed at the medulla, resulting in uncrossed aberrant corticospinal tract projections to ipsilateral motor neurons, as well as contralateral projections (Depienne et al., 2011; Méneret et al., 2017). Since the netrin-1 mutation causes the abnormal projection of cortical spinal tract, it is plausible that netrin-1 is not relevant to cortical migration. On the other hand, netrin-1 is expressed in neurons and oligodendrocytes in the spinal cord and regulates radial and tangential neuronal migration (Manitt et al., 2001; Junge et al., 2016). Therefore, it is possible that the abnormal positioning of neurons indirectly affects the distribution of the corticospinal tract. Also, short-range netrin-1 effects might be associated with the maintenance of appropriate neuronal and axon–oligodendroglial interactions and/or maintenance of synaptic interactions and plasticity in the mature nervous system.

Netrin-1 is expressed in the marginal zone and intermediate zone in the mid-gestation stage of mouse, but its expression disappears thereafter (**Figure 2**) (Livesey and Hunt, 1997). However, limited netrin-1 expression is observed in the medial prefrontal cortex, which dopaminergic neurons innervate from the midbrain (Manitt et al., 2011). Notably, several psychiatric disorders, including schizophrenia, depression, and drug abuse, are associated with altered organization and function of mPFC circuitry (Tan et al., 2007; Davey et al., 2008; Feil et al., 2010). Indeed, a genome-wide methylation study of twins revealed that *Netrin-1* had an altered methylation status in patients with depression (Roberson-Nay et al., 2020). Another study showed an association between a SNP in Netrin-1 (rs8081460) and neuroticism (Smith et al., 2016).

Recent genome-wide association studies have revealed that a growing number of *DCC* mutations are associated with psychiatric disorders, such as mood instability, neuroticism, schizophrenia, and depression (Ward et al., 2017; Kibinge et al., 2020; Li et al., 2020; Torres-Berrío et al., 2020; Vosberg et al., 2020). In patients with depression, the expression of DCC is abnormally high in the dorsolateral prefrontal cortex, which connects to the thalamus, caudate nucleus, hippocampus, orbitofrontal cortex, and other cortical areas (Li et al., 2020). Furthermore, *DCC* mRNA levels in prefrontal cortex were ∼40% higher in patients who committed suicide (Manitt et al., 2013; Torres-Berrío et al., 2017). A murine model with depression-like symptoms induced by chronic social defeat stress also exhibits higher levels of DCC in the prefrontal cortex (Torres-Berrío et al., 2017). Individuals with *DCC* haploinsufficiency exhibit reduced striatal volumes and impaired connectivity between the substantia nigra and the ventral tegmental area and ventromedial prefrontal cortex, resulting in lower novelty-seeking scores (Vosberg et al., 2018).

Although there is no direct evidence that aberrant cortical migration caused by DCC mutations is involved in the above mentioned phenotypes, a cortical migration defect is known to cause psychiatric disorders, such as schizophrenia (Muraki and Tanigaki, 2015) and decreased novelty recognition (Hamada et al., 2017). Indeed, miRNA knockdown of the psychiatric illness risk gene DISC1 affects the tangential migration of interneurons (Steinecke et al., 2012). Therefore, DCC-associated psychiatric disorders may be caused by abnormal cortical migration. In patients with schizophrenia, abnormal cortical layers or cell distributions have been reported. Iritani et al. (1999) reported that calbindin-D28K positive cells are distributed abnormally in the prefrontal cortex (Brodmann area 9). Cajal–Retzius cells, which produce reelin signals, are more numerous in the lower third of layer I in schizophrenia patients (Kalus et al., 1997). Again, since netrin-1 is expressed in layer I and IZ, and involved in interneuron migration during the gestation stage (**Figure 2**), these abnormal distributions may explain the correlation between psychiatric disorders associated with *Netrin-1* and *DCC* mutations (Vosberg et al., 2020).

Single nucleotide polymorphisms or abnormal expression of *NTNG1* and *NTNG2* are related to psychiatric disorders such as autism, Rett syndrome, schizophrenia, and bipolar disorder in human and murine models (Fukasawa et al., 2004; Aoki-Suzuki et al., 2005; Chuang et al., 2015; Huang and Hsueh, 2015; Heimer et al., 2020). The levels of *NTNG1* mRNA, especially isoform G1c, and *NTNG2* are decreased in patients with bipolar disorder (Eastwood and Harrison, 2008). However, the same group later reported elevations of netrin G1d and G1f isoforms and of netrin G2 in patients with bipolar disorder, indicating that an alteration of netrin G1 levels is critical for susceptibility to the disease (Eastwood and Harrison, 2010). Abnormally high expression of netrin G2 was observed in the temporal lobes of patients with intractable epilepsy, as well as in a rat model involving the use of pilocarpine (Pan et al., 2010). Mutations in the *NTNG1* gene are linked to Rett syndrome with epileptic seizures of early onset (Archer et al., 2006; Nectoux et al., 2007), and a more recent study revealed that netrin G2 dysfunction is associated with a Rett-like phenotype with areflexia (Heimer et al., 2020).

A large-scale single nucleotide polymorphism analysis of chromosome 4 revealed that *UNC5C* is one of the susceptibility genes for schizophrenia, bipolar disorder, and depression (Tang et al., 2019). Moreover, several studies revealed that single nucleotide polymorphism of *UNC5C* is relevant to Alzheimer's disease (Sun et al., 2016; Cukier et al., 2017; Yang et al., 2017). The level of Unc5c was decreased in the dorsolateral prefrontal cortices of patients with Alzheimer's disease-related cerebral amyloid angiopathy (Yang et al., 2017). In addition, a rare mutation, T835M, of *UNC5C* was identified from parametric linkage analysis of late-onset Alzheimer's disease (Wetzel-Smith et al., 2014). Using a mouse model, the authors revealed that T835M-expressing neurons were more vulnerable to Aβ-induced neurotoxicity than controls. However, whether there is a link between cortical migration and the onset of the these diseases remains an open question.

FUTURE DIRECTIONS

The last two decades of research have seen an expansion of the involvement of netrins in axon guidance to multiple physiologic and pathophysiologic functions such as synapse formation/plasticity, learning/memory, neuronal migration, and psychiatric disorders. However, little is known about how these multiple events are spatially and temporally coordinated in axons and dendrites for proper wiring of neuronal networks after radial and tangential migration, which is orchestrated by the guidance molecules and other extracellular cues. "Multi-omic" studies of netrins and other related genes, including transcriptomic, proteomic, and metabolomics approaches, could help identify other key targets impacting neuronal migration and its downstream events. In addition, spatially and temporally confined deletion of the guidance genes could shed light on crucial mechanisms involved in the dynamic regulation of cortical migration.

Interestingly, many mutations in netrins and their related molecules cause psychiatric disorders, such as schizophrenia, bipolar disorder, mood instability, neuroticism, depression, autism, and Rett syndrome, as mentioned above. However, the detailed molecular mechanisms via which mutations in these

genes cause these diseases remain unclear. Although netrins and their receptors are known risk factors for these diseases, other extracellular molecules such as laminins and proteoglycans may also interact with netrin signaling cascades and lead to the onset of these diseases. Further investigations are needed to fully understand whether or not abnormalities in cortical migration contribute to psychiatric disorders. The results of such investigations combined with the identification of netrin signaling targets may open new avenues for understanding and treating neurological disorders.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Subtle Roles of Down Syndrome Cell Adhesion Molecules in Embryonic Forebrain Development and Neuronal Migration

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Down Syndrome (DS) Cell Adhesion Molecules (DSCAMs) are transmembrane proteins of the immunoglobulin superfamily. Human DSCAM is located within the DS critical region of chromosome 21 (duplicated in Down Syndrome patients), and mutations or copy-number variations of this gene have also been associated to Fragile X syndrome, intellectual disability, autism, and bipolar disorder. The DSCAM paralogue DSCAM-like 1 (DSCAML1) maps to chromosome 11q23, implicated in the development of Jacobsen and Tourette syndromes. Additionally, a spontaneous mouse DSCAM deletion leads to motor coordination defects and seizures. Previous research has revealed roles for DSCAMs in several neurodevelopmental processes, including synaptogenesis, dendritic self-avoidance, cell sorting, axon growth and branching. However, their functions in embryonic mammalian forebrain development have yet to be completely elucidated. In this study, we revealed highly dynamic spatiotemporal patterns of *Dscam* and *Dscaml1* expression in definite cortical layers of the embryonic mouse brain, as well as in structures and ganglionic eminence-derived neural populations within the embryonic subpallium. However, an in-depth histological analysis of cortical development, ventral forebrain morphogenesis, cortical interneuron migration, and cortical-subcortical connectivity formation processes in Dscam and Dscaml1 knockout mice ($Dscam^{del17}$ and $Dscaml1^{GT}$) at several embryonic stages indicated that constitutive loss of *Dscam* and *Dscaml1* does not affect these developmental events in a significant manner. Given that several *Dscam*- and *Dscaml1*-linked neurodevelopmental disorders are associated to chromosomal region duplication events, we furthermore sought to examine the neurodevelopmental effects of *Dscam* and *Dscaml1* gain of function (GOF). *In vitro*, *ex vivo*, and *in vivo* GOF negatively impacted neural migration processes important to cortical development, and affected the morphology of maturing neurons. Overall, these findings contribute to existing knowledge on the molecular etiology of human neurodevelopmental disorders by elucidating how dosage variations of genes

encoding adhesive cues can disrupt cell-cell or cell-environment interactions crucial for neuronal migration.

Keywords: Dscam, Dscaml1, neuronal migration, cell adhesion, telencephalic development, radial migration, interneuron migration

INTRODUCTION

Down Syndrome (DS) Cell Adhesion Molecules (DSCAMs) represent a small group of transmembrane proteins of the immunoglobulin superfamily comprising, in vertebrates, DSCAM and its paralogue DSCAM-like 1 (DSCAML1) (Yamakawa et al., 1998; Agarwala et al., 2001). These molecules owe their name to the location of human *DSCAM* within the DS critical region of chromosome 21 (Yamakawa et al., 1998; Schmucker and Chen, 2009), which is considered to be crucially involved in the emergence of cognitive phenotypes associated with DS (Delabar et al., 1993; Korenberg et al., 1994; Belichenko et al., 2009, 2015; Aziz et al., 2018). Higher DSCAM levels have been observed in post-mortem brain tissue preparations/cultures from DS-affected patients and fetuses (Saito et al., 2000; Bahn et al., 2002), as well as in the central nervous system (CNS) of DS mouse models (Alves-Sampaio et al., 2010).

In addition to trisomy 21, mutations, single-nucleotide polymorphisms (SNPs), and transcriptional dysregulation of this gene have also been associated to other neurodevelopmental and neuropsychiatric disorders, including Fragile X syndrome (Brown et al., 2001; Darnell et al., 2011; Ascano et al., 2012; Cvetkovska et al., 2013), intellectual disability (Wei et al., 2016; Aleksiuniene et al., 2017; Monies et al., 2017; Stessman et al., 2017), autism (Iossifov et al., 2014; Turner et al., 2016; Wang et al., 2016; Varghese et al., 2017), bipolar disorder (Amano et al., 2008), and epilepsy (Shen et al., 2011; Wei et al., 2016). Animal models further substantiate a causal relation between variations in *Dscam* gene dosage and CNS dysfunction. A spontaneous *Dscam* null mutation occurring in mice ($Dscam^{del17}$) leads to the early post-natal emergence of uncoordinated movements; as adults, these animals additionally display severe hydrocephalus, seizures, aberrant locomotion, and impaired motor learning (Fuerst et al., 2008; Xu et al., 2011). Similarly, mice carrying a different *Dscam* null mutant allele ($Dscam^{2J}$) present dystonic hypertonia and deficits in locomotor coordination related to abnormalities in central sensorimotor circuitry (Fuerst et al., 2010; Lemieux et al., 2016; Thiry et al., 2016, 2018; Laflamme et al., 2019). Viability of *Dscam* null mutant mice is highly affected by their genetic background, leading to early post-natal lethality in a C57BL/6 background but survival to adulthood in an inbred C3H background, which suggests that modifier genes partly compensate for early developmental roles of DSCAM (Fuerst et al., 2010). In *Drosophila*, a third copy of the *Dscam* gene results in sensory perception impairments mirroring those found in flies lacking the Fragile X Mental Retardation gene, in which Dscam levels are elevated, and that in the latter animals can be rescued by reducing *Dscam* expression (Cvetkovska et al., 2013).

On the other hand, *DSCAML1* has been mapped to the 11q23 region, implicated in the pathophysiology of neurodevelopmental disorders including Jacobsen, Gilles de la Tourette, and distal trisomy 11q syndromes which points to *DSCAML1* as a potential causative gene, although a clear causation has not been proven (Agarwala et al., 2001; Pauls, 2003; Mattina et al., 2009; Chen et al., 2014; Choi et al., 2015).

As cell adhesion molecules, DSCAM and DSCAML1 engage in homophilic interactions at the cell membrane, which ensures cell interaction specificity. In arthropods, alternative splicing yields tens of thousands of DSCAM1 isoforms from one gene locus, a process known to be instrumental in achieving self-recognition critical to neural wiring as well as innate immunity (Schmucker et al., 2000; Schmucker and Chen, 2009). This staggering complexity is an insect innovation, as vertebrates can only produce single DSCAM and DSCAML1 isoforms. The higher neural network complexity shown by vertebrate species is thus hypothesized to result from the expansion of other cell adhesion molecule classes with similar characteristics, such as clustered Protocadherins (Jin and Li, 2019).

Previous research in vertebrates and invertebrates has revealed roles for DSCAMs in several neurodevelopmental processes, including synaptogenesis, neural proliferation, dendritic self-avoidance, cell sorting, and axon growth, guidance, and branching (Chen et al., 2006; Fuerst et al., 2008, 2009; Li et al., 2009; Liu et al., 2009; Maynard and Stein, 2012; He et al., 2014; Dascenco et al., 2015; Thiry et al., 2016; Laflamme et al., 2019; Sachse et al., 2019). In the mouse retina, loss of *Dscam* or *Dscaml1* leads to excessive dendritic fasciculation and somatic clustering of the cell types that normally express these molecules, demonstrating a role in dendritic self-avoidance and tiling (Fuerst et al., 2008, 2009). In addition, conditional loss of *Dscam* in the retina produces a decrease in programmed cell death of the targeted population (Fuerst et al., 2012).

Whether these functions are retained and contribute to mammalian forebrain development has yet to be completely elucidated. Research in mouse has shown that *Dscam* loss of function (LOF) results in a transient, early post-natal decrease in the thickness of upper cortical layers; notably, this phenotype could not be attributed to an increase in cell death, nor to a reduction in progenitor proliferation during embryonic development (Maynard and Stein, 2012). Whether the generation of different cortical layers during embryonic brain development is also affected remains unclear. Knockdown of either *Dscam* or *Dscaml1* in the cortex impairs radial migration of projection neurons and leads to a partial mispositioning of presumptive layer II/III neurons in layers IV/V observable for more than 2 weeks after birth. In addition, this partial loss of *Dscam* or *Dscaml1* function in the cortex reduces the midline-oriented extension of callosal axons, which at later post-natal time-points results in a decrease in axon terminals in contralateral cortical regions, supporting the idea that DSCAM and DSCAML1

are important for axon extension, and perhaps also guidance (Zhang et al., 2015). Given the expression of DSCAM and DSCAML1 during embryonic forebrain development, our aim was to further investigate whether these molecules are implicated in the migration of both cortical neurons and interneurons, the patterning/morphogenesis of embryonic telencephalic structures, and the early establishment of forebrain connectivity. Using constitutive loss-of-function models, we demonstrate that loss of DSCAM or DSCAML1 only has minor effects on these processes. However, as in human increased dosage of DSCAM or DSCAML1 seems to be more detrimental to neurodevelopment, we also implemented gain-of-function approaches to study potential roles in neuronal migration and morphological maturation. Our data indicate that overexpression of either DSCAM or DSCAML1 reduced migration distances traveled by immature cortical interneurons, while DSCAML1 overexpression selectively affected neurite branching. Future investigations should reveal the molecular mechanisms at the basis of these phenotypes.

MATERIALS AND METHODS

Animals

All animal procedures were performed in accordance with Belgian and EU regulations on the use of animals for scientific purposes (Royal Decree of 29 May 2013, Directive 2010/63/EU) and approved by the KU Leuven Ethical Committee for Animal Experimentation (project licenses 267/2015 and 005/2017).

All experiments were performed on embryonic brains obtained from C57BL/6J mice (wild-type) (Jackson Laboratories), a *Dlx5/6-Cre-IRES-EGFP* reporter line (Stenman et al., 2003) bred on a CD1 background, a C57BL/6J strain carrying a null mutation in the *Dscam* gene consisting of a 38 bp deletion within exon 17 ($Dscam^{del17}$) (Fuerst et al., 2008), and a *Dscaml1* null mutant C57BL/6J strain ($Dscaml1^{GT}$). In the latter case, LOF was achieved by the insertion of a gene-trap vector in the 3rd *Dscaml1* intron, resulting in the production of a non-functional N-terminal DSCAML1–β-galactosidase fusion protein (Fuerst et al., 2009). $Dscam^{del17}$*; Dlx5/6-Cre-IRES-EGFP* ($Dscam^{del17}$*; Dlx5/6-CIE*) and $Dscaml1^{GT}$*; Dlx5/6-Cre-IRES-EGFP* ($Dscaml1^{GT}$*; Dlx5/6-CIE*) mutant mice were generated by crossing $Dscam^{del17}$ and $Dscaml1^{GT}$ lines with the *Dlx5/6-Cre-IRES-EGFP* reporter line. Mouse colonies were maintained in a 14/10 h light-dark cycle, in a humidity- and temperature-controlled pathogen free animal unit.

Pregnant females for embryo collection were obtained via timed matings. Embryonic age was calculated considering the day of vaginal plug detection as E0.5. Mouse brains were dissected in cold phosphate-buffered saline (PBS) and fixed in 4% w/v paraformaldehyde (PFA)/PBS for 16–24 h at 4°C, unless they were processed for X-gal staining. Following fixation, specimens were washed once in PBS for 30–60 min at 4°C, and stored at this temperature for up to 9 months in storage buffer (0.01% w/v thimerosal/PBS). Mouse tail samples (∼5 mm) were also collected for DNA extraction and genotyping.

To verify the absence of DSCAM protein in the DSCAM knockout mouse (**Supplementary Figure 1I**), protein was extracted from E17.5 brains from knockout and wildtype mice using TRIS-HCL SDS-buffer (65 mM Tris-HCL, 2% SDS) containing cOmplete™ Protease Inhibitor Cocktail (Roche). Tissue lysates were cleared by centrifugation and proteins were heat denatured in a mixture of XT sample buffer 4x and XT reducing agent 20x, separated on 4–12% Bis-Tris precast polyacrylamide gels (Criterion XT Bis-Tris Precast Gel, Bio-Rad) in MOPS buffer, and immuno-blotted to nitrocellulose membranes (Trans-Blot Turbo Midi 0.2 μm Nitrocellulose Transfer Packs, Bio-Rad) using a Trans Blot Turbo system (Bio-Rad). Standard protein detection was performed using rabbit anti-DSCAM antibodies (1:250; HPA019234, Sigma-Aldrich). After 2 h blocking in 5% w/v non-fat dry milk/TBST (WB buffer) at RT, o/n incubation at 4°C in primary antibody diluted in WB buffer, and washing in TBST, transfer membranes were incubated for 45 min in HRP-conjugated anti-rabbit secondary antibodies (Bio-Rad) diluted 1:10,000 in WB buffer. Protein bands were visualized with a ChemiDoc MP imaging system (Bio-Rad) after incubation in ECL substrate (Thermo Fisher Scientific).

Genotyping

Tissue samples were digested overnight (o/n) at 56°C in a 1:100 Proteinase K solution (10 mg/mL in 40% glycerol/nuclease-free H_2O; Thermo Fisher Scientific) in lysis buffer (50 mM Tris-HCl pH 8.5, 2.5 mM EDTA pH 8, 50 mM NaCl, 1% SDS). Genotyping PCR reactions were prepared using a small aliquot of the digestion solution, a PCR mix (KAPA2G Fast HotStart ReadyMix with dye, KAPA Biosystems) containing dNTPs, a Taq polymerase and a loading dye, and primers for the genes of interest (see **Supplementary Table 1**).

In situ Hybridization

In situ hybridization (ISH) experiments were performed on 20 μm cryosections or 6 μm paraffin sections from E13.5 and E16.5 wild-type brains. To obtain frozen tissue samples, after PFA fixation brains were incubated in 30% sucrose/PBS at 4°C until sinking, submerged in Optimal Cutting Temperature compound (Tissue-Tek, Sakura Finetek) for 1–2 h at 4°C, fast frozen in liquid nitrogen, and maintained at −20°C until sectioning. Paraffin-embedded specimens were first dehydrated by o/n incubation in 50% ethanol/saline at 4°C, then processed for paraffinization (Excelsior AS Tissue Processor, Thermo Fisher Scientific) and embedding (HistoStar Embedding Workstation, Thermo Fisher Scientific). Sectioning of frozen or paraffin-embedded brains was performed with a Microm HM560 cryostat or a Microm HM360 rotary microtome (Thermo Fisher Scientific), respectively; sections were collected on SuperFrost Plus slides (Thermo Fisher Scientific).

Plasmids for the synthesis of antisense *Gad1* riboprobes were a gift from Prof. Brian Condie (University of Georgia) (Maddox and Condie, 2001). *Dscam, Dscaml1* and *Ebf1* ISH probe sequences were amplified from an embryonic cDNA pool with primer pairs 5′-TCAGGAAGTTCACTTGGAACC-3′/5′-TGGAGAATCCCATTCAAGGC-3′ (*Dscam*), 5′-CTTTGT TGTACGAAAGAAGAGGAAG-3′/5′-CATAGATGTCATACTG TCAGCGTTC-3’ (*Dscaml1*), and 5′-CAGGAAAGCATCCAAC GGAGTGG-3′/5′-GCCCGTGCTTGGAGTTATTGTGG-3′

(*Ebf1*), respectively. Amplicons (521 bp, 747 bp, and 691 bp) were blunt-cloned in pCRII-TOPO vectors using a TOPO TA Cloning Kit (Thermo Fisher Scientific); following transformation of DH5α chemocompetent cells and blue/white screening, successfully transformed colonies were sequenced to determine the quality and orientation of the inserts. Plasmid DNA from selected colonies' cultures was purified using a PureLink HiPure Plasmid Maxiprep Kit (Invitrogen, Thermo Fisher Scientific).

Digoxigenin (DIG)-labeled antisense riboprobes for *Dscam*, *Dscaml1*, *Ebf1*, and *Gad1* ISH were produced from plasmid templates linearized overnight at 37°C. An *in vitro* transcription reaction was prepared with 1 μg of linearized plasmid template using a SP6/T7 DIG RNA Labeling Kit (Roche). The synthesized RNA was purified with Micro Bio-Spin P-30 Gel Columns (Bio-Rad) and quantified using a SimpliNano spectrophotometer (Biochrom).

ISH was performed for all section types on a DISCOVERY automated staining platform (Ventana Medical Systems, Roche). Section were first processed for deparaffinization, fixation, pre-treatment, and post-fixation using RiboMap Kit solutions (Roche). The probes of interest were diluted in RiboHybe (Roche) to a final concentration of 150–300 ng/slide, denatured at 90°C for 10 min, and hybridized at 70°C for 6 h. After a series of stringency washes at 68°C in saline-sodium citrate buffer, specifically bound probes were detected by incubation in a 1:1,000 dilution of AP-conjugated sheep anti-DIG antibody in PBS (30 min at 37°C), and visualized using a BlueMap Detection kit (Roche) (7 h substrate incubation at 37°C). At the end of the ISH protocol, all sections were dehydrated in a graded ethanol dilution series (70%, 2 min; 96%, 2 min; 100%, 3 min; 100%, 3 min) and finally washed twice in xylene for 5 min each. Coverslips were applied using Eukitt Quick-hardening mounting medium (Sigma-Aldrich). Brightfield images of the ISH experiments were acquired using a Leica DM6 B microscope connected to a digital CMOS camera (DMC2900, Leica) with the LAS X software suite (Leica). Images were further processed with the Fiji distribution of the open source program Image J (Schindelin et al., 2012) and Adobe Photoshop CC 2018.

Immunohistochemistry

Vibratome-processed brain sections were stained using a free-floating IHC protocol, in a 12-well plate, and using a shaking platform for all washes/incubations. Serial 60 μm free-floating brain sections were obtained from PFA-fixed brains embedded in 4% w/v agarose/PBS cut with a Microm HM650V vibratome (Thermo Fisher Scientific), and collected in storage buffer. Tissue pre-treatment was then performed by incubation for 1–2 h at room temperature (RT) in a blocking and permeabilization buffer (10% normal donkey serum, 0.3% Triton X-100 in PBS). If heat-induced antigen retrieval was recommended by the manufacturers of the primary antibodies employed, an additional 20–40 min incubation in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) pre-heated and maintained at 85°C in a hybridization oven, followed by a 20 min cool-down step at RT, was performed before blocking and permeabilization.

Following pre-treatment, the sections were incubated with primary antibodies diluted in storage buffer for 24–48 h at 4°C. Primary antibodies used were rat anti-CTIP2 (1:500, ab18465, Abcam), mouse anti-Islet1 (1:50; 39.4D5, Developmental Studies Hybridoma Bank), rabbit anti Nkx2.1 (1:1,000; sc-13040, Santa Cruz Biotechnology), mouse anti-neurofilament 165 kD (1:100; 2H3, Developmental Studies Hybridoma Bank), chicken anti-GFP (1:1,000; ab13970, Abcam), rabbit anti-TBR1 (1:400; AB10554, Merck-Millipore), mouse anti-SATB2 (1:200; ab51502, Abcam), rabbit anti-RFP (1:2,000; 600-401-379, Rockland Immunochemicals), and mouse anti-HA tag (1:1,000; 6E2, Cell Signaling Technology). The monoclonal anti-neurofilament 165 kD (2H3) and anti-Islet1/2 homeobox (39.4D5) antibodies, developed by respectively by T.M. Jessell and J. Dodd, and by T.M. Jessell and S. Brenner-Morton, were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa.

After four 10 min washes in PBS at RT, the sections were subsequently incubated for 2 h at RT, or overnight at 4° C, with donkey-derived secondary antibodies conjugated with Alexa Fluor® dyes (Jackson Immunoresearch or Invitrogen) diluted 1:500 in storage buffer. Next, the tissue samples were washed in PBS at RT in four 10 min cycles, counterstained with 4′,6-Diamidino-2-Phenylindole (DAPI) (Sigma-Aldrich), and finally mounted on SuperFrost Plus slides in Mowiol (Sigma-Aldrich) mounting medium (30% w/v glycerol, 12% w/v Mowiol, 0.1 M Tris-HCl pH 8.5).

Slides were examined with a Leica DM6 B epifluorescence microscope digital CCD camera (DFC365 FX, Leica) or an Olympus FLUOVIEW FV1000 confocal laser scanning microscope. Images acquired using the LAS X or FV10-ASW Viewer v. 4.2c (Olympus) software packages, respectively, and processed as previously described.

X-Gal Stainings

Whole mount X-gal stainings were performed on freshly dissected brains from *Dscaml1*^{GT} mice pre-incubated in X-gal fixative (1% formaldehyde, 0.2% glyceraldehyde, 0.5% Triton X-100 in PBS) at 4°C on a shaker. Incubation time was adjusted according to brain size to respectively, 20 and 35 min for E13.5 and E16.5 specimens. After three 20–30 min washes in cold PBS, pre-fixed brains were subsequently incubated for 24–48 h in the dark at 37°C in freshly prepared staining buffer (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.1% Triton-X, 0.01% sodium deoxycholate in PBS) containing 1 mg/mL X-gal (Applichem). Next, samples were repeatedly washed in PBS at 4°C until washout looked completely clear, post-fixed o/n in 4% PFA at 4°C, and vibratome-sectioned as detailed in section Immunohistochemistry. The obtained sections were counterstained with a Nuclear Fast Red–aluminum sulfate 0.1% solution (Sigma), mounted on glass microscope slides, cover-slipped with Mowiol mounting medium, and dried o/n at RT before imaging. Brightfield microscope images were acquired and processed as described in section *in situ* Hybridization.

Neuroanatomical Tracings

Mixed retrograde and anterograde tracing of reciprocal connections between distinct thalamic nuclei and either

the primary visual (occipital) or primary somatosensory (parietal) cortex in wild-type, *Dscam*^{del17} and *Dscaml1*^{GT} embryonic mouse brains were performed by inserting 0.1–0.3 mm crystals of, respectively, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Biotium) and 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide (DiA; Biotium) in the superficial cortical layers of E17.5 brain hemispheres, with the aid of a tungsten dissecting probe (World Precision Instruments). Following the insertion of dye crystals, brains were kept in 1% PFA/PBS at RT in the dark for 3–4 weeks to allow diffusion of the carbocyanine tracers in the axonal tracts and thalamic populations of interest.

At the end of their incubation period, brains were vibratome-sectioned as detailed in section Immunohistochemistry. Sections were counterstained with DAPI, mounted in Mowiol mounting medium onto SuperFrost Plus slides, and imaged using the epifluorescence microscope setup also described in section Immunohistochemistry within 48 h after sectioning, to avoid artifacts due to local dye diffusion at the sections' surfaces.

Expression Plasmid Production and Testing

Expression vectors used in electroporation experiments were synthetized starting from a pCAGGS-IRES-EGFP plasmid backbone (Megason and McMahon, 2002) (a gift from P. Vanderhaeghen, Université libre de Bruxelles), wherein EGFP was replaced by TdTomato. Full length *Dscam* and *Dscaml1* cDNA sequences tagged in frame at the 3′ end with EYFP- and HA tag-encoding sequences were blunt-end cloned into this pCAGGS vector from pcDNA5-FRT-TO-GW-DSCAM-HA, pcDNA5-FRT-TO-GW-DSCAM-EYFP-HA, pcDNA5-FRT-TO-GW-DSCAML1-HA, and pcDNA5-FRT-TO-GW-DSCAM-EYFP-HA plasmids (Sachse et al., 2019) to produce tagged DSCAM/DSCAML1 and tdTomato co-expression constructs. Control pCAGGS vectors were obtained by sub-cloning only EYFP-HA coding sequences. Correct cloning in all novel expression constructs produced was verified by sequencing.

Expression vectors were first tested by transfection in Neuro 2a mouse cells (Sigma). As culture supports, 35 mm glass bottom dishes (Ibidi) pre-coated with Geltrex (Gibco, Thermo Fisher Scientific) at least 3 h before seeding were used for confocal microscopy imaging, while 6-well plates were used for all other applications. 24 h before transfection cells were seeded to a density of 5×10^5 (6-well plate) or 5×10^4 (cell dish) cells, and maintained in a humidified incubator at 37°C using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, and 50 U/mL Penicillin/50 μg/mL Streptomycin (all from Gibco, Thermo Fisher Scientific). On transfection day, cell adherence and confluency were checked under a microscope. Transfection was performed using a Lipofectamine 3000 transfection kit (Thermo Fisher Scientific). Briefly, Lipofectamine 3000 reagent was diluted in Opti-MEM medium (Gibco, Thermo Fisher Scientific) according to manufacturer instructions for 6-well or 24-well (cell dish) plates. 5 μg of plasmid DNA were diluted in Opti-MEM, and subsequently 2 μL of P3000 reagent per 1 μg DNA were added to generate the DNA master mix. The master mix was then combined 1:1 with Opti-MEM-diluted Lipofectamine, and incubated for 30 min at room temperature. The resulting DNA-lipid complex mix was added to each well/dish in volumes recommended by the manufacturer, and cells were re-transferred to a humidified incubator at 37°C. Transfection efficiency was examined after 24 and 48 h under a fluorescent microscope; confocal imaging (see section Immunohistochemistry) of cells cultured on glass bottom dishes was performed 48 h after transfection, following o/n fixation in 4% PFA/PBS at 4°C and washing in storage buffer.

To further verify the synthesis of tagged DSCAM/DSCAML1 proteins, Neuro 2a cells were harvested 48 h post-lipofection and lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS) containing cOmplete™ Protease Inhibitor Cocktail (Roche). Cell lysates were cleared by centrifugation, and proteins were heat-denatured in Laemmli sample buffer containing 50 mM dithiothreitol, separated on 4–20% polyacrylamide gels (Criterion TGX Stain-Free Protein Gel, Bio-Rad) in Tris-glycine-SDS buffer, and immuno-blotted to nitrocellulose membranes (Trans-Blot Turbo Midi 0.2 μm Nitrocellulose Transfer Packs, Bio-Rad) using a Trans Blot Turbo system (Bio-Rad). Standard protein detection was performed using mouse anti-HA tag antibodies (1:1,000; 6E2, Cell Signaling Technology). After 2 h blocking in 5% w/v non-fat dry milk/TBST (WB buffer) at RT, o/n incubation at 4°C in primary antibody diluted in WB buffer, and washing in TBST, transfer membranes were incubated for 1 h in HRP-conjugated anti-mouse secondary antibodies (Jackson Laboratories or Agilent Technologies) diluted 1:10,000 in WB buffer. Protein bands were visualized with a ChemiDoc XRS+ imaging system (Bio-Rad) after incubation in ECL substrate (Pierce).

In utero Electroporation

In utero electroporation (IUEP) of mouse embryonic brains was performed at E14.5 in an aseptic environment. Pregnant females were sedated via intramuscular injection of ketamine (75 mg/kg, Eurovet) and medetomidine (1.0 mg/kg, Orion Pharma), and peri-operative analgesia was provided by a subcutaneous injection of meloxicam (5.0 mg/kg, Boehringer Ingelheim). Once sedation was achieved, an ophthalmic ointment (Terramycin, Pfizer) was applied on the animal's eyes, the abdominal fur was removed, and the exposed skin was disinfected with a povidone-iodine solution. All surgical materials were sterilized using a hot bead sterilizer (FST 250, Fine Science Tools) immediately before laparotomy.

After placing the mouse on a heat mat, two incisions of ∼2 cm along the linea alba abdominis were made consecutively through the abdominal skin and the muscle/peritoneum tissue layers. To keep the uterus and peritoneal cavity hydrated, a sterile saline solution pre-heated at 37°C was applied as necessary. The uterine horns were gently pulled out of the abdominal cavity and placed on a sterile gauze. 2 μg/μL solutions of DSCAM-EYFP-HA, DSCAML1-EYFP-HA, or EYFP-HA expression constructs diluted in Opti-MEM medium, supplemented at a 1:30 ratio with a Fast Green FCF dye solution (1 mg/mL, Sigma-Aldrich)

for visualization purposes, were micro-injected in the embryo's lateral ventricles with glass microcapillary needles (Harvard Apparatus) produced with a magnetic puller (PN-31, Narishige), and connected to a filtered aspirator tube assembly (Drummond). Following bilateral injections, CUY650P5 tweezer electrodes connected to a NEPA21 electroporator (Nepa Gene) were washed with saline solution and positioned at the sides of the embryo's head for electroporation (see **Supplementary Table 2** for IUEP parameters) (**Figure 6A**). The injection and electroporation steps were repeated for a maximum of 8 embryos per female.

At the end of the procedure, the uterus was re-positioned within the abdominal cavity, and the abdominal incisions were closed using non-absorbable suture (PERMA-HAND silk, Ethicon). A povidone-iodine solution and an antibiotic cream (Fucidin, Leo Pharma) were applied on the sutured wound, and atipamezole (0.5–1.0 mg/kg, Orion) was finally injected intramuscularly to reverse anesthesia. After the surgery, mice were allowed to recover o/n in cages placed on a heating pad at 37°C, and provided with fresh bedding material, food and water. All operated pregnant females were kept in the animal facility until E18.5, when they were sacrificed for embryonic brain collection.

To better detect the transfected neurons in the obtained brains, free-floating IHC using primary antibodies against tdTomato and EYFP, and secondary antibodies matching the excitation/emission spectrum of the respective fluorescent protein, were performed on coronal vibratome sections from the electroporated brains before confocal imaging. Immunohistochemistry was performed as described in section Immunohistochemistry. Confocal imaging equipment and procedures are detailed in section Immunohistochemistry and Phenotype Quantification and Statistical Analysis.

Medial Ganglionic Eminence Electroporation and Explant Culture

Medial ganglionic eminence (MGE) electroporation (MEP) and MGE explant cultures were performed under sterile conditions on E13.5 brain tissue from *Dlx5/6-Cre-IRES-EGFP* mouse embryos. Pregnant females were euthanized by cervical dislocation to collect E13.5 embryos, the heads of which were dissected in cold Leibovitz's L-15 medium (Gibco, Thermo Fisher Scientific) supplemented with 35 mM D-glucose (Merck Millipore) and 2.5 mM HEPES (Gibco, Thermo Fisher Scientific) (L15++). For each isolated EGFP+ embryonic head, the MGEs were exposed by incisions at dorsal cortical level, and 2 μg/μL solutions of overexpression or control plasmids were injected with in 8–10 discrete MGE sites per hemisphere; next, injected brains were electroporated with CUY650P5 tweezer electrodes connected to a BTX electroporator (Harvard Apparatus) (see section *in utero* Electroporation for solution composition and injection material details, and **Supplementary Table 3** for MEP parameters) (**Figure 7A**).

Electroporated heads were left in L15++ medium for a minimum of 3 h on ice to allow recovery of neural cells. Subsequently, each MGE was dissected under a stereomicroscope to obtain ∼8 similarly sized explants (∼400–500 μm of diameter), which were transferred to cold Neurobasal Medium containing 2.5 mM HEPES, 2 mM L-glutamine, 100 U/mL Penicillin-Streptomycin, 1x B-27 supplement (all from Gibco, Thermo Fisher Scientific) (Complete Neurobasal Medium, CNB). Each explant was embedded on ice in ∼20 μL of Matrigel (Corning Life Sciences) diluted 1:1 in CNB, using as a support 35 mm glass bottom cell culture dishes (Ibidi). Embedded explants were briefly incubated at 37°C to enable Matrigel polymerization, covered with 500 μL of CNB, and cultured for 48 h at 37°C, 5% CO_2 in a humidified incubator (**Figure 7A**). Finally, explant cultures were fixed o/n in 4% PFA/PBS at 4°C, and preserved in storage buffer until imaging.

Phenotype Quantification and Statistical Analysis

For cortical lamination and thickness analyses in E17.5 *Dscam*$^{del17/del17}$, *Dscaml1*$^{GT/GT}$, or wild-type brains, cell counts per marker and radial measurements were obtained from single plane confocal images of three sections (representative of rostral, intermediate and caudal positions on the rostro-caudal axis) per specimen with the ImageJ Cell Counter plugin and Measure function. Data was statistically analyzed via a mixed ANOVA test, with rostro-caudal position as a within-subject factor and genotype as a between-subject factor, using Greenhouse-Geisser corrections if the assumption of sphericity was violated.

Interneuron distribution along the cortical radial axis of E18.5 *Dscaml1*$^{GT/GT}$; *Dlx5/6-CIE* and *Dscaml1*$^{+/+}$; *Dlx5/6-CIE* brains was assessed following IHC against eGFP, on coronal sections at a rostral and caudal level. In total three *Dscaml1*$^{GT/GT}$; *Dlx5/6-CIE* and two *Dscaml1*$^{+/+}$; *Dlx5/6-CIE* brains were analyzed, each including two individual technical replicates. A rectangle of 200μm x 550μm (rostral) and 200 × 500 μm (caudal) in the same medio-lateral region in the cortex was delineated, and further divided into 10 bins (bin1 = pial to bin10 = ventricular) of equal size using Fiji. Integrated density was quantified per bin, and normalized for area.

Radial migration following IUEP was assessed by measuring the distribution of TdTomato fluorescence along the cortical radius in confocal images (4 μm step maximum Z-stacks projections) of coronal brain sections. TdTomato fluorescence intensity values were acquired within 200 um dorso-lateral cortex sectors, divided in 10 equal bins, with the ImageJ Plot Profile function. A mixed model ANOVA test with bin as a within-subject factor and expression construct as a between-subject factor was carried out to compare means per bin across treatment groups. Greenhouse-Geisser corrections were applied when the assumption of sphericity was violated by data.

MGE explant migration was quantified by measuring linear distances from explant edge of TdTomato+ neurons on mixed brightfield-fluorescence confocal images (2 μm step maximum Z-stacks projections) (**Figures 7B–E**) with the ImageJ Measure function. Morphological analysis of MGE explant-derived neurons was performed using the SNT ImageJ plugin (Longair et al., 2011; Arshadi et al., 2020). A minimum of 9 explants obtained from at least two experimental replicates were analyzed per treatment group. Means per experimental group were

compared with a one-way ANOVA followed by a Tukey *post-hoc* test. If data did not meet basic requirements for use with parametric models, a Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's pairwise tests with Bonferroni adjustments were employed instead. Frequencies per neuron category were compared across experimental conditions with a Pearson's Chi-square test followed by a *post-hoc* residuals analysis, applying a Bonferroni correction for multiple comparisons.

RESULTS

Dscam and *Dscaml1* Are Dynamically Expressed During Embryonic Forebrain Development

Dscam and *Dscaml1* expression patterns in the mouse telencephalon during embryonic development have been so far poorly characterized. Thus, to better understand in which cellular and neurostructural context they might provide crucial functions, the spatiotemporal dynamics of *Dscam* and *Dscaml1* expression were first analyzed by *in situ* hybridization and X-gal staining.

At embryonic day (E) 13.5 ($n = 5$), *Dscam* was found to be strongly expressed in postmitotic layers of the developing cortex [the marginal zone (MZ) and the preplate (PP)/cortical plate (CP)], and in mantle regions of the ventral telencephalon (vTel) surrounding the internal capsule (IC), comprising the subpallial corridor dorsally and the globus pallidus ventrally. Sparse transcription was also observed in the presumptive striatum and amygdala, and in pial surface layers (**Figures 1A,B**). At E16.5 ($n = 5$), *Dscam* expression appeared to have extended to all cortical layers, except for the ventricular zone (VZ), and was found to be particularly robust in deeper cortical plate regions. Similarly, in the E16.5 vTel *Dscam* mRNA could also be clearly detected in progenitor zones, particularly the subventricular zone (SVZ), and additionally in the piriform cortex (**Figures 1E,F**).

Concerning *Dscaml1* expression, ISH at E13.5 ($n = 5$) revealed similar transcriptional patterns to those of *Dscam*; however, high mRNA levels were additionally observed in subventricular layers of both cortex and vTel, and expression at the cortical MZ and the vTel pial surface appeared stronger and denser at more rostral levels (**Figures 1C,D**). At E16.5 ($n = 5$), high transcriptional activity was still observed in both cortical and subpallial SVZs, and vTel ventral surface regions corresponding to the piriform cortex and cell populations delineating the lateral olfactory tract (LOT); sparse expression could be further detected at the VZs, the subplate, the deep CP, the MZ, the vTel VZ, the striatum, and the amygdala (**Figures 1G,H**). These findings align with expression patterns collected in the Allen Developing Mouse Brain Atlas (Thompson et al., 2014).

In the absence of sufficiently specific and sensitive antibodies against DSCAML1 epitopes for IHC purposes, DSCAML1 protein expression patterns in the developing mouse forebrain were investigated by whole mount X-gal staining on *Dscaml1*$^{+/GT}$ and *Dscaml1*$^{GT/GT}$ brains at E13.5 and E16.5 ($n = 3$ per genotype for both time-points). In these mice, the insertion of a gene-trap vector in the 3rd *Dscaml1* intron resulted in the production of a non-functional N-terminal DSCAML1–β-galactosidase fusion protein (Fuerst et al., 2009). While overlapping domains of protein synthesis and mRNA expression were found, there were also significant discrepancies in their pattern. At E13.5, X-gal stainings highlighted translation only in a few areas where transcription was detected by ISH, namely the MZ, the pial surface of the vTel, the pallidum, and a vTel mantle region delineating the pallial-subpallial boundary (**Supplementary Figures 1A–D**). Similarly, E16.5 samples indicated protein production resembling mRNA expression in the dorsal pallium, but distinct in the caudate-putamen, the amygdala, demonstrating remarkably high levels in areas surrounding the LOT, in particular the nucleus of the LOT (nLOT), the anterior and central amygdaloid areas, and the cortical, medial, and basolateral amygdaloid nuclei (**Supplementary Figures 1E–H**). Whether this is due to differences in protein half-life and/or post-transcriptional regulation remains to be determined.

Striatal Development Occurs in an Overall Normal Manner in *Dscaml1* Null Mutants

Expression of *Dscaml1* seemed prominent in the ventral telencephalon (**Figures 1C,D** and **Supplementary Figures 1A–H**). To test whether the development of striatal cell populations and the striatal cytoarchitecture were affected by loss of *Dscaml1* function, the expression patterns of distinct striatal neural markers were examined in *Dscaml1*$^{GT/GT}$ vs. wild-type embryonic brain at E17.5 and E18.5, which corresponds to a peak in SVZ-specific matrix neurogenesis (van der Kooy and Fishell, 1987; Hamasaki et al., 2001). The first marker analyzed was Ebf1, a transcription factor predominantly labeling postmitotic neurons of the matrix component, and providing essential functions in normal striatal development (Garel et al., 1999; Lobo et al., 2008; Faedo et al., 2017; Tinterri et al., 2018). ISH with *Ebf1* mRNA-specific antisense probes revealed that at E18.5 ($n = 4$ per genotype) expression of this striatal marker occurred comparably in wild-type (**Figures 2A,B**) and Dscaml1GT/GT (**Figures 2C,D**) brains; transcription appeared preserved throughout postmitotic neurons of the caudate-putamen region in *Dscaml1*$^{GT/GT}$ specimens, and, like in wild-type sections, concentrated in the striatal matrix compartment. Moreover, at dorsal vTel level, in the SVZ/upper mantle area, neurons expressing *Ebf1* at E18.5 delineated a compact cell layer in both wild-type and *Dscaml1*$^{GT/GT}$ rostral brain sections, suggesting a correct distribution of striatal neurons at the site where loss of X-gal staining in *Dscaml1*$^{GT/GT}$ was previously detected.

Since Ebf1 represents a striatal subpopulation-specific cell fate marker, protein synthesis patterns of CTIP2, a transcription factor crucially involved in medium spiny neuron (MSN) differentiation and striatal cytoarchitecture establishment (Arlotta et al., 2008; Tinterri et al., 2018), were further investigated to acquire a broader view of caudate-putamen development in *Dscaml1*$^{GT/GT}$ embryonic brains. Comparison of immunostainings on E17.5 brain sections from wild-type and

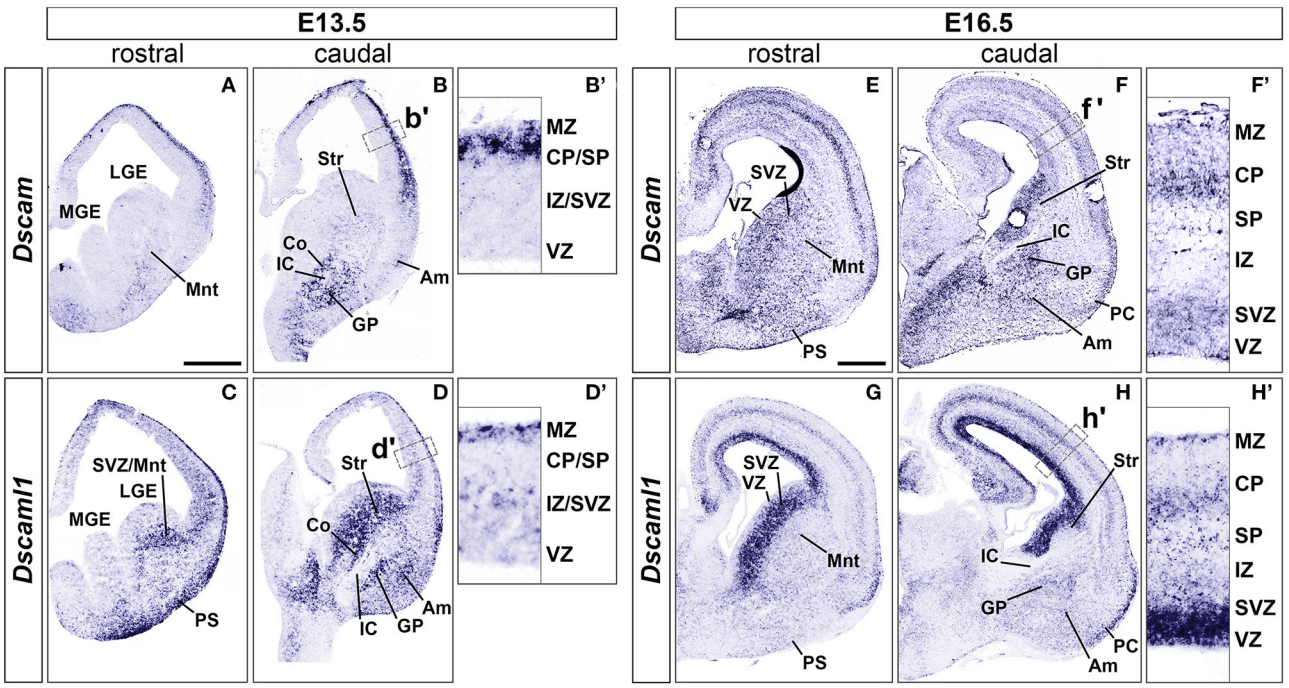

FIGURE 1 | Expression of *Dscam* and *Dscaml1* mRNA in the developing mouse forebrain. Levels of mRNA expression were detected by ISH with antisense RNA probes. **(A–D)** *Dscam* and *Dscaml1* expression in coronal sections of wild-type E13.5 mouse brains. **(A,B)** *Dscam* is restrictedly expressed in post-mitotic regions of both E13.5 dorsal and ventral telencephalon across the rostro-caudal axis. In the subpallium, high transcription levels are specifically observed in structures surrounding the IC, corresponding to the corridor and the GP; sparse expression is also detected throughout the mantle region, in presumptive striatal and amygdalar territories **(B)**. Within the developing cortex, *Dscam* mRNA is highly present in the MZ and upper CP **(B')**. **(C,D)** *Dscaml1* transcription at E13.5 occurs mostly in post-mitotic areas of the dorsal and ventral telencephalon, as well as subventricular progenitor zones. Robust expression is observed in territories where *Dscam* mRNA is also present, e.g., in subpallial cell populations of the corridor, GP, presumptive striatum and amygdala, and is additionally detected in the SVZ (D). In the dorsal pallium, *Dscaml1* mRNA is observed in some cells of the MZ and IZ/SVZ **(D')**. **(E-H)** *Dscam* and *Dscaml1* expression in coronal sections of wild-type E16.5 mouse brains. **(E,F)** At E16.5, *Dscam* remains robustly, but sparsely expressed in subpallial areas surrounding the IC; transcription furthermore extends to the pial surface ventrally, and the VZ/SVZ dorsally across the rostro-caudal axis. In this latter region, expression reaches high levels comparable to those found in IC-adjacent areas **(F)**. In the dorsal forebrain, *Dscam* mRNA is detected in sparse cells occupying most cortical layers; highest transcription levels can be observed in the CP and the SVZ **(F')**. *Dscam* expression can also be observed at PC level. **(G,H)** In the E16.5 telencephalon, *Dscaml1* shows maximum subpallial expression in the SVZ; sparse cells presenting moderate transcription can be additionally detected ventrally to this region, particularly in GP, presumptive amygdala, and presumptive striatum populations **(H)**. At cortical level, *Dscaml1* mRNA is detected at high levels in most cells of the VZ and SVZ; sparse cells showing robust transcription can be observed throughout the radial extension, with a higher accumulation at the SP, CP, and MZ **(H')**. Am, amygdala; Co, corridor; CP, cortical plate; GP, globus pallidus; IC, internal capsule; IZ, intermediate zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Mnt, mantle; MZ, marginal zone; PC, piriform cortex; PS, pial surface; SP, subplate; Str, striatum; SVZ, subventricular zone; VZ, ventricular zone. Scale: **(A–D)**, 500 μm; **(E–H)**, 500 μm.

Dscaml1$^{GT/GT}$ specimens ($n = 4$ per genotype) (**Figures 2E,F**) indicated a normal spatiotemporal transcription of CTIP2 of the *Dscaml1*$^{GT/GT}$ vTel, and a proper distribution of MSNs in the dorsal striatum with *Dscaml1* LOF.

Based on analysis of striatal markers Ebf1 and CTIP2, no gross abnormalities could be detected in the embryonic development of caudate and putamen nuclei in DSCAML1-deficient mouse brains.

Subpallial Cytoarchitecture and Internal Capsule Tracts Remain Properly Established With *Dscam* or *Dscaml1* Loss of Function

ISH and IHC experiments highlighted the expression of both *Dscam* and *Dscaml1* in both pallial and subpallial domains delineating pathways where IC axonal tracts, including cortical-subcortical connections such as the thalamocortical and corticothalamic axons (TCA and CTA), elongate during embryonic development. These projections extend in the vTel starting as early as E11.5 until ∼E15.5, supported in their navigation by intermediate subpallial targets expressing critical guidance cues, such as the corridor region and the striatum (Auladell et al., 2000; Molnár et al., 2012; Garel and López-Bendito, 2014). The presence of DSCAM and DSCAML1 in these structures therefore hinted at possible roles in the embryonic establishment of forebrain connectivity, likely by contributing to vTel morphogenetic processes.

To first test this hypothesis, the correct formation of subpallial territories allowing TCAs and CTAs to proceed toward the cortex within the IC was investigated by double IHC for the transcription factors Islet1 and Nkx2.1. At

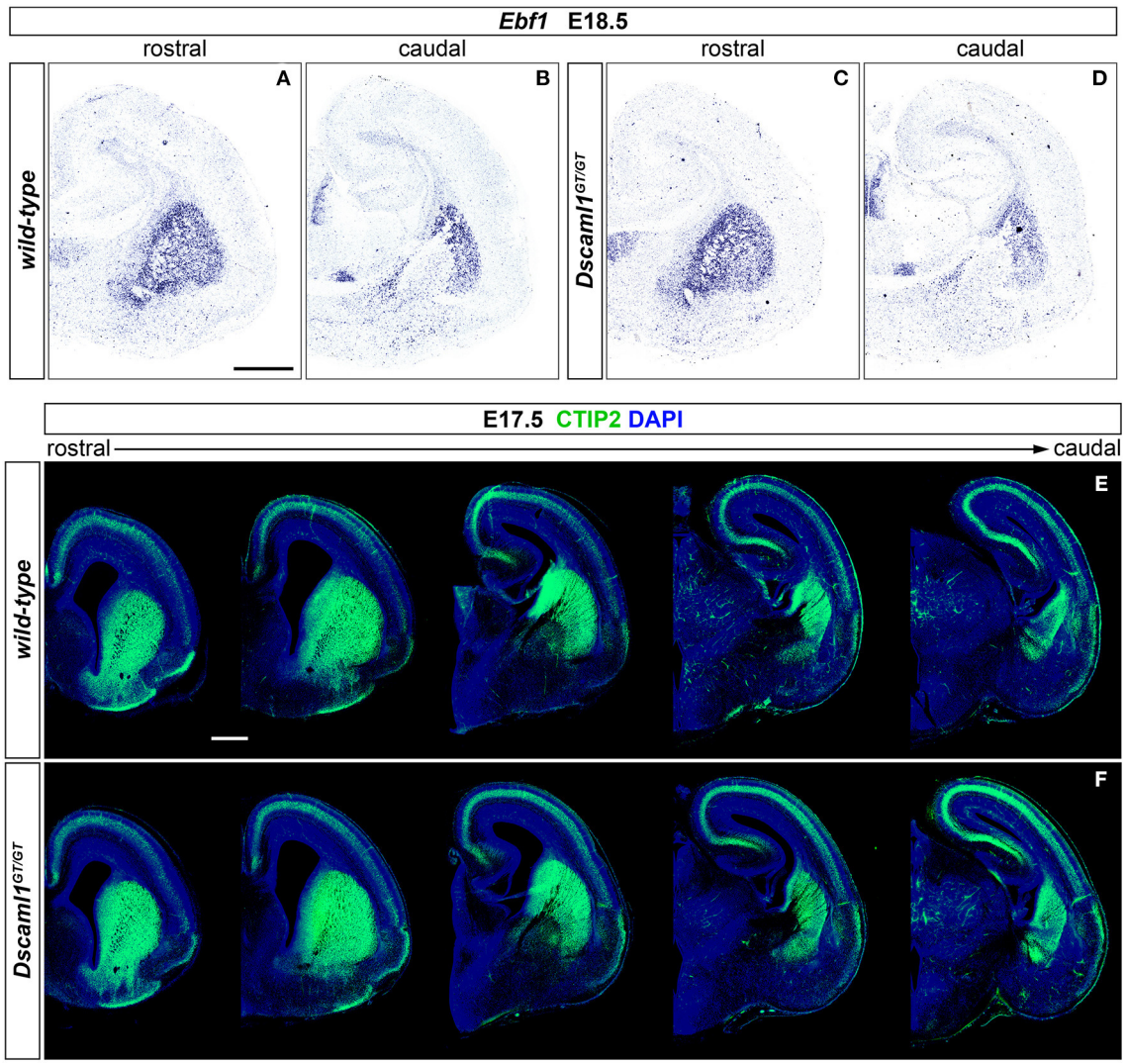

FIGURE 2 | Normal striatal development in embryonic *Dscaml1* null mutant mouse brains. **(A–D)** *Ebf1* mRNA expression, detected by ISH with antisense RNA probes, in coronal sections of wild-type and *Dscaml1*$^{GT/GT}$ E18.5 mouse brains. Spatial expression patterns of *Ebf1*, a marker of striatal postmitotic neurons mostly populating the matrix compartment, detected in Dscaml1GT/GT specimens **(C,D)** are comparable to those observed in wild-type brain tissue **(A,B)** across the rostro-caudal axis. In both cases, *Ebf1*-expressing neurons span the ventral SVZ and mantle regions of the vTel in a compact manner rostrally **(A,C)**, and delineate the corridor region dorsal to the IC caudally **(B,D)**. Moreover, ISH results highlight the preservation of the striatosome/matrix cytoarchitecture in the striatum of *Dscaml1*$^{GT/GT}$ animals (A), as compared to wild-type mice **(C)**. **(E,F)** IHC for the striatal medium spiny neuron marker CTIP2 (green) on coronal E18.5 brain sections reveals similar patterns of expression between wild-type (E) and *Dscaml1*$^{GT/GT}$ embryos **(F)**. As expected in normal development, *Dscaml1*$^{GT/GT}$ CTIP2+ neurons are present at high density in SVZ and mantle subpallial regions; at intermediate rostro-caudal levels, CTIP2+ cells populate the corridor region, while being mostly absent from the GP. Scale: 500 μm.

intermediate stages of TCA and CTA development (E12–E15), these proteins mark two distinct vTel populations, the LGE-derived corridor and striatal cells and the MGE-derived globus pallidus neurons, respectively permissive and repellent to TCA growth (López-Bendito et al., 2006). IHC performed on wild-type, *Dscam*$^{del17/del17}$, and *Dscaml1*$^{GT/GT}$ brain sections at E13.5 ($n = 3$ per genotype) (**Figures 3A–C**), when corridor neurons are expected to have fully migrated from the LGE to the MGE mantle area dorsal to the globus pallidus, demonstrated similar Islet1 and Nkx2.1 immunostaining patterns between wild-type and mutant specimens. At IC level, Islet1 was detected in a narrow band of cells lining a pathway for TCAs between the Nkx2.1+ globus pallidus and the SVZ of the MGE, corresponding to the proper location of corridor neurons at this developmental stage. Additionally, immunostaining could be clearly observed in LGE-derived striatal regions. Thus, Islet1/Nkx2.1 IHCs provided evidence for an appropriate cytoarchitectural development and cellular

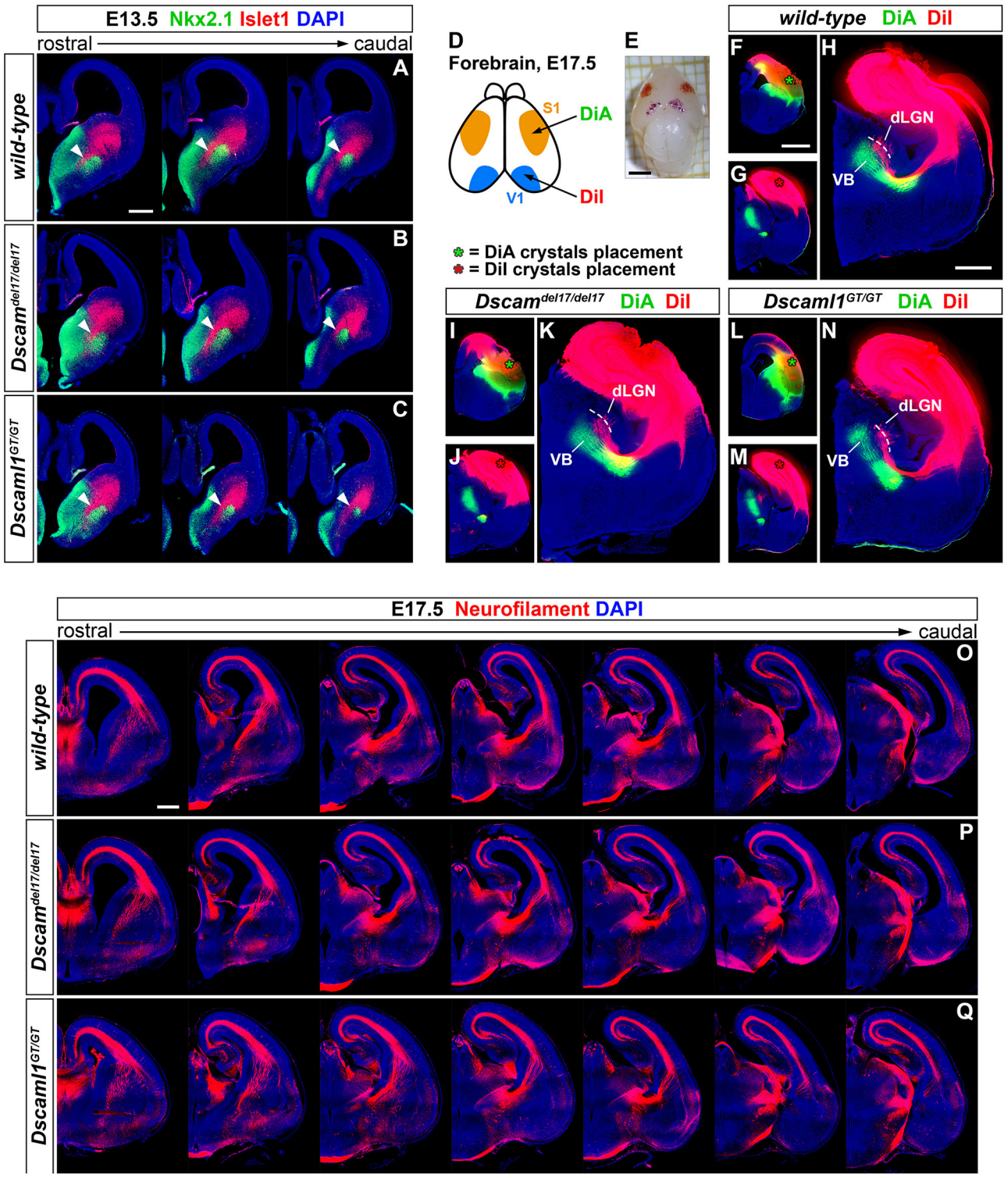

FIGURE 3 | Preserved subpallial cytoarchitecture and IC connectivity in embryonic *Dscam* and *Dscaml1* null mutant mouse brains. **(A–C)** Double IHC for Nkx2.1 (green) and Islet1 (red) on E13.5 coronal brain sections indicates the normal organization, at this stage, of Islet1+ and Nkx2.1+ cell domains in the developing vTel of *Dscam*$^{del17/del17}$ **(B)** and *Dscaml1*$^{GT/GT}$ **(C)** mouse brains, as compared to wild-type specimens **(A)**. LGE-derived, Islet1-expressing neurons can be observed sparsely in the SVZ, and at high density throughout the striatum; Islet1+ cells additionally form a narrow band (i.e., the subpallial corridor; white arrowheads) dorsally adjacent to the GP, which is characterized by cells derived from MGE progenitors and thus expressing Nkx2.1. **(D–N)** Neuroanatomical tracing of TCAs and CTAs with

(Continued)

FIGURE 3 | two distinct carbocyanine dyes from the visual (occipital) and the somatosensory (parietal) cortex areas in E17.5 wild-type, *Dscam*$^{del17/del17}$, and *Dscaml1*$^{GT/GT}$ mouse brains. **(D,E)** Schematic representation of cortical dye placement sites in E17.5 brain hemispheres **(D)**. DiA (green) and DiA (red) crystals are placed respectively within parietal (S1) and occipital (V1) regions of the cortex, as shown in the sample brain illustrated **(E)**. **(F–N)** Insertion of DiA crystals in parietal cortical areas of wild-type **(F)**, *Dscam*$^{del17/del17}$ **(I)**, and *Dscaml1*$^{GT/GT}$ **(L)** brains results in retrograde labeling of thalamic neurons of the ventro-medial VB nucleus comparably across all genotypes examined **(H,K,N)**. Likewise, retrograde tracing from parietal cortical sites in wild-type **(G)**, *Dscam*$^{del17/del17}$ **(J)**, and *Dscaml1*$^{GT/GT}$ **(M)** brains using DiI crystals leads to similar labeling of thalamic neurons in dLGN and dorso-lateral VB in all samples analyzed **(H,K,N)**. **(O–Q)** Immunostaining for neurofilament (NF; red) in coronal E17.5 brain sections confirms the absence of gross abnormalities in IC tracts' development and morphology in either *Dscam*$^{del17/del17}$ **(P)** or *Dscaml1*$^{GT/GT}$ mutant embryos **(Q)**, as compared to wild-type specimens **(O)**. NF+ TCAs and CTAs traverse the diencephalic-telencephalic boundary in a tight bundle, within the IC, spread in a fan-like shape at striatal level, and compactly elongate within the cortex in the IZ after crossing the pallial-subpallial boundary. dLGN, dorsal lateral geniculate nucleus; VB: ventrobasal complex. Scale: **(A–C)**, 200 μm; **(E)**, 2 mm; **(F,G,I,J,L,M)**, 1 mm; **(H,K,N)**, 600 μm.

differentiation of subpallial territories required for TCA and CTA axon guidance.

While proper vTel morphogenesis was observed in *Dscam* and *Dscaml1* knockout mice, it could not be excluded that the function of both corridor and striatal territories might be altered in these animals, and thus still give rise to topographical IC axonal sorting issues. Moreover, the presence of both DSCAM molecules in the developing cortex suggested potential TCA navigation and targeting functions at pallial level. To explore the possibility of reciprocal connectivity alterations between neocortical areas and dorsal thalamic nuclei arising due to defects in TCA/CTA guidance with *Dscam* or *Dscaml1* LOF, targeted axonal tracing experiments were performed in wild-type and mutant mouse brains at late embryonic development stages, at which major axonal tracts have been established. Mixed retrograde and anterograde double-tracing experiments were carried out by placing crystals of the carbocyanine dyes DiI and DiA in, respectively, occipital and parietal cortical areas of *Dscam*$^{del17/del17}$, *Dscaml1*$^{GT/GT}$, and wild-type E17.5 mouse brains ($n = 3$ per genotype) (**Figures 3D–N**). Neuroanatomical tracings indicated that connectivity between different TCA subsets and their cognate cortical domains is preserved in both *Dscam*$^{del17/del17}$ and *Dscaml1*$^{GT/GT}$ mice. Like in wild-type mouse brains, DiA crystals placed in the parietal cortex of knockout specimens, at the level of somatosensory areas, led to the back-labeling of a medial ventrobasal complex (VB) cell population. Furthermore, DiI crystals placed in the occipital cortex, diffusing within visual and auditory processing regions, retrogradely traced somas in a dorsal VB neuronal subset and within the dorsal lateral geniculate nucleus in both wild-type and mutant brains.

To better investigate the formation and spatial organization of IC axonal tracts in mouse brains lacking *Dscam* or *Dscaml1*, IHC for the 165 kDa neurofilament (NF) subunit, a pan-axonal marker, was additionally performed on *Dscam*$^{del17/del17}$, *Dscaml1*$^{GT/GT}$, and wild-type E17.5 brain sections ($n = 3$ per genotype) (**Figures 3O–Q**). Consistently with previous findings, TCAs and CTAs were observed to correctly navigate the vTel in both *Dscam* and *Dscaml1* knockout forebrains, traversing the medial subpallium in a tight axonal bundle (i.e., the IC), while extending in a fan-like shape in the lateral subpallium. At cortical level, thalamocortical and corticofugal projections elongated in a compact tract within the IZ, as normally expected. Moreover, the examination of striatonigral and nigrostriatal connections, which also express NF (Uemura et al., 2007) and elongate within the IC, revealed a preserved spatial navigation of other IC projections with *Dscam* and *Dscaml1* LOF.

Interneuron Migration Is Grossly Preserved in Developing Brains Lacking *Dscam* or *Dscaml1*

ISH and X-gal experiments highlighted the presence of DSCAM and DSCAML1 in forebrain regions corresponding to neural territories where immature cortical interneurons (INs) originate (e.g., the subpallial SVZ and mantle) or migrate into (e.g., the cortical MZ and IZ) during embryonic development. To investigate whether *Dscam* or *Dscaml1* LOF affects the navigation of GABAergic neurons toward their cortical targets, INs were first studied in *Dscam*$^{del17/del17}$ and *Dscaml1*$^{GT/GT}$ vs. wild-type embryonic brains via ISH with DIG-labeled antisense *Gad1* probes. ISH experiments were performed at E13.5 in *Dscam*$^{del17/del17}$ brains, and at E16.5 in *Dscaml1*$^{GT/GT}$ brains, based on X-gal staining results suggesting the absence of DSCAML1 transcription in most subpallial and cortical areas around E13.5. ISH results indicated that the absence of DSCAM does not affect cortical IN generation or population of the cortex by cortical IN: at E13.5, *Gad1*-expressing cells were abundantly found as normally expected in post-mitotic regions of the subpallium, and from this area MZ and SVZ/IZ streams of migrating INs could be clearly detected in both *Dscam*$^{del17/del17}$ and wild-type coronal brain sections (**Supplementary Figures 2A,B**). These streams could be observed to extend tangentially within the developing cortex to a comparable degree and density in *Dscam*$^{del17/del17}$ and wild-type specimens ($n = 3$ per genotype). At E16.5, loss of *Dscaml1* also did not seem to impair IN entry in the dorsal pallium (**Figures 4A–D**), as *Gad1*-expressing cells were detected throughout post-mitotic vTel territories, and delineated MZ and SVZ/IZ streams of INs extending uniformly from the subpallium to the cortical hem. Within the cortex, GABAergic neurons were found at high densities within the aforementioned streams, and in addition more sparsely across the cortical radius, in particular within the CP layer.

As *Dscaml1* expression occurs robustly in the vTel SVZ (a progenitor zone for cortical INs) throughout later stages of embryonic development, and is also maintained at the level of both cortical IN streams, GABAergic cell migration was further investigated at a peri-natal developmental time-point, E18.5, in

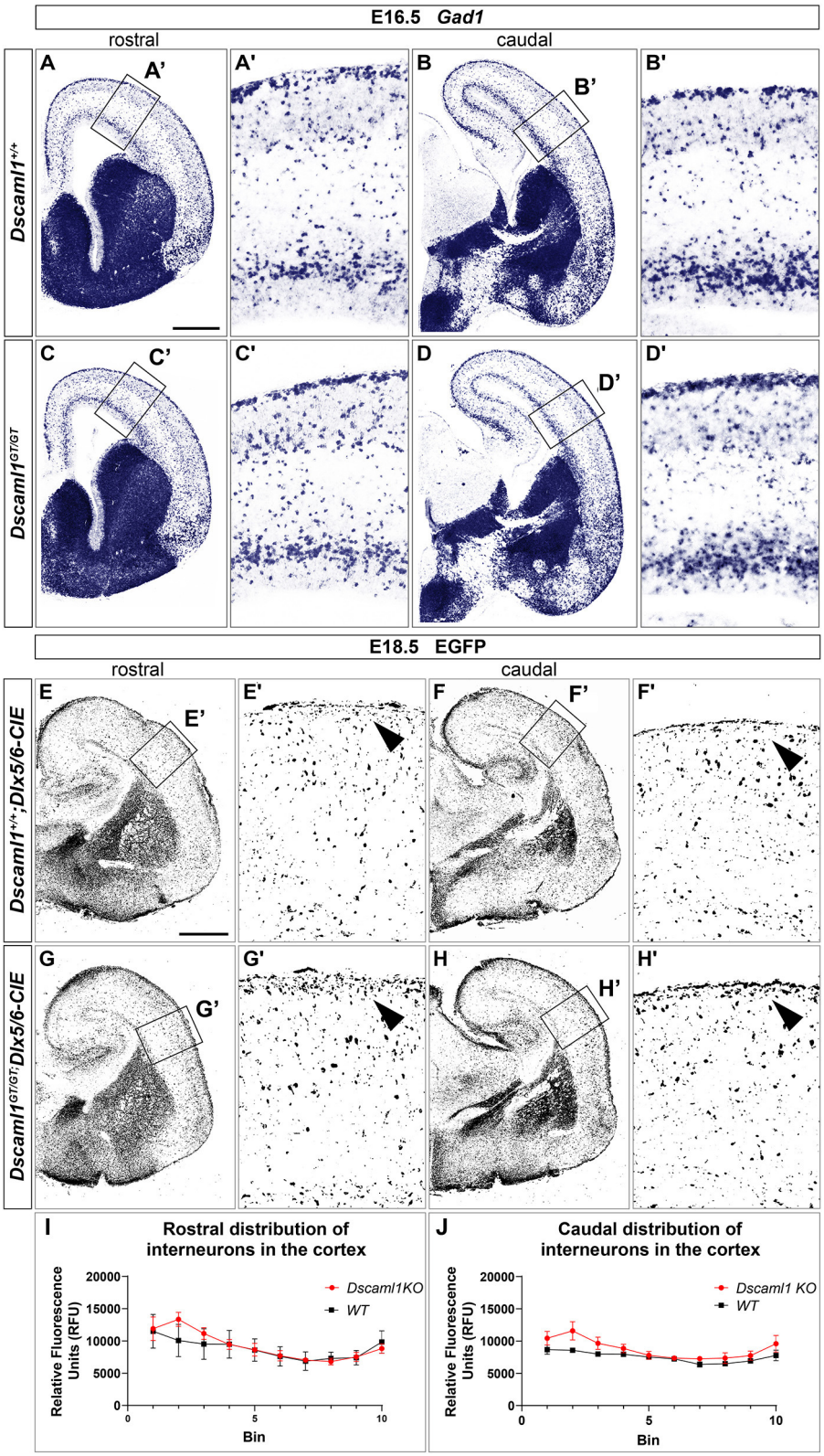

FIGURE 4 | Interneuron migration in *Dscaml1* null mutant mouse brains. **(A-D)** *Gad1* mRNA expression, detected by ISH with antisense RNA probes, in coronal sections of wild-type **(A,B)** and *Dscaml1*$^{GT/GT}$ E16.5 **(C,D)** mouse brains. In both cases, *Gad1*-expressing neurons can be observed from SVZ to pial surface regions

(Continued)

FIGURE 4 | of the vTel; at the pallial-subpallial boundary, labeled GABAergic cortical interneurons tangentially invade the developing cortex in two main streams within the SVZ/IZ and MZ. At this developmental stage, cortical INs spread radially within the cortex toward their presumptive target layers, and can be found across all cortical laminae, particularly in the CP. *Gad1*-expressing cell densities and distribution patterns within the cortex are comparable between genotypes **(A'–D')**. **(E–H)** IHC using anti-EGFP antibodies on coronal sections from *Dscaml1*$^{+/+}$; *Dlx5/6-CIE* and *Dscaml1*$^{GT/GT}$; *Dlx5/6-CIE* E18.5 brains. EGFP-labeled cortical interneurons, derived from subpallial territories expressing *Dlx5/6,* similarly distribute in the cortex of wild-type **(E,F)** and *Dscaml1*$^{GT/GT}$ mutant specimens **(G,H)** in both tangential and radial directions. An accumulation of *Dlx5/6*-labeled neurons is observable at MZ level in *Dscaml1*$^{GT/GT}$ brain sections compared to wild-type sections [**(E'–H')**, black arrowheads]. **(I,J)** Quantification of interneuron distribution on coronal sections from *Dscaml1*$^{+/+}$*; Dlx5/6-CIE* and *Dscaml1*$^{GT/GT}$*; Dlx5/6-CIE* E18.5 brains. In both rostral **(I)** and caudal **(J)** sections, EGFP-labeled interneuron accumulation is confined to Bin 2 (with Bin 1 and 10 corresponding, respectively, to the most superficial and deeper radial bins the cortex is divided into) in Dscaml1GT/GT brains compared to wild-type brains. Scale: **(A–D)**, 500 μm; **(E–H)**, 500 μm.

Dscaml1$^{GT/GT}$*; Dlx5/6-Cre-IRES-EGFP* (*Dscaml1*$^{GT/GT}$; *Dlx5/6-CIE)* and *Dscaml1*$^{+/+}$; *Dlx5/6-Cre-IRES-EGFP (Dscaml1*$^{+/+}$*; Dlx5/6-CIE)* mutant mice, in which cortical INs are endogenously labeled by EGFP. IHC for EGFP on coronal brain sections (**Figures 4E–H**) confirmed previous ISH findings at E16.5: in *Dscaml1* null mutant specimens, GABAergic INs reached the cortex, and were distributed similarly to the control within the cortical field. However, compared to wild-type, INs were found to accumulate more densely within the MZ in *Dscaml1*$^{GT/GT}$ mutant brains at E18.5 (**Figures 4E–H**, arrowheads, quantification in **Figures 4I,J**). Thus, findings overall indicated that *Dscaml1* LOF might subtly affect the distribution of cortical INs close to the marginal zone at late embryonic developmental stages.

Embryonic Cortical Development and Lamination Are Unaffected by *Dscam* or *Dscaml1* Loss of Function

Expression data suggested that DSCAM and DSCAML1 are consistently present, albeit in different patterns, within the developing murine neocortex, and previous studies reported changes in cortical migration and thickness with *Dscam* or *Dscaml1* transcriptional suppression. These phenotypes were observed at early post-natal stages, implicating embryonic processes in the emergence of such defects. Thus, the development of cortical layers in *Dscam*^{del17} and *Dscaml1*^{GT} mutant mice was investigated at E17.5 *via* immunostaining with antibodies against the transcription factors Tbr1, Ctip2 (Bcl11b), and Satb2, markers, respectively of subplate and early born, early born, and late born cortical pyramidal neurons (Bulfone et al., 1995; Hevner et al., 2001; Leid et al., 2004; Arlotta et al., 2005; Britanova et al., 2008; Fishell and Hanashima, 2008) (**Figures 5A–D**). In parallel, cortical thickness was measured in all specimens to detect potential reductions in radial expansion. However, comparisons across *Dscam*$^{del1/del17}$, *Dscaml1*$^{GT/GT}$, and wild type mutant brains ($n = 3$ per genotype, three sections across the rostro-caudal axis per brain) did not unveil any significant differences in terms of total number of immunostained cells per 100-μm-wide tissue sector (**Figure 5E**) [$F_{\text{genotype}(2,6)} = 0.126$, $p = 0.884$], number of either Satb2+, Ctip2+, or Tbr1+ neurons per 100-μm-wide tissue sector (**Figure 5F**) [$F_{\text{genotype}(2,6)} = 0.301$, $p = 0.750$; $F_{\text{genotype}(2,6)} = 0.070$, $p = 0.933$; $F_{\text{genotype}(2,6)} = 0.069$, $p = 0.934$], and cortical thickness (**Figure 5G**) ($F_{\text{genotype}(2,6)} = 0.008$, $p = 0.992$). Moreover, statistical analysis of these measures at either anterior, intermediate, or posterior positions on the rostro-caudal axis yielded similar results, with a non-significant effect of genotype on cortical thickness (**Supplementary Figure 3A**) [$F_{\text{position} \times \text{genotype}(4,12)} = 0.300$, $p = 0.805$], total number of immunostained cells *per sector* (not shown) [$F_{\text{position} \times \text{genotype}(4,12)} = 0.596$, $p = 0.673$], and number of Satb2+, Ctip2+, or Tbr1+ cells *per sector* (**Supplementary Figures 3B–D**) ($F_{\text{position} \times \text{genotype}(4,12)} = 0.071$, $p = 0.990$; $F_{\text{position} \times \text{genotype}(4,12)} = 1.226$, $p = 0.351$; $F_{\text{position} \times \text{genotype}(4,12)} = 1.209$, $p = 0.357$) at each rostro-caudal level. Taken together, these results point to a preserved overall development and lamination of the embryonic murine cortex in the absence of DSCAM or DSCAML1.

In summary, despite clear expression of DSCAM or DSCAML1 in cortical and subcortical areas, constitutive loss-of-function on the C57BL/6J background did not strongly affect cortical lamination, interneuron migration nor corticothalamic circuitry formation.

Dscam or *Dscaml1* Gain of Function Affects the Embryonic Migration of Cortical Projection Neurons *in vivo*

Modeling DSCAM and DSCAML1 CNS overexpression in a mammalian species has the potential to unravel how disorders such as Down syndrome, in which DSCAM levels are known to be elevated in the fetal brain, and distal trisomy 11q, which involves the duplication of a chromosomal region including DSCAML1, develop in humans. Given the reduced cell number and anomalous neuronal organization observed within specific neocortex regions and layers of DS brains already at mid-to-late gestational stages (Colon, 1972; Becker et al., 1991; Golden and Hyman, 1994; Haydar and Reeves, 2012; Lott, 2012), we sought to examine the effects of *Dscam* and *Dscaml1* gain of function (GOF) within the developing mammalian neocortex. In particular, we focused on the process of radial migration of pyramidal neurons, since knockout/knockdown experiments in mouse brains pointed to possible roles of DSCAM and DSCAML1 in this context (Maynard and Stein, 2012; Zhang et al., 2015).

GOF was thus modeled in the murine embryonic cortex *in vivo via in utero* electroporation (Tabata and Nakajima, 2001) of C-terminally-tagged DSCAM/DSCAML1 and tdTomato expression vectors (pCAGGS-DSCAM-EYFP-HA-IRES-tdTomato, pCAGGS-DSCAML1-EYFP-HA-IRES-tdTomato), while constructs driving the production of EYFP-HA and TdTomato were used in control specimens (pCAGGS-EYFP-HA-IRES-tdTomato). All constructs were pre-tested *in vitro* in mouse Neuro 2a cells, which were transfected and cultured

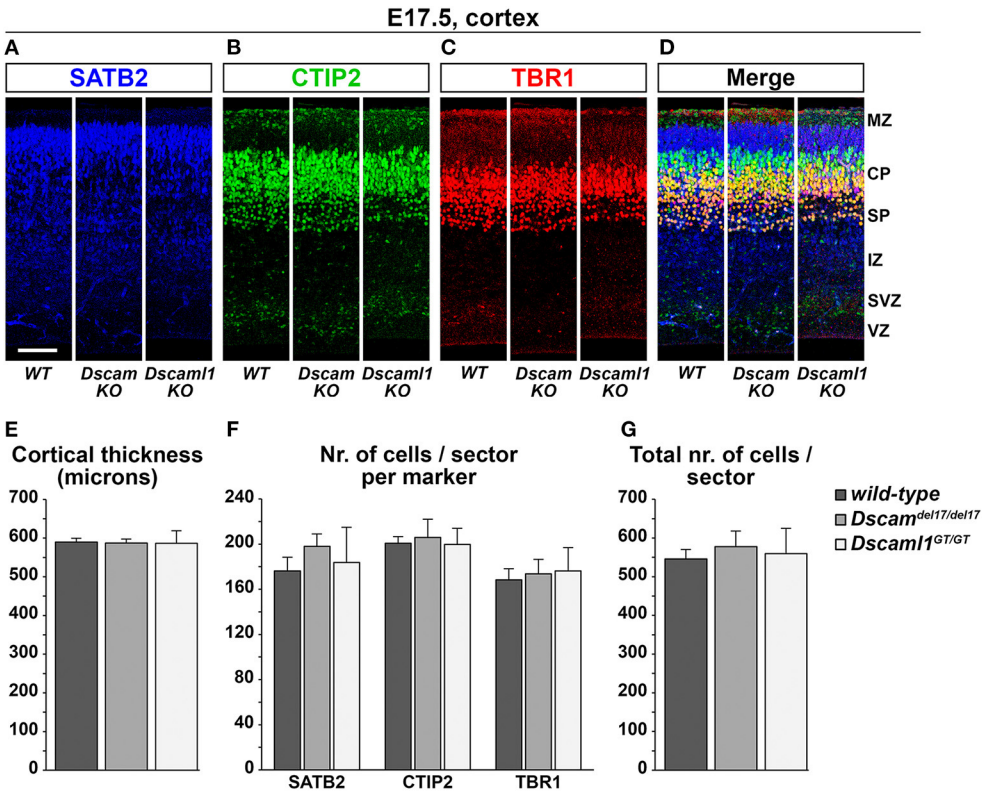

FIGURE 5 | Normal cortical development and lamination in embryonic *Dscam* and *Dscaml1* null mutant mouse brains. **(A–D)** Triple immunostaining for the cortical markers SATB2 [upper layer neurons, blue; **(A)**], CTIP2 [deep layer neurons, green; **(B)**], and TBR1 [deep layer and subplate neurons, red; **(C)**] in coronal sections of wild-type (*WT*), *Dscaml1*$^{del17/del17}$ (*Dscam KO*), and *Dscaml1*$^{GT/GT}$ (*Dscaml1 KO*) E17.5 mouse brains. Panels represent radial sectors of the dorso-lateral cortex. **(E)** Histogram depicting average cortical thickness values measured across the rostro-caudal axis in wild-type, *Dscaml1*$^{del17/del17}$, and *Dscaml1*$^{GT/GT}$ E17.5 coronal mouse brain sections. No significant differences are detected across genotypes ($n = 3$ brains/group, mixed ANOVA test). **(F)** Histogram representing average numbers of SATB2+, CTIP2+, and TBR1+ cell measured in 100 μm-wide radial sectors of wild-type, *Dscaml1*$^{del17/del17}$, and *Dscaml1*$^{GT/GT}$ E17.5 coronal mouse brain sections across the rostro-caudal axis. No significant differences are detected across genotypes ($n = 3$ brains/group, mixed ANOVA test). **(G)** Histogram illustrating average total numbers of SATB2, CTIP2, and TBR1 immunolabeled cells measured in 100 μm radial sectors of wild-type, *Dscaml1*$^{del17/del17}$, and *Dscaml1*$^{GT/GT}$ E17.5 coronal mouse brain sections across the rostro-caudal axis. No significant differences are detected across genotypes ($n = 3$ brains/group, mixed ANOVA test). All graphs represent mean ± S.E.M values. CP, cortical plate; IZ: intermediate zone; MZ: marginal zone; SP: subplate; SVZ: subventricular zone; VZ: ventricular zone. Scale: **(A–D)**, 100 μm.

for 2 days before imaging and protein extraction. Inspection of cellular resolution confocal images of successfully transfected (i.e., tdTomato-labeled) cells confirmed the synthesis and correct localization at cytoplasmic and membrane level of EYFP-tagged proteins (**Supplementary Figures 4A–D**); further investigation via Western Blot demonstrated the production of full length, EYFP- and HA-tagged DSCAM and DSCAML1 in the transfected Neuro 2a cells (**Supplementary Figure 4E**). *In utero* electroporation was performed at E14.5, leading to targeting of layer II/III and IV neurons (Takahashi et al., 1999; Taniguchi et al., 2012). Layer II/III neurons were reported to be the most affected around birth by DSCAM and DSCAML1 loss-of-function in the work of Maynard and Stein (2012) and Zhang et al. (2015), and layer II/III and IV neurons also show abnormalities in DS (Ross et al., 1984; Wisniewski and Rabe, 1986; Lott, 2012). Migration of tdTomato+ neurons was assessed in dorso-lateral cortical regions 4 days post-electroporation, at E18.5 (**Figure 6A**), once electroporated neurons had reached the uppermost regions of the CP in control conditions (**Figures 6B,E**). To verify the production and localization of the tagged DSCAM and DSCAML1 proteins in the transfected projection neurons, immunolabeling of EYFP in tdTomato+ neurons was examined in confocal images at cellular resolution. This analysis demonstrated the synthesis of EYFP-labeled proteins with both tagged DSCAM and DSCAML1 expression construct transfection, and an accumulation of these two molecules within cytoplasmic and plasma membrane compartments of the immature neurons' leading processes, particularly in soma-proximal areas (**Supplementary Figures 4F,G**).

Compared to EYFP-HA and tdTomato expressing neurons, which mostly accumulated in upper CP layers, cells expressing DSCAM-EYFP-HA or DSCAML1-EYFP-HA with tdTomato seemed to distribute across the cortical radial extension more sparsely (**Figures 6B–G**). To quantify this variation in distribution of tdTomato+ cells, tdTomato fluorescence

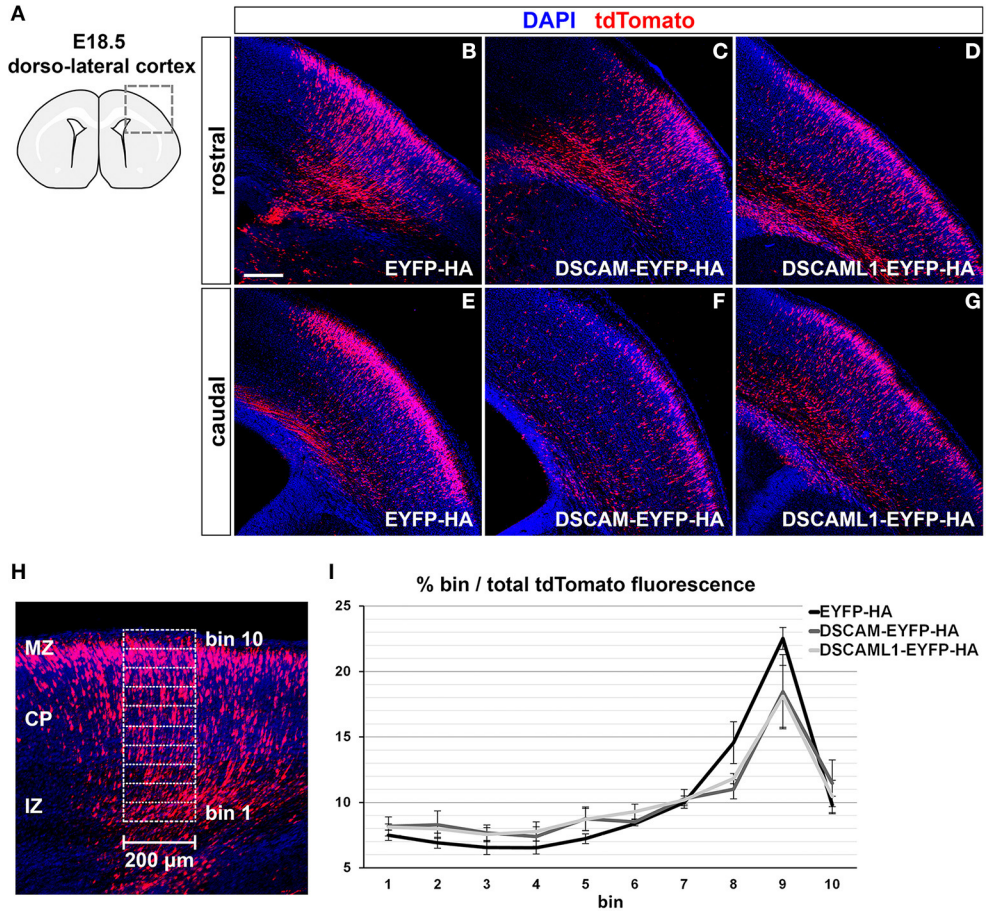

FIGURE 6 | Effect of DSCAM and DSCAML1 *in vivo* gain of function in cortical projection neurons transfected with EYFP- and HA-tag labeled protein expression constructs. **(A–G)** Radial migration of cortical neuron electroporated with EYFP-HA (control) **(B,E)**, DSCAM-EYFP-HA **(C,F)**, or DSCAML1-EYFP-HA **(D,G)** expression constructs at E14.5. Electroporated cells are detected by co-expression of tdTomato in coronal sections of electroporated E18.5 mouse brains. Section are immunostained for tdTomato before analysis for signal enhancement. **(A)** Schematic representation of the dorso-lateral cortical region targeted via IUEP and represented in panels **(B–G)**, at two positions of the rostro-caudal axis. Radial distributions of tdTomato+ cells show mild differences between control and DSCAM/DSCAML1 expression construct-transfected specimens: tdTomato+ cells accumulate at the uppermost cortical layers in control conditions, whereas DSCAM and DSCAML1-overexpressing neurons spread more evenly across cortical zones. **(H,I)** Analysis of electroporated neurons' migration in E18.5 coronal brain sections. **(H)** tdTomato fluorescence intensity after immunostaining is measured in 200 μm-wide columns from the upper IZ to the MZ, subdivided radially in ten equally-sized bins. **(I)** Graph depicting average tdTomato fluorescence intensity levels per radial bin, expressed as percentages of total fluorescence intensity quantified over all ten bins, measured from brains electroporated with EYFP-HA (control), DSCAM-EYFP-HA, or DSCAML1-EYFP-HA expression constructs. A comparison of fluorescence distribution profiles highlights subtle, non-statistically significant differences in radial migration between control and tagged DSCAM/DSCAML1 expression conditions (n = 5–6 brains/group, mixed ANOVA test). Graph data represent mean ± S.E.M values. CP, cortical plate; IZ: intermediate zone; MZ: marginal zone. Scale: **(B–G)**, 200 μm.

was measured in columnar sectors, divided radially in 10 equal bins, of the electroporated cortices from the upper IZ to the MZ, and expressed for each bin as a percentage of total fluorescence (EYFP-HA group: n = 6; DSCAM-EYFP-HA group: n = 5; DSCAML1-EYFP-HA group: n = 5) (**Figure 6H**). While overall the tdTomato fluorescence distribution profiles across bins reflected the observed differences between control and DSCAM/DSCAML1 overexpression vector-transfected neurons (**Figure 6I**), statistical analysis indicated these profiles to non-significantly differ across electroporation groups over all bins ($F_{\text{expression construct}(2,13)} = 0.518$, $p = 0.608$; $F_{\text{bin}\times\text{expression construct}(4.222,27.443)} = 1.423$, $p = 0.252$). Our data suggest that DSCAML1 and DSCAM overexpression only has a mild effect on radial migration of projection neurons in the cortex during late embryonic development.

Dscam or *Dscaml1* Gain of Function Impairs Migration and Process Development of Cortical Interneurons *in vitro*

In DS, defects in cortical layers II and III are associated to a striking reduction in the number of small granular cells, likely related to the GABAergic aspinous stellate cell type (Ross et al., 1984). Indeed, GABA neurotransmitter levels are

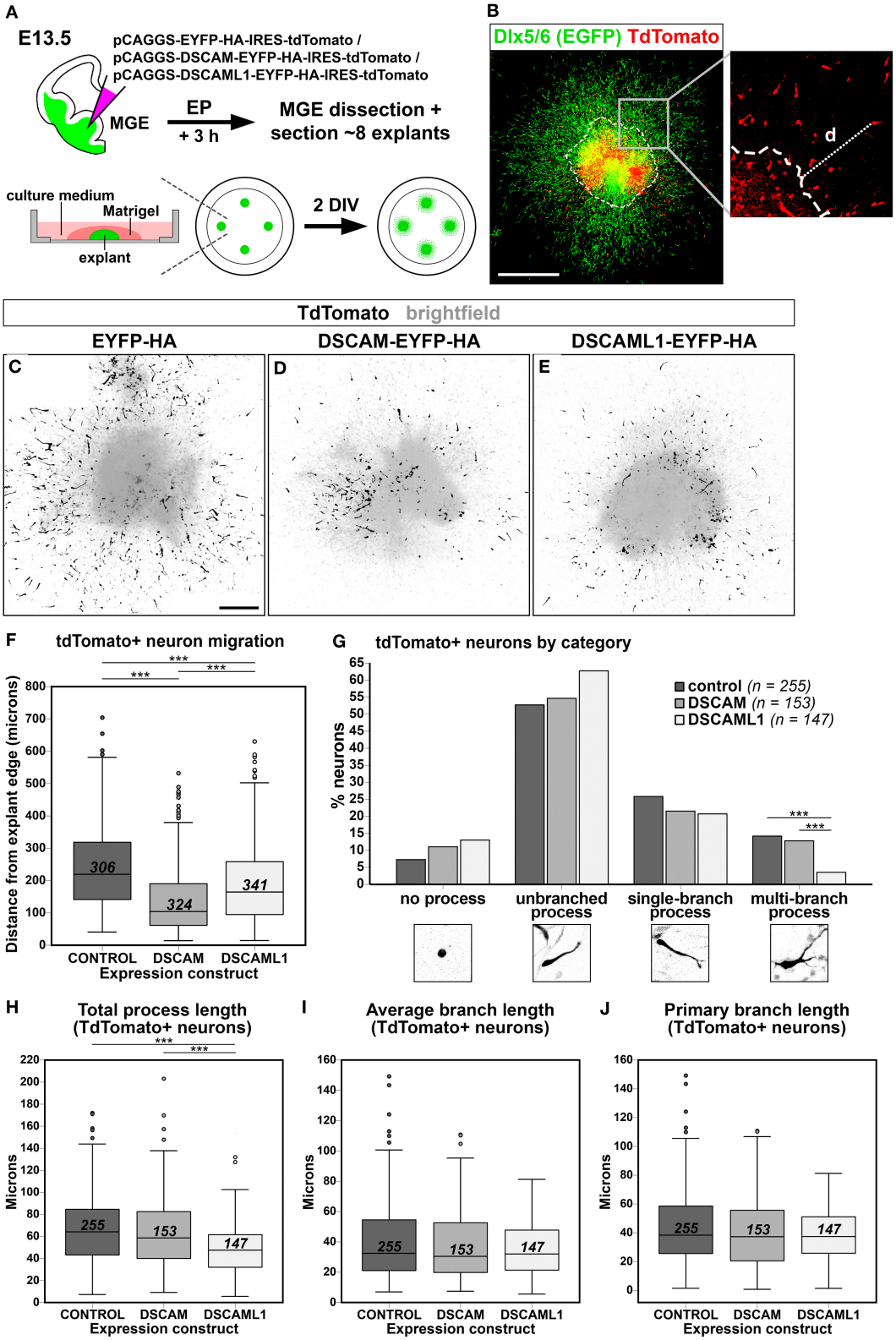

FIGURE 7 | Effect of DSCAM and DSCAML1 *in vitro* gain of function in MGE-derived interneurons transfected with EYFP- and HA-tag labeled protein expression constructs. **(A)** Schematic illustration of the *ex vivo* MGE explant electroporation model used to study DSCAM and DSCAML1 overexpression effects on IN migration.

(Continued)

FIGURE 7 | E13.5 *Dlx5/6-Cre-IRES-EGFP* whole brains maintained in culture medium are injected at MGE level with expression constructs, electroporated, and dissected 3 h later to obtain MGE explants. The explants are embedded in Matrigel, supplemented with culture medium, and cultured for 48 h before analysis. **(B)** Analysis of IN migration in *Dlx5/6-Cre-IRES-EGFP* MGE explants 48 h post-electroporation with constructs driving co-expression of tagged proteins and tdTomato. The minimum distance from explant edge "d" is used as an indirect measure of migration capabilities of transfected (tdTomato+, red) INs, identifiable by EGFP expression (green). **(C–E)** Example images of cultured explants 48 h after electroporation of EYFP-HA (control) **(C)**, DSCAM-EYFP-HA **(D)**, or DSCAML1-EYFP-HA **(E)** expression constructs. **(F)** Boxplot chart depicting tdTomato+ neuron-associated "distance from explant edge" values measured in EYFP-HA (control), DSCAM-EYFP-HA (DSCAM), or DSCAML1-EYFP-HA (DSCAML1) expression constructs-transfected MGE explants. Comparisons across experimental groups reveal significantly shorter distances measured in either DSCAM or DSCAML1 expression construct-transfected vs. control construct-transfected explants, and in DSCAM vs. DSCAML1 expression construct-transfected explants (Kruskal-Wallis test with Dunn-Bonferroni pairwise comparisons, $^{***}p < 0.001$). **(G)** Histogram representing percentages of INs classified in one of four morphological categories, calculated on total number of INs analyzed per experimental group, in EYFP-HA (control), DSCAM-EYFP-HA (DSCAM), or DSCAML1-EYFP-HA (DSCAML1) expression constructs-transfected MGE explants. IN categories based on the type of leading process presented are illustrated on the x axis. Comparisons across experimental groups highlight a significantly lower percentage of cells possessing multi-branched processes in DSCAML1 expression construct-transfected vs. control or DSCAM expression construct-transfected explants (Pearson's Chi-square test and residuals analysis with Bonferroni correction; $^{***}p < 0.001$). **(H–J)** Boxplot chart illustrating tdTomato+ neuron-associated "total process length" **(H)**, "average branch length" **(I)**, and "primary branch length" **(J)** values measured in EYFP-HA (control), DSCAM-EYFP-HA (DSCAM), or DSCAML1-EYFP-HA (DSCAML1) expression constructs-transfected MGE explants. While no significant differences in average and primary branch length variables are detected across experimental groups, significantly shorter total process length are measured in DSCAML1 expression construct-transfected vs. either control or DSCAM expression construct-transfected explants (Kruskal-Wallis test with Dunn-Bonferroni pairwise comparisons, $^{***}p < 0.001$). In boxplot charts, numbers within boxes represent the total number of cells analyzed per experimental group; horizontal black lines within boxes denote median values; box edges indicate the 25th and 75th percentile of each group's distribution of values; whiskers represent highest and lowest values within 1.5 interquartile range measures per group; dots denote outliers. DIV, days *in vitro*. Scale: **(B)**, 500 μm; **(C–E)**, 200 μm.

decreased in DS brains at fetal developmental stages (Whittle et al., 2007). Moreover, in *Dlx1/2* double knockout mutant mice, in which immature INs migrating to the neocortex show an abnormal morphology in correspondence to an impaired tangential migration ability, embryonic subpallial *Dscam* mRNA levels are increased (Cobos et al., 2007). These findings suggest the possibility that overexpression of DSCAM proteins during embryonic development might impact the tangential migration or laminar positioning of cortical INs. We therefore modeled *Dscam* and *Dscaml1* GOF in mouse INs via *ex vivo* E13.5 MGE-targeted electroporation of C-terminally tagged DSCAM/DSCAML1 expression constructs (see section Dscam or Dscaml1 Gain of Function Affects the Embryonic Migration of Cortical Projection Neurons *in vivo*); subsequently, transfected IN migration was examined in Matrigel-embedded MGE explant cultures maintained *in vitro* for 2 days (**Figure 7A**). To easily identify MGE-derived INs, electroporation was performed in brains from *Dlx5/6-Cre-IRES-EGFP* mutant embryos, in which all postmitotic neurons express EGFP throughout embryonic development (Stenman et al., 2003). Migration was quantified by measuring distances traveled by transfected (i.e., tdTomato+) neurons from the edge of the explants (**Figure 7B**) in EYFP-HA (control; $n = 306$), DSCAM-EYFP-HA (DSCAM; $n = 324$), and DSCAML1-EYFP-HA (DSCAML1; $n = 341$) expression conditions. Example images of resulting explants are depicted in **Figures 7C–E**.

Statistical analysis indicated an overall difference in "distance from explant's edge" measures across treatment groups [$H_{(2)} = 130.194$, $P < 0.001$; mean distance rank scores: control = 612.49; DSCAM = 357,75; DSCAML1 = 488,96]. Pairwise *post-hoc* tests revealed that both overexpression of tagged DSCAM and DSCAML1 induced a significant reduction in IN spreading ($p < 0.001$ vs. control for both comparisons), with DSCAM overexpression leading to a more severe phenotype than DSCAML1 overexpression ($p < 0.001$) (**Figure 7F**).

Directed IN migration relies on the extension of a leading process (LP), which undergoes branching as the neuron explores the surrounding environment, paired with nucleokinesis; in turn, these processes are dependent on dynamic, extracellular cue-modulated microtubule and actin cytoskeleton remodeling events (Métin et al., 2006; Guo and Anton, 2014). Proper morphological development of the LP is thus essential for correct IN navigation, and interestingly several studies have provided a link between DSCAM-dependent intracellular signaling pathways or interactors and molecular networks controlling cytoskeletal remodeling (Liu et al., 2009; Purohit et al., 2012; Kamiyama et al., 2015; Okumura et al., 2015; Pérez-Núñez et al., 2016; Huo et al., 2018; Sachse et al., 2019). Thus, to gain insight into potential mechanisms underlying the migration defect observed in the explant EP assay previously described, we performed a morphological analysis of MGE-derived INs transfected with either control, tagged DSCAM, or tagged DSCAML1 expression constructs ($n = 255, 153, 147$). In particular, total LP length, average branch length, and primary branch length were measured for all neurons (**Figures 7H–J**), and each cell was categorized according to the morphology of the LP as "no process," "unbranched process," "single-branch process," and "multi-branch process" (**Figure 7G**). This analysis overall revealed a significant effect of tagged DSCAML1 overexpression on IN morphology *ex vivo*. First, compared to control and DSCAM expression construct-transfected neurons, a significantly smaller percentage of INs transfected with DSCAML1 expression constructs represented "multi-branch process" cells [$\chi^2_{(6,N=616)} = 12.895757$, $p < 0.004$]. Secondly, total LP length was found to be affected by the type of construct transfected [$H_{(2)} = 40.467$, $P < 0.001$]; *post-hoc* pairwise comparisons revealed this measure to be significantly reduced in DSCAML1 vs. control or DSCAM expression construct-transfected INs ($p < 0.001$), whereas no differences could be detected between DSCAM and control transfection groups ($p = 0.380$). However, no effect of expression

construct transfected was observed on either average branch length [$H_{(2)} = 1.436$, $P = 0.488$] or primary branch length [$H_{(2)} = 1.286$, $P = 0.526$] measures.

In summary, while increased DSCAM levels significantly reduced IN spreading, we could not link this phenotype to obvious neurite growth or morphology defects. In contrast, we found the impaired migration resulting from DSCAML1 overexpression to be associated with a lower IN neurite branching complexity, suggesting different, yet related mechanisms of action of these molecules in INs.

DISCUSSION

This study first aimed to survey potential effects of generalized loss of DSCAM or DSCAML1 on cortical development, including lamination, patterning, and connectivity. The impact of such a loss seemed to be rather minimal. The absence of significant defects in forebrain patterning in DSCAM and DSCAML1 knockout mice was quite unexpected. Studying the cortical morphology of *Dscam*^{del17} mice, Maynard and Stein observed that homozygous mutant neonates exhibit an early post-natal (P1 to P10) reduction in cortical thickness attributable to a thinning of Cux1+ layers II and III, suggesting a specific role of DSCAM in the development of pyramidal neurons born around and after E14. This cortical phenotype was not accompanied by either an embryonic decrease in cell proliferation, tested via the administration of a BrdU pulse at E16.5, or an increased programmed cell death rate, assessed with a TUNEL assay at E16.5 and P1 (Maynard and Stein, 2012). As this reduced thickness was transient, it might have been caused by a delayed radial migration of the upper cortical layers. In a study where cortical expression of either *Dscam* or *Dscaml1* was suppressed *in vivo* via RNA interference, knockdown at E15.5 was found to impair radial migration of projection neurons at early post-natal stages, leading to a partial mispositioning of presumptive layer II/III neurons in layers IV/V observable for more than 2 weeks after birth (Zhang et al., 2015). Our data however could not substantiate a reduced thickness of upper cortical layers in the absence of DSCAM/DSCAML1, nor a significant defect in radial migration of late-born cortical neurons upon DSCAM/DSCAML1 overproduction, ruling out a major early role for DSCAMs in this process. The seeming discrepancy with the study of Maynard and Stein that detected reduced upper layer thickness at P1 might root in the different mouse background used, or in a specific defect in neurons populating the upper layers between E17.5 and P1. Whether DSCAMs might have a role in radial migration of a specific subpopulation of neurons, labeled by Cux1 but not Satb2 (Leone et al., 2015), needs further investigation. Considering the subtlety of the radial migration-related phenotypes examined, it is also possible that significant effects of a neuronal positioning delay/impairment due to *Dscam*/*Dscaml1* acute dosage variations embryonically might be detectable only at later developmental stages. Data relative to our IUEP-induced DSCAM/DSCAML1 overexpression experiments in particular suggest small, but evident changes in cortical migration dynamics being present 4 days after transfection with expression constructs. The high variability in results obtained via targeted electroporation, coupled with the limited time-frame within which additional DSCAM/DSCAML1 molecules were active, could have masked the impact of our genetic manipulations. Thus, it would be important to analyze the migration of transfected neurons during the first post-natal week in future research.

In the study by Zhang et al. (2015), acute downregulation of DSCAM or DSCAML1 during embryonic development also negatively affected callosal axonal outgrowth. Our findings indicate that loss of DSCAMs does not affect development of thalamocortical connectivity, suggesting that the negative impact of *Dscam* LOF on callosal connectivity might be a specific defect related to upper layer cortical neurons, rather than a general axonal outgrowth problem emerging in the absence of DSCAMs.

Absence of DSCAMs (particularly DSCAM) in vertebrates has been linked to either increased or decreased neurite outgrowth and branching, depending on the cellular context. Loss of DSCAM function in pyramidal cortical neurons drives a transient post-natal apical dendrite-associated branching and overall length increase, but a basal dendrite-associated branching and overall length decrease, *in vivo* (Maynard and Stein, 2012). Moreover, *Dscam* or *Dscaml1* knockdown results in an impaired axonal growth in cultured cortical neurons (Zhang et al., 2015). In retinal ganglion cells (RGCs), DSCAM downregulation leads to reduced axon extension and complexity levels in *Xenopus* (Santos et al., 2018), and delayed optic nerve outgrowth and thalamic targeting, accompanied by axonal fasciculation impairments, in mice (Bruce et al., 2017). Likewise, chick spinal cord interneurons present reduced axonal fasciculation levels upon DSCAM knockdown (Cohen et al., 2017). On the other hand, DSCAM knockout in *Xenopus* tectal neurons *in vivo* is associated with increased dendritic growth and branching rates. Interestingly, disruptive effects on axonal/dendritic growth and branching have been also observed upon DSCAM GOF. In mouse cortical neurons, *in vitro* overexpression of full-length DSCAM also impairs axonal outgrowth and branching dose-dependently, and increased expression of the DSCAM intracellular domain alone also results in a reduced overall neurite growth (Jain and Welshhans, 2016; Sachse et al., 2019). DSCAM overexpression additionally impairs dendritic branching and extension in mouse cultured hippocampal neurons (Alves-Sampaio et al., 2010) and in tectal neurons of *Xenopus* tadpoles (Santos et al., 2018). However, *Dscam* GOF is associated to RGC axonal overgrowth in mouse (Bruce et al., 2017). Overall, research on vertebrate development indicates that DSCAMs play important roles in outgrowth and branching of both dendrites and axons, and influence these processes in a markedly cell type-specific, and sometimes cellular structure-specific, manner. In our study, genetic manipulation of immature cortical inhibitory neurons *in vitro* resulted in a significant reduction of total LP length only upon DSCAML1 overexpression, an effect likely related to a concomitant impairment of LP branching. Nevertheless, both *Dscam* and *Dscaml1* GOF negatively impacted the migration process of post-mitotic INs away from progenitor cell territories in our explant model. The reduction of distances observed from

neurons to explant edges with DSCAML1 overexpression could be directly due to the lower complexity and extension of the LP. Since this structure serves, similarly to the axonal growth cone, as an extracellular cue sensor that primarily orients the movement of migrating INs, defects in LP growth and branching can perturb the probing function of the LP, and lead to delayed and/or disorganized migration (Kappeler et al., 2006; Métin et al., 2006; Nasrallah et al., 2006; Martini et al., 2009; Valiente and Martini, 2009).

Interestingly, cortical INs derived from DS patient induced pluripotent stem cells (iPSC) have been reported to display smaller sizes, less complex neurite morphologies, and migration deficits *in vitro* as well as *in vivo* after transplantation in the mouse medial septum. Analysis of molecular pathways in these DS iPSC-derived GABAergic INS has revealed upregulation of PAK1, leading to increased phosphorylated cofilin levels. Pharmacological inhibition of this pathway restored DS iPSC-derived IN migration *in vitro*, suggesting a causal relation between PAK1 pathway dysregulation and migration defects (Huo et al., 2018). However, our explant EP results exclude significant morphological differences being determined by DSCAM overexpression in migrating INs. The different cell-autonomous effects observed upon DSCAM vs. DSCAML1 overexpression are consistent with the divergence of their intracellular domains, which are estimated to be only 45% identical at protein level and present unique interaction motifs (Agarwala et al., 2001; Fuerst et al., 2009; Cui et al., 2013; Pérez-Núñez et al., 2016). Indeed, the PAK1 binding domain located at the DSCAM C-terminal is one of the most divergent regions between DSCAM and DSCAML1 (Agarwala et al., 2001), thus it is unlikely that DSCAML1 would too activate this signaling pathway. Reduced distance of transfected INs from the explant core could derive from issues in cell-environment or cell-cell interactions. Imbalances in DSCAM-modulated signaling or adhesion might translate in an uncoordinated, non-linear IN migration away from the explant. Furthermore, *Dscam* GOF might affect other intracellular aspects of the migration process. Additional research is needed to investigate whether, for instance, IN nucleokinesis, saltatory motion dynamics, and centrosome positioning (Polleux et al., 2002; Bellion et al., 2005; Yanagida et al., 2012; Silva et al., 2018) are affected by dosage increases of both DSCAMs.

A general discrepancy in results between our experiments using constitutive LOF and transient, local overexpression models, as well as between our study and previous research, might point toward a mechanistic difference due to the nature of the models themselves, rather than the induced molecular dosage changes. Acute up- or downregulation via electroporation of specifically designed constructs creates a mosaic situation in which some cells have lost or gained DSCAM/DSCAML1, while untargeted cells in the local environment have not. Considering that DSCAMs interact homophilically, such a situation creates a transmembrane signaling protein-related imbalance across neurons that might exacerbate some phenotypes, as a complete LOF/GOF would not affect local cell-cell or cell-environment interaction dynamics, or might be compensated for by other membrane-bound molecules. In addition, electroporation of knockdown or overexpression constructs might induce undesirable toxicity effects on the targeted cells that need to be carefully controlled for.

Taken together, our findings suggest that DSCAM/DSCAML1 are rather dispensable in embryonic cortical development processes. Nevertheless, it is conceivable that dosage levels of a given cell might need to be in balance with those of neighboring cells to allow cell type-specific homophilic interactions. Future research will elucidate the molecular downstream effectors determining the subtle phenotypes observed in this study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethische Commissie Dierproeven, KU Leuven, project licenses 267/2015 and 005/2017.

AUTHOR CONTRIBUTIONS

ES, DS, and MM conceived the research project, and designed the experiments. MM performed all experiments together with AP, in case of MGE explant electroporation and culture assays. TA collected samples and helped in the revision. MM analyzed experimental results and data. SS produced plasmid constructs for the subcloning of *Dscam-EYFP-HA* and *Dscaml1-EYFP-HA* sequences. RV and LN provided technical support in the performance of ISH and IHC experiments, and carried out genotyping of all tissue samples. MM and ES wrote the manuscript in consultation with DS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Neuronal Delamination and Outer Radial Glia Generation in Neocortical Development

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During neocortical development, many neuronally differentiating cells (neurons and intermediate progenitor cells) are generated at the apical/ventricular surface by the division of neural progenitor cells (apical radial glial cells, aRGs). Neurogenic cell delamination, in which these neuronally differentiating cells retract their apical processes and depart from the apical surface, is the first step of their migration. Since the microenvironment established by the apical endfeet is crucial for maintaining neuroepithelial (NE)/aRGs, proper timing of the detachment of the apical endfeet is critical for the quantitative control of neurogenesis in cerebral development. During delamination, the microtubule–actin–AJ (adherens junction) configuration at the apical endfeet shows dynamic changes, concurrent with the constriction of the AJ ring at the apical endfeet and downregulation of cadherin expression. This process is mediated by transcriptional suppression of AJ-related molecules and multiple cascades to regulate cell adhesion and cytoskeletal architecture in a posttranscriptional manner. Recent advances have added molecules to the latter category: the interphase centrosome protein AKNA affects microtubule dynamics to destabilize the microtubule–actin–AJ complex, and the microtubule-associated protein Lzts1 inhibits microtubule assembly and activates actomyosin systems at the apical endfeet of differentiating cells. Moreover, Lzts1 induces the oblique division of aRGs, and loss of Lzts1 reduces the generation of outer radial glia (oRGs, also called basal radial glia, bRGs), another type of neural progenitor cell in the subventricular zone. These findings suggest that neurogenic cell delamination, and in some cases oRG generation, could be caused by a spectrum of interlinked mechanisms.

Keywords: neuronal delamination, Lzts1, neural progenitor cell, outer radial glial cell, adherens junction, AKNA, neocortical development

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INTRODUCTION

The vertebrate central nervous system originates from the neuroepithelium lining the embryonic neural tube. Neuroepithelial (NE) cells have polarized morphology along the radial axis, spanning the apical surface to the basal side at the basement membrane, and behave as neural progenitor cells. In the early period of mammalian cerebral wall development, neural progenitor cells (NE cells) undergo symmetric, proliferative division to expand the progenitor pool (**Figure 1A**). In the neurogenic period, the primary type of neural progenitor cell is called the apical radial glial cell, or aRG (also called apical progenitor cells, APs) (Miyata et al., 2001; Noctor et al., 2001; Uzquiano et al., 2018). Along with the progression of the cell cycle, aRGs undergo interkinetic nuclear migration

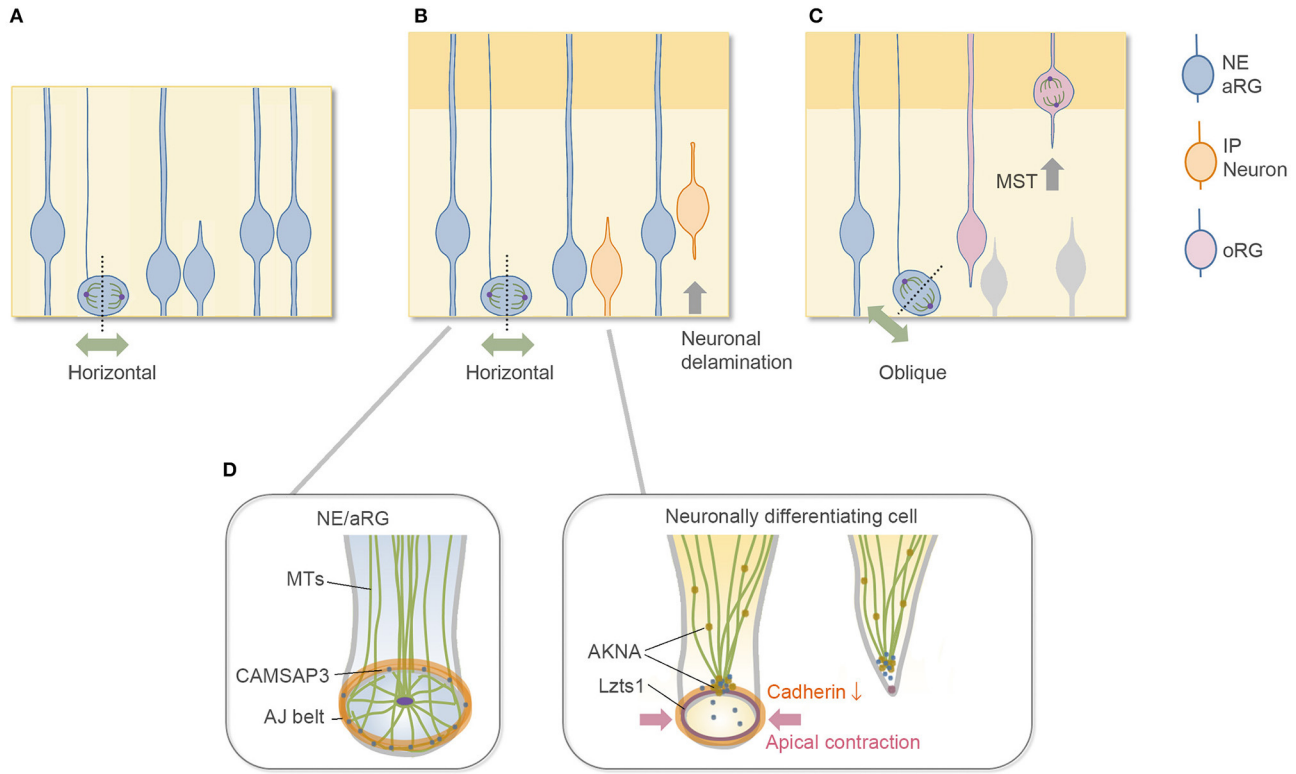

FIGURE 1 | Division mode of aRGs and cytoskeletal architecture of apical endfoot. **(A)** Symmetric proliferative division of aRG at the early embryonic stage. **(B)** Neurogenic asymmetric division of aRG at the mid-embryonic, neurogenic stage. Most aRGs divide horizontally to allow both daughter cells to inherit the apical membrane. The neuronally differentiating daughter cell detaches its apical endfoot and starts to migrate basally (neurogenic cell delamination). **(C)** oRG-generating oblique division. The newly generated basal daughter cell does not inherit the apical junctional complex and migrates to the SVZ to become oRGs. **(D)** Cytoskeletal remodeling in the detachment of the apical endfoot during neurogenic cell delamination and its regulators. NE, neuroepithelial cell; aRG, apical radial glial cell (apical progenitor cell); IP, intermediate progenitor cell; oRG, outer radial glial cell (basal radial glial cell); MST, mitotic somal translocation; MTs, microtubules.

(INM) in the ventricular zone (VZ) and divide at the apical surface (**Figure 1B**) to generate cells that differentiate to become an ordered series of neuron types. These differentiative aRG divisions are mostly asymmetric in terms of daughter cell fate; i.e., an aRG division generates one aRG and one neuronally differentiating cell, which are neurons for direct neurogenesis or intermediate progenitor cells (IPs) for indirect neurogenesis (Delaunay et al., 2017; Uzquiano et al., 2018). IPs have limited proliferative potential in rodent and typically undergo terminal mitosis to produce a pair of neurons in the subventricular zone (SVZ) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). In mouse embryos, indirect neurogenesis substantially contributes to cortical expansion (Kowalczyk et al., 2009; Vasistha et al., 2015; Cárdenas et al., 2018). In both (direct and indirect) cases, these differentiative divisions typically occur horizontally along the apical surface with a cleavage along the apicobasal axis (Kosodo et al., 2004; Konno et al., 2008; Uzquiano et al., 2018), through which they inherit the apical membrane at birth (Shitamukai et al., 2011). Then, the newborn, neuronally differentiating daughter cells retract their apical processes to delaminate from the cadherin-based adherens junction (AJ) belt (Hatta and Takeichi, 1986) that packs the apical endfeet of VZ cells together (**Figure 1B**). When the daughter cell is a neuron, this delamination is the first step of neuronal migration, by which the daughter cells escape from the influence of extracellular cues at the apical side of the VZ.

This review article briefly describes the subcellular architecture of the apical endfeet, which provides an environment for proper neurogenesis from aRGs, and then summarizes our current knowledge on the molecular mechanisms underlying delamination. This review further discusses the common features of neurogenic cell delamination and outer radial glial cell (oRG) generation. oRGs, also called basal radial glial cells (bRGs), are another type of undifferentiated neural progenitor cell with long radial fibers extending to the basal side, and their cell body exists in the SVZ, where they divide multiple times (Fietz et al., 2010; Hansen et al., 2010; Wang et al., 2011; Pilz et al., 2013; Uzquiano et al., 2018). oRGs are first generated from aRGs, typically by oblique division at the apical surface (Shitamukai et al., 2011; LaMonica et al., 2013; Martínez-Martínez et al., 2016), and they migrate to the SVZ without inheriting the apical structure (**Figure 1C**). In this sense, oblique division is another step for daughter cells to disconnect and depart from the apical surface in addition to neurogenic cell delamination. Although the typical, major division patterns are summarized in **Figures 1A–C**, a relatively low proportion of neuronally differentiating cells

may be generated by the oblique division in the rodent brain (Kosodo et al., 2004; Shitamukai et al., 2011), and it is unclear whether oRGs can be generated by the direct detachment of the apical processes. The relationship between the division angle of aRGs and their daughter cell fate is relatively complicated with differences at different developmental stages and in different species (Shitamukai and Matsuzaki, 2012; Gertz et al., 2014; Uzquiano et al., 2018).

Many studies have shown that the apicobasal (AB) polarity of aRGs is important for the maintenance of neural progenitor cells (or aRGs). Impaired AB polarity or apical protein complexes of aRGs induce cell cycle exit, precocious neuronal differentiation, and pathological delamination (Stocker and Chenn, 2009; Zhang et al., 2010; Hatakeyama et al., 2014; Camargo Ortega et al., 2019). This review does not discuss in detail AB polarity and its perturbations in neurodevelopmental disorders, as there are excellent reviews regarding these topics (Singh and Solecki, 2015; Arai and Taverna, 2017; Uzquiano et al., 2018; Hakanen et al., 2019).

APICAL CYTOSKELETAL ARCHITECTURE MAINTAINS NEURAL PROGENITOR CELLS

The apical surface of the developing brain walls is formed by the apical endfeet of NE/aRG cells or VZ cells, which are tightly connected to each other by AJs with the cell adhesion molecule cadherin (Hatta and Takeichi, 1986; Nagasaka et al., 2016; Veeraval et al., 2020). The actin cytoskeleton is selectively concentrated and forms a dense and dynamic filament belt to support AJs of the apical endfeet (Lian and Sheen, 2015; Veeraval et al., 2020). The pharmacological inhibition of actomyosin at AJs reduces the concavity (Shinoda et al., 2018) and the stiffness (Nagasaka et al., 2016) of the apical surface, indicating that the actomyosin system contributes to these properties. Microtubule-based cellular organelles, such as centrosomes and primary cilia, are also positioned at the apical side of the NE/aRGs and are important for their morphology and cellular dynamics (Uzquiano et al., 2018; Park et al., 2019; Meka et al., 2020; Shao et al., 2020). Furthermore, the CAMSAP3 protein, which anchors non-centrosomal microtubules at the adhesion belt of cadherin-based AJs in epithelial cells (Meng et al., 2008), is also enriched at the AJs of the apical endfeet in the developing cortex (Camargo Ortega et al., 2019). These cytoskeletal architectures form a complex configuration at the apical endfeet (**Figure 1D**). In the NE cells of the chick spinal cord, a centrosome-nucleated wheel-like microtubule configuration aligns with the apical actin cable and AJs (Kasioulis et al., 2017), and a similar microtubule ring and intricate organization of the centrosome have been reported in the aRGs of the developing mammalian cortex (Shao et al., 2020).

These apical cytoskeletal architectures provide the environment for the proper proliferation and maintenance of NE/aRG cells. For example, from the apical surface, the cells receive signaling by soluble factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), Neuregulin, and Shh, from cerebrospinal fluid (CSF) filling with the ventricle (Ferent et al., 2020). The direct physical contact of the apical endfeet provides the niche for activating Wnt–β-catenin signaling at the Cdh2 (N-cadherin) complex (Zhang et al., 2010) and Notch signaling (Hatakeyama et al., 2014). Additionally, Shao et al. showed that apical centrosome-organized microtubules maintain proper stiffness or tension of the apical membrane, which regulates aRG proliferation and neurogenesis through activation of YAP, a transcriptional coactivator in the HIPPO signaling pathway (Shao et al., 2020).

DYNAMIC CYTOSKELETAL AND AJ REMODELING IN CELL DELAMINATION

Neuronally differentiating cells generated by the horizontal division of aRGs inherit the apical membrane at birth, and then, they detach their apical endfeet from the cadherin-based AJ belt. Upon this delamination, the microtubule–actin–AJ cytoskeletal architecture at the apical endfeet shows dynamic changes (Das and Storey, 2014; Kasioulis et al., 2017; Camargo Ortega et al., 2019), concurrent with the constriction of the AJ ring at the apical endfeet and downregulation of cadherin expression at the AJs (**Figure 1D**). The constriction of the apical AJ ring primarily occurs by activation of the actomyosin system. In the chick spinal cord, this apical constriction allows the delaminating neurons to leave behind their apical tip with the primary cilia ("apical abscission"). Then, the primary cilia are rapidly reassembled in the differentiating neurons during the apical process retraction. These cilium dynamics may switch the Shh signaling pathway from canonical to noncanonical (Das and Storey, 2014; Kasioulis et al., 2017; Toro-Tapia and Das, 2020). In mice, the apical plasma membrane protrusions of the NE cells and Prominin-1 (CD133)-enriched extracellular membrane particles in the ventricular fluid were observed (Dubreuil et al., 2007; Corbeil et al., 2010), providing the possibility that the apical abscission-like phenomenon might also occur in the developing cerebrum. Unlike in the chick neural tube, however, the apical abscission that leaves behind the primary cilia (Das and Storey, 2014) has not been reported yet in the developing mouse brain; instead, the basolateral cilia are formed by nascent differentiating cells before delamination (Wilsch-Bräuninger et al., 2012; Tozer and Morin, 2014). Such basolateral cilium possibly reduces the exposure to luminal mitogen such as Shh (Arai and Taverna, 2017), but the experimental loss of primary cilia after around embryonic day (E) 11 in mice does not alter cortical neurogenesis (Shao et al., 2020). Overall, these results suggest the evolutionarily or regionally different cilium dynamics and functions in the delamination and early differentiation steps.

APICAL DETACHMENT AND NEUROGENESIS

Since the environment established by the subcellular architecture at the apical endfeet is crucial for maintaining the NE/aRGs as described above, the experimentally induced detachment of the apical processes of the cells sometimes promotes the differentiation cascade in the rodent brain (Arimura et al., 2020).

For example, if Cdh2 expression is experimentally eliminated *in vivo*, abnormal rapid delamination and differentiation of aRGs are observed (Zhang et al., 2010; Hatakeyama and Shimamura, 2019). Furthermore, as nascent differentiating cells express Dll1, a ligand of Notch signaling, at their apical endfeet, their detachment itself changes the microenvironment around the cells during delamination (Kawaguchi et al., 2008; Hatakeyama et al., 2014). If the apical endfeet retention period before delamination is experimentally lengthened, neuronal production from aRGs is decreased during a certain period (Hatakeyama and Shimamura, 2019). These observations suggest that proper detachment timing of the apical endfeet is critical for the quantitative control of neurogenesis in cerebral development.

In physiological scenarios, however, the inheritance of the apical epithelial structure or detachment of apical endfeet themselves seems not to determine the daughter cell's identity (neuronally differentiating or undifferentiating) in neocortical development. For example, at the early developmental stage, during which NE cells undergo symmetric proliferative division, both daughter cells retain the apical endfeet (**Figure 1A**), and if one cell becomes detached from the apical surface during division, it regenerates the apical endfeet (Fujita et al., 2020). This phenomenon contributes to the robust epithelial structure at the early stage but is not observed in daughter cells during the neurogenic stages. Another example is the oRG generation, in which the daughter cells to become oRGs are detached from the apical surface but still undifferentiated. In addition to the basal processes, the cell intrinsic and extrinsic cues contribute to the maintenance and proliferation of the oRGs in a species-different manner (Tsunekawa et al., 2012; Uzquiano et al., 2018; Penisson et al., 2019; Kalebic and Huttner, 2020).

MOLECULES LINKING COMMITMENT AND DELAMINATION

Cell delamination is the dynamic event with cytoskeletal remodeling of the apical microtubule–actin–AJ configuration (Kasioulis et al., 2017). This step is mediated by transcriptional suppression of AJ-related molecules and multiple cascades to regulate cell adhesion and cytoskeletal architecture in a post-transcriptional manner (Camargo Ortega et al., 2019; Kawaue et al., 2019; Arimura et al., 2020). Moreover, knockdown of cell-surface molecule TAG-1 results in the retraction of the basal processes of progenitors, which induces overcrowding of the subapical region to evoke cell departures with retraction of the apical processes. This observation suggests passive forces from neighboring crowding cells also regulate the departure of cells (Okamoto et al., 2013). These redundant regulatory mechanisms of delamination will contribute to robust brain histogenesis.

Recent advances have added to the molecules that link neuronal commitment and delamination as below.

Transcription Factors

Since fate decisions of daughter cells likely occur prior to or during cell division of aRGs (Uzquiano et al., 2018), neuronal commitment is thought to proceed before detachment of the apical endfeet in one of the daughter cells in the case of neurogenic asymmetric division (**Figure 1B**): thus, proneural gene(s) expression is a candidate for the switch that starts the delamination cascades. The proneural genes Neurogenin 2 (Neurog2) and Ascl1 activate the Rho GTPases Rnd2 and Rnd3, respectively, to reorganize the actin cytoskeleton by inhibiting Rho activity in migrating neurons (Ge et al., 2006; Heng et al., 2008; Pacary et al., 2011); therefore, these proneural genes might also be implicated in delamination by modulating the cytoskeleton.

Neurog2 and several transcription factors downstream of Neurog2 are reported to be involved in delamination through transcriptional suppression of cadherins and AJ-related molecules (Pacary et al., 2012; Itoh et al., 2013b; Singh and Solecki, 2015). The overexpression of Neurog2 represses Cdh1 (E-cadherin) transcription in cultured cortical neural progenitor cells (Itoh et al., 2013a). In the spinal cord, Foxp2 and Foxp4, known as transcriptional repressors, promote neuronal delamination through direct transcriptional suppression of Cdh2, and Foxp4-mutant and Foxp-misexpression studies suggest similar functions of these molecules in delamination in the developing cortex (Rousso et al., 2012). Tbr2 (Eomes) promotes the detachment of cells from the apical surface and their differentiation (Sessa et al., 2008). Tavano et al. showed that another transcription factor, insulinoma-associated 1 (Insm1), is upregulated by Neurog2 in neuronal commitment and promotes delamination by repressing the AJ belt-specific protein Plekha7 (Farkas et al., 2008; Tavano et al., 2018; Kalebic and Huttner, 2020). The epithelial–mesenchymal transition (EMT)-related transcription factors Scratch1 and Scratch2, members of the Snail superfamily, are also expressed upon neuronal fate commitment by upregulation of proneural genes such as Neurog2 and induce delamination by transcriptional repression of the adhesion molecule Cdh1 (Itoh et al., 2013a).

Slit-Robo Signal

In the developing cerebral cortex, the absence of Robo receptors (Robo1/2 mutant) decreases Hes1 messenger RNA (mRNA) levels and produces an excess of IPs (Borrell et al., 2012; Cárdenas et al., 2018). Interestingly, a large proportion of Robo1/2 mutant Ips fail to retract their apical processes from the apical surface. This mutant phenotype is accompanied by enhanced thickness of the apical band in Cdh2 and ß-Catenin immunoreactivity (Borrell et al., 2012). Thus, Robo signaling inhibits cadherin-based adhesions at apical processes, similar to retinal ganglion cells (Wong et al., 2012), whereas its molecular link to the cytoskeletal architecture of apical Ajs is still unknown.

AKNA

Recently, Camargo Ortega et al. reported that the centrosome protein AKNA is localized at the interphase centrosome of neuronally differentiating cells and SVZ progenitors in the developing cerebrum at the neurogenic stage (Camargo Ortega et al., 2019). The authors further demonstrated that AKNA overexpression induced rapid delamination, and conversely, AKNA loss-of-function impairs delamination, indicating that AKNA plays a crucial role in delamination. The

delamination processes are primarily mediated by AKNA's effect on microtubule dynamics that destabilize apical microtubule–actin–AJ complexes, which promote constriction of the apical endfeet (Camargo Ortega et al., 2019).

In TGFb1-treated murine mammary gland epithelial (NMuMG) cells during EMT, AKNA recruits the microtubule minus-end binding protein CAMSAP3 (Tanaka et al., 2012) from junctional microtubules to the centrosome (Camargo Ortega et al., 2019), suggesting that this molecular mechanism underlying EMT (Pongrakhananon et al., 2018) also regulates delamination in neocortical development (**Figure 1D**). Moreover, a transcription factor SOX4, which regulates EMT of NMuMG cells (Tiwari et al., 2013), upregulates *Akna* mRNA in NMuMG cells in EMT and neural stem cell line N2A cells (Camargo Ortega et al., 2019), and SOX4 overexpression generates SVZ progenitors in the developing brain (Chen et al., 2015). These observations further support that AKNA regulates neurogenic cell delamination through EMT-like molecular mechanisms.

Lzts1

Our research group has recently found that leucine zipper putative tumor suppressor 1 (Lzts1) (also known as FEZ1 and PSD-Zip70) (Konno et al., 2002) acts as a master modulator of neurogenic cell delamination (Kawaue et al., 2019). Lzts1 is reported as a microtubule-associated protein that inhibits microtubule polymerization (Ishii et al., 2001) and is implicated in several human cancers (Vecchione et al., 2007). Notably, Lzts1 expression is upregulated by Neurog1/2 and closely localizes at the AJ belts of the apical processes of differentiating newborn cells (Kawaguchi et al., 2008; Kawaue et al., 2019). Overexpression of Lzts1 induces apical contraction with a decrease in the expression of Cdh2 at AJs, which results in detachment of the apical processes. In contrast, loss of Lzts1 impairs the differentiating cells from departing the apical surface. Thus, local Lzts1 expression at endfeet AJs has a unique function that positively controls neurogenic cell delamination in the developing cortex.

Lzts1-induced apical contraction is mediated by activation of the actomyosin system (Kawaue et al., 2019), whereas apical contraction by the activation of myosin II does not solely reduce cadherin expression and is not sufficient to induce detachment (Das and Storey, 2014). Therefore, the function of Lzts1 in delamination is likely caused by the coordinated cytoskeletal rearrangement of the microtubule–actin–AJ complex at the apical endfeet mediated by both inhibiting microtubule polymerization and activating actomyosin systems (Kawaue et al., 2019) (**Figure 1D**).

DSCAM

In the mouse dorsal midbrain, down syndrome cell adhesion molecule (DSCAM) has been shown to control neuronal delamination. DSCAM starts to be expressed in differentiated neurons only before migration and locally suppresses the RapGEF2–Rap1–Cdh2 cascade at their apical endfeet to delaminate (Arimura et al., 2020).

COMMON MECHANISMS IN NEUROGENIC CELL DELAMINATION AND oRG GENERATION

oRGs can be produced by the oblique (or perpendicular) cell divisions of aRGs (LaMonica et al., 2013; Gertz et al., 2014; Martínez-Martínez et al., 2016). With the oblique division, the newly generated basal daughter cells do not inherit the apical junctional complex and can migrate to the SVZ to become oRGs (or oRG-like cells) (**Figures 1C**, **2A**). Even though they lack apical anchoring, these basal daughter cells still have proliferative potential, and their basal processes are considered a key morphological feature underlying this capacity (Tsunekawa et al., 2012; Uzquiano et al., 2018; Kalebic and Huttner, 2020). Many genes and extracellular factors contribute to the amplification of oRGs in the SVZ, and some oRG-specific genes that are present only in humans or primates, such as ARHGAP11B, are thought to explain the evolutional expansion of the neocortex (Florio et al., 2015; Penisson et al., 2019).

The molecular mechanisms regulating oRG generation at the apical surface have been partially uncovered. In the VZ during the restricted period for massive oRG generation, *Cdh1* mRNA is expressed at a significantly lower level than that during the other periods. Reduced Cdh1 function increases oRG generation by both weakening cell adhesion and promoting oblique division in the ferret brain (Martínez-Martínez et al., 2016). Furthermore, in the epithelial cells, the cell division orientation is shown to be coupled to cell–cell adhesion by the LGN–Cdh1 complex (Gloerich et al., 2017). These evidences suggest that AJ-related molecules are involved in the regulation of spindle orientation in oRG generation.

RNA-seq analysis suggested that neuronally differentiating cells and some oRGs might share common molecular features (Johnson et al., 2015), and forced Neurog2 expression in the ferret brain induced the generation of oRG-like cells *in vivo* (Johnson et al., 2015). These observations raise an intriguing possibility that proneural genes or delamination cascades may underlie the generation of a subset of oRGs.

In line with this, we found that Lzts1, a key molecule of neurogenic cell delamination, also induces oRG generation by the oblique division of aRGs (Kawaue et al., 2019). Single-cell analysis (Okamoto et al., 2016) shows that in the E14 mouse VZ, when oRG-like cells are generated from aRGs, some aRGs weakly express *Lzts1* mRNA. Weakly forced-expressed Lztz1 localizes to the cell cortex of aRGs in mitosis and induces oblique division. Conversely, loss of Lzts1 decreases the oblique division frequency in mice and reduces oRG generation in mice and ferrets. Currently, the precise molecular mechanisms underlying Lzts1-mediated oblique division are unclear. Live imaging of the Lzts1-expressed aRG suggests that Lzts1 inhibits the anchoring of centrosomes to the subapical (basolateral) portion of the process during M phase (Kawaue et al., 2019) (**Figure 2B**). On the other hand, the basolateral localization of LGN, which binds Numa to orient the mitotic spindle by anchoring spindle astral microtubules (Konno et al., 2008), is maintained in the Lzts1-induced obliquely dividing aRGs, suggesting that the localized LGN–Cdh complex

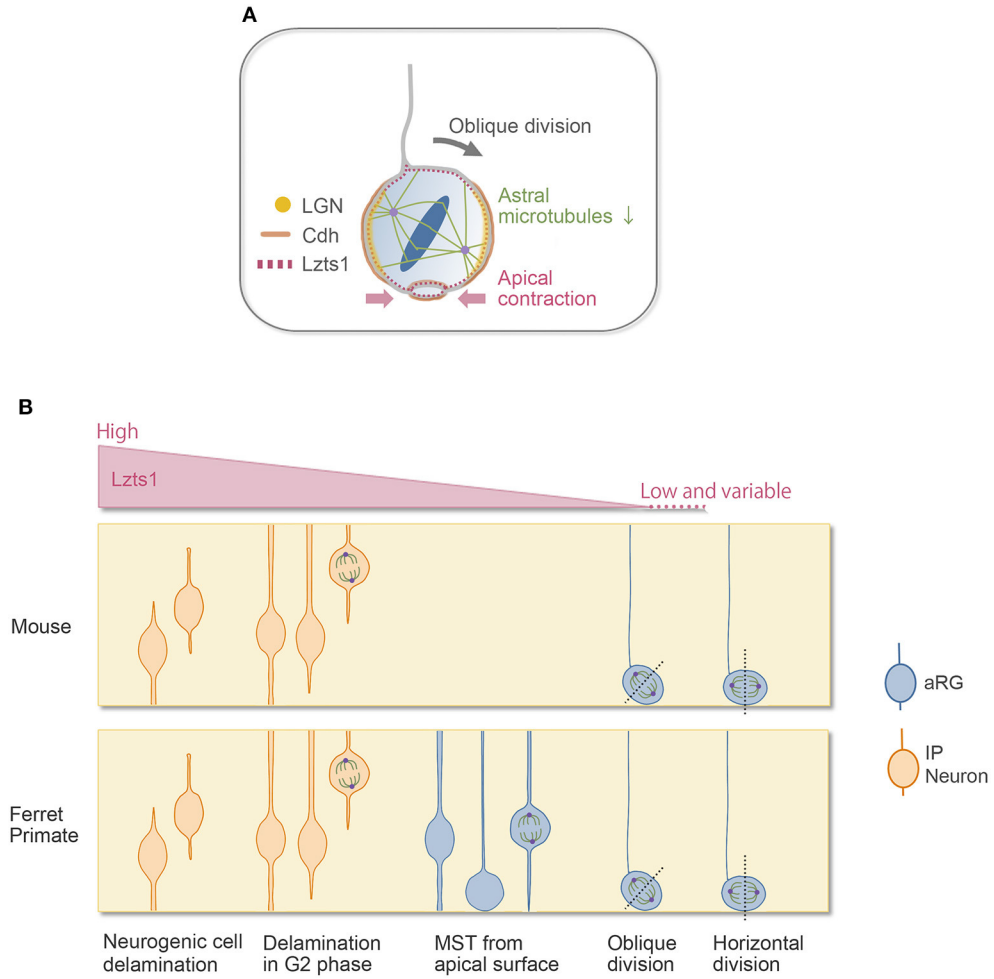

FIGURE 2 | Lzts1 controls both neuronal and progenitor cell delamination. **(A)** Weak Lzts1 expression induces oRG-generating oblique division by inhibiting centrosome anchoring to the lateral side in mitosis (model). The apical contraction induced by Lzts1 may also contribute to oblique division. Lzts1 induces MST of basal daughter cells by activating the actomyosin system. **(B)** Lzts1 controls both neurogenic cell delamination and oRG generation as a master modulator of the cytoskeleton. In the developing cerebrum, aRGs/IPs show various behaviors to generate their daughter cells. In addition to the typical detachment of the differentiating daughter cells (**Figure 1B**), some IP cells shed their apical processes during G2 and then show MST and divide in the SVZ. There is also a rare pattern in human and ferret oRG generation, where MST occurs from the apical surface (Gertz et al., 2014). Experimental Lzts1 expression levels correlate with these diverse cellular behaviors. *In vivo*, Lzts1 is expressed at high levels in neuronally differentiating cells, including nascent neurons and IPs, whereas in the aRG, Lzts1 exhibits variable and weak expression.

might be relatively maintained. Since Lzts1 has inhibitory effect on the microtubule assembly (Ishii et al., 2001), low-level Lzts1 in mitotic aRGs may perturb the formation of astral microtubules and inhibit the astral microtubule–LGN–AJ interaction, which may induce oblique division (Kawaue et al., 2019) (**Figure 2A**). Consistently, Btg2::GFP+ neuronal progenitors, which should express *Lzts1* mRNA (Kawaguchi et al., 2008; Schenk et al., 2009), show more variable spindle orientation with relatively small astral microtubules than those of proliferating progenitors (Mora-Bermúdez et al., 2014). Moreover, the function of Lzts1 on apical contraction may also be involved in inducing oblique division (Kawaue et al., 2019) (**Figure 2A**). The latter mechanism might link the apical process retraction with the spindle orientation change in some experimental conditions manipulating a certain number of genes (Lancaster and Knoblich, 2012; Mora-Bermúdez and Huttner, 2015).

Overall, these observations suggest that, in the case of Lzts1, the oblique division that generates oRGs is controlled by a molecular mechanism similar to that of delamination in the context of the microtubule–AJ complex. Therefore, the junctional proteins would play critical roles both in maintaining epithelial structure at the apical endfeet (Zhang et al., 2010; Veeraval et al., 2020) and, as in the case of the epithelial cells (Gloerich et al., 2017), in controlling the spindle orientation in aRGs. It is an open question whether the adhesion molecules, Cdh1 and Cdh2, differently play these two roles in aRGs.

Lzts1 function in oblique division may explain some of the diverse, contradictory conclusions of previous studies on spindle orientation and fate determinant (Lancaster and Knoblich, 2012; Mora-Bermúdez and Huttner, 2015): if the experimentally manipulated molecules have some functions in the maintenance of aRGs, their depletion increases the expression levels of neuronal molecules (molecules upregulated under proneural transcription factors) including Lzts1 in the dividing aRGs, which will increase the frequency of oblique division. This interpretation may explain why the oblique or perpendicular divisions of aRGs are correlated with the progenies' neuronal fate under some experimental conditions, which is distinct from the physiological situation in which most differentiative divisions occur horizontally (Shitamukai and Matsuzaki, 2012; Uzquiano et al., 2018). If the experimental conditions have no or weak effect on the maintenance of aRGs but strongly impair the apical AJ complex, aRGs would detach or delaminate without neuronal differentiation.

A CONTINUOUS SPECTRUM OF MECHANISMS CONTROLLING DELAMINATION

Unlike AKNA, which primarily affects microtubule dynamics (Camargo Ortega et al., 2019), Lzts1 activates actomyosin systems in addition to its inhibitory effect on microtubules. The activating effect of Lzts1 on the actomyosin system does not seem to require its inhibitory effect on microtubule assembly because cellular stiffness measurement by atomic force microscopy (AFM) reveals that in Lzts1-overexpressing NIH3T3 cells, even under Taxol (microtubule stabilizer) treatment, Lzts1 increases cellular stiffness by activating myosin II (Kawaue et al., 2019). Furthermore, live imaging of the Lzts1-expressing cerebral walls shows that Lzts1 strongly induces mitotic somal translocation (MST), in which the soma rapidly translocates basally before cytokinesis (Kawaue et al., 2019). MST is the characteristic behavior observed in oRG or IP migration (Hansen et al., 2010; Gertz et al., 2014; Ostrem et al., 2014) (**Figure 1C**). MST requires the activation of the Rho–ROCK–myosin II pathway but not microtubule motors or centrosomal guidance (Ostrem et al., 2014, 2017).

In normal neocortical development, there are various cell departure patterns from the apical surface in the developing cerebral wall, as shown in **Figure 2B**. Interestingly, these diverse cellular behaviors appeared in response to the level of overexpressed Lzts1, suggesting that the various cellular departure events might be understood as a continuous phenomenon linked to common molecular mechanisms, likely as a spectrum (Kawaue et al., 2019) (**Figure 2B**). Further research is needed to elucidate the precise molecular mechanisms by which Lzts1 orchestrates cytoskeletal dynamics to induce neuronal differentiation, MST, and oRG generation in neocortical development.

In mice with lissencephalic brains, the number of oRGs is small, and their self-renewal potential in the SVZ is relatively limited (Wang et al., 2011) (thus, sometimes they are interpreted as "oRG-like" cells). In contrast, in species with gyrencephalic brains, such as ferrets and primates, oRGs are more abundant and self-renew, producing many IPs and neurons (Hansen et al., 2010; García-Moreno et al., 2012; Reillo and Borrell, 2012; Betizeau et al., 2013; Gertz et al., 2014). The unique cellular behaviors related to oRG generation, i.e., oblique division, and MST show evolutionary changes in their frequency and distance (LaMonica et al., 2013; Ostrem et al., 2014, 2017). Lzts1 expression is weak and variable in the aRG population in mice, and its expression levels are likely regulated by the oscillatory/variable expression of Hes1 and proneural genes (Shimojo et al., 2008; Kawaue et al., 2019; Kageyama et al., 2020). Since it is still unknown whether the differential expression of Lztz1 in neural progenitor cells might be involved in the differential cell behaviors between species, it would be interesting to address this question in the future.

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