MECHANISMS OF NEURONAL MIGRATION DURING CORTICOGENESIS

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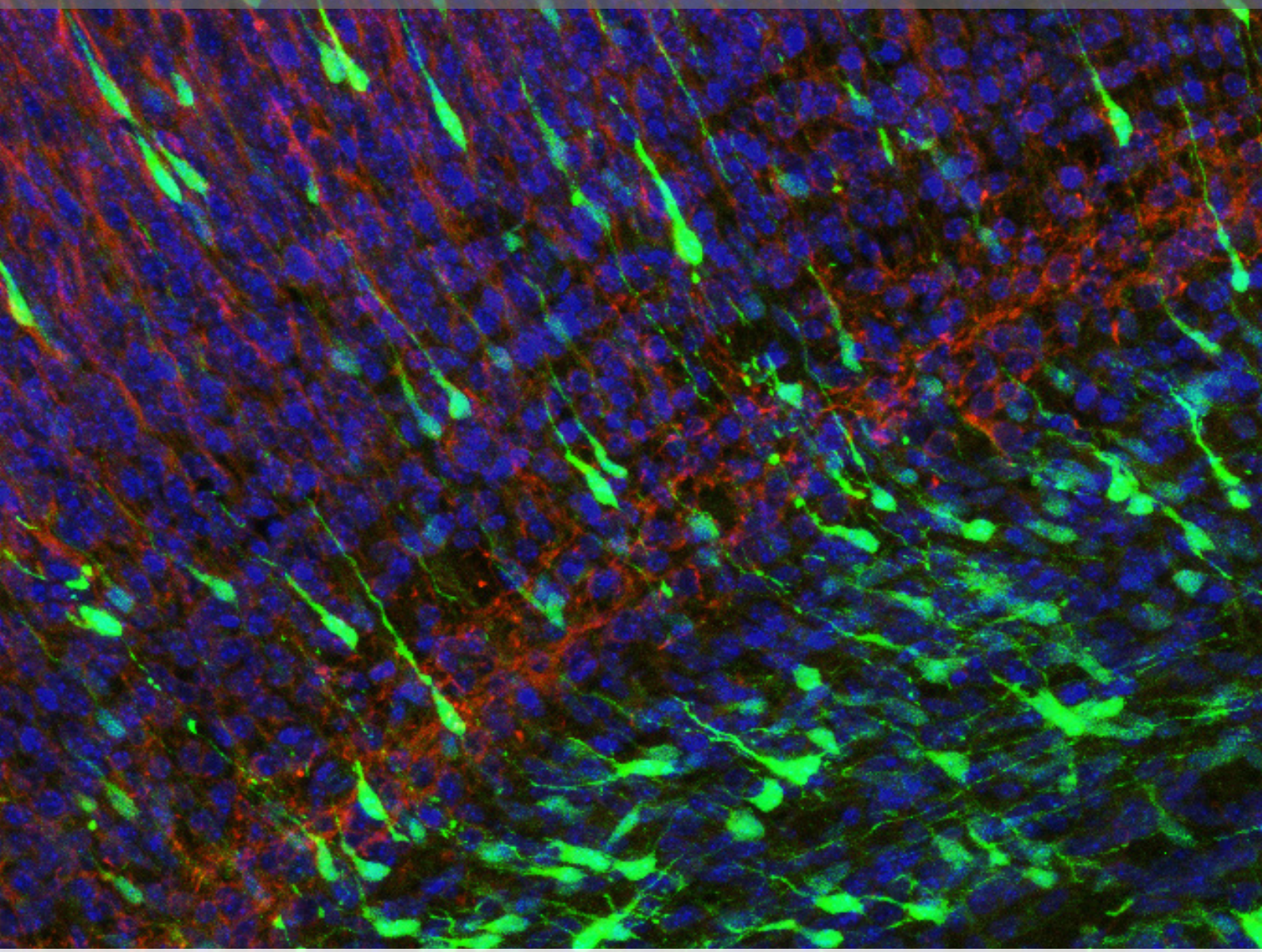

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MECHANISMS OF NEURONAL MIGRATION DURING CORTICOGENESIS

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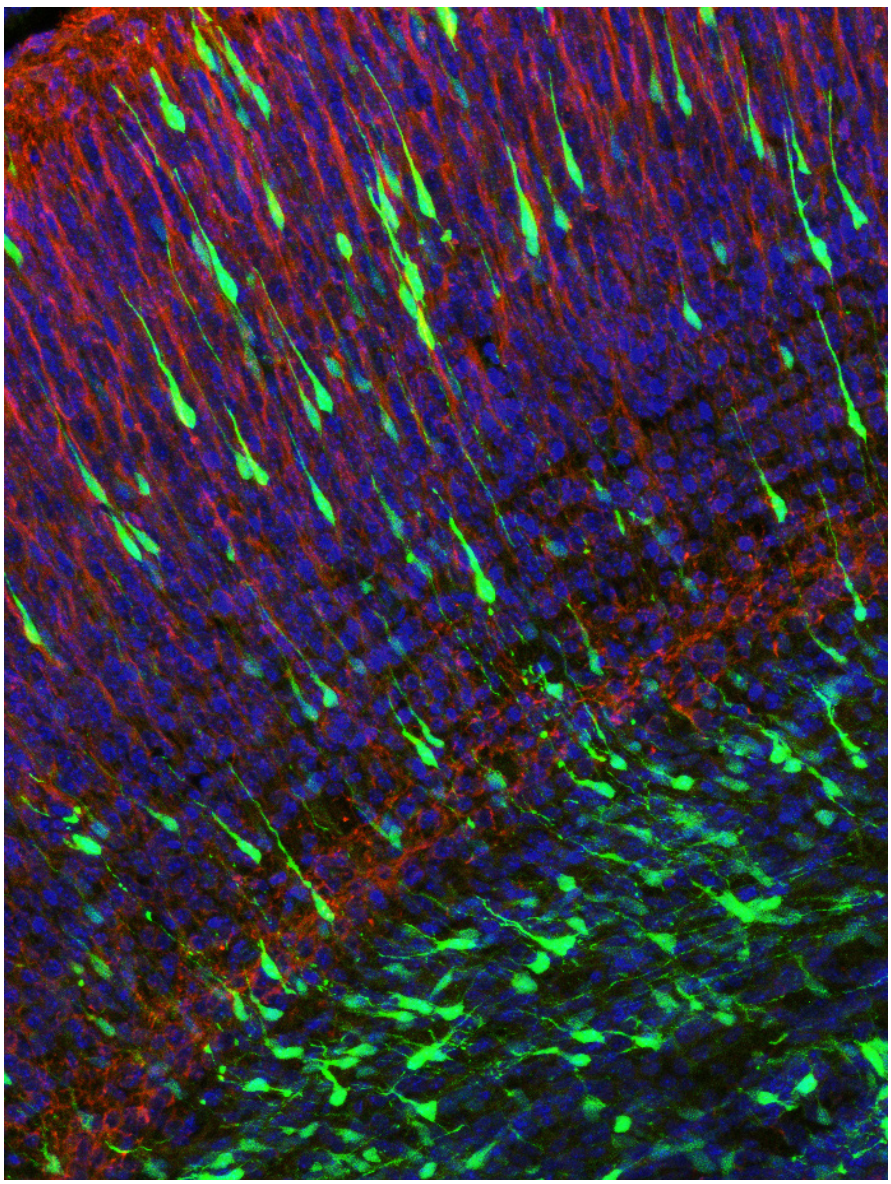

Newborn pyramidal neurons electroporated with GFP-expression plasmid(green) are migrating toward the pial surface of the cerebral cortex. The section was stained with DAPI (blue) for nuclei and the antibody against microtubule-associated protein 2 (MAP2) (red) to visualize subplate and cortical plate neurons. Photo by Noe Kaneko (Tokyo Metropolitan Institute of Medical Science).

The cerebral cortex plays central roles in many higher-order functions such as cognition, language, consciousness, and the control of voluntary behavior. These processes are performed by the densely interconnected networks of excitatory pyramidal neurons and inhibitory interneurons, and the balanced development of these two types of neuron is quite important. During cortical development, pyramidal neurons and interneurons show quite different migratory behaviors: radial migration and tangential migration, respectively. Pyramidal neurons are generated in the ventricular zone of the dorsal telencephalon, and migrate radially along radial glial fibers toward the pial surface, forming a six-layered cortical structure in an " inside-out" manner. On the other hand, cortical interneurons are generated in the medial and caudal ganglionic eminence in the ventral telencephalon, and follow long tangential migratory paths into the cortex. Defects in these migration processes result in abnormalities in the cortical layer structure and neuronal networks, which may cause various neurological and psychiatric conditions such as epilepsy and schizophrenia. Accordingly, besides basic scientific interest, elucidation of the mechanism of neuronal migration is essential for understanding the pathogenesis of these diseases. This Research Topic includes a series of articles ranging from the basic mechanism of neocortical development to the malformation and evolution of the neocortex. We do hope that the present ebook will further stimulate the interest in the fascinating investigations of neuronal migration and corticogenesis.

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EDITORIAL
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Editorial: Mechanisms of Neuronal Migration during Corticogenesis

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The Editorial on the Research Topic

Mechanisms of Neuronal Migration during Corticogenesis

The mammalian neocortex shows an extremely well-organized structure that underlies higher brain functions such as cognition, language, and memory. The neocortex consists of a six-layered structure, in which excitatory and inhibitory neurons form complex neural circuits in concert with glial cells. As a result of recent technological innovations in live imaging and *in utero* electroporation, the processes involved in neocortical development, especially the mechanism of neuronal migration, have been successively revealed. Furthermore, it has been recognized recently that defects in neuronal migration lead to brain malformations and diverse psychiatric and neurological disorders including schizophrenia, epilepsy, and autism. Accordingly, it is important to elucidate the molecular mechanism of neuronal migration in the neocortex, in order to understand not only the basic principles of brain development but also the pathological processes of these disorders. In this special issue, we attempt to cover topics ranging from the basic mechanisms of neocortical development to the malformation and evolution of the neocortex, with a special focus on neuronal migration.

Radial glial cells (RGCs) are primary progenitors capable of generating various types of neurons and glial cells, which include Cajal-Retzius cells, subplate neurons, pyramidal neurons, interneurons, oligodendrocytes, and astrocytes. Thus, it is important to know how these diverse types of cells are generated from RGCs and integrated into complex neocortical circuits. Toma and Hanashima reviewed the mechanisms that regulate the changes in RGC competency and neuronal subtype transitions, focusing on the regulatory networks of various transcription factors including Foxg1. At the earlier stage of neocortical development, RGCs predominantly produce a large number of neurons, but later they change into glia-restricted progenitors. After the discovery of the importance of astrocytes in synaptic plasticity and blood flow, the mechanisms of glial development have attracted increasing interest for many neuroscientists. Tabata reviewed the mechanism controlling the production of diverse types of astrocytes and their migration behavior, demonstrating the multiple origins of glial cells in the neocortex.

Neocortical circuits consist of highly interconnected excitatory glutamatergic and inhibitory GABAergic neurons, which are generated from distinct pools of RGCs. The excitatory neurons are generated from RGCs localized in the ventricular zone of the dorsal telencephalon and migrate radially toward the pial surface in an inside-out manner (radial migration). On the other hand, inhibitory neurons mainly originate from the ventral telencephalon and migrate tangentially into the neocortex (tangential migration). In spite of such different developmental origins, both

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excitatory and inhibitory neurons go through the multipolar stage with several minor processes in the neocortex before axon extension. Then, they undergo dramatic morphological changes to initiate axon formation, namely, neuronal polarization. Sakakibara and Hatanaka reviewed the sequential events in polarization processes of both excitatory and inhibitory neurons, and they discussed the underlying molecular mechanisms.

At the multipolar stage, the excitatory neurons transiently use a multipolar migration mode, namely migration with no fixed direction, in the subventricular and intermediate zones. Then, they adopt a bipolar shape during neuronal polarization and migrate quickly toward the pial surface along RGC processes, which is called locomotion mode. Many kinds of molecules are involved in these dynamic changes in the morphology and behavior of neurons. Small GTP binding proteins belonging to the Rho family play critical roles in cytoskeletal regulation during such dynamic processes. Azzarelli et al. reviewed the roles of Rnd proteins, "atypical" Rho family members, in neuronal migration and discussed its upstream and downstream pathways. The functions of many cytoplasmic proteins including cytoskeletal components are regulated by phosphorylation and dephosphorylation processes. Ohshima focused on protein kinases, including CDK5 and JNKs, and reviewed their regulatory roles in cytoskeletal organization during multipolar-bipolar transition and radial migration. Ohtaka-Maruyama and Okado comprehensively summarized the molecular pathways involved in these developmental processes, emphasizing the importance of subplate neurons in the development and evolution of the six-layered neocortical structure.

It is apparent that neuronal migration and wiring are regulated by various secreted factors such as growth factors, chemokines, and extracellular matrix molecules, although their mechanisms are poorly understood. Kondo et al. demonstrated that subplate neurons transiently express high levels of secretary proteins such as connective tissue growth factor, neuroserpin, and insulin-like growth factor binding protein 5, which may be involved in cortical circuit formation. Greenman et al. reported a novel finding that autotaxin (ENPP2), a secretary enzyme bearing lysophospholipase D activity, regulates the localization and adhesion of neural progenitor cells independent of its catalytic activity. Maeda reviewed the roles of proteoglycans in neuronal polarization and migration and discussed the possibility that extracellular matrix regulates the distribution and activity of multiple secreted factors in the developing neocortex. In addition to the long-range gradient of secreted factors, axon pathfinding is also regulated by short-range guidance cues and direct cell-cell contacts mediated by guidepost cells. Squarzoni et al. reviewed the roles of already known guideposts such as Cajal-Retzius cells for entorhinal-hippocampal axons and corridor cells for thalamocortical axons, and further proposed a new class of guidepost cells, microglia, in the cortex.

Hippocampal formation has a close relationship with the neocortex both functionally and structurally, but it shows a distinct arrangement of pyramidal neurons from that of the neocortex. Hayashi et al. reviewed the differences in the migratory behaviors of neocortical and hippocampal neurons, which lead to the formation of distinct layered structures in these two cortical regions. Defects in the migration of excitatory and inhibitory neurons can lead to the various neurological and psychiatric disorders. Kato reviewed recent development in the understanding of the genetic bases of neuronal migration disorders in terms of genotype-phenotype correlations, focusing mainly on lissencepahaly. Muraki and Tanigaki discussed the possible relationship between neuronal migration defects and behavioral abnormalities relevant to schizophrenia based on studies using genetically defined animal models. The evolutionary approaches should greatly deepen our understanding of the mechanisms underlying neocortical development. Nomura et al. established the method of *in ovo* electroporation and *ex ovo* culture of reptilian embryos. Comparative studies using this method will provide significant insights into the origin of the mammalian neocortex.

It is hoped that the special issue entitled "Mechanisms of Neuronal Migration during Corticogenesis" will serve as a valuable resource for many neuroscientists to promote their research perspectives. Finally, as topic editors, we would like to express our sincere appreciation to all the authors for their outstanding contributions and to all the reviewers for their insightful comments on the papers. We also thank the editorial office and the production staff for their unceasing efforts and dedication.

AUTHOR CONTRIBUTIONS

CO wrote the manuscript. KN, AP, and NM revised the manuscript.

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REVIEW
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Switching modes in corticogenesis: mechanisms of neuronal subtype transitions and integration in the cerebral cortex

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Information processing in the cerebral cortex requires the activation of diverse neurons across layers and columns, which are established through the coordinated production of distinct neuronal subtypes and their placement along the three-dimensional axis. Over recent years, our knowledge of the regulatory mechanisms of the specification and integration of neuronal subtypes in the cerebral cortex has progressed rapidly. In this review, we address how the unique cytoarchitecture of the neocortex is established from a limited number of progenitors featuring neuronal identity transitions during development. We further illuminate the molecular mechanisms of the subtype-specific integration of these neurons into the cerebral cortex along the radial and tangential axis, and we discuss these key features to exemplify how neocortical circuit formation accomplishes economical connectivity while maintaining plasticity and evolvability to adapt to environmental changes.

Keywords: neocortex, cell fate specification, neurogenesis, Cajal-Retzius cell, subplate, layer

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Introduction

Information processing in the neocortex relies on a highly ordered cytoarchitecture and its neuronal assembly to serve higher cognitive functions, such as perceptions, voluntary movements, and language. Neocortical neurons are organized into six major layers along the radial axis, which are further modified tangentially across areal and columnar subdivisions. These laminar and tangential organizations are key aspects of the cerebral cortex and are conserved among mammalian species, and they are thought to underlie the increase in neuronal numbers and expansion of the neocortex during evolution (Rakic, 2009). While the distinguishing feature of cellular organization of the cerebral cortex was acknowledged over a century ago (Meynert, 1868; Brodmann, 1909), the molecular mechanisms underlying the development and assembly of each neuronal component of the neocortex have rapidly begun to unravel over the past decade.

A major challenge in neocortical development is to efficiently recruit diverse cell types into its circuitry through the cost-effective production and wiring of individual neuronal elements. As dendrites and axons occupy the dominant fraction of the neocortical volume (Braitenberg and Schuz, 1998), minimizing neuronal process length in cortical network while maximizing their coverage is a key strategy in recruiting diverse neuron types in a restricted cortical capacity. In theory, this aim could be achieved through the reduction of molecular and wiring components

while optimizing their networks; however, in a broader context, such effective topology and energy-saving construction is ideally adaptable to environmental and evolutionary changes.

For this purpose, the construction of the neocortical circuit becomes a highly dynamic process, which involves two fundamental steps that regulate the temporal and spatial behavior of cells during the progenitor and postmitotic stages. First, diverse neocortical neurons are generated from a restricted pool of progenitor cells within the ventricular and subventricular zones (VZ and SVZ), which differ in their connectivity, dendritic morphology, and molecular character. Second, the movement of cells from their place of birth to their final destination is an essential step to recruit these diverse neurons into the circuit and accommodate massive numbers of neurons within a restricted head volume.

In early development, the cerebral cortex starts from a simple neuroepithelial sheet at the anterior neural tube. This sheet gives rise to two major cell types of the neocortex, neurons and glia. The former are further classified into glutamatergic projection neurons and GABA (γ-aminobutyric acid)-ergic interneurons, which participate directly in the cortical circuit through the excitation and inhibition of distal and proximal target neurons, respectively. The glia, in turn, which include astrocytes and oligodendrocytes, play pleiotropic roles in shaping the cortical circuit by modulating its activity (Muller and Best, 1989; Chung et al., 2013). At a glance, the neocortical cytoarchitecture can be defined by its glutamatergic neuron components (Brodmann, 1909). In this review, we focus exclusively on the glutamatergic subtypes of the neocortex and reveal the organizing principles of the neocortical circuit through understanding the mechanisms by which neuronal subtype identity and integration are instructed in the cerebral cortex.

Key Elements of the Neocortical Scaffold

Radial Glial Cells and Transition from Symmetric to Asymmetric Cell Divisions

Genetic fate-mapping and loss-of-function studies have shown that neocortical excitatory neurons arise from neuroepithelial cells of the dorsal telencephalon, which confer glutamatergic over GABAergic transmitter identity through the sequential induction of *Pax6*, *Neurog1/2*, and *NeuroD* expressions (Fode et al., 2000; Gorski et al., 2002; Schuurmans et al., 2004; Kroll and O'Leary, 2005; Louvi et al., 2007). These cells then give rise to radial glial cells (RGCs), which possess characteristic apical and basal processes that make contact with the ventricular and pial surface, respectively. RGCs are the principal progenitor cells of the cerebral cortex (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001) and also serve as scaffolds for the oriented migration of later-born neurons through their elongated processes. The progenitors contribute to cortical expansion in gyrencephalic mammals through the diversification of its subtypes (Hansen et al., 2010). RGCs undergo cell divisions at the ventricular surface that typically produce a pair of progenitors or a progenitor and a neuron. The former process is called symmetric cell division and expands the number of neural stem cells, whereas the latter is called asymmetric cell division and contributes to neurogenesis while maintaining the progenitor pool, owing to its output of both progenitor cells and neurons (and later glia). These progenitors are more fate-restricted in the sense that they have a limited capacity to undergo self-renewal.

The transition from neuroepithelial cells to RGCs is instructed through multiple signaling molecules. Fgf10, which is expressed in the apical surface of the VZ, exhibits a rostral-high to caudal-low gradient within the telencephalon, and genetic deletion of *Fgf10* results in delayed onset of RG markers, brain lipid binding protein (BLBP) and glutamate transporter (GLAST) in the rostral cortex. This delay results in the tangential expansion of prefrontal areas in the *Fgf10* mutants (Kang et al., 2009; Sahara and O'Leary, 2009), implying that the differential timing of neuroepithelial cell to RGC conversion may also contribute to the regulation of neuronal numbers in an area-dependent manner. Similarly, retinoic acid (RA) expressed in the meninges (Siegenthaler et al., 2009) instructs the conversion of division modes. Mutants that lack *Foxc1* fail to establish the meninges, which through contact with the end-feet of neuroepithelial cells propagate RA signaling, which is necessary for the transition from symmetric to asymmetric divisions. Lack of RA signaling derived from the meninges results in a significant decrease in neuronal output and thus prolonged neuroepithelial cell stage and symmetric cell divisions (Siegenthaler et al., 2009). Recently, a single-cell clonal analysis in mouse neocortex using retroviral vectors has demonstrated that, while the timing of transitions from symmetric to asymmetric cell divisions varies from clone to clone, within each clone, once the progenitors enter the asymmetric division phase, their progenies produce a remarkably fixed number of neurons (Gao et al., 2014). These observations revealed that following the conversion to asymmetric cell division mode, progenitor cells may undergo a stereotypic program in their proliferation and neurogenic output.

Cajal-Retzius Cells and Subplate Cells in Cortical Scaffolding

When RGCs switch to asymmetric cell division, progenitor cells begin to produce the first cohort of neurons, which serve as essential scaffolds for the construction of the neocortical cytoarchitecture. These neurons consist of Cajal-Retzius (CR) cells and subplate (SP) neurons and form a transient structure called the preplate (PPL) above the VZ. CR cells were first recognized through their expression of secretory glycoprotein, Reelin (Reln) (D'Arcangelo et al., 1995; Ogawa et al., 1995), and the functional study of CR cells has largely focused on their regulation of radial migration in subsequent-born projection neurons through diffusive cues. However, recent reports have also revealed their roles in instructing radial migration via cell contact-mediated signaling (Gil-Sanz et al., 2013). Heterophilic cell adhesions mediated by nectin1-expressing CR cells stabilize the leading processes of nectin3-expressing migrating projection neurons to anchor to the MZ, facilitating their somal translocations toward the cortical surface. CR cells extend long horizontal axons within the MZ and also act as surface docking sites of synaptic contacts with branches of apical

dendrites (Marin-Padilla, 1998; Meyer et al., 1999; Soriano and Del Rio, 2005). Recent reports have revealed the roles of CR cells in areal patterning and neurogenesis (Griveau et al., 2010; Teissier et al., 2012), indicating that CR cells have multimodal roles in instructing the early steps of cortical assembly.

In turn, the roles of SP cells in neocortical scaffolds were first revealed through ablation studies, in which SP cells in cats were eliminated using kainate. These experiments demonstrated that lateral geniculate neuron (LGN) axons fail to innervate their normal targets, which are layer 4 thalamorecipient neurons in the visual cortex (Ghosh et al., 1990). An interesting experiment to shift the tangential alignment of SP and overlaying primary somatosensory area (S1) layer 4 neurons through the electroporation of Fgf8 in the E11.5 mouse neocortex has revealed that thalamocortical axons can still recognize and innervate layer 4 cells via contact with SP neurons, albeit in a positionally shifted manner (Shimogori and Grove, 2005). Together with the observation that thalamic axons relay through superficially mispositioned SP cells in the *reeler* mutants (Molnar et al., 1998), these results indicate the primary roles of SP cells in guiding thalamic axons to enter the cortical plate (CP) and respond to cues provided by layer 4 neurons. SP cells also act as a gateway for neurons to enter the overlaying CP and accommodate massive numbers of neurons during and after their migration, thereby serving as a physical border between the CP and the intermediate zone (IZ). Perturbations in the expression of multiple genes in postmitotic cells result in halted migration and accumulation of neurons in the IZ (Miyoshi and Fishell, 2012; Ohtaka-Maruyama et al., 2013). SP cells are also required to assemble the functional neocortical circuit, where the ablation of SP cells disrupts the formation of ocular dominance columns (Ghosh and Shatz, 1992; Kanold et al., 2003). Although the molecular functions of SP cells have yet to be identified, extensive transcriptome analysis has revealed multiple cell surface components and secretory molecules that are expressed in both mouse and human SP cells, including CTGF, Cdh18, Efna5 (Mackarehtschian et al., 1999; Oeschger et al., 2012; Hoerder-Suabedissen and Molnar, 2013; Miller et al., 2014).

These tangentially coordinated CR cells and SP cells, with vertically oriented RGC fibers, form a perpendicular meshwork that enables the efficient weaving (integration) of newly generated layers of 6 to 2/3 neurons above their recently diverged siblings. In this view, the longitudinal radial glia serve as the warp and horizontally piled layer neurons serve as the weft to enable compacted neuronal accumulation and stratified CP. This process facilitates the efficient compression of massive number of neurons within a hard-boned skull-constrained space. RGCs, CR cells, and SP cells are also characteristic cell types of mammalian vertebrates, indicating that the appearance of these scaffolds instructed a neocortex-type laminated brain structure specifically in mammals. The numbers of CR cells and SP cells also expand during the course of mammalian evolution, suggesting that these neurons may have contributed to robust intercortical connectivity in primates (Smart et al., 2002; Molnar et al., 2006; Cabrera-Socorro et al., 2007). While many of these scaffolding cells are eliminated during the early postnatal period (el Rio et al., 1995; Price et al., 1997; Soda et al., 2003), a proportion of CR cells and SP cells survive in the postnatal neocortex, suggesting that these neurons also play roles in modulating the mature neocortical circuit (Kostovic and Rakic, 1980; Chowdhury et al., 2010; Judas et al., 2010).

Molecular Mechanisms of Neuronal Identity Transitions

Following the dispositions of the preplate cells and conversion from symmetric to asymmetric cell division, RGCs begin to produce layer projection neurons through sequential rounds of cell cycles (Takahashi et al., 1999). Neurons are successively generated and migrate past the pre-existing neurons to occupy the more superficial layers, resulting in an inside-out lamination of the neocortex (Angevine and Sidman, 1961). Therefore, neuronal birthdate is highly correlated with final laminar fate, in which neurons that occupy the same radial positions are typically generated within the same temporal window and share common projection targets. Deep-layer (DL) neurons, which include layers 5 and 6, consist mainly of corticofugal projection neurons and project to subcortical targets. These neurons express transcription factors Fezf2, Ctip2, Tbr1, or Sox5 (Hevner et al., 2001; Arlotta et al., 2005; Kwan et al., 2008; Lai et al., 2008; Han et al., 2011; McKenna et al., 2011), according to their projection subtypes, including the spinal cord, tectum, and thalamus (Hirata et al., 2004; Inoue et al., 2004; Chen et al., 2005a,b, 2008; Molyneaux et al., 2005; Molnar and Cheung, 2006; Yoneshima et al., 2006). In turn, upper-layer (UL) neurons, which include layer 2/3 projection neurons and layer 4 thalamorecipient neurons process higher-order information through intracortical connections. Layer 2/3 neurons typically express the transcription factors Cux1/2, Brn1/2, Satb2 (McEvilly et al., 2002; Sugitani et al., 2002; Nieto et al., 2004; Alcamo et al., 2008; Britanova et al., 2008; Franco et al., 2012) and project their axons to the ipsilateral and contralateral cortex, thereby establishing bilateral cortical connections and information integration. Layer 4 neurons, in turn, are recipient cells for thalamocortical inputs and act as a gateway for processing information from peripheral sensory organs. Layer 4 neurons typically exhibit unique cellular arrangements in the primary sensory areas, maintaining topographic organization mediated through sensory transfer. Here, we focus exclusively on understanding the mechanisms that regulate the specification and transitions between the major layer subtypes of the neocortex.

Cell Competence and Lineage Restrictions

The earliest assessment of temporal neurogenesis in the cerebral cortex was achieved through birthdating studies using tritiated thymidine injection in mice and monkeys. These experiments revealed that neocortical layer neurons are produced in a fixed temporal order (Angevine and Sidman, 1961; Rakic, 1974), implying that once progenitors switch to asymmetric cell division mode, they undergo progressive changes in competence to generate distinct layer subtypes (**Figure 1A**) (Takahashi et al., 1999). This strictly ordered production has raised several hypotheses concerning the mechanisms by which distinct

layer subtypes arise from a small number of progenitor cells. McConnell and colleagues were the first to experimentally test the temporal differentiation capacity of cortical progenitors, using a series of isochronic and heterochronic cell transplantation in ferret cortices. The major findings from these studies were that, while early-born DL progenitors can adopt later (UL) cell fates upon transplantation to an older host environment, the converse manipulation could not induce later-born UL progenitors to adopt an earlier (DL) fate (McConnell, 1988; McConnell and Kaznowski, 1991; Frantz and McConnell, 1996). While subtype-specific markers were unavailable at the time, these studies were the first to demonstrate that the differentiation potency of progenitor cells is progressively restricted throughout the course of corticogenesis.

Aside from these transplantation experiments, examining the segregation mechanisms between laminar-specific subtypes involved complementary approaches to test their lineage relationships. Hence, extensive clonal analyses in mouse and rat cortex were performed to assess when and how the layer subtypes diverge during development. These studies revealed that at least a portion of progenitor cells, if not the majority, contribute to generating clones that encompass neurons of both deep and upper cortical layers (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988, 1992; Reid et al., 1995; Yu et al., 2009; Gao et al., 2014). Furthermore, cell culture models testing the differentiation capacity of cortical progenitor cells *in vitro* also provided the basis for intrinsic and extrinsic mechanisms involved in these subtype transitions. *In vitro*, cortical cells also followed the general trend observed *in vivo*: DL neurons were commonly generated after fewer cell divisions than UL neurons in isolated cortical progenitors, and progenitors from later-stage embryos were more restricted in their ability to generate earlier-born neuronal subtypes (Shen et al., 2006). Furthermore, both mouse and human embryonic stem cell (ESC)- and induced pluripotent stem cell (iPSC)-derived cortical progenitors recapitulated the sequential generation of principal layer subtypes: preplate, DL, and UL neurons (Eiraku et al., 2008; Gaspard et al., 2008; Shi et al., 2012). These studies implied that the defined temporal order of projection neuron subtypes in the neocortex is controlled by temporal cues provided within the cortical cells themselves. Here, we discuss the identity of such cues that regulate the transitions between the major layer subtypes.

CR Cells to Deep-layer Neurons

Both *in vivo* and *in vitro*, the appearance of preplate neurons precedes the appearance of all other layer subtypes (Hevner et al., 2003; Eiraku et al., 2008; Gaspard et al., 2008; Shi et al., 2012). Here, preplate neurons are mainly CR cells based on the pan-CR cell marker Reln; thus far, no common marker for SP cells has been identified to test their differentiation capacity *in vitro*. Because of their earliest differentiation, a simple explanation concerning the ontogeny of CR cells may be that CR cell progenitors represent the default state of all cortical progenitors, thereby requiring minimum cues for their induction. However, several reports are discordant with this view: fate-mapping studies demonstrated that CR cells arise from discrete spatial domains, including the cortical hem, ventral pallium, thalamic eminence and septum, and these spatially distinct CR subtypes exhibit different molecular expressions (Bielle et al., 2005; Yoshida et al., 2006; Teissier et al., 2010; Zimmer et al., 2010). These observations implied that CR cells themselves already consist of different subtypes upon their differentiation. This discrepancy was later resolved through independent studies that assessed the temporal and spatial competence of CR cells, revealing that the distinct CR origins were commonly repressed by transcription factors Foxg1 (Kumamoto et al., 2013) and Lhx2 (Roy et al., 2014). Through a series of gene knockout studies, the removal of either of Foxg1 and Lhx2 at developmental onset resulted in the expansion of CR origins of cortical hem-, ventral pallium- and thalamic eminence-derived character (Hanashima et al., 2007; Mangale et al., 2008; Kumamoto et al., 2013; Roy et al., 2014). Interestingly, these transcription factors appear to act largely independently of each other, where their temporal knockout studies revealed an earlier competence window of neocortical progenitors to revert to CR regional identities upon the loss of Lhx2 (E10.5–E11.5) compared to the loss of Foxg1 (E13) (Hanashima et al., 2007; Mangale et al., 2008; Chou et al., 2009; Kumamoto et al., 2013; Roy et al., 2014). These results were consistent with the distinct consensus binding sequences of these two transcription factors (Hatini et al., 1994; Wilson et al., 2008).

The termination of early CR cell production is instructed through combinatorial repression by Foxg1 and Lhx2; however, the mechanisms by which progenitor cells switch from CR cell to DL neuron production required further mechanistic insights. Although the primary targets of Lhx2 involved in this event remain to be identified, the transcriptional regulatory network underlying this early subtype transition was revealed through an experiment in which Foxg1 expression onset was synchronously manipulated in cortical progenitors *in vivo*. When Foxg1 was induced at a progressively later stage during the corticogenesis period, progenitors converted to producing DL neurons (Kumamoto et al., 2013), enabling the examination of the temporal gene expression dynamics within the progenitors involved in this transition. These genome-wide studies revealed that the switch from CR cells to DL neurons involves the rapid repression of multiple transcription factors, followed by the delayed induction of upregulated transcription factors (Kumamoto et al., 2013). These results also demonstrated that the progenitor cells of CR cell and DL neuron fates share a common competence window, in which Foxg1 is both necessary and sufficient to confer the DL neuron fate over the CR cell fate. Taken together, the earliest transition of CR-to-DL neurons requires two sequential steps, which are mediated through the suppression of CR cell identity and the switch to projection neuron fate through the Foxg1 downstream cascade followed by cross-regulatory determination within layer neurons through subtype-specific determinants. Foxg1 itself is induced by FGF8 expressed in the anterior neural ridge (Shimamura and Rubenstein, 1997) and subsequently expands caudally, thus the onset of Foxg1 expression represses multiple transcription factors in an opposing rostral-to-caudal gradient, resulting in a spatiotemporal switch from CR cell to DL neuron identity (Kumamoto et al., 2013). This process also implies that the

expansion timing of Foxg1 determines the total number of CR cells produced in the cortex, which provides a mechanism to generate sufficient numbers of CR cells to cover the entire surface area prior to the onset of DL neurogenesis and to instruct the migration of later-born projection neurons.

Deep-layer to Upper-layer Neurons

In contrast to the transition from CR cells to DL neurons, which is mediated by Foxg1 and its downstream gene network, the switch from DL to UL neurons appears to utilize multiple regulatory cascades. In the aforementioned *Foxg1* conditional mutant mice, the induction of Foxg1 at progressively later stages during development (E14.5–E16.5) showed that UL progenitors are unable to bypass DL competence for their production even at the latest period of corticogenesis (Toma et al., 2014) (**Figure 1B**). The emergence of UL neurons was also assessed through lineage studies, in which Foxg1 and Cre constructs were introduced into *Foxg1*$^{-/-}$*; Rosa26*-stop-*YFP* mice, thereby labeling all progeny of Foxg1-introduced progenitors. These studies revealed that both DL and UL neurons were labeled with YFP, which determined that UL neurons emerge from cells with a Foxg1-lineage after the onset of Foxg1 expression (Toma et al., 2014). Birth-dating studies further confirmed that UL generation followed DL neurogenesis in these cells, demonstrating that the cascade downstream of Foxg1 triggers the sequence of DL and UL neuron production. These results also indicated that neocortical progenitors were biased toward DL over UL neuron fate upon Foxg1 induction.

The molecular logic underlying this DL neuron fate bias of progenitors was again uncovered through Foxg1 downstream transcriptome analysis. Of the layer transcription factors, Tbr1, which is expressed in the majority of early-born neurons (Hevner et al., 2001) and establish the corticothalamic projection neuron identity within the layer-subtype transcriptional network (Han et al., 2011; McKenna et al., 2011; Srinivasan et al., 2012), exhibited a significant downregulated response to Foxg1 induction. A reporter assay revealed that this repression was mediated through a 4-kb Tbr1 promoter region consisting of multiple conserved Foxg1 binding sequences. The introduction of *Foxg1* into E14.5 *Foxg1*$^{-/-}$ cortices demonstrated that this downregulation of Tbr1 preceded the onset of Ctip2 and Fezf2 protein induction (Toma et al., 2014). Collectively, these data show that Tbr1 repression by Foxg1 confers the sequence of DL and UL competence by establishing the bias to DL (Fezf2^{ON}/Satb2^{OFF}/Ctip2^{HI}) identity (blue cells indicated in **Figure 1F**).

The subsequent transition from DL to UL neurogenesis requires the repression of DL determinants to terminate DL competence, which involves both negative feedback and epigenetic regulations. In this regard, in experiments with the ablation of post-mitotic DL neurons *in vivo*, the relative DL neuron:UL neuron ratio was maintained despite the ablation of a significant number of DL neurons. The injection of EdU to monitor the neurons that were born from progenitors in post-ablated cortices revealed that the ablation of DL neurons prolonged the production period of DL neurons themselves, and UL neurons born at E14.5 also decreased alongside increased DL production (**Figure 1C**). Collectively, these results demonstrate that the onset of UL neuron generation is controlled by the termination of DL competence, which is propagated through post-mitotic DL neurons (Toma et al., 2014). Interestingly, this signal appears to act qualitatively rather than quantitatively *in vivo*, where only a few postmitotic DL neurons are required to induce UL neurogenesis (Toma et al., 2014), in contrast to the requirements *in vitro* (Shen et al., 2006; Eiraku et al., 2008; Gaspard et al., 2008; Kadoshima et al., 2013). These observations raise the possibility that this feedback signaling may be propagated by short-range signaling through cell–cell interactions.

While these studies showed that both DL and UL lineages are generated downstream of the Foxg1 cascade, whether the generation timing differences between the DL and UL neurons are achieved through temporal changes in competence within common progenitors (Guo et al., 2013; Gao et al., 2014; Eckler et al., 2015) or through extended mitosis specifically in early UL-committed cells (Franco et al., 2012; Gil-Sanz et al., 2015) is unclear. Because the termination of DL competence is required for both cases, negative feedback from postmitotic neurons appears to be the primary source of this cue, whereas in the latter model, additional mechanisms are required to extend mitosis in UL-committed cells. Although the decrease in UL neurons generated with extended DL neurogenesis upon ablation of DL neurons suggests the presence of common progenitors that can contribute to both DL and UL neurons, it is possible that the prolonged DL production in DL-ablated cortices may result in extended proliferative cues for UL cells. This regulation has been suggested in Sip1-expressing postmitotic neurons that maintain low expression levels of multiple secretory protein genes, including Ntf3 (Seuntjens et al., 2009; Parthasarathy et al., 2014). The accumulation of these proteins may be required to induce the differentiation of UL progenitors (Seuntjens et al., 2009) (**Figure 1D**). In this case, since *Ntf3* knockout alone or *Ntf3; Sip1* double knockout mice do not exhibit changes in the Ctip2^{+} neuron:Satb2^{+} neuron ratio compared with wild-type or *Sip1* knockouts, respectively (Parthasarathy et al., 2014), Sip1 may act through the repression of additional molecule(s) in this event (factor X indicated in **Figure 1F**). The extended DL neurogenesis achieved through the ablation of DL neurons may itself sustain low levels of these signaling molecules, thereby maintaining the UL-committed cells as progenitor cells for a prolonged period of time. As UL projection neurons mediate higher-order information processing, and their numbers expand in gyrencephalic mammals (Aboitiz and Montiel, 2003; Schoenemann et al., 2005), these feedback mechanisms also provide a new perspective as to how cell type transitions adapt to increases in cortical size, gestational period, cell cycle, and division modes (Fietz and Huttner, 2011; Lui et al., 2011) to balance the production of UL with DL neurons in different mammalian species (Striedter, 2005; Abdel-Mannan et al., 2008).

Studies have indicated that the transition from DL to UL neurogenesis is also controlled by epigenetic mechanisms. Ring1B, a component of the polycomb-repressing complex, represses Fezf2 expression in the late corticogenesis phase to shift the progenitor competence from DL to UL neurons. In

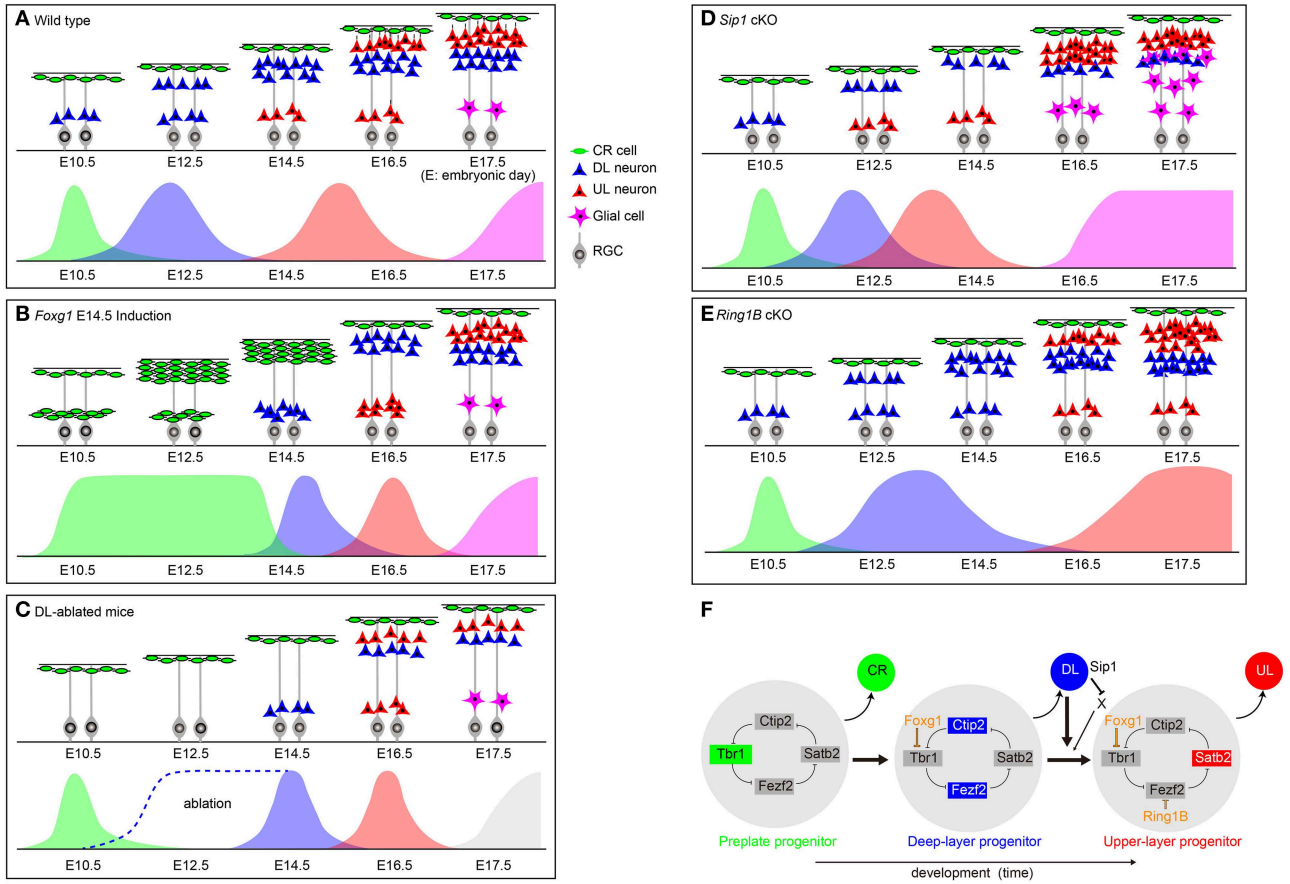

FIGURE 1 | Mouse mutants that exhibit shifts in temporal subtype transitions. (A–E) Boxes indicate temporal progression of neurogenesis in wildtype and conditional knockout mice and in DL-ablated mutant mice. Bottom scheme in each box indicates production of respective subtypes based on representative birthdating experiments depicted from each mutant analysis. **(B)** Foxg1 E14.5 induction: analysis from E9.5 to 14.5 Foxg1 OFF in *Foxg1*^{tetOFoxg1} mice (Kumamoto et al., 2013; Toma et al., 2014), **(C)** DL-ablated mice: mice in which newly-born DL neurons were ablated through consecutive tamoxifen administration at E11.5, E12.5, E13.5 in *Neurog2*$^{CreER/+}$; *Rosa*-stop-*DTA* mice (Toma et al., 2014). These mutants have not been assessed for glial production. **(D)** *Sip1* cKO: analysis from *Nestin-Cre*; *Sip1*$^{flox/flox}$ or *NEX-Cre*; *Sip1*$^{flox/flox}$ conditional knockout mice (Seuntjens et al., 2009), **(E)** Ring1B cKO mice: analysis from *NestinCreERT2; Ring1B*$^{flox/flox}$ mice administered tamoxifen at E13.0 (Morimoto-Suzki et al., 2014) and E13.5 (Hirabayashi et al., 2009). CR, Cajal-Retzius; DL, deep-layer; UL, upper-layer; RGC, radial glial cell; cKO, conditional knockout. **(F)** Molecular mechanisms of neuronal subtype transitions during corticogenesis. Cortical progenitor cells at earliest stage express multiple transcription factors including Tbr1 and differentiate to CR cells. Induction of Foxg1 by FGF8 represses Tbr1 in the layer transcriptional network, switching the progenitor fate to DL production. The transition from DL to UL neurons is regulated by signals propagated from postmitotic DL neurons, terminating DL production through negative feedback. However, DL neurons also express Sip1, which represses DL to UL transition through presumptive downstream molecule(s) X, in which the progressive accumulation of these molecule(s) may facilitate DL to UL and subsequent UL to gliogenesis transitions. The H3K27me3 level and Ring1B binding at the *Fezf2* promoter also increases over time, facilitating the DL-UL transition.

knockouts that disrupt the expression of Ring1B, Ctip2^{+} DL neurons are increased and Cux1^{+} UL neurons are decreased (Morimoto-Suzki et al., 2014) (**Figure 1E**). During this process, the H3K27me3 epigenetic mark is increased on the promoter region of Fezf2, and Ring1B binds to this marked region to suppress *Fezf2* gene expression (**Figure 1F**). In turn, in mutants in which ESET histone methyltransferase was ablated, the population of UL neurons expands at the expense of DL neurons (Tan et al., 2012). This accelerated UL production, however, prematurely decelerates at E16.5, which is the peak of normal UL neurogenesis. As a result, the production of UL neuron numbers is not significantly affected. Because neuronal survival and proliferation is also affected in ESET cKO mice, ESET may regulate the transition from DL to UL neurons indirectly through these events (Tan et al., 2012). In the future, studies that examine gene locus-specific and time-dependent mechanisms that regulate chromatin modification will likely provide further insights into the epigenetic mechanisms that govern temporal neuronal identity transitions.

Upper-layer Neurons to Gliogenesis

The switch from UL neurons to gliogenesis represents the latest transition in corticogenesis; as this step involves the termination of neurogenesis, the timing of its transition determines the overall

number of neurons produced in the neocortex. Here, we mainly refer to the transition from UL neurons to astrocytes, which are generated earlier than their glial counterparts, oligodendrocytes (Bayer and Altman, 1991; Jacobson, 1991). Dissociated cells from embryonic rodent brains revealed highly reproducible timing of the appearance of neurons and glia *in vitro*, and the generation of glia required fewer rounds of cell division in older cortex-derived progenitors than in progenitors from younger cortex (Abney et al., 1981; Qian et al., 2000), demonstrating that this neuron-glia sequence was also preserved outside the cortical environment. The timing of the appearance of gliogenic clones and the relative proportions of neurons and glia that arise from a single cortical progenitor were also assessed through *in vivo* clonal analysis using retroviral vectors (Reid et al., 1995; Mione et al., 1997; Costa et al., 2009; Gao et al., 2014) and transgenic mice (Magavi et al., 2012). These studies indicated that both neuron-restricted and bipotent (that produce neurons and glia) progenitor cells appear early in the developing cortex (E10–E13 in mice) (Costa et al., 2009; Gao et al., 2014). Of all these labeled clones, approximately 16% were bipotent (Gao et al., 2014), implying that 1 out of 6 asymmetrically dividing clones proceed to gliogenesis after neurogenesis. In turn, glia-restricted progenitors were observed mainly in later stages of corticogenesis (Costa et al., 2009).

The sequential appearance of neurons and glia in isolated cortical cells has suggested several possible mechanisms underlying the transition from neurogenesis to gliogenesis. In particular, the behavior of these cells outside the cortical environment has demonstrated that temporal cues provided in culture were sufficient to drive these transitions. In this regard, key molecular pathways that direct progenitors toward neurons or astrocyte fate have been identified. Basic helix-loop-helix (bHLH) genes play redundant roles in repressing astrocyte identity during early- to mid-stage corticogenesis, where compound knockout of *Neurog2* and *Mash1* shows precocious astrocyte production at the expense of neurons (Nieto et al., 2001), and exogenous Neurog1 can increase the number of neurons and repress astrocyte differentiation (Sun et al., 2001). In turn, the differentiation of astrocytes is mainly activated through the Janus kinase-signal transducer and activator of transcription 3 (JAK-STAT3) pathway (Bonni et al., 1997). However, both JAK-STAT signaling components and activation ligands are present even during the neurogenesis phase (Molne et al., 2000), implying that the temporal switch from repression to activation of this pathway is crucial for the UL neuron to glia transition.

In this regard, polycomb group (PcG) protein-mediated epigenetic mechanisms play key roles in this transition. PcG proteins, which repress the *Neurog1* promoter in a developmental stage-dependent manner, suppress the *Neurog1* locus to restrict the neuronal competence of progenitors and promote the transition from neurogenesis to gliogenesis (Hirabayashi et al., 2009). The inactivation of PcG by knocking out *Ring1B* and *Ezh2* genes extends the neurogenesis period and delays the transition to astrocyte genesis (Hirabayashi et al., 2009). Interestingly, this shift in neuron-to-glia transition appears to depend on the time window of *Ezh2* removal: whereas conditional knockout of *Ezh2* at E12.5 results in a prolonged neurogenesis and delayed gliogenesis (Hirabayashi et al., 2009), the removal of *Ezh2* before the onset of neurogenesis results in the accelerated neurogenesis and also early onset of gliogenesis (Pereira et al., 2010). Thus, Ezh2 may independently regulate the switch from symmetric to asymmetric cell divisions in RGCs, which later alters the timing of neuron-to-glia switch in cortical progenitors. STAT signaling increases during the later corticogenesis phase through a positive autoregulatory feedback mechanism, thereby facilitating astrocyte production during the perinatal stages. The repression of astrocyte-specific genes during the neurogenesis period is also mediated through DNA methylation, in which DNA methyltransferase gene *DNMT1* knockout results in the upregulation of JAK–STAT signaling and early transition to astrocyte differentiation. Interestingly, the progenitor potential to switch from neurogenesis to gliogenesis is also regulated through a progressive global condensation of chromatin. The overexpression of the high-mobility group A proteins HMGA1 and HMGA2 in the E15.5 mouse neocortex maintains progenitors that express Tbr2, a marker for immature neuronal precursors, at a significantly late stage of corticogenesis (Kishi et al., 2012).

The latest transition from neurogenesis to gliogenesis also requires feedback mechanisms that instruct progenitors to switch competence from neurogenic to gliogenic progenitors. It has been reported that Fgf9, which is upregulated in postmitotic neurons during the later phase of the corticogenesis period, enhances the switch to gliogenic competence. In this regard, Sip1, which suppresses the expression of Fgf9 during the neurogenic period, is gradually downregulated during the progression of corticogenesis, which derepresses Fgf9 expression and facilitates the gliogenic competence transition (Seuntjens et al., 2009). Cardiotrophin-1 (CT-1), a member of the interleukin-6 family of neurotrophic cytokines, is also expressed in post-mitotic neurons and instructs the cortical progenitors to generate astrocytes through the gp130-JAK-STAT pathway. The introduction of this neurotrophic cytokine induces premature gliogenesis, whereas perturbations in the gp130-JAK-STAT pathway delay the onset of gliogenesis (Barnabé-Heider et al., 2005). Collectively, the transition from neurogenesis to gliogenesis utilizes compound regulatory cascades to progressively restrict the neurogenic potential of progenitor cells during the late stage of corticogenesis.

Subtype-specific Integration and Neocortical Assembly

Following the generation of diverse cell types, the precise integration of these cells is essential to the formation of the neocortical circuit. The migration of diverse neurons to a location away from their place of origin enables efficient wiring between distinct classes of neurons and promotes connection between the subtypes along the radial and tangential axis. Following the exit from the cell cycle, many neocortical neurons migrate along a stereotypic route from their place of origin to their final allocation; however, growing evidence has shown that distinct subtypes dynamically change patterns of migration en route by switching their responsiveness to temporal and spatial guidance

cues. Here, we highlight such features that involve subtype-specific modes of neuronal integration during the assembly of the neocortex.

Migration Modes of Preplate Neurons

The patterns of neuronal migration of early-born preplate neurons have begun to be rapidly uncovered over the past years, illuminating their various integration routes upon entering the neocortical primordium. These disparate features likely reflect their molecular diversity acquired through their distinct spatial origins (Meyer et al., 1999, 2002; Griveau et al., 2010; Pedraza et al., 2014). As preplate neurons are the earliest neurons to migrate into the neocortex, they have the flexibility to move without much physical restriction in the absence of abundant radial glia or axonal fibers. While the spread of these neurons is clearly distinct from radial migration of later-born projection neurons, it is also somewhat different from a directional tangential migration, in which neurons exhibit a coordinated migration along the defined route, as observed in GABAergic interneurons from the ganglionic eminence to the cerebral cortex. The experimental evidence on the migration patterns of CR cells came first from fate-mapping studies of these neurons by the *exo utero* electroporation of lacZ-expressing plasmids in distinct regions of the pallium, where cells labeled in the dorsomedial pallium with lacZ migrate over the cortical surface through tangential dispersion (Takiguchi-Hayashi et al., 2004). These features were further confirmed by the genetic fate-mapping of distinct CR subtypes cells using *Wnt3a* and *Dbx1* knock-in mice (Bielle et al., 2005; Yoshida et al., 2006). These studies revealed that in addition to CR cells of the cortical hem (medial pallium) origin, the ventral pallium-derived CR cells also migrate along the surface of the developing cortex (Bielle et al., 2005). This behavior suggests that the "tangential spreading" property may be a fundamental feature of most CR cells generated from distinct sources.

Although CR cells arise from a relatively small district at the pallial border (**Figures 2A,C,D**), the unique surface spreading feature that enables CR cells to cover the entire cortical surface implies that the dispersion of these neurons may be achieved through either self-repulsive behavior and/or attractive cues provided along the route of their migration. Indeed, studies indicate that CR cells utilize both repellent and attractive cues to facilitate their dispersion along the tangential axis (**Figures 2B,E**). Here, both whole-mount cortical culture and mathematical modeling indicate that contact-mediated repulsion is necessary to optimize the cortical coverage of CR cells (Villar-Cerviño et al., 2013). In this study, CR cells of homotypic or heterotypic origins (i.e., cortical hem and ventral pallium or septum) (**Figures 2C,D**) exhibit similar repulsive responses, indicating that the CR cells of distinct sources can recognize each other to form spatial territories, mediated through the expression of multiple ephrin signaling molecules (Villar-Cerviño et al., 2013) (**Figure 2B**). This mechanism is consistent with the observation that CR cell coverage from distinct origins is highly compensatory, where ablation of either cortical hem-derived CR cells (Yoshida et al., 2006), septum-derived CR cells (Griveau et al., 2010), or combinatorial CR cell ablation of multiple sources (Tissir et al., 2009) results in the redistribution of alternative subtypes along the tangential axis. In these experiments, even upon the ablation of 84% of CR cells, Reln expression was still detectable at the cortical surface (Tissir et al., 2009), underpinning the highly compensatory features of Reln-expressing cells upon developmental perturbation. By contrast, the loss of septum-derived CR cells results in a shift in areal positioning during the postnatal stages (Griveau et al., 2010), suggesting that regional subtypes and their territorial disputes may be an important feature of neocortical tangential organization. In addition to these self-repulsive "tiling" properties, reports have indicated that CR cells also utilize attractive guidance cues. In particular, the chemokine CXCL12, expressed in the meninges, exerts its action through both of its receptors CXCR7 and CXCR4 to facilitate the surface migration of CR cells that express these receptors (Borrell and Marin, 2006; Trousse et al., 2014) (**Figure 2E**). The spatiotemporal expression of these receptors is slightly different: CXCR7 is expressed in most CR cells by E11.5 and later downregulated, whereas CXCR4 is predominantly expressed in cortical hem-derived CR cells at E11.5 and onward (Schönemeier et al., 2008; Tiveron et al., 2010), and knockout of either of these genes results in the ectopic distribution of a fraction of Reln-positive CR cells to deeper positions in the CP. Interestingly, CXCL12/CXCR4 signaling appears to be further modulated through Sema3E/PlexinD1 signaling, where the loss of PlexinD1 facilitates the migration of cortical hem-derived CR cells to more dorsomedial regions (Bribian et al., 2014) (**Figure 2E**).

In contrast to CR cells, the migration and integration properties of SP cells are worthy of further exploration. While the ontogeny of SP neurons has not been fully clarified, fate-mapping studies imply that these neurons contain at least two distinct lineages (Gao et al., 2014; Pedraza et al., 2014). Retroviral lineage tracing revealed a proportion of SP cells co-labeled with DL and UL neurons in the neocortex, indicating the common lineage between these subtypes and the cortical VZ origin of SP cells (Gao et al., 2014) (**Figure 2E**). However, SP cells have also been observed at the pallial boundary; specifically, a subpopulation of SP cells arises from the rostromedial pallium (Pedraza et al., 2014) and migrates dorsally to invade the cortex (**Figure 2C**). The diversity in their molecular repertoire and ontogeny (Miller et al., 2014) implies that SP cells may also possess subtype-specific integration and function during cortical assembly, and merits further study.

Radial Integration of Neocortical Subtypes

The lamination of the cerebral cortex is largely attributed to the unique radial migrating feature of projection neurons in the mammalian brain system, in which identical migration modes have not been observed thus far in other amniote cortices (Nomura et al., 2008, 2013b; Lui et al., 2011; Jarvis et al., 2013; Montiel and Molnar, 2013). This feature contributes to the distinctive cytoarchitecture of the neocortex and neural processing in mammalian vertebrates, despite the conserved components of neuronal subtypes based on gene expression and connectivity patterns (Suzuki et al., 2012; Jarvis et al., 2013; Nomura et al., 2013a).

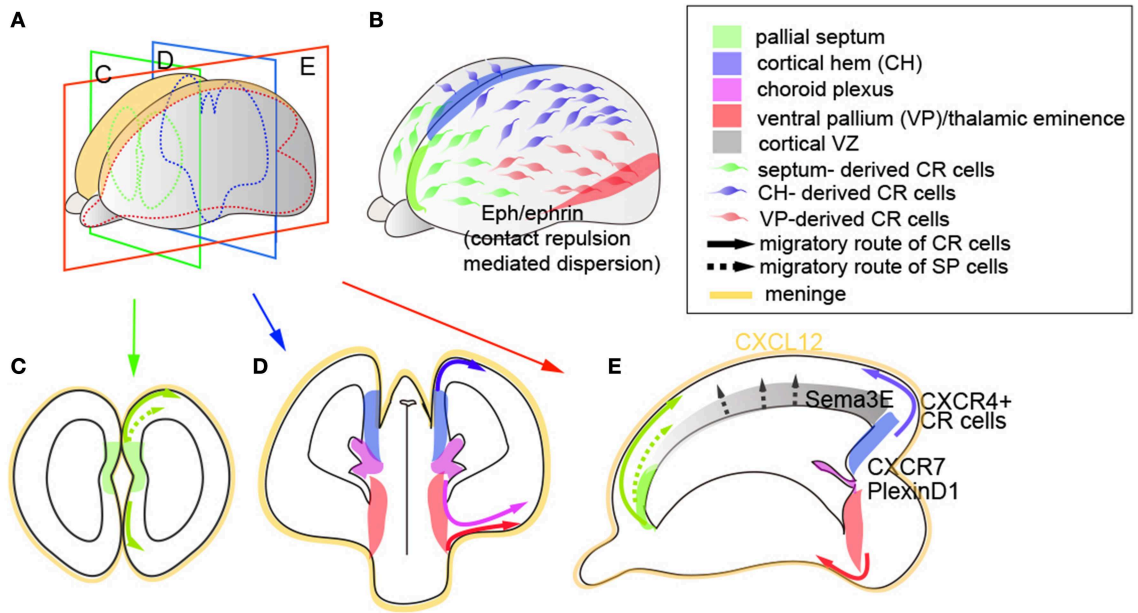

FIGURE 2 | Birth and integration of early-born preplate neurons. **(A,B)** Whole view of the mouse neocortex. **(C,D)** Indicate coronal sections of **(A)** at rostral **(C)** and caudal **(D)** levels, **(E)** indicates tangential sections of **(A)**. Colored regions indicate respective domains of CR cell origins (pallial septum, cortical hem, choroid plexus, ventral pallium/thalamic eminence) and SP cell origins (pallial septum and cortical VZ). Lines and their colors indicate the migration routes of CR cells (solid lines) and SP cells (dashed lines) arising from respective regions. Yellow regions indicate meninges, which are the primary source of CXCL12 ligands. CXCR7 and PlexinD1 are expressed in most CR cells, whereas CXCR4 is predominantly expressed in cortical hem-derived CR cells. In addition, CR cells express multiple ephrin ligands and receptors, which act as contact-dependent repulsive cues within both homotypic and heterotypic CR cell subtypes. Sema3E is expressed in a caudomedial-high to rostrolateral-low gradient in the cortical VZ, which controls the pace of migration of CR cells that express PlexinD1.

In general, the patterns of birth and migration of cortical projection neurons are considered to conform the following rules: each layer of neurons arises from the VZ and SVZ progenitors and moves radially toward the pial surface via multi-step guided migration processes. Broadly, this process involves a series of migration and positioning events, including multipolar-to-bipolar transition (Tabata and Nakajima, 2003; Noctor et al., 2004; Tabata et al., 2009), radial glia-guided locomotion (Rakic, 1972; O'Rourke et al., 1992; Nadarajah et al., 2001), detachment from radial glia (Pinto-Lord, 1982; Gongidi et al., 2004; Elias et al., 2007), and terminal somal translocation (Nadarajah et al., 2001; Sekine et al., 2011). The repetition of these events by sequential cohorts of neurons enables newly born neurons to migrate past their predecessors and take a more superficial position within the CP, establishing an "inside-out" neuronal distribution pattern (Angevine and Sidman, 1961; Rakic, 1974). The earliest evidence that layer projection neurons may utilize a subtype-specific migration mode came from a time-lapse imaging study of mouse cortical slices obtained from different developmental stages (E13–16) and labeled with Oregon Green to visualize individual neurons (Nadarajah et al., 2001). These experiments revealed that early-born subtypes predominantly undergo somal translocation to move toward the pia, which is later replaced with radial glia-guided locomotion events. The switch in these events is correlated with the overall increase in distance from the ventricular zone to the pial surface, where early-generated DL neurons require a shorter distance to migrate using extended basal processes. Consistent with this view, DL and UL progenitors appear to use distinct molecular machineries to enter the CP, in which UL but not DL neurons are susceptible to the loss of cyclin-dependent kinase 5 (Cdk5) activity (Hatanaka et al., 2004) (**Figure 3**). Furthermore, while Reln is required for both DL and UL neuron migration, its signal propagation appears to be mediated through distinct receptors between these subtypes; apolipoprotein receptor 2 (ApoER2) knockout mice exhibit a defect in Cux2-positive UL neurons but not ER81-positive DL neurons (Hack et al., 2007). Consistent with this observation, a recent expression study has demonstrated that ApoER2 protein is predominantly upregulated in postmitotic cells during the UL neurogenesis period (Hirota et al., 2015) (**Figure 3**).

Studies have also suggested that the timing of the CP entry of cortical projection neurons may also be instructed through subtype-specific mechanisms. Expression and loss-of-function studies have indicated that the UL neurons of the neocortex include at least two subpopulations, Satb2$^+$ and Unc5d$^+$ neurons; whereas Satb2$^+$ neurons migrate toward the CP immediately after their cell cycle exit, Unc5D-positive cells undergo a longer waiting period (3–4 days) within the SVZ (Tarabykin et al., 2001; Britanova et al., 2008) (**Figure 3**). The

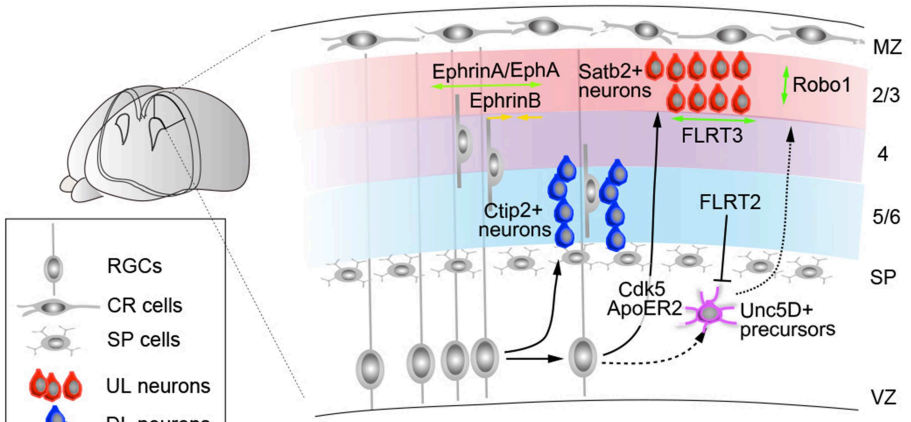

FIGURE 3 | Molecules that control subtype-dependent cortical neuron integration. The migration and distribution along the radial and tangential axis are regulated by multiple ligand/receptor molecules expressed in neocortical subtypes. In deep layers, Ctip2-positive subcerebral projection neurons form periodic organizations in layer 5, observed in both the mouse and human neocortex. In humans, the expression segregation of NOS1 in these columns is regulated by FMRP. Upper-layer neurons require Cdk5 and ApoER2 for their migration. Within the upper-layers, Satb2-, and Unc5d-positive neurons represent distinct subpopulations. The latter exhibits delayed integration into the CP through repulsive interactions, with high FLRT2 expression at E15.5 that decreases perinatally. In turn, FLRT3 regulates the tangential dispersion of E15.5-born UL neurons through adhesive interactions. In turn, the radial distribution of Satb2-positive UL neurons is regulated by Robo1. Apart from these molecules, the tangential integration of neocortical neurons is regulated through multiple ephrin-As, which facilitate lateral dispersion of both DL and UL neurons. Ephrin-B1 reverse signaling, in turn, is required to limit the tangential dispersion of ontogenic columns derived from E13.5-born progenitor cells. MZ, marginal zone; SP, subplate.

knockout mutant of both *Unc5D* and its interacting fibronectin and leucine-rich transmembrane protein-2 (*FLRT2*) exhibits the acceleration of these neurons to migrate toward the CP (Yamagishi et al., 2011), implying that the timing of integration of UL neurons is determined through subtype-dependent molecular cues.

Currently, increasing numbers of molecules have been identified that control the early phase of radial migration (Caviness and Rakic, 1978; Gupta et al., 2002; Nadarajah and Parnavelas, 2002; Tsai and Gleeson, 2005; Cooper, 2008; Huang, 2009; Honda et al., 2011); however, little is known about how the terminal positioning of neuronal subtypes is established after they arrive at the surface of the CP. The conditional ablation of genes encoding the alpha subunits of heteromeric G proteins G_{12} and G_{13} has shown that neurons cause overmigration at the cortical surface despite the intact organization of CR cells, RGCs, and basal lamina (Moers et al., 2008). In these mutants, the positioning defect appear only in a restricted number of neurons, suggesting that alternative mechanisms may also contribute to this event. In this context, Robo1, a member of the family of Roundabout receptors, regulates the radial dispersion of UL neurons in the neocortex (**Figure 3**). In a series of knockout and knockdown studies, the suppression of Robo1 was shown to result in E15-born neurons predominantly localizing to the uppermost part of layers 2/3, in contrast to control cells that were distributed radially in these layers. The sequential electroporation of fluorescent reporter constructs revealed that Robo1-suppressed cells fail to establish the characteristic inside-out neuronal distribution and accumulate beneath the marginal zone, also resulting in a thinner CP, as observed in *Robo1* knockouts. Temporal analysis also reveals that E14.5-born cells, unlike E15.5 or E16.5 neurons, do not exhibit changes in their positioning upon Robo1 suppression. As the majority of E14-born neurons adopt a layer 4 fate (Takahashi et al., 1999) and normally do not express detectable levels of Robo1 (Gonda et al., 2013), these results imply that Robo signaling acts in a subtype-restricted manner, where layer 4 neurons are refractory to loss of Robo1 expression. Collectively, these studies suggest that the mechanisms by which projection neurons migrate and integrate to their radial positions are regulated through subtype-specific codes that refine the formation of neocortical layers.

Tangential Dispersion of Neocortical Neurons

Following extensive histological studies of Golgi impregnated brains, the periodic neuronal arrangements within the cerebral cortex have motivated scientists to decipher the spatial and functional codes that drive the circuit of the neocortex. However, in contrast to the discernible laminar organization of neocortical neurons (Brodmann, 1909), the existence of definable anatomical cellular organization across tangential dimensions has remained less clear. Following Lorente de No's hypothesis of translaminar cellular modules, Mountcastle (1957) proposed that vertical columns of neurons in the cerebral cortex are fundamental processing units of the neocortex, a theory inherited by Hubel and Wiesel, leading the concept of cortical modules and

receptive fields. Although electrical recordings have revealed functional clustering and neuronal interactions along the cortical tangential dimensions, whether such modules could be defined by their anatomical and molecular character has remained elusive. However, it is increasingly becoming clear that multiple molecules may contribute to the efficient tangential mixing of neocortical projection neurons.

The functional analysis of Ephrin signaling has demonstrated that Eph receptor A (EphA) and ephrin A (Efna) signaling are essential for the assembly of cortical columns through the lateral dispersion of clonally related neurons (Torii et al., 2009) (**Figure 3**). Furthermore, a recent study revealed that ephrin-B1 also regulates the tangential motility of projection neurons, where gain-of-function of ephrin-B1 results in abnormal neuronal clustering. Conversely, ephrin-B1 knockouts display a wider lateral dispersion, resulting in the enlargement of ontogenic columns (Dimidschstein et al., 2013) (**Figure 3**). Similarly, FLRT-mediated signaling has also been shown to regulate the early tangential spread of projection neurons, in which abnormal neuronal clustering of E15.5-born neurons was observed in the tangential but not the radial axis in *FLRT3* conditional knockout mice. Together, these observations established the molecular basis that facilitates the tangential arrangement of neocortical projection neurons in general (**Figure 3**).

In this context, several reports have also indicated subtype-specific mechanisms for tangential neuronal dispersions. DL projection neurons, particularly the subcerebral projection subtypes within layer 5, that express markers including CTIP2 and FEZF2 and nitric oxide synthase 1 (NOS1) are segregated in periodic arrangements across the tangential dimensions (Maruoka et al., 2011; Kwan et al., 2012) (**Figure 3**). In both the developing mouse and human cortex, these neurons also exhibit high expression correlation with the neuronal activity marker c-Fos (Maruoka et al., 2011; Kwan et al., 2012). In mice, these microcolumns appear to comprise multiple clones, in agreement with clonal studies indicating more radially dispersed neurons of sister neurons arising from a single progenitor origin (Yu et al., 2009). Interestingly, in humans, this periodic segregation of layer 5 gene expression appears to be instructed in an area-specific manner, through the translational regulation of NOS1 by RNA-binding protein FMRP. Whereas, NOS1 mRNA is ubiquitously expressed, NOS1 protein is transiently co-expressed with FMRP during the early synaptogenesis period in layer 5 neurons of the prospective Broca's area and orofacial motor cortex (Kwan et al., 2012). The translation of NOS1 is activated by FMRP via interactions with binding motifs that are absent in mouse *Nos1* mRNA, implying that while periodic arrangements are common features of mouse and human subcerebral projection neurons, subsets of their gene expressions may be regulated in a species- and area-dependent manner. These alterations to gene expression regulation in the developing neocortical circuit may also contribute to cognitive dysfunctions in X fragile syndrome caused by mutations in FMRP coding gene *FMR1* (Ashley et al., 1993).

Studies have demonstrated that Reln, in addition to their roles in instructing radial neuronal migration, also plays important roles in the tangential migration of layer projection neuron subtypes (Britanova et al., 2006). Migration assay using wildtype mouse brain slices revealed that $Satb2^+$ projection neurons, derived from local neocortical progenitors, migrate tangentially within the upper IZ over long distances; however in *reeler* mice this migration was impaired, resulting in the reduced number of $Satb2^+$ cells in the subiculum (Britanova et al., 2006). Because the tangential migration of interneurons is not affected in *reeler* mice (Hevner et al., 2004), Reln appears to be specifically required for the tangential migration of $Satb2^+$ projection neuron subtypes. Furthermore, a recent study demonstrated that the disruption of *Reln* or its receptor *Dab1* expression, or overexpression of Ephrin-A signaling components, all disrupted the preferential electrical coupling between the radially aligned sister excitatory neurons, which are normally observed during development (Yu et al., 2009, 2012; He et al., 2015). Thus, the extent of tangential dispersion of newborn neurons within and across the cortical subtypes, may be a critical determinant for instructing the neuronal connectivity during the initial phase of cortical circuit assembly.

Areal Patterning of Neocortical Neurons

In addition to the segregation of the laminar subtypes, which is achieved through cross-repressive interactions between multiple transcription factors, it is becoming increasingly evident that transcription factors also play pivotal roles in establishing the regional identity of the neocortex, referred to as cortical arealization. Seminal work examining the function of transcription factors Emx2, Pax6, and Sp8, have revealed that the graded expression of these genes within cortical progenitors and their genetic interactions is required for establishing the topographic organization of neocortical areas (Bishop et al., 2000; Muzio et al., 2002; Hamasaki et al., 2004; Sahara et al., 2007; Zembrzycki et al., 2007, 2013). Notably, the regional characters acquired in cortical progenitors are susceptible to subsequent gene expression changes in post-mitotic neurons. Conditional knockout of *COUP-TFI*, an orphan nuclear receptor expressed in a caudal-high to rostral-low gradient in the developing forebrain (Qiu et al., 1994), results in the expansion of the frontal cortex at the expense of a compressed occipital cortex (Armentano et al., 2007). Interestingly, this caudal-to-rostral shift in cortical identity is also observed in mouse mutants in which *COUP-TFI* was specifically removed in post-mitotic neurons (Alfano et al., 2014). Conversely, the expression of *COUP-TFI* in post-mitotic neurons appears necessary and sufficient to restore the area-specific expression patterns of genes including *Cadherin-8*, *Bhlhb5*, and *Id2* (Alfano et al., 2014). Similarly, *Bhlhb5*, a bHLH gene expressed in a caudomedial-high to rostrolateral-low gradient in the post-mitotic neurons, is required to establish the regional expression of *COUP-TFI*, *RORb*, *Id2,* and *Cadherin-8* (Joshi et al., 2008). Therefore, COUP-TFI and Bhlhb5 are not only responsible for establishing areal patterning of neocortical neurons, but are also reciprocally required for their regional and laminar-specific gene expressions (Joshi et al., 2008; Alfano et al., 2014). Although the downstream mechanisms by which these transcription factors confer the area-specific neuronal distribution remain to be explored, these results suggest that cortical layer subtypes utilize region-specific cues to integrate

into distinct cortical areas, which may contribute to different laminar thicknesses among neocortical areas.

Perspectives

Neurological Disorders Associated with Cortical Assembly Defects

The increased number of genes identified in their functions for the generation and integration of neocortical subtypes, has provided molecular link between neurological disorders with corresponding gene mutations and mechanisms underlying pathogenesis. Apart from the aforementioned fragile X syndrome causative gene *FMR1*, perturbations of genes that play key roles in the differentiation of neocortical layer subtypes have been associated with a wide spectrum of neurological phenotypes. Screening for *de novo* mutations in patients with intellectual disability have identified Foxg1 and Tbr1, two of the transcriptional regulatory network components for layer subtype specification (see Section Deep-layer to Upper-layer Neurons and **Figure 1F**) as altered in their gene sequences (Hamdan et al., 2014). Loss-of-function variants (point mutations, deletions, and *de novo* translocations) and gene duplications of *FOXG1* have been associated with phenotypes including developmental epilepsy, agenesis of the corpus callosum, microcephaly, and speech impairment (Shoichet et al., 2005; Bisgaard et al., 2006; Papa et al., 2008; Yeung et al., 2009; Bahi-Buisson et al., 2010; Mencarelli et al., 2010; Brunetti-Pierri et al., 2011). In turn, its repression target *TBR1* has also been identified as one of the genes with recurrent *de novo* mutations in autism spectrum disorders (ASD) (O'Roak et al., 2012). Coexpression network analysis to identify the time period and regional convergence of high-confidence ASD genes, revealed *TBR1* as the most connected ASD gene within the key convergence point in human midfetal layers 5/6 projection neurons (Willsey et al., 2013). The functional implications of the identified *de novo* mutations, were assessed by introducing the corresponding *TBR1* gene mutations into HEK293 and SHSY5Y cell lines (Deriziotis et al., 2014). These experiments resulted in the disruption of subcellular localization of TBR1 and interaction with CASK, a membrane-associated guanylate kinase also involved in ASD (Moog et al., 2011). Similarly *SATB2*, an evolutionary conserved chromatin remodeling gene that is activated in UL neurogenesis and required for callosal projection subtype determination (Section Deep-layer to Upper-layer Neurons and **Figure 1F**), is a key gene for the 2q33.1 microdeletion syndrome (Rosenfeld et al., 2009), and *SATB2* haploinsufficiency has been associated with significant speech delay and cognitive defects (FitzPatrick et al., 2003; Leoyklang et al., 2007; Usui et al., 2013; Döcker et al., 2014).

Taken together, subtle mutations in the corresponding genes can result in profound neurodevelopmental disorders in humans; however, studies in mouse neocortex have also revealed a high compensatory feature of neurogenesis upon robust ablation of its subpopulations. Up to 84% of CR cell ablation does not demolish Reln expression in the neocortex (Tissir et al., 2009), and ablation of a significant number of DL neurons still preserves the DL:UL neuron ratio at later stages of corticogenesis (Toma et al., 2014). These features imply that while the differentiation of laminar subtypes relies on the precise regulation of spatiotemporal expression and expression levels of the key genes, the procedure of neocortical neurogenesis and assembly is robust. Such an adaptable system would enable cells to respond to extrinsic cues provided within and outside the neocortex, which may underlie the significant cortical expansion during evolution.

Future Directions

Neocortical assembly is a highly intricate process that requires multiple layers of regulation in cell behavior at the progenitor and postmitotic cell stages. The emerging picture of neocortical assembly is that while the identities of neuronal subtypes are largely determined at birth, the mechanisms by which these neurons are navigated to their final positions involve cell type- and context-dependent combinatorial codes that enable their precise integration into the neocortical circuit. While the original finding indicated that neural stem cells undergo progressive restrictions in cell competence to sequentially produce the principal layer types (Frantz and McConnell, 1996; Desai and McConnell, 2000), the molecular logic underlying these subtype transitions has only begun to unravel over the past years. Importantly, these studies also provided new insights into how the timing and quantity of the production of each neuron subtype are controlled. While the appearance of RGCs and the elaboration of early preplate cells were likely the driving force of neocortical cytoarchitecture that enabled its tangential expansion during evolution (Pollard et al., 2006; Abellan and Medina, 2009), our current understanding of the mechanisms of neocortical assembly relies heavily on the regulatory molecules and their functions identified through mouse studies. However, in an evolutionary context, the timing of production and integration of each of the neuronal subtypes must be coordinated on a species-specific developmental time scale. This process is a particular challenge for gyrencephalic mammals with an enlarged cortex, which have increased gestational period, cell cycle or division modes. Growing evidence now demonstrates that the transitions between sequential layer subtypes utilize a regulatory system that integrates both intrinsic and extrinsic mechanisms. This system not only provides qualitative cues for the migration and integration of neurons at the correct timing but quantitatively calibrates the numbers of each subtype based on the presence of their counterparts. Such hierarchical transcriptional and intercellular network organization promotes the cost-effective production and wiring of neurons during development and evolution. Continuous efforts to decipher the molecular mechanisms of subtype-specific neuronal differentiation and their integration, would facilitate our understanding of the logic that balance between economical brain assembly and vulnerability to pathological conditions.

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Diverse subtypes of astrocytes and their development during corticogenesis

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Astrocytes are one of the most abundant cell types in the mammalian central nervous system, and are known to have a wide variety of physiological functions, including maintenance of neurons, formation of the blood brain barrier, and regulation of synapse functions. Although the migration and positioning of neurons has been extensively studied over the last several decades and many aspects have been uncovered, the process underlying glial development was largely unknown until recently due to the existence of multiple subtypes of glia and the sustained proliferative ability of these cells through adulthood. To overcome these difficulties, new gene transfer techniques and genetically modified mice were developed, and have been gradually revealing when and how astrocytes develop during corticogenesis. In this paper, I review the diversity of astrocytes and summarize our knowledge about their production and migration.

Keywords: astrocyte, oligodendrocyte, cerebral cortex, subventricular zone, gliogenesis, cell specification

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Introduction

Astrocytes are among the most abundant types of glia, and the ratio of astrocytes to neurons has been shown increase with primate evolution (Bass et al., 1971). Recent studies have indicated that astrocytes not only provide support to neurons, but also actively regulate the physiological functions of the brains, and that astrocyte dysfunction can lead to developmental and/or psychiatric disorders (Molofsky et al., 2012; Burda and Sofroniew, 2014; Sloan and Barres, 2014). Despite their existence in abundance and their physiological importance, the processes underlying the development of astrocytes are largely unknown. This is partly due to the occurrence of diverse subtypes of astrocytes. The morphologies and functions of these cells differ among sites of the brain and among species. In addition, the cells have multiple origins and their proliferation persists into adult life, making analysis of the fates of these cells more complex. However, the recent introduction of novel techniques, including mice expressing region-specific Cre recombinase and *in utero* electroporation of transposon vectors have helped in revealing, at least in part, the process of normal development of astrocytes in the brain. In this brief review article, I focus on the development of astrocytes in the cerebral cortex. I first summarize the subtypes of astrocytes and their functions in rodents and primates. I then describe the migration of these subtypes from the cortical ventricular zone (VZ), and from other sites. I also describe in brief the process of development of oligodendrocytes, and compare it to that of astrocytes.

Heterogeneity of Astrocytes

The existence of two basic subtypes of astrocytes in rodents, the protoplasmic and fibrous astrocytes, has been established beyond doubt (Miller and Raff, 1984). Protoplasmic astrocytes posses highly branched bushy processes and are widely distributed in the gray matter. They extend endfeet to blood vessels and enwrap them to form the glial limiting membrane, which is the outermost wall of the blood brain barrier (BBB). They are also closely associated with synapses with its processes and play diverse roles, such as clearance of glutamate (Rothstein et al., 1996; Oliet et al., 2001), modulation of synaptic functions (Henneberger et al., 2010; Uwechue et al., 2012), and regulation of local blood flow in response to synaptic activities (Simard et al., 2003; Takano et al., 2005). Protoplasmic astrocytes have also been reported to participate in the formation and elimination of synapses (Pfrieger, 2010; Kucukdereli et al., 2011). Interestingly, the processes of two adjacent protoplasmic astrocytes are mutually exclusive, and occupy non-overlapping domains (Bushong et al., 2002; Ogata and Kosaka, 2002; Halassa et al., 2007). The domain of a single astrocyte covers about 100,000 synapses in mice (Bushong et al., 2002), and these synapses can be simultaneously regulated by one astrocyte as a synaptic island (Halassa et al., 2007).

On the other hand, fibrous astrocytes possess straight and long processes and are mainly located in the white matter. In this cell type, the expressesion level of glial fibrillary acidic protein (GFAP), an intermediate filament protein, is higher than that in the protoplasmic astrocyte, in which the GFAP protein is sometimes found only in the endfeet on the blood vessels (Oberheim et al., 2009). The functions of fibrous astrocytes are not clear. At least, these cells associate with the blood vessels via their processes just like the protoplasmic astrocytes (Marín-Padilla, 1995). In addition to these basic cell types, there are specialized astrocytes in Layer 1 of the murine cerebral cortex that show a bushy morphology similar to that of protoplasmic astrocytes in the gray matter, but strongly express GFAP like fibrous astrocytes. Their processes cover the outer surface of the brain parenchyma just under the pia matter and form the glial limiting membrane, which continues into the other part of the glial limiting membrane formed by the endfeet of the protoplasmic astrocytes, as described above (**Figure 1**). GFAP-positive fibroblast-like cells have been reported to exist on the pial surface, (García-Marques and López-Mascaraque, 2013; Martín-López et al., 2013). These cells also cover the outer surface of the brain with their cell bodies to participate in the formation of the glial limiting membrane. Although the subtypes of astrocytes described above, namely

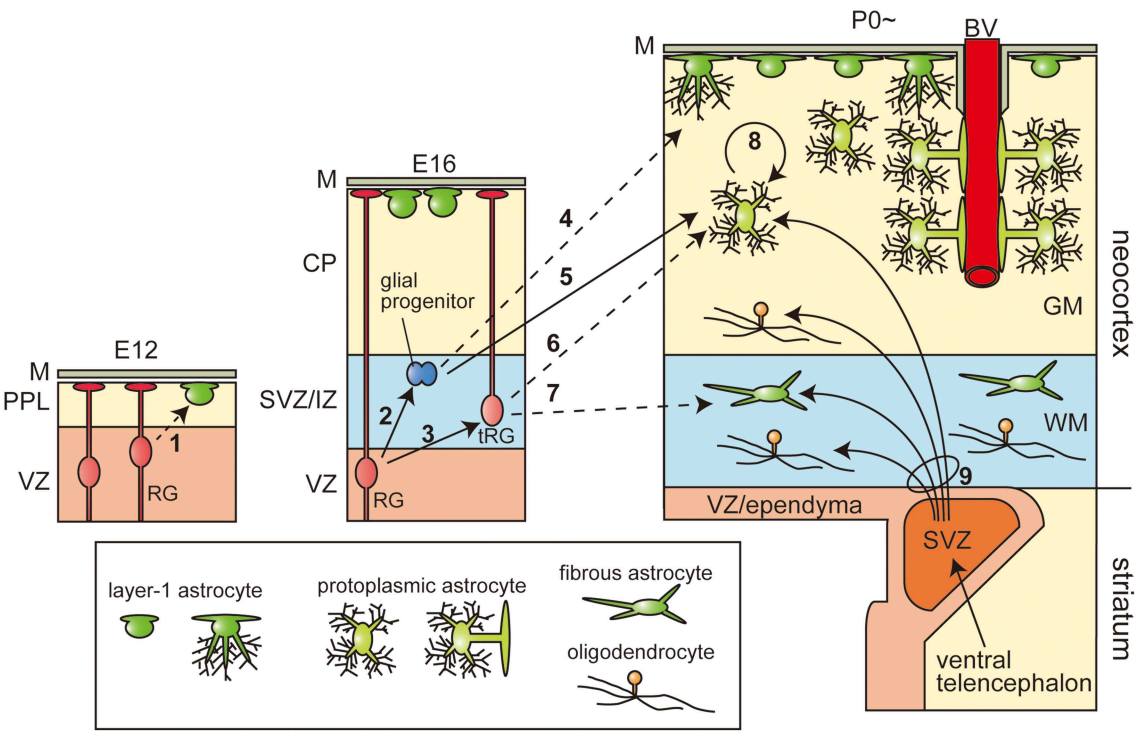

FIGURE 1 | Heterogeneity of astrocytes and the multiplicity of their origin. The three pictures represent the production and final positioning of the astrocytes and oligodendrocytes in the developmental stages. The stages in the mice are given above each picture (E, embryonic day; P, postnatal day). Arrows with solid lines indicate the cell lineages confirmed by lineage tracing experiments. Arrows with broken lines show the hypothetic cell lineages by histological investigations, but not confirmed by precise lineage tracing. Neurons and OPCs are not shown. GM, gray matter; WM, white matter; M, meninges or pia matter; PPL, primordial plexiform layer; VZ, ventricular zone; SVZ, (embryonic or postnatal) subventricular zone; IZ, intermediate zone; CP, cortical plate; BV, blood vessel.

fibrous, protoplasmic and Layer-1 astrocytes, are widely found in mammalian brains, there are at least two specific subtypes for human or other primates (Colombo and Reisin, 2004; Oberheim et al., 2009; Sosunov et al., 2014). In Layer 1 of the primate cerebral cortex, there are densely packed $GFAP^+/CD44^+$ astrocytes called interlaminar astrocytes (Colombo and Reisin, 2004). These cells extend straight and poorly branched processes that are about a millimeter long into the cortical gray matter, frequently terminating on the blood vessels in Layers 2–4 (Sosunov et al., 2014). This subtype appears after birth, and in the fetal stages the glial constituents in the Layer 1 are similar to that of rodents, and thus, transformation of Layer-1 astrocytes with short processes to interlaminar astrocytes has been suggested (Marín-Padilla, 1995; Colombo et al., 1997). The second subtype that is primate-specific is the varicose projection astrocytes, which are also $GFAP^+/CD44^+$ and are situated mainly in Layers 5 and 6. This cell type extends many straight 100-μm long processes and one to five up to 1-mm long processes with many varicosities (Oberheim et al., 2009; Sosunov et al., 2014), which may terminate in the neuropil or on the vasculature. In human, protoplasmic and fibrous astrocytes also exhibit unique structure. They have been reported to be 2∼2.5-fold larger in diameter in the human cortex than in the mouse (Oberheim et al., 2009). Human protoplasmic astrocytes also form exclusive domains like the cells in rodents, and a single domain covers about 2,000,000 synapses. In the deeper layers of the human cortex, protoplasmic astrocytes and varicose projection astrocytes coexist, and their processes are intermingled, suggesting that they are distinct subtypes of cells with differing functions.

Glial Production in the Cortical VZ

Astrocytes in the cerebral cortex are produced from the cortical ventricular zone (VZ) or from the ventral forebrain. In the cortical VZ of mammalian embryonic/fetal brains, there are cells called radial glia (RG), which extend long ascending processes called radial fibers to the pial surface and act as a scaffold for neurons migrating from the VZ toward the pial surface. RG were labeled as "glia" because they show several features of astrocytes, such as glycogen granules (Schmechel and Rakic, 1979; Gressens et al., 1992) and express GFAP, especially in the human fetus (Levitt et al., 1981; Cameron and Rakic, 1991). However, they are actually not differentiated glia, but neural stem cells, which generate neurons during the early to late cortical development, and later, glia (Fujita, 1963; Miyata et al., 2001; Noctor et al., 2001). There is a longstanding debate on whether RG in the cortical VZ are homogeneous and whether their potential changes from neuronal production to glial production during the course of development, or whether the RG population includes neuron-restricted progenitors and glia-restricted progenitors even from the early stage of cortical development and the glial progenitors are in a dormant state until the late stages. Although several lines of evidence support the latter (Levitt et al., 1981; McCarthy et al., 2001), recent lineage tracing experiments using mixed retroviruses (Costa et al., 2009) and the Mosaic Analysis with Double Markers (MADM) technique (Gao et al., 2014) have shown no significant numbers of glia-restricted progenitors in the early stages. Recently, a new glial lineage tracing system using transposon plasmid vectors, which integrates into the host genome in the presence of transposase (Kawakami and Noda, 2004; Sato et al., 2007), has been developed. It has been demonstrated that introduction of the transposon vector together with the transposase expression vector by *in utero* electroporation (Fukuchi-Shimogori, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001) successfully labeled glial cells (Yoshida et al., 2010). Using this technique, Siddiqi et al. demonstrated that the RG were first exclusively $GLAST^+/Nestin^+$ and produced neurons preferentially, and then $GLAST^+/Nestin^-$ progenitors emerged within the RG population in the later stages, that preferentially produced astrocytes (Siddiqi et al., 2014), demonstrating a potential shift from neuronal to glial production from RG. Moreover, Noctor et al. directly observed that the neural stem cells first produced neurons by asymmetric cell divisions and then the same cells differentiated into astrocytes in long-term live imaging on slice culture (Noctor et al., 2004). Based on the aforementioned evidence, the former hypothesis is now widely accepted.

After specification of the glial lineage, the glial progenitors migrate into cortical gray matter and white matter and differentiate into protoplasmic and fibrous astrocytes, respectively. The most accepted model of such migration of glial progenitors is the direct transformation of RG (**Figure 1**, arrow-3, 6, 7), in which the radial fibers are retracted to elevate the cell soma from the VZ. This cell movement is similar to that identified in the neuronal migration process called "somal translocation" (Nadarajah et al., 2001), and the cells under such transformation are called transforming RG (tRG). The morphology of tRG has been observed repeatedly by Golgi staining, immunostaining for GFAP, and carbocyanine dye (DiI) staining (Schmechel and Rakic, 1979; Voigt, 1989; Gressens et al., 1992; deAzevedo et al., 2003). The differentiation of tRG cells into astrocytes has been directly shown by live imaging on slice culture (Noctor et al., 2004). On the other hand, astrocytes are also thought to arise from proliferative glial progenitors in the subventricular zone (SVZ; **Figure 1**, arrow-2, 4, 5). It would be of interest to know which progenitors produce which subtypes of astrocytes. Gressens et al. administrated [3H]-thymidine to E17 mice, after completion of neurogenesis, and observed that the GFAP- or RC2-positive [3H]-thymidine labeled cells (proliferative glial progenitors) were first found in the SVZ or IZ and gradually shifted toward the pial surface and positioned themselves in the upper cortical plate, but not in the white matter. Moreover, they administrated methylazoxymethanol acetate (MAM), which eliminates proliferative cells, to E17 and E18 mice, and observed the greatly reduced number of protoplasmic astrocytes in the upper cortical plate, with no significant effect on the generation of the fibrous astrocytes in the white matter (Gressens et al., 1992), suggesting that the proliferative glial progenitors in the SVZ only differentiate into protoplasmic astrocytes (**Figure 1**, arrow-5). On the other hand, Cai et al. demonstrated that postnatal genetic deletion of Olig2, a transcription factor known to be essential for glial differentiation (Ono et al., 2008), resulted in a severe deficit in the formation of fibrous astrocytes, but no significant difference in the number of protoplasmic astrocytes in the upper cortical plate (Cai et al., 2007), indicating that these

two classical subtypes are generated in different ways. Recently, the multi-color lineage tracing system for astrocytes, called the "Star Track" method, has been developed by modifying the transposon vector system (García-Marques and López-Mascaraque, 2013; Martín-López et al., 2013). Consistent with the results of the traditional retrovirus lineage tracing experiments (Price and Thurlow, 1988; Levison et al., 1993), Star Track also demonstrated that most of the clones were either exclusively protoplasmic or exclusively fibrous astrocytes, suggesting that these two types of astrocytes are generated from independent progenitors, although it remains unknown as to which progenitors they might be.

The process of generation of Layer-1 astrocytes was also found to be unique. By intensive observations using Golgi staining, Marin-Padilla proposed that the Layer-1 astrocytes are produced in two waves (Marín-Padilla, 1995). In the very early stage of cortical development, the primordial plexiform layer (PPL), which is also called preplate, is formed just outside the VZ. In this stage, a subset of VZ-derived cells move onto the basal lamina underling the pia matter, and differentiated into first Layer-1 astrocytes and form the subpial glial limiting membrane (**Figure 1**, arrow-1). As development proceeds the population of Layer-1 astrocytes adopts newly generated astrocytes probably derived from the SVZ (**Figure 1**, arrow 4). It is not clear whether these two different origins of the Layer-1 astrocytes correspond to two types of Layer-1 astrocytes, namely fibroblast-like and protoplasmic-like astrocytes. Nevertheless, the Star Track analyses revealed that the clones of these subtypes of Layer-1 astrocytes are highly exclusive of each other (García-Marques and López-Mascaraque, 2013; Martín-López et al., 2013). It has been reported that a subset of protoplasmic astrocytes arises from the Layer-1 astrocytes or multipotent progenitors in the layer 1 of the cerebral cortex (Marín-Padilla, 1995; Costa et al., 2007).

As the brain increases in size during the first 20 postnatal days in mice, the number of glia increases dramatically (Bandeira et al., 2009). However, direct transformation of RG may produce a limited number of astrocytes, and the production of astrocytes from the proliferative glial progenitors in the SVZ almost ends by P14 (Levison et al., 1993), suggesting the additional cell-amplifying system. By using two-photon microscopy, Ge et al. observed the frequent cell divisions of the protoplasmic astrocytes in P5 hGFAP-GFP mice with an open skull, but an intact pial surface (Ge et al., 2012) (**Figure 1**, arrow-8). The dividing cells were not migrating glial progenitors, but differentiated protoplasmic astrocytes settled in the cortical gray matter. These dividing astrocytes extended highly branched processes, contacting the blood vessels with their endfeet, and coupled with surrounding mature astrocytes with gap junctions. This local production was estimated as the major source of protoplasmic astrocytes in the adult brain.

Multiple Origins of Glia

Glia of the cerebral cortex are also produced from the postnatal SVZ, a specialized reservoir of glial and neuronal progenitors. The postnatal SVZ is represented by the wedge-shaped structure between the pallium and subpallium, and is composed of Zebrin II (aldolase C)-positive cortical VZ-derived cells, mainly located in the periphery, and Dlx2-positive ventral telencephalon-derived cells populating the center (Marshall and Goldman, 2002). Lineage tracing after direct injection of a retrovirus into the postnatal SVZ revealed that while the neurons migrated anteriorly to the olfactory bulb and differentiated into granular and periglomerular interneurons (Alvarez-Buylla and Garcia-Verdugo, 2002), the glial progenitors migrated dorsally and differentiated into both astrocytes and oligodenderocytes in the gray and white matter (Levison and Goldman, 1993; Parnavelas, 1999; Marshall and Goldman, 2002) (**Figure 1**, arrow-9). The proportions of astrocytes and oligodendrocytes produced from this structure show temporal changes. The glial progenitors of the P2 SVZ in the neonatal rat gave rise to astrocytes mostly in the cortical gray matter, while the P14 SVZ cells mainly differentiated into oligodendrocytes in the white matter (Levison et al., 1993). Within the MGE-derived cell population, Olig2 acts as a determinant of the glial fate. Overexpression of wild-type Olig2 using retrovirus increased the production of both astrocytes and oligodendrocytes, while overexpression of the dominant-negative form of Olig2 increased the production of neurons (Marshall et al., 2005).

As another possible source of astrocytes, oligodendrocyte progenitors (OPCs) cannot be ignored. OPCs express several specific markers, such as NG2 and platelet-derived growth factor receptor α (PDGFRA), and are distributed widely in the late embryonic and postnatal brains. OPCs collected from the rat optic nerve using A2B5 mononclonal antibody, which binds to an early OPC-specific ganglioside (Eisenbarth et al., 1979; Schnitzer and Schachner, 1982; Raff et al., 1983a), were shown to differentiate into $GFAP^+$ astrocytes in culture in the presence of serum factors (Raff et al., 1983b). The resulting astrocytes from the OPCs in culture are called type 2 astrocytes, while those from the cortical VZ are called type 1 astrocytes, because they exhibit different morphologies. The ability of cultured OPCs to produce astrocytes *in vivo* was shown by transplantation. When human $A2B5^+$, $PSA\text{-}NCAM^-$ cells taken from 17- to 23-week forebrains were expanded in culture with fetal bovine serum and grafted into newborn mice at P0 or P1, they gave rise to astrocytes as well as NG2 cells and oligodendrocytes (Windrem et al., 2004, 2008, 2014; Han et al., 2013). These observations indicate the potential of OPCs to produce astrocytes. However, differentiation of OPCs into astrocytes during the course of normal development of brains seems minor, if any. When OPCs were cultured in serum-free medium and grafted into P5 rats, OPCs differentiated only into oligodendrocytes but not astrocytes (de los Monteros et al., 1993). Moreover, lineage tracing experiments using transgenic mice that express Cre recombinase in the OPCs (NG2-Cre and PDGFRA-CreERT2) revealed that OPCs produce oligodendrocytes in the gray and white matter, but not astrocytes in the neocortex, although some astrocytes were produced in the ventral forebrain (Zhu et al., 2007, 2012; Rivers et al., 2008).

As described above, astrocytes in the cerebral cortex have multiple origins, and are functionally and morphologically diverse. This raised the question of whether the progenitors at different sites of the brain produce functionally identical populations of astrocytes and compensate cell numbers, or produce different

subtypes of astrocytes. The results of an experiment using the Cre-loxP lineage tracing system showed that oligodendrocytes in the cerebral cortex are also produced from different sites depending on the developmental stages (Kessaris et al., 2006). The first wave of production begins around E12.5 from Nkx2.1-expressing precursors in the MGE and anterior entopeduncular area (AEP). The second wave begins around E15 from Gsh2-expressing LGE and the caudal ganglionic eminence (CGE), and finally, local production begins in the Emx1-expressing cortical VZ around birth. When any one of these production sites is eliminated by expressing diphtheria toxin A fragment (DTA) under the control of the same Cre driver mouse lines, the OPCs from the other sites cover the deficient area (Kessaris et al., 2006). Furthermore, the oligodendrocyte lineage cells derived from the Nkx2.1-progenitors decreased during postnatal life, and were replaced with newly generated Gsh2- and Emx1-derived cells. Hence, oligodendrocytes derived from three different progenitor domains are functionally replaceable by each other, and compete to populate the limiting space in the cerebral cortex. This situation is referred to as "oligodendrocyte wars" (Richardson et al., 2006). However, this is not the case for astrocytes, especially in the spinal cord. Astrocytes in the spinal cord are produced from different progenitor domains arrayed in a dorsal to ventral pattern in the VZ. When one of the progenitor domains is eliminated by specific expression of DTA, neighboring astrocytes or their progenitors do not enter the deficient area to cover the functions (Tsai et al., 2012). In the cerebral hemispheres, however, substantial amounts of glial progenitors migrate from the MGE and differentiate into astrocytes as mentioned above. In fact, the astrocytes derived from Dlx2-expressing progenitors in the postnatal SVZ were reported to extend their endfeet onto blood vessels (Marshall and Goldman, 2002), indicating that they are functionally equivalent to the cortical VZ-derived astrocytes. Moreover, in the transplantation experiments of A2B5$^+$/PSA-NCAM$^-$ human glia progenitors, astrocytes in the host mouse brains were gradually replaced by human astrocytes derived from the donor cells (Han et al., 2013; Windrem et al., 2014), suggesting cell-cell competition among the astrocytes for their exclusive domains in the limited space of the cerebral hemispheres. This situation should be called "astrocyte wars." Interestingly, the implanted human glial progenitors developed in a cell-autonomous manner in the host mouse brains, and generated protoplasmic astrocytes of larger diameter than the host cells and also varicose projection astrocytes having several long unbranched processes with many varicosities. Surprisingly, the resulting humanized chimeric mice represented higher LTP and higher learning ability than the control mice (Han et al., 2013), suggesting that the higher intellectual activity of humans is, at least a part, due to the human-type astrocytes.

Perspectives

In this article, I have described the heterogeneity of astrocytes among different sites of the cerebral cortex and among different animal species. I have also described the several distinct origins of astrocytes. As at present, the relationships between the origins and subtypes of astrocytes are not yet fully clarified. For example, the development and specification of protoplasmic and fibrous astrocytes are still not clear, even though they are the most basic subtypes of astrocytes. Recent studies have revealed many aspects of the physiological importance of astrocytes, such as the regulation of synapse functions and blood flow, which has drawn a lot of attention to the process of glial development, maturation and plasticity. Moreover, novel methods of lineage tracing and gene transfer for glial progenitors have been developed using transposon or Cre-loxP systems, and these modern techniques are now greatly accelerating the accumulation of knowledge in this field. Interestingly, many glia-specific genes have been identified as genes related to developmental and/or psychiatric disorders. To understand the mechanisms underlying the development of these diseases and to develop new clinical treatments, further knowledge of glial development is important.

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REVIEW
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Neuronal polarization in the developing cerebral cortex

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Cortical neurons consist of excitatory projection neurons and inhibitory GABAergic interneurons, whose connections construct highly organized neuronal circuits that control higher order information processing. Recent progress in live imaging has allowed us to examine how these neurons differentiate during development *in vivo* or in *in vivo*-like conditions. These analyses have revealed how the initial steps of polarization, in which neurons establish an axon, occur. Interestingly, both excitatory and inhibitory cortical neurons establish neuronal polarity *de novo* by undergoing a multipolar stage reminiscent of the manner in which polarity formation occurs in hippocampal neurons in dissociated culture. In this review, we focus on polarity formation in cortical neurons and describe their typical morphology and dynamic behavior during the polarization period. We also discuss cellular and molecular mechanisms underlying polarization, with reference to polarity formation in dissociated hippocampal neurons *in vitro.*

Keywords: neuron, polarization, axon, cerebral cortex, imaging, excitatory cortical neuron, inhibitory cortical neuron

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Introduction

Neurons are highly polarized cells that typically exhibit a single axon and several dendrites. Dendrites receive incoming signals at the synapse and convey them to the soma. These signals trigger action potentials at the level of soma, which propagate along the axon and are transmitted to target cells at a presynaptic site. A critical question in neurobiology is how neurons acquire axon-dendrite polarity, a property required for directional information flow in the nervous system.

Axon-dendrite polarization has been historically examined using cultured, dissociated hippocampal neurons (Dotti et al., 1988). These neurons initially appear symmetric, extending and retracting several immature neurites of similar length. Elongation of a single process, the one that will become the axon, breaks this symmetry. Thus, based on this model, neuronal polarity formation has been believed to result from a stochastic symmetry-breaking event. However, more recent morphological and imaging studies *in vivo* or *in situ* (in *in vivo*-like conditions that maintain an intact three-dimensional structure surrounding immature neurons) suggest that several types of neurons establish neuronal polarity by inheriting apicobasal polarity from neuroepithelial progenitors or maintaining front-rear polarity in migrating cells (Barnes and Polleux, 2009; Hatanaka et al., 2012). Therefore, it remained uncertain whether these activities occurred *in vivo* and, if so, whether they were regulated by similar events as that appear in cultured hippocampal neurons.

The cerebral cortex is evolutionary the youngest and the most complex region of the brain. It is composed primarily of excitatory neurons, which are glutamatergic, and by a smaller proportion of inhibitory neurons, which are GABA (γ-aminobutyric acid)-ergic. During development, excitatory

neurons originate from the dorsal telencephalon (Molyneaux et al., 2007), while inhibitory neurons originate from the ventral telencephalon (Gelman and Marin, 2010). Both subtypes are then integrated into the cerebral cortex and extend axons and dendrites to establish functional cortical circuitry. Interestingly, recent imaging studies have revealed how these dynamic developmental processes occur *in vivo* or *in situ*. Those studies suggest that most cortical neurons likely establish an axon via an initial symmetry-breaking event, a process similar to that observed in cultured hippocampal neurons.

In this review, we first give an overview of current knowledge about the developmental process of axon and dendrite formation of excitatory and inhibitory neurons in the cerebral cortex. Then we focus on the dynamic behavior underlying axon formation of these neurons, with reference to dissociated hippocampal neurons. Although there is evidence that some neurons may inherit some aspects of polarity emerged at a stage prior to axon formation, the model based on hippocampal cells still predominates in this field and could explain behavior of cortical neurons. We thus further summarize both intracellular signals and cytoskeletal dynamics underlying polarity formation in dissociated hippocampal neurons and in cortical neurons *in vivo* or *in situ*.

Axon-Dendrite Polarization of Excitatory and Inhibitory Cortical Neurons

The cerebral cortex is composed of the neocortex and allocortex. The neocortex, which is a six-layered structure unique to mammals, is phylogenetically the youngest brain region and comprises most of the cortex. In contrast, the allocortex is phylogenenetically older and characterized by fewer layers than the neocortex. The development of polarity by neocortical cells is the major focus of this review.

In rodents, cortical neurons are comprised of 70–80% excitatory and 20–30% inhibitory neurons. Excitatory projecting neurons convey cortical output to subcortical structures and to other cortical areas. In general, neurons exhibiting corticofugal projections, which extend axons away from the cortex, reside in deep layers; by contrast, neurons that project intracortically extend axons to areas in the ipsilateral and/or contralateral cortex, reside in upper layers and to a lesser extent in deep layers (Greig et al., 2013). Depending on layer location and projection, excitatory neuron morphology varies. However, many excitatory neurons resemble so-called "pyramidal cells": their soma is shaped like a pyramid with a base facing the apical aspect of the cortex, and these cells extend a single axon and two separate apical and basal dendrites (Jones, 1984). Their axons extend toward the white matter (WM) where they typically turn and continue to project tangentially, while their apical dendrites extend toward the pial surface. Inhibitory neurons, on the other hand, are mostly local-circuit neurons that contribute to intracortical information processing by modulating excitability and thus shaping cortical output. Inhibitory neurons also extend a single axon and multiple dendrites, but their morphologies are highly diverse: they include basket cells, chandelier cells, Martinotti cells, double bouquet cells, neurogliaform cells, and at least 10 others (Kubota, 2014). Until now, however, only a few reports have described how axons or dendrites emerge from these neurons (e.g., Kawaguchi, 1993).

Axon Formation Is the Initial Step of Cortical Neuronal Polarization

Excitatory and inhibitory cortical neurons originate in distinct brain regions: the former emerge from the pallium and the latter primarily from the subpallium (Molyneaux et al., 2007; Gelman and Marin, 2010). Recent advances in cell labeling techniques, including use of genetically-modified mice and *in utero* electroporation methods, allow us to label these neurons accurately. Furthermore, advanced imaging techniques have revealed dynamic processes underlying their development.

Development of Excitatory Cortical Neurons

Excitatory cortical neurons originate predominantly from radial glial progenitors in the cortical ventricular zone (VZ) (**Figure 1**). Asymmetric division of these cells generates both self-renewing progenitors and young neurons or intermediate progenitors, and those intermediate progenitors then further divide to increase neuronal number (Miyata et al., 2001, 2004; Noctor et al., 2001, 2004; Pontious et al., 2008). Newly-generated neurons migrate through the subventricular zone (SVZ) and intermediate zone (IZ) to reach the cortical plate (CP). There, later-generated neurons migrate past neurons generated earlier and eventually occupy more superficial positions, resulting in an inside-first/outside-last neurogenetic gradient (Angevine and Sidman, 1961). Following completion of this migration, these activities give rise to a sixed-layered cortical structure (Bayer and Altman, 1991).

Morphologically, bipolar progenitor cells are extraordinarily slender and extend a long apical process toward the pial surface and a short basal process toward the ventricle. During asymmetric cell division, the daughter cell destined to become a neuron assumes a multipolar shape from which emanates multiple short, thin processes in the SVZ/IZ (Tabata and Nakajima, 2003; Noctor et al., 2004). After repeated extension and retraction of processes over several hours, one process suddenly extends tangentially within the IZ (Hatanaka and Yamauchi, 2013; Namba et al., 2014; Sakakibara et al., 2014). That process continues to elongate and eventually becomes an axon, as indicated by its length, morphology, and accumulation axonal markers such as Kif5c560 in the tip (Hatanaka and Yamauchi, 2013; see also Section Intracellular Mechanisms). Immature axonal processes also contain bidirectional microtubule fibers (Sakakibara et al., 2014), and in this aspect exhibit the kind of mixed microtubule polarity typically seen in the trailing process of migrating cerebellar granule cells, which also becomes an axon (Rakic et al., 1996). The remaining short processes gradually transform into thick leading processes directed toward the pia (Hatanaka and Yamauchi, 2013; Sakakibara et al., 2014). By the time cell bodies reach the CP, they appear bipolar, extending a trailing process "behind" (opposite the direction of migration) and a leading process "in front" of the nucleus. Cells then migrate with glia-guided locomotion mode (Rakic, 1972; Nadarajah and Parnavelas, 2002) characterized by

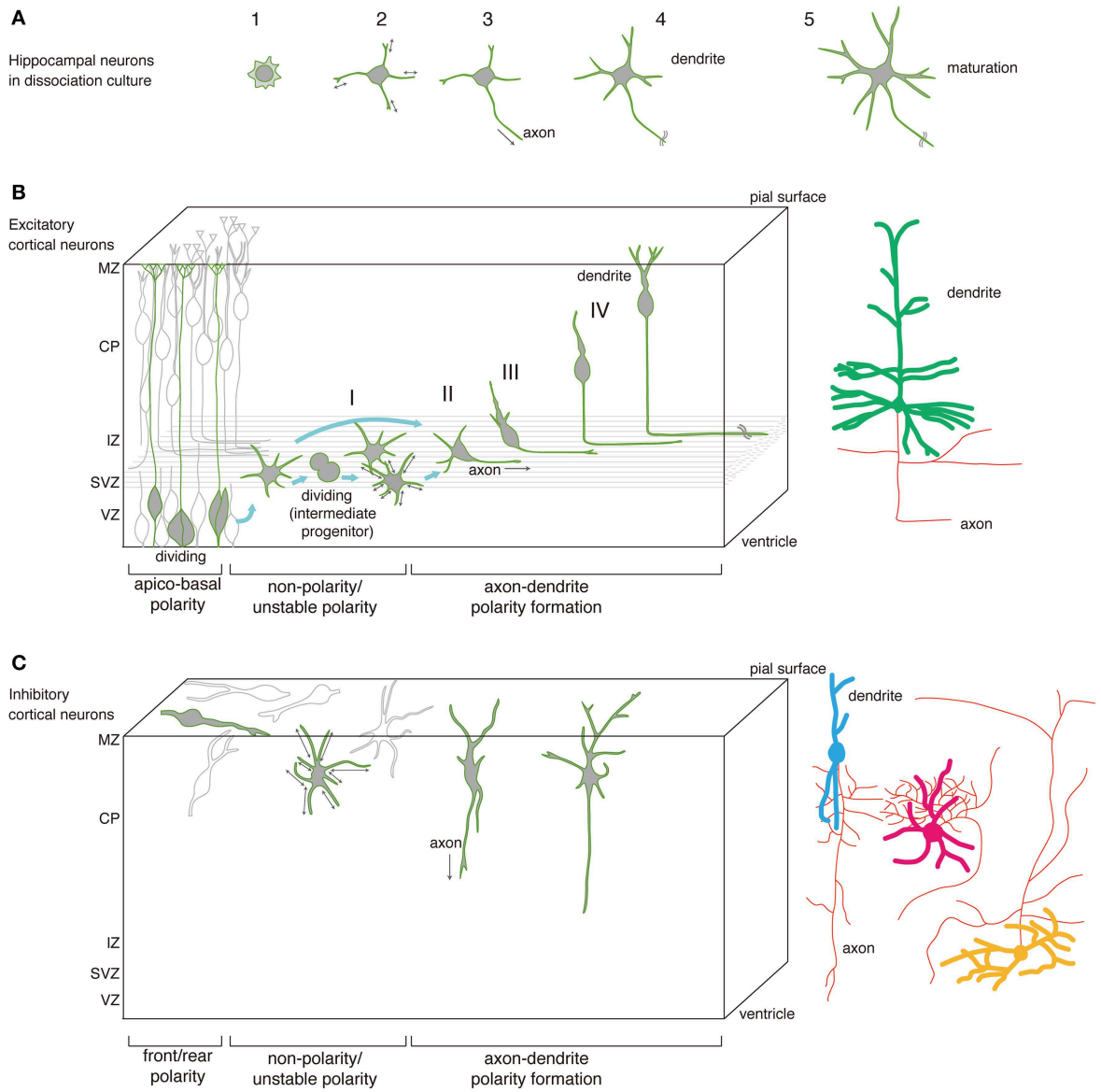

FIGURE 1 | Sequential events of polarity formation as seen in hippocampal neurons *in vitro* and excitatory and inhibitory cortical neurons *in vivo*. Axon outgrowth processes are similar *in vivo* and *in vitro*, as axons emerge from non-polarized cells. **(A)** Schematic drawing showing neuronal polarity formation in dissociated hippocampal neurons in culture. (1) Immature neurons actively form filopodia and lamellipodia and then (2) extend multiple minor processes that randomly extend and retract their tips. (3) After several hours in culture, a minor process begins to grow rapidly and transform into an axon (symmetry break). (4) That axon further extends, and remaining processes differentiate into dendrites. (5) Finally, these differentiated processes mature. **(B)** Schematic drawing showing acquisition of neuronal polarity by excitatory cortical neurons. (I) Young neurons differentiated from ventricular zone (VZ) cells or through intermediate progenitors transform into multipolar cells, whose short processes repeatedly extend and retract in the subventricular zone (SVZ)/intermediate zone (IZ) over several hours. (II) A new process, which will become the axon (symmetry break), suddenly elongates tangentially. (III) The remaining processes, which will become dendrites, transform into a pia-directed leading process. (IV) Neurons gradually change shape, become bipolar, and migrate radially toward the pia, with the elongating axon as the trailing process. After reaching their final destination, axonal and dendritic processes mature. Most excitatory neurons differentiate into pyramidal cells (dendrites, green; axons, red). **(C)** Schematic drawing showing neuronal polarity formation by inhibitory neurons. These neurons are generated in the subpallium and migrate to the cortex. There they reach the marginal zone and execute multidirectional tangential migration, exhibiting a bipolar shape with a leading and trailing process. As development proceeds, they localize in the cortical plate, alternately extend and retract short processes, and exhibit low motility of somata. An axon then emerges (symmetry break) extending primarily toward the ventricle. Inhibitory neuron morphology is highly diverse: these subtypes include basket cells (the major inhibitory cortical neuron; dendrites, pink), Martinotti cells (the second major type; dendrites, yellow), double bouquet cells (the third major type; dendrites, blue) and others (Kubota, 2014). Whether dynamic process of axon formation depicted here correspond to all inhibitory neurons or subtypes of inhibitory neurons remains unknown.

repeated short extensions and contractions of the leading process accompanied by saltatory cell movement. Once the leading process reaches the marginal zone (MZ), cells switch to somal translocation as their final mode of movement (Nadarajah and Parnavelas, 2002). The leading process then differentiates into a dendritic arbor-like structure (Hatanaka and Murakami, 2002), which probably develops into an apical dendrite. Since radial migration is accompanied by sustained trailing process elongation in the IZ (the future WM), this dynamic behavior eventually results in the typical pyramidal cell morphology in which an axon extends from the bottom of the soma toward the WM and an apical dendrite orients toward the pia. Thus, for excitatory cortical neurons, migration is closely related to establishment of prospective neuronal polarity.

Development of Inhibitory Cortical Neurons

Tangential migration of neurons that transgress the corticostriatal boundary and enter the neocortex was first reported by de Carlos et al. (1996). Collectively, several subsequent studies using transplantation, genetic fate mapping, and cell labeling analysis *in vivo* and *in vitro* established that tangentially migrating neurons include inhibitory cortical neurons. Moreover, most, if not all, inhibitory cortical neurons in mouse are reportedly generated embryonically from regions in the subpallium, including the medial and caudal ganglionic eminences and the preoptic area (Gelman and Marin, 2010), although some investigators have called into question whether cells emerge from the preoptic area (Ceci et al., 2012). Inhibitory neurons from these regions are further subdivided into distinct morphological subtypes exhibiting specific axonal arbors and dendritic patterns. Each subtype displays a unique combination of neurochemical markers and firing properties (Gelman and Marin, 2010; Bartolini et al., 2013; Kubota, 2014). Although each inhibitory neuron subtype originates in a distinct region, their overall migration behavior appears similar: in general, immature neurons migrate tangentially over long distances toward the cortex (Nadarajah and Parnavelas, 2002; Tanaka et al., 2003; Lopez-Bendito et al., 2004). They enter the CP from the SVZ, pass through it, and reach the MZ (Tanaka et al., 2009), where they further execute multidirectional tangential migration and become dispersed throughout the cortex (Tanaka et al., 2006, 2009; Inada et al., 2011; Yanagida et al., 2012). In mouse, these neurons settle into their final positions in the CP postnatally (Hevner et al., 2004; Tanaka et al., 2009).

During tangential migration, inhibitory neurons exhibit a bipolar shape with either an unbranched or branched leading process and a short trailing process. Currently, knowledge of dynamic developmental processes of these neurons is limited. However, Yamasaki et al. (2010) used electroporation with *gfp* or *DsRed* plasmid to label cells in the medial ganglionic eminence at E12.5 to assess morphological changes in mouse inhibitory neurons. Perinatally, as those labeled neurons moved from the MZ to CP, they appeared to transform into a multipolar, "sea urchin"-like shape, exhibiting multiple, thin processes (Yamasaki et al., 2010); no further information relevant to cellular dynamics during this transition is yet available. These processes repeatedly extended and retracted for several hours until one process became unusually long; most of these long processes extended toward the WM, while a minority extended toward the pia. Based on elongation, length and growth dynamics, it is likely that all of these processes represent prospective axons. Currently, it is not known what kind of inhibitory neuron these cells differentiate into; however, the medial ganglionic eminence can produce a variety of inhibitory neurons including Martinotti cells, which extend an axon oriented perpendicular to the pial surface (Gelman and Marin, 2010; Kubota, 2014). The remaining processes of multipolar cells likely become dendrites; however, details of their maturation remain to be elucidated.

It remains uncertain whether these behaviors of inhibitory neurons originating in the medial ganglionic eminence at E12.5 occur in all inhibitory cortical neurons. Nonetheless, these analyses indicate that a subset of inhibitory neurons initiates their polarization from a multipolar cell stage. Future analysis using tools such as genetically-modified mice expressing subtype-specific markers or Cre/CreER drivers (Taniguchi et al., 2011) should determine whether polarity can be established via alternate mechanisms.

Cortical Neurons *in situ* and Hippocampal Neurons in Dissociation Culture Show Similar Polarity Formation

The morphological dynamics of neurons undergoing polarization *in vitro* has been well-studied using time-lapse imaging of hippocampal neurons in dissociated culture (Dotti et al., 1988). After plating, hippocampal neurons typically develop axons and dendrites in five stages (**Figure 1**): (1) initially round cells form filopodia and lamellipodia; (2) cells extend and retract multiple minor processes; (3) one process transforms into an axon; (4) that axon extends and remaining processes differentiate into dendrites; and (5) differentiated processes mature. The first three stages in particular are key to establishment of polarity.

Evidence indicates that the initial minor processes of cells grown in culture have equal potential to differentiate into an axon: for example, if one experimentally cuts off a process that has grown longer than the others (presumably the future axon), a new potential axon emerges *de novo* (Dotti and Banker, 1987). Thus, polarity can be definitively established after an axon becomes apparently stable. However, some neurons *in vivo* are polarized at the time of generation and likely retain some aspects apico-basal polarity of progenitors in the neuroepithelum, or they have front-rear polarity of migrating immature neurons (Hatanaka et al., 2012). Therefore, these neurons *in vivo* do not need to redefine polarity but rather can inherit aspects of polarity. Indeed, retinal ganglion cells (Zolessi et al., 2006; Randlett et al., 2011) and bipolar cells (Morgan et al., 2006) appear to inherit apicobasal polarity of their progenitors. In retinal ganglion cells, not only the appearance but also the polarized distribution of intracellular components, such as the centrosome and Golgi apparatus, exhibit inheritance of polarity during axon formation (Zolessi et al., 2006; Randlett et al., 2011). Migrating neurons use polarized cellular components to form a leading and a trailing process required for directed movement (Evsyukova et al., 2013). Currently, there are no imaging studies *in vivo* or *in*

situ that directly demonstrate the dynamics of these components during axon formation of migrating neurons. Nonetheless, pontine nucleus neurons form an axon from their leading process (Kawauchi et al., 2006; Watanabe and Murakami, 2009; Shinohara et al., 2013), and trailing processes of cerebellar granule neurons transform into axons (Komuro et al., 2001), indicating that these neurons inherit some elements of front/rear (leading/trailing process) polarity. These mechanisms differ from polarity formation seen in dissociated hippocampal neurons, in which an axon emerges *de novo* from non-polarized cells.

In contrast, as described above, in both excitatory and inhibitory cortical neurons *in vivo* or at least *in situ*, polarity formation primarily occurs in multipolar cells in a manner similar to that seen in dissociated hippocampal neurons. In the next section, we focus on early polarity events that occur during multipolar cell stages prior to axon formation.

Dynamic Processes of Polarity Formation in Excitatory Cortical Neurons

Radial glial cells, the main progenitor population of excitatory cortical neurons, are neuroepithelial cells that exhibit apicobasal polarity. However, young neurons and intermediate progenitors appear to lose that polarity by retracting apical and basal processes during asymmetric cell division and assuming a multipolar shape (Tabata and Nakajima, 2003; Noctor et al., 2004; Hatanaka and Yamauchi, 2013). Furthermore, intermediate progenitors retract all visible processes and round up prior to division (Miyata et al., 2004; Noctor et al., 2004, 2008), suggesting that they do not inherit the apicobasal polarity exhibited by their progenitors. In addition, multipolar cells do not exhibit stable front-rear polarity, which is seen in actively migrating cells, but instead show highly dynamic behavior, alternately extending and retracting multiple short processes usually $<50\,\mu m$ in length. These cells also show unsteady somal movement (Tabata and Nakajima, 2003; Sakakibara et al., 2014), and some apparently form transient thick processes used to change migration direction (Sakakibara et al., 2014). Random distribution of the centrosome in multipolar cells reported in an imaging study (Sakakibara et al., 2014) and in fixed preparations (Shoukimas and Hinds, 1978) also support the idea that polarity is undetermined at these neuronal stages. After a prolonged period of this activity, a new thin process suddenly emerges and elongates tangentially. Occasionally, a cell retracts that process, even after it reaches $>50\,\mu m$, and extends another (Hatanaka and Yamauchi, 2013), an activity also observed in hippocampal neurons prior to polarity establishment (Dotti et al., 1988). Once a process exceeds $100\,\mu m$, it will likely become an axon and continue to elongate (Hatanaka and Yamauchi, 2013; Namba et al., 2014; Sakakibara et al., 2014). Thus, axon formation of excitatory neurons mostly occurs during the multipolar period while exhibiting unstable or fluctuating polarity.

Recently, several papers have reported that signaling and cytoskeletal proteins function in the multipolar-bipolar transition of excitatory neurons in the IZ (reviewed in Cooper, 2014). To further understand mechanisms governing axon formation in these neurons, it will be important to determine if loss-of-function of those factors merely locks neurons into a multipolar state or also prevents them from forming an axon.

Dynamic Processes of Polarity Formation in Inhibitory Cortical Neurons

During multidirectional tangential migration in the MZ, inhibitory cortical neurons extend a leading process in the direction of their movement (Tanaka et al., 2009; Inada et al., 2011; Yanagida et al., 2012). After long periods of migration in the MZ (estimated >1d; Tanaka et al., 2009), these neurons descend to the CP (Tanaka et al., 2009). Concomitantly, many transform into multipolar cells that extend numerous short processes, most $<50\,\mu m$ in length, although some are longer (Yamasaki et al., 2010). Because cells in the multipolar stage do not translocate (that is, their soma does not change position significantly), they appear to terminate their migration and lose front-rear polarity. Their short processes repeatedly extend and retract and show no preferential direction of extension. After a prolonged period of this activity, one process abruptly elongates (initial axon formation). As observed in dissociated hippocampal neurons in culture and in excitatory neurons *in situ*, other processes occasionally extend up to 150–200 μm but fail to extend further, and eventually only one exceeds $200\,\mu m$ in length and differentiates into an axon (Yamasaki et al., 2010). Thus, at multipolar stages inhibitory neurons likely do not have fixed polarity, and axon formation occurs in these cells *de novo*. Further study examining dynamic movement of cellular components in multipolar cells during axon formation should validate this view.

Modes of Polarity Establishment

There are minor differences between behavior of cortical neurons *in situ* and hippocampal neurons *in vitro*. Although in both cases neurons initially appear multipolar, the mode of "random growth and retraction" of processes differs slightly. First, minor processes of hippocampal neurons *in vitro* show alternate increases and decreases in length, while those of cortical neurons *in situ* often show alternate appearance and disappearance of processes. Therefore, potential sites of axon initiation seem to be set at the very beginning of the polarization process *in vitro*. Second, the location of a hippocampal neuron cell body *in vitro* appears fixed during the multipolar stage, while that of excitatory or inhibitory cortical neurons *in situ* does not. These activities may be due to microenvironmental differences, such as adhesive properties: hippocampal neurons interact with a positively-charged planar substrate, while cortical neurons do not. Some of the activities one sees in hippocampal neurons in *in vitro* might be artifacts.

Centrosome Positioning during Polarity Formation

In vitro and *in vivo* studies suggest an instructive role for centrosome positioning in axon specification (Lefcort and Bentley, 1989; Zmuda and Rivas, 1998; de Anda et al., 2005, 2010; Andersen and Halloran, 2012). However, recent time-lapse observations of centrosomes in polarizing excitatory cortical neurons *in situ* reveal that different mechanisms may govern axon formation in these cells (Sakakibara et al., 2014; reviewed in Sakakibara et al., 2013). The centrosome tends to move toward the

most actively growing process (the so-called "dominant process") and that the initiating axon does not always behave as the dominant process. Neurons undergoing multipolar migration in the IZ form an axon by extending a dominant process toward which the centrosome orients. Thus, the centrosome positions at the base of initiating axon (Sakakibara et al., 2014). Similarly, in polarizing hippocampal neurons *in vitro*, one minor process becomes an axon and then behaves as the dominant process. In both cases, the centrosome attracted to the growing axon. On the other hand, neurons in the CP at later migration stages exhibit a leading process oriented toward the brain surface, which then behave as the dominant process and attract the centrosome. When an axon forms at the rear of these cells *in situ*, the centrosome does not translocate toward the initiating axon but rather remains oriented toward the leading process (Sakakibara et al., 2014). Although the latter mode of axon formation may not be primarily observed *in vivo*, these observations suggest that centrosome positioning is passively controlled by a balance of protrusive activities among processes and does not play an instructive role in excitatory cortical neurons *in vivo*. In migrating inhibitory cortical neurons, the primary cilium, whose basal body is formed from a centriole, reportedly regulates Sonic hedgehog-mediated reorientation of the leading process (Baudoin et al., 2012). However, the function of the primary cilium in extracellular cue-oriented axonogenesis in these neurons remains unclear. Clarification of the contribution of centrosome/primary cilium to neuronal polarization *in vivo* may further prompt our understanding of microtubule function underlying axonal morphogenesis.

Cellular Mechanisms Underlying Neuronal Polarization

Axon specification in cortical neurons is driven by intracellular and extracellular mechanisms (**Figure 2**). Intracellular signaling molecules relevant to polarization have been identified primarily in *in vitro* studies of hippocampal neurons, although *in vivo* studies validating these findings have also been reported. Extracellular mechanisms regulating cortical neuron polarization have been studied by *in vivo* analyses of knockout phenotypes or gene manipulation in embryonic mouse brain, although most of these studies have been confined to excitatory cortical neurons.

Intracellular Mechanisms

Cytoskeletal changes dependent on protein phosphorylation are required for axon specification; thus, axon formation can be assessed using markers recognizing differential phosphorylation states of cytoskeletal proteins (Sternberger and Sternberger, 1983; Mandell and Banker, 1996). Stable microtubules within axons confer distinct characteristics based on their organization (Witte et al., 2008; Conde and Cáceres, 2009), and signaling molecules like LKB1 and SAD-A/B kinases trigger axonogenesis by changing the phosphorylation state of microtubule-associated proteins (MAPs), such as Tau and DCX (Kishi et al., 2005; Barnes et al., 2007; Shelly et al., 2007). In excitatory cortical neurons, PKA reportedly activates LKB1, leading to phosphorylation of SAD kinases. Consequent downstream signaling of SAD kinases phosphorylates Tau at S262, an event thought to initiate axon formation (Kishi et al., 2005).

The aPKC/Par complex plays a central role in axon specification: for example, aPKC inhibition suppresses axon formation in hippocampal neurons (Zhang et al., 2007). aPKC/Par complex function is differentially regulated by Par3 phosphorylation via multiple kinase pathways (Funahashi et al., 2013; Yang et al., 2014). TGF-ß signaling reportedly increases Par6 phosphorylation, which is required for axon formation by excitatory cortical neurons (Yi et al., 2010). MARKs/Par-1, which acts downstream of the aPKC/Par complex, controls microtubule-binding affinity of DCX (Sapir et al., 2008). The DLK-JNK pathway also regulates DCX phosphorylation and that of other MAPs as well as SCG10/stathmin-2 (Gdalyahu et al., 2004; Eto et al., 2010; Hirai et al., 2011; Westerlund et al., 2011). DOCK7 activation of the small GTPase Rac controls MT stability in axons by inactivation of stathmin/Op18 (Watabe-Uchida et al., 2006). Thus, concerted regulation of microtubule function by multiple kinases and their effectors likely underlies axon formation.

Several small GTPases function differentially in neuronal polarization (Arimura and Kaibuchi, 2007; Gonzalez-Billault et al., 2012). Local activation of the Rap1-Cdc42 pathway has been observed at the tip of an initiating axon in hippocampal neurons *in vitro* (Schwamborn and Püschel, 2004). Cdc42 reportedly remodels the actin cytoskeleton via cofilin phosphorylation (Garvalov et al., 2007). Rac/Cdc42 also controls retrograde movement of F-actin via phosphorylation of downstream effectors such as PAK1 and Shootin1 (Toriyama et al., 2013). Interestingly, Shootin1 is implicated in potential crosstalk between the L1-cell adhesion molecule, F-actin, and microtubules in regulating growth cone dynamics during neuronal polarization (Shimada et al., 2008; Sapir et al., 2013), suggesting that coordinated regulation of actin and microtubules is critical for axon formation.

Axon formation also requires directed transport of membrane vesicles and other cargos along polarized microtubules (reviewed in Conde and Cáceres, 2009; Hirokawa et al., 2010; Stiess and Bradke, 2011; Sakakibara et al., 2013). Polarized transport by plus-end-directed motors, such as kinesin-1 (KIF5) and kinesin-2 (KIF3), plays a central role in establishing a single axon (Nakata and Hirokawa, 2003; Jacobson et al., 2006). Identification of several cargo molecules suggests that directed accumulation of signaling and scaffold proteins, such as CRMP-2, PAR-3, and JIP1, is important for axon specification (Nishimura et al., 2004; Kimura et al., 2005; Dajas-Bailador et al., 2008). Localized acetylation of microtubules may regulate cargo/microtubule affinity during axon specification (Reed et al., 2006). PIP_3 transport by GAKIN/KIF13B functions in axon formation, suggesting a role for accumulated PIP_3 in positive feedback regulation of Rac and Cdc42 small GTPases (Horiguchi et al., 2006). Shootin1 also is known as a cargo of Kif20b (Sapir et al., 2013).

Recent studies show that regulators of microtubule dynamics are required for neuronal polarization. Control of microtubule minus-end dynamics by CAMSAP2 is essential for polarization of cortical neurons *in vivo* (Yau et al., 2014). Altered plus-end dynamics induced by depletion of microtubule regulators, such

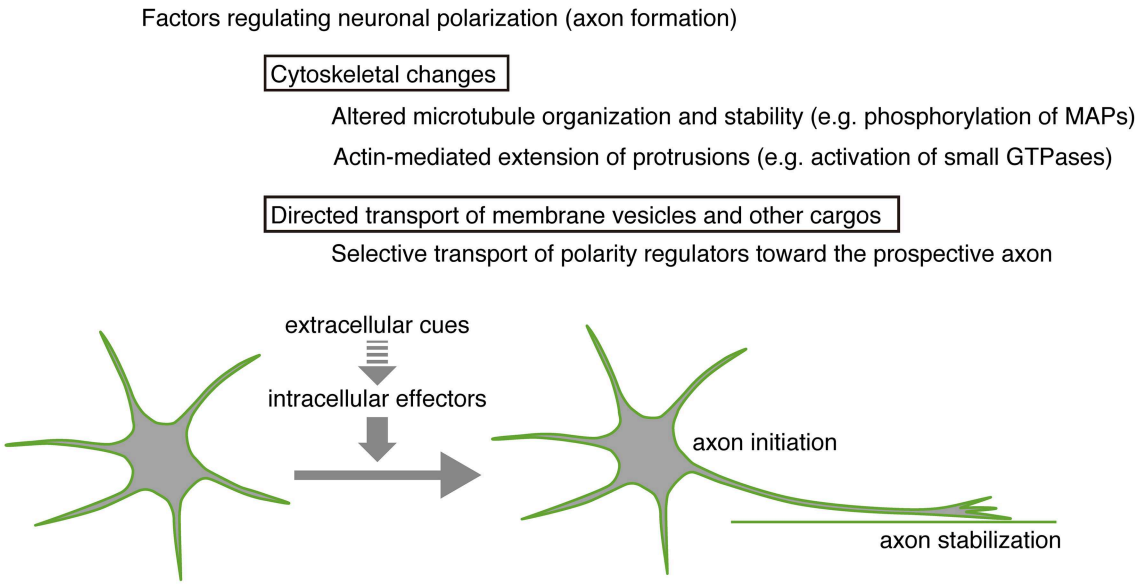

FIGURE 2 | Regulation of cortical neuron polarization. Axon formation *in vivo* is influenced by extracellular cues. Intracellular effectors regulating cytoskeletal dynamics and membrane vesicle transport function in the initiation, stabilization, and subsequent elongation of a single axon.

as, SLAIN1/2, chTOG/XMAP215, and CLASP2, reportedly underlie polarization defects (Beffert et al., 2012; van der Vaart et al., 2012). These observations suggest that properly controlled microtubule growth, which also underlies microtubule-dependent directional transport, is essential to shape axons.

Extracellular Mechanisms

Interestingly, when the growth cone of an immature process of a hippocampal neuron encounters a preferred substrate *in vitro*, its growth rapidly increases, while that of other immature processes does not (Esch et al., 1999; Shelly et al., 2007). Observations such as this indicate that external cues also govern polarity formation. Contact with laminin in the basal lamina also triggers retinal ganglion cell axon formation (Zolessi et al., 2006; Randlett et al., 2011). In the case of excitatory cortical neurons, integrity of the surrounding microenvironment may greatly impact axonal specification. Indeed, recent work reveals that these neurons establish directed tangential axon outgrowth due to instructive cues presented on pre-existing efferents: close contact with the cell adhesion molecule TAG-1 on these efferents in the lower IZ stimulates axon formation by multipolar cells, an event mediated in part by downstream Lyn-kinase (Namba et al., 2014). In addition, several extracellular factors, such as the homotypic cell adhesion protein N-cadherin (Gärtner et al., 2012), diffusible protein TGF-ß (Yi et al., 2010) and neurotrophins (Nakamuta et al., 2011), reportedly function as polarization signals for excitatory cortical neurons *in vivo*. Some investigators propose that a single axon is specified via positive feedback signals that stabilize process extension (Arimura and Kaibuchi, 2007; Inagaki et al., 2011), suggesting that external cues, either contact-mediated or locally diffused, have a stabilizing effect on polarity. In the case of inhibitory neurons, the surrounding environment indeed appears to influence polarity formation: inhibitory neurons do not assume a multipolar shape in dissociated culture. Instead, one of the two processes emerging from these neurons elongates and eventually becomes an axon (Hayashi et al., 2003). Possible cell-cell interactions, such as tiling interactions between neighboring inhibitory neurons, might partially contribute to shape cells *in vivo*. Because excitatory cortical neurons under the same conditions appear multipolar (Hayashi et al., 2003), intrinsic mechanisms governing axon formation in these two types of neurons may differ despite their similar behavior *in vivo*.

Concluding Remarks

Here, we have reviewed recent evidence suggesting that cortical neurons, both excitatory and inhibitory, establish polarity *de novo*. These neurons initiate axons after assuming a multipolar stage, in which no fixed polarity is exhibited. Although these neurons appear similar during axon formation, it is important, especially in the case of inhibitory neurons, to examine the dynamics of cellular components to validate this view. Also, it will be interesting to examine whether these neuronal subtypes share signaling pathways governing polarity. It should be noted that inhibitory cortical neurons are in fact diverse and consist of multiple morphological subtypes with different spatial and temporal origins. Thus, future investigations are needed to determine whether these subtypes share a common mechanism of axon initiation. In addition, it is important to define dynamic processes governing dendrite formation by both excitatory and inhibitory cortical neurons. These analyses will likely require a combination of genetic labeling of specific excitatory and inhibitory neuronal subtypes with live imaging both *in situ* and *in vivo*.

Finally, analysis of dissociated hippocampal neurons has set the foundation for our current understanding of polarization processes and their molecular basis. Although some of this knowledge is applicable to cortical neurons *in situ*, care should be taken in generalizing these mechanisms to other neuronal types. Proper understanding of polarity formation in the cerebral cortex requires identification of the key processes that underlie external cue-mediated polarization *in vivo*.

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Function and regulation of Rnd proteins in cortical projection neuron migration

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The mammalian cerebral cortex contains a high variety of neuronal subtypes that acquire precise spatial locations and form long or short-range connections to establish functional neuronal circuits. During embryonic development, cortical projection neurons are generated in the areas lining the lateral ventricles and they subsequently undergo radial migration to reach the position of their final maturation within the cortical plate. The control of the neuroblast migratory behavior and the coordination of the migration process with other neurogenic events such as cell cycle exit, differentiation and final maturation are crucial to normal brain development. Among the key regulators of cortical neuron migration, the small GTP binding proteins of the Rho family and the atypical Rnd members play important roles in integrating intracellular signaling pathways into changes in cytoskeletal dynamics and motility behavior. Here we review the role of Rnd proteins during cortical neuronal migration and we discuss both the upstream mechanisms that regulate Rnd protein activity and the downstream molecular pathways that mediate Rnd effects on cell cytoskeleton.

Keywords: Rho GTPases, Rnd, cortical development, neuronal migration, Plexin

INTRODUCTION

During the development of the central nervous system, neural progenitors undergo a sequence of distinct cellular events to give rise to the vast array of neurons that populate the entire brain. In the cerebral cortex, excitatory projection neurons, which constitute the majority of cortical neurons, are generated from neural stem/progenitor cells located in the areas lining the lateral ventricles, the ventricular (VZ) and subventricular zones (SVZ) of the dorsal telencephalon (Franco and Muller, 2013; Marin and Muller, 2014). Soon after birth, young neuroblasts leave the proliferative areas and migrate to the cortical plate (CP), where they distribute into six horizontal layers and they establish local and long-range connections (Rakic, 1988; Nadarajah and Parnavelas, 2002; Martynoga et al., 2012; Greig et al., 2013). It is now increasingly evident that a highly motile cellular behavior is crucial for different aspects of cortical neurogenesis, including, but not restricted to radial migration of post-mitotic neurons. Indeed, progenitor cells in the VZ also exhibit motile characteristics, such as the migration of their nuclei in coordination with cell cycle progression.

The sequential steps of neurogenesis and migration are promoted by the extensive and dynamic remodeling of the cell cytoskeleton. It is indeed the rapid re-organization of the actin filaments and microtubule network that ultimately regulates the motility behavior of nuclei in cycling progenitors and of migrating neurons (Lambrechts et al., 2004; Heng et al., 2010; Taverna and Huttner, 2010). The importance of the control of cytoskeletal remodeling for cortical neurogenesis is highlighted by the fact that most of the genes mutated in human patients with cortical malformations produce cytoskeletal proteins or their regulators (Guerrini and Parrini, 2010; Friocourt et al., 2011).

Members of the Rho family of small GTPases are key regulators of cell cytoskeleton in various cell types (Ridley, 2001). Rho proteins act as molecular switches capable of fast cycles of activation and inactivation, which represent an ideal system to regulate the dynamic changes of the cytoskeleton during migration. Also, the spatial and temporal control over Rho GTPase activity within the cell enables differential regulation of cytoskeletal components in distinct subcellular compartments, driving for example protrusion formation at the front of a migrating cell and cell retraction at the rear. The Rho family includes not only the classical members, which cycle between an active GTP-bound state and an inactive GDP-bound state, but it also contains "atypical" members like the Rnd subfamily, which possess low or no intrinsic GTPase activity and are therefore considered to be constitutively active (Nobes et al., 1998; Chardin, 2006; Riou et al., 2010). Since Rnd proteins do not undergo the classical GTPase cycle, gene expression, protein post-transcriptional modifications and subcellular localization are predominant mechanisms that control Rnd activity. Interestingly, Rnd proteins evolved relatively recently and they are present only in vertebrates, indicating that they might be involved in more specialized neuronal functions than the other Rho GTPases (Chardin, 2006; Boureux et al., 2007). The role of Rnd proteins in cortical development has become subject of intensive research only recently. Here we review the functions and regulation of Rnd small GTPases during progenitor

nuclear migration and during radial migration of cortical neurons.

NEURONAL MIGRATION IN THE CEREBRAL CORTEX

INTERKINETIC NUCLEAR MIGRATION OF NEURAL PROGENITORS

After closure of the neural tube, the epithelium lining the ventricles becomes a specialized neuroepithelium that consists of a single sheet of progenitors called neuroepithelial cells. At the onset of neurogenesis (~E10 in mouse), these cells self-renew to expand the progenitor pool and then convert into cells with glia-like features, the radial glial cells. A typical feature of these two populations of progenitors is the apico-basal movement of their nuclei in coordination with cell cycle progression, a phenomenon known as interkinetic nuclear migration (INM) (Sauer and Walker, 1959) (**Figure 1A**). In neuroepithelial cells, INM spans the entire apico–basal axis of the cell whereas in radial glia cells, this behavior is confined to the portion of the cell in the VZ. During G1 phase of the cell cycle, the nuclei of neural progenitor cells migrate from the apical to the basal side, where DNA replication occurs, whereas during G2 phase of the cell cycle, the nucleus moves toward the ventricular surface and undergoes mitosis at the most apical side. Since neural progenitors are not synchronized in their cell cycle and as a consequence of INM, the nuclei are found scattered in different apico-basal positions and the single layered neuroepithelium and the VZ appear pseudo-stratified. Interestingly, several lines of evidence indicate that INM is not required for cell cycle progression, whereas alteration of cell-cycle parameters may interfere with INM (Taverna and Huttner, 2010).

Although the contribution of INM to cortical neurogenesis has not yet been fully understood, it is possible that INM may allow packing more progenitor cells within a limited surface in order to maximize the mitoses of progenitor cells. Alternatively, INM may regulate progenitor fate by controlling the exposure of progenitor nuclei to proliferative vs. neurogenic signals (Taverna and Huttner, 2010; Spear and Erickson, 2012).

The translocation of the nucleus during INM requires dynamic changes of the cell cytoskeleton, with both actin and microtubule (MT) networks involved in this process (Taverna and Huttner, 2010). The relative contribution of MT- and actin-dependent mechanisms depends on the model organism and on the brain region investigated (Lee and Norden, 2013). In the developing rodent cortex, the basal-to-apical nuclear migration involves MT-based motors, whereas the apical-to-basal migration seems to depend on both actomyosin and MT-based motors (Schenk et al., 2009; Tsai et al., 2010). In addition, recent work proposes that the regulation of apical-to-basal nuclear migration during G1 is not an active, cell-autonomous process, but it involves a passive component (Kosodo et al., 2011). Kosodo and colleagues suggested that the basal nuclear movement during G1 is indirectly driven by the opposite movement of G2-phase nuclei migrating apically (Kosodo et al., 2011). Thus, the mechanisms underlying basal-to apical and apical-to-basal INM seem to exhibit profound differences.

RADIAL MIGRATION OF PROJECTION NEURONS

The majority of cortical neurons are excitatory glutamatergic cells that extend long projections toward cerebral and subcerebral targets. The first cohort of cortical neurons that migrate out from the VZ determine the formation of the preplate, a primitive structure that becomes soon divided into the superficial marginal zone and the deeper subplate by a subsequent wave of migrating neurons (Luskin et al., 1988). At the stage of preplate formation and early born neuron production, between E12 and E14 in the mouse, the main mode of migration is somal translocation (Miyata et al., 2001; Nadarajah et al., 2001) (**Figure 1B**). The cells that undergo somal translocation are born from radial glia cells at early developmental stages and they possess both apical attachment and basal radial process at the time of birth. After detachment form the ventricular surface, the continuous advancement of the nucleus and the concomitant retraction of the basal process determine a fast migratory behavior. Early-born neurons eventually occupy deep cortical layers since later-born neurons migrate and pass earlier born cells in order to settle progressively in upper cortical layers in an "inside-out" fashion.

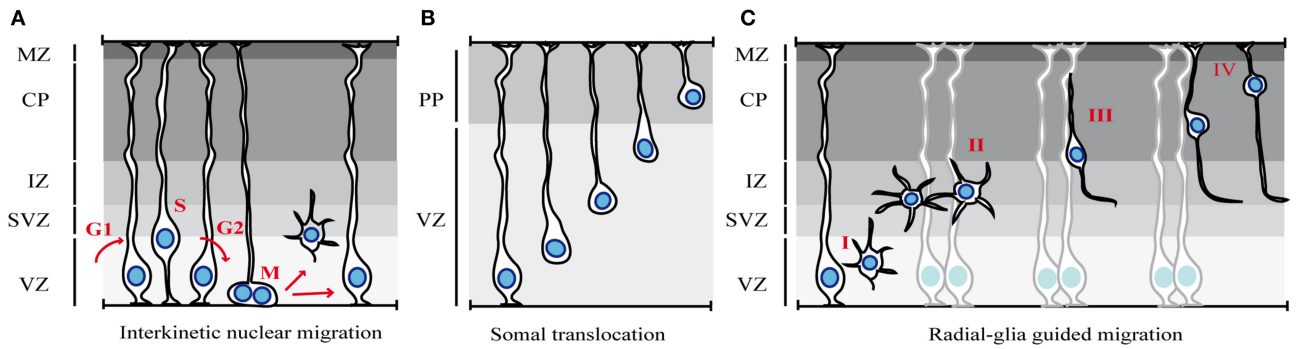

FIGURE 1 | Modes of migration in the cortex. (A) Interkinetic nuclear migration. The nuclei of neuroepithelial cells or radial glia cells occupy different positions along the apical-basal axis depending on the phase of the cell cycle (see text for details). **(B)** Somal translocation of early-born cortical neurons. Newborn neurons lose their apical attachment and reach the PP by translocation of the soma and progressive shortening of the basal process. **(C)** Glia-guided radial migration of cortical neurons. Four phases of radial migration can be distinguished. Newborn neurons leave the proliferative areas **(I)** and reach the SVZ/IZ, where they acquire a multipolar morphology **(II)**. After pausing in the SVZ/IZ, cells migrate toward the CP, using locomotion **(III)**. At the end of their migration, cortical neurons switch to soma translocation (**IV**). MZ, marginal zone; CP, cortical plate; PP, preplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

At later developmental stages, after E14, when the cortical wall progressively increases in its thickness, neurons predominantly use a mode of radial migration called glia-guided migration (Rakic, 1972; Noctor et al., 2001) (**Figure 1C**). In contrast to somal translocation that is independent from radial glia fibers, glia-guided migration strictly relies on the radial glia scaffold. Young neurons that use this mode of migration lose contact with both the ventricle and the basal lamina and "embraced" the radial fiber during their migration. Newborn neurons usually migrate along the fiber of their mother radial glia, although they can jump from one fiber to another during migration, a process that regulates intermixing of neuronal clones within the cortex.

This entire process of radial migration can be subdivided into distinct phases (**Figure 1C**) (Nadarajah and Parnavelas, 2002; Noctor et al., 2004), in which neurons undergo rapid changes in cell polarity, morphology and speed of migration, as they progress from the VZ to the CP. The first step is characterized by the detachment of cells from the apical/ventricular surface in order to leave the proliferative zones and reach the SVZ and the intermediate zone (IZ) (**Figure 1C-I**). Then, post-mitotic neurons pause for a variable amount of time in the SVZ/IZ (maximum time recorded of 24 h), where they acquire a multipolar shape (**Figure 1C-II**). In this phase, neurons actively extend and retract dynamic processes, but they do not move significantly (Tabata and Nakajima, 2003). After sojourning in the IZ, neurons enter the CP. However, some neurons take a path toward the VZ, before reversing their direction of migration toward the CP (Noctor et al., 2004). The purpose of this migratory behavior is poorly understood. Once in the CP, neurons become bipolar, extending a leading process toward the pial surface and a trailing process in the direction of the IZ (**Figure 1C-III**), and migrate toward the upper layer of the CP. During this phase, nascent neurons use glia-guided migration (also called glia-guided locomotion), which is characterized by repetitive migratory cycles of extension of the leading process, translocation of the nucleus, and retraction of the trailing process. However, since the trailing process of migrating cortical neurons will become the future axon, it has been proposed that neurons do not retract the trailing process at the end of each migratory cycle, but rather extend their axon as they move (Noctor et al., 2004; Tabata et al., 2009; Hatanaka and Yamauchi, 2013). Finally, when projection neurons reach their destination, they undergo a last nuclear translocation without leading process extension, indicating that locomoting cells switch to somal translocation at the end of their migration (Nadarajah et al., 2001) (**Figure 1C-IV**).

CLASSICAL AND ATYPICAL Rho GTPases

Rho (Ras homologous) GTPases belong to the large superfamily of small GTP binding proteins, whose founding member is Ras (Jaffe and Hall, 2005; Heasman and Ridley, 2008). Ras superfamily contains more than 150 members, which are grouped into 5 categories according to their major functions: Ras, Rho, Rab, Arf, and Ran (**Table 1**). Mammalian Rho GTPases comprise a family of 20 molecules that regulate actin and microtubule components of the cytoskeleton (**Figure 2A**). By controlling cytoskeletal dynamics, Rho GTPases affect many cellular processes, including cell polarity, cell shape and migration (Hall and Nobes, 2000; Ridley, 2001). The most extensively studied members of the Rho family are RhoA (Ras homologous member A), Rac1 (ras related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42). Rac1 and Cdc42 promote the formation of cellular protrusions, such as lamellipodia or filopodia, respectively. RhoA instead is involved in acto-myosin contraction and stress fiber formation (Ridley, 2001). The overexpression of constitutively active or dominant negative forms of Rho proteins in the embryonic cortex, together with more recent analysis of conditional knockout mice have revealed a crucial role for Rac1 and Cdc42 during INM (Cappello et al., 2006; Minobe et al., 2009) and for RhoA, Rac1, and Cdc42 during radial migration in the cortex (Kawauchi et al., 2003; Konno et al., 2005; Cappello et al., 2012). (For recent reviews see (Govek et al., 2011; Shah and Puschel, 2014).

Table 1 | Members of the Ras superfamily and their major functions.

Family	Members	Function
Ras	Ha-Ras, K-Ras, N-Ras, R-Ras, M-Ras, RalA, RalB, Rap1A, Rap1B, Rap2A, TC21, Rit, Rin, Rad, Kir/Gem, Rheb, KB-Ras1, KB-Ras2	Control of cell proliferation
Rho	RhoA, RhoB, RhoC, RhoD, Rif (RhoF), Rnd1 (Rho6), Rnd2 (Rho7, RhoN), Rnd3 (Rho8, RhoE), TTF (RhoH), Rac1, Rac2, Rac3, RhoG, Cdc42, TC10 (RhoQ), TCL (RhoJ), Wrch1 (RhoV), Chp/Wrch2 (RhoU), RhoBTB1, RhoBTB2	Control of cell cytoskeleton
Rab	Rab proteins from Rab1 to Rab33	Control of vesicle trafficking
Arf	Arf1, Arf2, Arf3, Arf4, Arf5, Arf6, Sar1a, Sar1b, Arl1, Arl2, Arl3, Arl4, Arl5, Arl6, Arl7, Ard1	Control of vesicle formation
Ran	Ran	Control of nuclear transport

Most Rho GTPases act as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound form (**Figure 2B**). When bound to GTP, Rho GTPases exhibit the correct structural conformation to interact with effectors and initiate downstream signaling (Raftopoulou and Hall, 2004). The GDP/GTP cycle is promoted by the activity of two classes of molecules, guanine nucleotide exchanging factors (GEFs) and GTPase activating proteins (GAPs). GEFs facilitate the exchange of GDP with GTP, resulting in protein activation. GAPs instead stimulate the intrinsic enzymatic activity of the GTPases, which promotes hydrolysis of GTP into GDP. GAP activity therefore ends the cycle and returns the GTPases in their inactive state (Bos et al., 2007). In addition, Rho GTPases can bind to proteins known as guanine-nucleotide dissociation inhibitors (GDIs). RhoGDIs sequester RhoGTPase in their inactive state and protect them from degradation (Dermardirossian and Bokoch, 2005; Boulter et al., 2010).

The GDP/GTP cycle and the regulation by GDI are common properties among Rho GTPases. However, the "atypical" Rho GTPases rarely follow this rule (Aspenstrom et al., 2007). Among

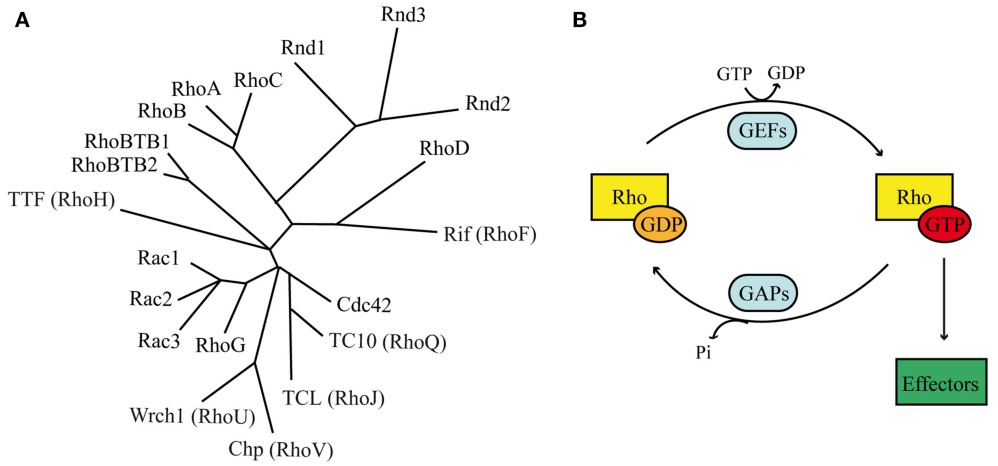

FIGURE 2 | Rho GTPases. (A) Phylogenetic tree based on alignment of the aminoacid sequences of the 20 Rho GTPases. Rnd proteins form a distinct branch, which is closely related to Rho members. **(B)** Classical Rho GTPases cycle between an inactive GDP-bound state and an active GTP-bound state. In their active conformation they transduce the signal to intracellular effectors. Two classes of molecules promote the regulatory cycle: GEFs stimulate the exchange of GDP with GTP, whereas GAPs stimulates the GTP hydrolysis.

them, the Rnd subfamily represents a distinct branch of the Rho family of small GTPases and consists of three different members: Rnd1/Rho6, Rnd2/Rho7, and Rnd3/Rho8/RhoE (Chardin, 2006; Riou et al., 2010) (**Figure 2A**). Interestingly, the Rnd subfamily is present only in vertebrates and not in other organisms such as worms or flies, suggesting that it might play important roles in biological processes that are specific to vertebrate organisms (Philips et al., 2003; Boureux et al., 2007). Rnd proteins have a core GTP-binding domain structurally similar to the other Rho proteins. However, they possess different biochemical properties due to amino acid substitutions at residues that are crucial for GTPase activity. In fact, in contrast to classical GTPases, Rnd proteins do not show any intrinsic or stimulated GTPase activity. In addition to their inability to hydrolize GTP into GDP, Rnd proteins exhibit a 100-times higher affinity for GTP than for GDP. Altogether, these properties suggest that Rnd are constitutively bound to GTP, therefore constitutively active (Foster et al., 1996; Nobes et al., 1998). Nobes et al. (1998) were the first to identify the three Rnd isoforms and shed light onto their function. By overexpressing Rnd1 and Rnd3 in cultured fibroblasts, the authors observed cell retraction from the substrate and cell rounding, hence their collective name round (Rnd). This phenotype is the result of Rnd1 and Rnd3 inhibitory functions on RhoA-mediated stress fiber formation and adhesion contact assembly. In contrast to Rnd1 and Rnd3, the expression of Rnd2 in fibroblasts does not modulate cytoskeletal reorganization, suggesting that Rnd2 acts via different and partially unknown mechanisms in these cells (Nobes et al., 1998). Recent evidences demonstrate that Rnd3 also plays a role in the control of cell proliferation via mechanisms that are independent from cytoskeletal remodeling (Villalonga et al., 2004; Poch et al., 2007; Pacary et al., 2013), indicating that Rnd proteins might have more pleiotropic functions that previously expected. Among the three members of the Rnd subfamily, only *Rnd2* and *Rnd3* show strong expression in the developing cerebral cortex. *Rnd2* is found in the preplate cells at early stages of cortical development and it is expressed in the SVZ/IZ at later stages (Heng et al., 2008). In contrast to *Rnd2*, *Rnd3* expression is widespread throughout the entire thickness of the cortical wall at early stages and it is later restricted to the VZ/SVZ, as well as to the CP (Pacary et al., 2011). The distribution of *Rnd2* and *Rnd3* transcripts in partially exclusive cortical domains suggests that they might play individual and non-redundant functions in distinct phases of cortical development and neuronal migration.

Rnd FUNCTIONS IN CORTICAL NEURON MIGRATION

Rnd3 ROLE IN INTERKINETIC NUCLEAR MIGRATION

The role of Rnd3 in INM has been recently studied *in vivo*, by *in utero* electroporation of the embryonic cortex with shRNA that specifically silences *Rnd3* expression (Pacary et al., 2013). The process of INM can be visualized and quantified after injection of 2-bromo-deoxyuridine (BrdU), which marks cells in S phase, followed by analysis of the position of the BrdU positive nuclei over time (Schenk et al., 2009). The nature of INM implies that cells that are in S phase at the time of BrdU injection are positioned in the most basal region of the VZ. BrdU labeled cells can be then followed when they subsequently undergo basal-to-apical nuclear migration to reach the apical surface, just before entering mitosis. Analysis performed 30 min after BrdU injection revealed that, in *Rnd3*-silenced cortices, a reduced fraction of $BrdU^+$ nuclei reach the apical side in comparison to control treated cortices, indicating delayed nuclear migration when *Rnd3* expression is decreased in progenitor cells (Pacary et al., 2013). Three hours after injection, control BrdU labeled cells have undergone cell division at the ventricular surface and the nucleus of the radial glia daughter cell begins to move again toward the basal side. In contrast, the delayed nuclei in *Rnd3* knock down cortices have just reached the apical side and start to divide, leading to an accumulation of cells at the ventricular surface. In addition, Rnd3-silenced VZ progenitors exhibit less elongated nuclei compared to control cells (Pacary et al., 2013), which is characteristic of INM impairment

(Sauer, 1935; Ge et al., 2010) Altogether these data show that Rnd3 is required during INM at least for the basal to apical movement. Importantly, the duration of the different phases of the cell cycle is unaffected by *Rnd3* silencing, indicating that the regulation of INM by Rnd3 is direct and not secondary to modification of cell-cycle progression in neural progenitors.

Rnd FUNCTIONS IN RADIAL MIGRATION OF PROJECTION NEURONS

The role of Rnd proteins in cortical neuron migration has been thoroughly investigated only in the last few years (Nakamura et al., 2006; Heng et al., 2008; Pacary et al., 2011; Azzarelli et al., 2014). As mentioned before, *Rnd2* and *Rnd3* are expressed in different cortical domains during embryonic development suggesting that they might control different phases of the migratory process. Accordingly, the *in vivo* knock down of Rnd2 and Rnd3 in the embryonic cortex produces migratory defects that are characterized by distinct morphological abnormalities.

As neurons progress from the VZ/SVZ to the CP, they transiently acquire a multipolar morphology in the IZ. *In vivo* knock down of *Rnd2*, but not of *Rnd3* expression, increases the fraction of neurons with a multipolar shape. This phenotype eventually leads to the accumulation of cells in the IZ of *Rnd2*-knocked down cortices and a concomitant reduction of neurons reaching the CP, in comparison to control cortices (**Figures 3A,B**) (Heng et al., 2008). Rnd2 thus regulates multipolar to bipolar transition in the IZ.

Rnd3-silenced neurons instead exhibit abnormal morphology during neuronal migration in the CP, i.e., during glia-guided locomotion. During this phase, migrating neurons in control condition exhibit a bipolar morphology with a leading process toward the CP and a trailing process in the direction of the IZ. In contrast, Rnd3-knocked down neurons display a grossly enlarged leading process and several thin processes protrude from the cell body (**Figure 3C**). Locomotion in the CP largely relies on the coordinated movement of nucleus and centrosome in the direction of migration. Neurons undergo cycles of extension of the leading process and forward movement of the nucleus toward the centrosome, which is located in a cytoplasmic dilation that forms in the proximal region of the leading process. When *Rnd3* expression is reduced by shRNA electroporation, the distance between the nucleus and the centrosome in bipolar neurons is increased. A possible role for Rnd3 in the regulation of nucleus-centrosome coupling during locomotion has been further supported by *ex vivo* time-lapse imaging, which clearly shows that the motility behavior of *Rnd3*-depleted neurons is not coordinated (Pacary et al., 2013).

Consistent with the aberrant migration in the CP, *Rnd3*-silenced neurons also exhibit a branched leading process, which have been previously associated with loss of adhesion between the leading process and the radial glia fibers (Gupta et al., 2003; Elias et al., 2007). Whether *Rnd3*-silenced neurons are detached from the radial glia scaffold and whether loss of adhesion is a primary effect or secondary to defective locomotion would be interesting issues to address in the future.

REGULATION OF Rnd PROTEINS

Rnd proteins are always present in the cell in their active conformation, capable to bind effectors. Since their activity is not affected by the GDP/GTP exchange or by interaction with RhoGDIs, other mechanisms must account for the regulation of their activity (Riou et al., 2010). Transcriptional regulation, subcellular localization and post-translational modifications have been shown to play crucial roles in the control of Rnd protein expression and function.

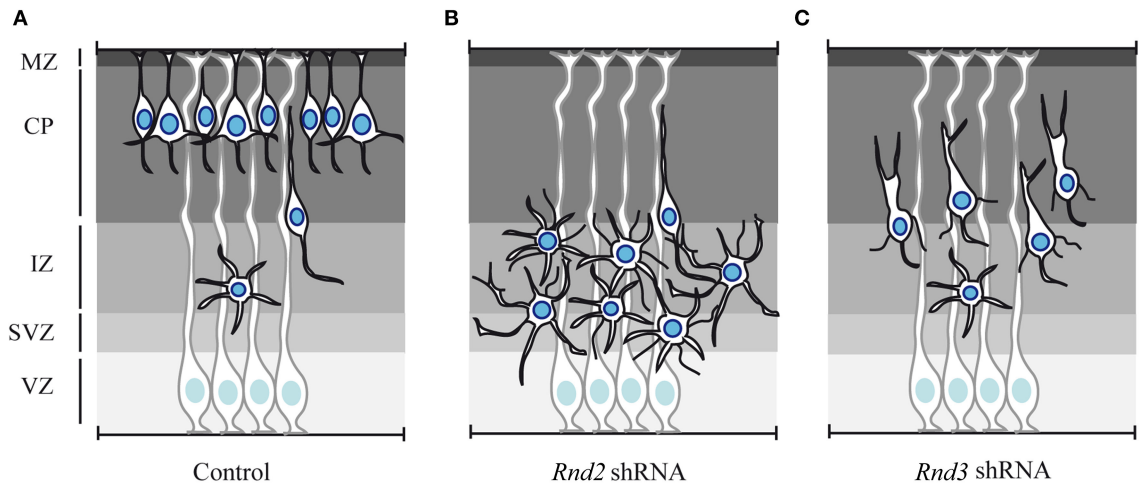

FIGURE 3 | Effect of *Rnd2* and *Rnd3* loss of function on cortical neuron migration. (A) Schematic representation of cortical radial migration in control condition. Newborn projection neurons undergo sequential steps of radial migration, which are characterized by distinct morphologies. At mid-end corticogenesis most of the neurons have reached the CP. Only few cells are still migrating and they exhibit multipolar morphology in the IZ and bipolar shape in the CP. **(B)** shRNA-mediated loss of function of *Rnd2* expression in the embryonic cortex produces an accumulation in the IZ of multipolar cells, which exhibit more and longer neuronal processes. **(C)** *Rnd3* knock down in the embryonic cortex interferes with the locomotion phase of migration in the CP. *Rnd3*-silenced neurons exhibit abnormal morphologies characterized by excessively enlarged and branched leading processes and by thin processes protruding from the cell body. MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

During the development of the cerebral cortex, *Rnd2* and *Rnd3* are under the transcriptional control of proneural factors that up-regulate their expression to control specific phases of neuronal migration. The proneural factors Neurogenin2 (Neurog2) and Ascl1 directly bind to E-box DNA sequences within enhancers located 3′ to the *Rnd2* and *Rnd3* gene, respectively (Heng et al., 2008; Pacary et al., 2011). Moreover, *Rnd2* expression in the developing brain is transcriptionally regulated by other factors, such as RP58, Scratch2, and COUP-TFI, which act as repressors (Alfano et al., 2011; Heng et al., 2013; Ohtaka-Maruyama et al., 2013; Paul et al., 2014). In particular, RP58 and Scratch2 regulate the 3′ enhancer previously identified as Neurog2 target, suggesting that the two repressors might compete with the proneural bHLH activator on *Rnd2* enhancer to fine-tune the levels of *Rnd2* in the cortex (Heng et al., 2013). Several other studies in different cell types have identified various stimuli that regulate Rnd2 and Rnd3 expression (**Table 2**).

Rnd proteins also undergo post-translational modifications that influence their subcellular localization and stability. Most Rho GTPases are modified at their C-terminus by addition of lipid moieties that promote their interaction with membranes (Seabra, 1998). Whereas most Rho-family proteins are geranylgeranylated, Rnd proteins are farnesylated, which consists in the addition of a 15-carbon farnesyl group on their C-terminal CAAX motif (where C represents cysteine, A is an aliphatic amino acid and X is any amino acid). This motif is important not only for membrane localization but also for Rnd activity. Indeed, mutation in the CAAX motif of Rnd3 ($Rnd3^{C241S}$) abolish its plasma membrane association and impairs its ability to rescue the migratory activity of *Rnd3*-silenced neurons, thus demonstrating that membrane association is required for Rnd3 activity in migrating neurons (Pacary et al., 2011). In addition to this motif, sequence elements positioned immediately upstream of the CAAX domain are important for membrane insertion (Roberts et al., 2008). Rnd2 and Rnd3 have similar CAAX motif, but distinct upstream sequences that are responsible for the different subcellular localization of Rnd2 and Rnd3. In fact, Rnd3 is preferentially associated to the plasma membrane, whereas Rnd2 is cytoplasmic or associated to endomembranes (Roberts et al., 2008). It has been recently shown that the replacement of the C-terminal domain of Rnd2, containing the CAAX motif and the upstream sequence, with that of Rnd3 is sufficient to recruit Rnd2 at the plasma membrane (Pacary et al., 2011). More importantly, although Rnd2 and Rnd3 cannot substitute for one another during cortical neuron

Table 2 | Mechanisms regulating Rnd expression.

Rnd	Stimuli or TFs that control expression	Cell type	Function	References
Rnd2	Neurog2	Cortical neurons	Migration	1
Rnd2	RP58*	Cortical neurons	Migration	2
Rnd2	COUPTFI*	Cortical neurons	Migration and differentiation	3
Rnd2	Scratch2*	Cortical neurons	Migration	4
Rnd3	Ascl1	Cortical neurons	Migration	5
Rnd3	PDGF	Fibroblast	Formation of stress fibers	6
Rnd3	HGF	MDCK	Motility	7, 8
Rnd3	Raf-MEK-BRF	MDCK	Transformation	9
		Melanoma cells	Invasiveness	10, 11
Rnd3	p53—chemoterapeutic agent or irradiation	Cancer cell line, keratinocytes	Pro-survival	12, 13
Rnd3	mTOR	Subependymal giant cell astrocytoma	Potential contribution to tumorigenesis	14
Rnd3	NF-kB	Prostate cancer	Potential contribution to tumorigenesis	15
Rnd2/3	MDMA and cocaine	Neurons in different brain regions	Potential contribution to dendritic branching and neurite outgrowth	16
Rnd3	mir200c mir200b*	Breast cancer cell	Invasive behavior	17, 18
Rnd1-2-3	Estradiol	Smooth muscle cells—myometrium	Decreased contraction	19
Rnd3	Estradiol*	Prostatic stromal cells	Unknown	20
Rnd3	MIC-1/GDF15	Prostate cancer cells	Decreased adhesion	21
Rnd3	CREB	Hippocampal neurons	BDNF-mediated synaptogenesis	22
Rnd3	HIF1a	Gastric cancer cells	Epithelial to mesenchymal transition and invasion	23
Rnd3	FOXD3*	Melanoma cells	Migration and invasion	24

*1 (Heng et al., 2008), 2 (Heng et al., 2013; Ohtaka-Maruyama et al., 2013), 3 (Alfano et al., 2011), 4 (Paul et al., 2014), 5 (Pacary et al., 2011), 6 (Riento et al., 2003), 7 (Guasch et al., 1998), 8 (Tanimura et al., 2002), 9 (Hansen et al., 2000), 10 (Klein et al., 2008), 11 (Klein and Aplin, 2009), 12 (Ongusaha et al., 2006), 13 (Boswell et al., 2007), 14 (Tyburczy et al., 2010), 15 (Nadiminty et al., 2010), 16 (Marie-Claire et al., 2007), 17 (Hurteau et al., 2006), 18 (Xia et al., 2010), 19 (Shimomura et al., 2009), 20 (Bektic et al., 2004), 21 (Liu et al., 2003), 22 (Lesiak et al., 2013), 23 (Zhou et al., 2011), 24 (Katiyar and Aplin, 2011). *Denotes factors that decrease Rnd expression.*

migration, the chimeric form of Rnd2 that exhibits a plasma membrane localization similar to Rnd3 can compensate for the loss of *Rnd3* (Pacary et al., 2011). Hence, when targeted to the correct subcellular region, Rnd2 can replace Rnd3 function in migrating neurons. Recent evidence also supports a role for the N-terminal region of Rnds in the control of subcellular localization. There is indeed a specific sequence at the N-terminal of Rnd1 and Rnd3, but not of Rnd2, that promotes their targeting to specialized membrane regions, the lipid rafts (Oinuma et al., 2012).

Another important post-translational modification that regulates Rnd protein activity is phosphorylation. Seven phosphorylation sites have been identified in Rnd3 (5 at the C-terminal end and 2 at the N-terminal end) and they have been shown to influence Rnd3 localization at the plasma membrane. Rnd3 can be phosphorylated by ROCKI or PKCα on multiple sites (Riento et al., 2005; Komander et al., 2008; Madigan et al., 2009) and upon phosphorylation, Rnd3 affinity for plasma membrane is reduced and the fraction of cytosolic Rnd3 increases. Interestingly, a non-phosphorylatable mutant form of Rnd3 ($Rnd3^{All\ A}$) that is preferentially associated to the plasma membrane is more efficient than wild-type Rnd3 in rescuing the cortical migration defects induced by *Rnd3* silencing (Madigan et al., 2009; Pacary et al., 2011). This result further demonstrates that the membrane association of Rnd3 regulates its activity in migrating neurons and determines the efficiency with which neurons migrate in the embryonic cortex.

Classical Rho proteins are generally solubilized from the plasma membrane and sequestered inactive in the cytosol, by interaction with RhoGDIs that mask the un-soluble hydrophobic group. Since Rnd proteins do not interact with RhoGDIs, an alternative mechanism has recently been proposed to explain how phosphorylated Rnd proteins become internalized and solubilized in the cytosol (Riou et al., 2013). Anne Ridley and colleagues have demonstrated that upon phosphorylation, the C-terminal region of the three Rnd interacts with the regulatory molecules 14-3-3. This interaction masks the lipid moiety of the Rnd protein and permit translocation from the plasma membrane to the cytosol. Whether the localization of Rnd2 and Rnd3 and thus their activity is controlled by this mechanism in cortical neurons is not known.

Lastly the levels of Rnd proteins in a cell can be controlled by their effectors through protein stabilization. It has been shown that the binding of Rnd3 to its effectors stabilizes Rnd3 proteins, suggesting that a positive feedback from effectors may contribute to extend the half-life of Rnd (Goh and Manser, 2012). This mechanism of regulation remains to be studied in migrating cortical neurons.

Altogether, the variety of factors that controls Rnd protein expression and localization reveal that Rnd activity is regulated by complex mechanisms, which substitute for the lack of the classical GDP/GTP molecular switch and GDI internalization.

MOLECULAR MECHANISMS MEDIATING Rnd ACTIVITY IN MIGRATING NEURONS

REGULATION OF RhoA SIGNALING AND CYTOSKELETON REMODELING

Experiments performed in non-neuronal cell types revealed that a mechanism commonly used by Rnd proteins to control cytoskeletal dynamics is the inhibition of RhoA signaling (Nobes et al., 1998; Wennerberg et al., 2003; Riou et al., 2010). Similarly, FRET analysis demonstrated that RhoA activity is increased in migrating neurons after *Rnd2* or *Rnd3* knockdown (Pacary et al., 2011). More importantly, in this study, coelectroporation of *Rnd3* shRNA together with a *RhoA* shRNA fully rescue the radial migration of *Rnd3*-silenced neurons, thus demonstrating that Rnd3 regulate radial migration in the cortex mostly by inhibiting RhoA activity. The same kind of experiment performed with *Rnd2* shRNA indicates that this RhoGTPase, in contrast to Rnd3, acts only partially through suppression of RhoA activity in migrating neurons.

In fibroblasts and epithelial cells, Rnd-mediated inhibition of RhoA activity induces cell rounding via disassembly of stress fibers, which are composed of bundles of actin filaments. Although neurons do not possess stress fibers, Rnd proteins have been shown to also control the dynamics of filamentous actin (F-actin) in migrating neurons (Pacary et al., 2011). In cultured primary cortical neurons, both *Rnd2* and *Rnd3* knock down produce an accumulation of F-actin in neuronal processes as well as in the cell body in the case of Rnd2. A common pathway that controls F-actin polymerization downstream of RhoA signaling is the ROCK (Rho Kinase)- LIMK (Lim Kinase)—cofilin pathway (Maekawa et al., 1999; Sumi et al., 1999; Peris et al., 2012). The activation of RhoA ultimately phosphorylates and inactivates cofilin, which is an actin-disassembling factor, thus resulting in local increase of F-actin. The co-electroporation in the embryonic cortex of a non-phosphorylatable form of cofilin ($cofilin^{S3A}$), which constitutively depolymerizes actin, together with *Rnd3* shRNA completely rescues the migration defects induced by *Rnd3* silencing. This suggests that when *Rnd3* is silenced, the RhoA-cofilin-mediated excessive polymerization of actin molecules hampers the motility behavior of migrating neurons. Interestingly, co-electroporation of the cofilin mutant with *Rnd3* shRNA also rescues the defects that *Rnd3* silencing produces during INM in VZ progenitor cells (Pacary et al., 2013), suggesting that similar basic molecular mechanisms may control nuclear translocation during INM and glia-guided locomotion.

In contrast, the migratory defects induced by *Rnd2* knockdown are not rescued by the mutated form of cofilin, indicating that Rnd2 promotes migration independently of its effect on the actin cytoskeleton. It is possible that accumulation of F-actin and aberrant cytoskeletal organization upon *Rnd2* knock down might be secondary to other events that impede migration. Rnd2 has been shown to be expressed in endosomes and to interact with molecules involved in the formation and trafficking of endocytic vesicles (Fujita et al., 2002; Tanaka et al., 2002; Wakita et al., 2011), raising the possibility that Rnd2 pro-migratory activity may involve the regulation of endocytosis. Further studies will be required to test this hypothesis.

Consistent with their different activities, Rnd2 and Rnd3 cannot replace one another, even if they both inhibit RhoA signaling. This apparent paradox can be explained by the fact that Rnd2 and Rnd3 interfere with RhoA activity in different subcellular compartments (Pacary et al., 2011). Indeed, Rnd3 preferentially localizes at the plasma membrane and inactivates RhoA in this compartment, whereas Rnd2 is expressed only in endosomes and cytosol, confining RhoA regulation to these internal structures

(Pacary et al., 2011). In accordance with these data, Rnd2 can replace Rnd3 function in migrating neurons if it is targeted to the plasma membrane by replacement of its C-terminal region with the one of Rnd3, as already mentioned. Importantly, the reduction of RhoA activity in endosomes has been shown to be essential for clathrin mediated endocytosis (Lamaze et al., 1996; Qualmann and Mellor, 2003; Ridley, 2006), further reinforcing the hypothesis that Rnd2 might control cortical neuron migration by regulating the trafficking of receptors or adhesion molecules which are essential for this process. However, it is worth noting that Rnd2 inhibition of RhoA signaling cannot fully explain Rnd2 pro-migratory activity and therefore Rnd2 might act in the cortex also via a different and RhoA-independent mechanism (Pacary et al., 2011).

MECHANISMS OF RhoA REGULATION BY Rnd PROTEINS

The molecular bases for Rnd-mediated RhoA inhibition are not yet completely understood, but many evidences suggest the existence of various mechanisms (**Figure 4**). Rnd3, for example, has been shown to antagonize RhoA signaling via three different pathways: (1) by promoting the activity of RhoA GAPs, which promote the hydrolysis of the GTP into GDP, such as p190RhoGAP (Wennerberg et al., 2003) (**Figure 4A**, **Table 3**), (2) by blocking the activity of RhoA effectors, such as the Rho kinase ROCKI (Riento et al., 2003) (**Figure 4B**, **Table 3**); (3) by inhibiting Rho GEFs, which exchange GDP with GTP on RhoA, such as Syx (Goh and Manser, 2010) (**Figure 4C**, **Table 3**).

The first mechanism, which involves the stimulation of p190RhoGAP by Rnd3, seems to be an important pathway of RhoA inhibition downstream of Rnd proteins, since Rnd1 and Rnd2 have also been shown to interact with p190RhoGAP (Wennerberg et al., 2003; Pacary et al., 2011). However, a mutant form of Rnd2 ($Rnd2^{T39V}$), which cannot bind to p190RhoGAP, is as active as wild type Rnd2 in rescuing the neuronal migration defects induced by *Rnd2* silencing in the cortex. Therefore, even if Rnd2 can interact with p190RhoGAP, this interaction does not mediate Rnd2 function in this context. It is possible that Rnd2 works via interaction with a different RhoGAP. One candidate is MgcRacGAP, which has been found associated to Rnd2 in male germ cells (Naud et al., 2003) and which is expressed in the developing cerebral cortex at the time of radial migration (Arar et al., 1999) (**Table 3**). MgcRacGAP (or RacGAP1) primary targets are Rac1 and Cdc42, but upon phosphorylation, MgcRacGAP turns its activity toward RhoA (Toure et al., 2008). Rather than promoting mere inhibition of RhoA activity, MgcRacGAP has been shown to control a RhoA GTPase flux at the site of furrow formation during cytokinesis (Miller and Bement, 2009). In the future, it would be interesting to understand whether MgcRacGAP mediates Rnd2 function in the endosomal compartments and to study whether also Rnd3 uses this different RhoGAP during early steps of corticogenesis, when Rnd3 is known to control INM, cleavage plane orientation, VZ integrity and SVZ progenitor proliferation (Pacary et al., 2013).

In contrast to Rnd2, Rnd3 requires the interaction with p190RhoGAP for its pro-migratory activity in the cortex. Indeed, an Rnd3 mutant form ($Rnd3^{T55V}$) that cannot bind to p190RhoGAP in rescue experiments failed to replace Rnd3 function in migrating neurons (Pacary et al., 2011). However, it has been recently shown that this Rnd3 mutant carries a mutation in the effector binding domain (Wennerberg et al., 2003), which not only prevents Rnd3 from binding to p190RhoGAP but also disrupts the ability of Rnd3 to bind to other candidate effectors, including a member of the Plexin family of axon guidance receptors, PlexinB2 (Azzarelli et al., 2014) (**Table 3**). Therefore, it is possible that Rnd3 activity in the cortex may also require the interaction with effectors other than p190RhoGAP. The binding of Rnd3 to the RhoA effector ROCKI is however not affected by the T55V mutation in the effector domain. Instead, ROCKI binds Rnd3 in a different position and ROCKI-Rnd3 interaction can be selectively disrupted by mutation of two sites present in the C-terminal region of Rnd3 protein ($Rnd3^{T173A+V192A}$) (Wennerberg et al., 2003). Selective disruption of Rnd3-ROCKI interaction does not interfere with Rnd3 function in migrating neurons, which indicates that blocking ROCKI does not account for Rnd3 inhibition of RhoA activity in this context. Finally, whether Rnd2 or Rnd3 also modulate RhoA activity in migrating neurons via Syx or other RhoGEFs remains unexplored.

A ROLE FOR PLEXINS

Over the past few years, Rnd proteins have been shown to constitute important functional components of the plexin-semaphorin signaling pathways (Chardin, 2006; Puschel, 2007). Plexins belong to a large family of transmembrane receptors, which are activated by their physiological ligands, the semaphorins. In

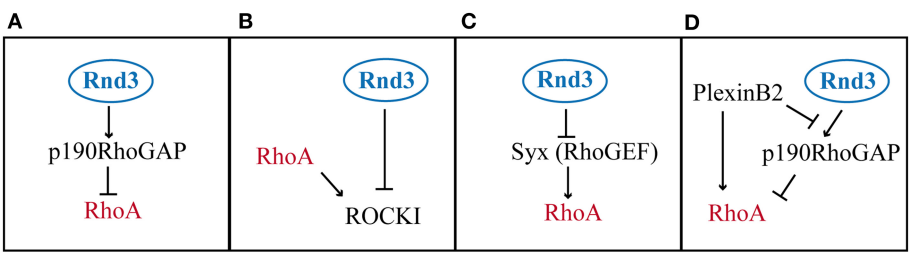

FIGURE 4 | Modes of RhoA activity regulation by Rnd3. (A) Rnd3 interacts with p190RhoGAP and promotes its activity of RhoA inactivation. **(B)** Rnd3 indirectly inhibits RhoA signaling, by blocking the RhoA downstream effector ROCKI. **(C)** Rnd3 inhibits a RhoA activator like Syx. **(D)** PlexinB2 interaction with Rnd3 disrupts Rnd3-p190RhoGAP binding, which lifts RhoA inhibition. In addition PlexinB2 directly activates RhoA via recruitment of RhoGEFs (not shown).

Table 3 | Rnd interacting partners and their functions.

Rnd partner	Rnd	Function	References
p190RhoGAP	Rnd1 Rnd2 Rnd3	Down-regulation of RhoA in stress fiber collapse and during cortical neuron migration	1, 2, 3, 4
ROCKI	Rnd3	Inhibition of ROCKI signaling in stress fiber disassembly; control of Rnd localization and stability through phosphorylation	5, 6
Syx	Rnd3	Down-regulation of RhoA in Zebrafish gastrulation	7
MgcRacGAP	Rnd2	Regulation of RhoGTPase flux during cytokinesis; control of male germ cell development	8, 9
PlexinB2	Rnd3	Regulation of neuronal migration by fine-tuning RhoA signaling	4
PlexinB1	Rnd1	Activation of RhoA and down-regulation of R-Ras in growth cone collapse	10, 11
PlexinD1	Rnd2	Down-regulation of R-Ras in axon outgrowth inhibition	12
PlexinA1	Rnd1	Activation of Rac1 and down-regulation of R-Ras in axonal repulsion	13, 14, 15
Rapostilin	Rnd2	Regulation of endocystosis and membrane invagination in neurite branching and spine formation	16, 17
Vps4A	Rnd2	Regulation of endosomal trafficking	18
FLRT3	Rnd1	Control of cadherin-mediated adhesion during Xenopus gastrulation	19, 20
Pragmin	Rnd2	Activation of RhoA in neurite outgrowth inhibition	21
SCG10	Rnd1	Control of microtubule stability in axon formation	22
Socius	Rnd1	Loss of stress fibers	23
FSR2a/b	Rnd1	Control of neurite extension downstream of FGF signaling	24
Grb7	Rnd1	Possible role in migration/invasion	25
STI1	Rnd1	Control of cytoskeletal collapse	26

1 (Foster et al., 1996), 2 (Wennerberg et al., 2003), 3 (Pacary et al., 2011), 4 (Azzarelli et al., 2014) 5 (Riento et al., 2003), 6 (Riento et al., 2005), 7 (Goh and Manser, 2010), 8 (Naud et al., 2003), 9 (Miller and Bement, 2009), 10 (Oinuma et al., 2003), 11 (Oinuma et al., 2004), 12 (Uesugi et al., 2009), 13 (Rohm et al., 2000), 14 (Toyofuku et al., 2005), 15 (Zanata et al., 2002), 16 (Fujita et al., 2002), 17 (Wakita et al., 2011), 18 (Tanaka et al., 2002), 19 (Karaulanov et al., 2009), 20 (Ogata et al., 2007), 21(Tanaka et al., 2006), 22 (Li et al., 2009), 23 (Katoh et al., 2002), 24 (Harada et al., 2005), 25 (Vayssiere et al., 2000), 26 (De Souza et al., 2014).

vertebrates, there are 9 plexin members, which can be divided into four classes, termed plexinA (A1–A4), B (B1–B3), C1 and D1 and 7 classes of secreted and membrane-bound semaphorins (Jackson and Eickholt, 2009). Although plexin-semaphorin signaling has been historically associated with regulation of axonal navigation, novel roles during brain developmental and neuronal migration have started to be characterized (Luo et al., 1993; Comeau et al., 1998; Kruger et al., 2005; Pasterkamp, 2012).

Plexins contain a binding site for Rho GTPases in the middle of their intracellular domain through which they recruit several Rho GTPases, including Rnd proteins. Several evidences indicate that preferential interactions occur between certain members of the Plexin and the Rnd families (**Table 3**). For example, PlexinB1 binds to Rnd1 and Rnd2, but not to Rnd3, which instead selectively interacts with PlexinB2 (Oinuma et al., 2003; Azzarelli et al., 2014); also, PlexinD1 has been found associated only with Rnd2, but not with Rnd1 or Rnd3 (Uesugi et al., 2009) and PlexinA1 interacts with Rnd1, but not with Rnd2 (Zanata et al., 2002). The functional relevance of the exclusive Plexin-Rnd interactions is not clear, but it is likely that the recruitment of specific Rnds may be important to differentially modulate plexin signaling.

Rnd1 binding to PlexinB1 has been shown to open the conformation of the receptor and to allow the transmission of the downstream signaling. This synergistic interaction is essential to drive cell contraction in COS cells and to induce growth cone collapse during axon guidance (Chardin, 2006). In migrating cortical neurons, the interaction between Rnd3 and PlexinB2 is crucial to fine-tune the levels of active RhoA. PlexinB2 recruitment of Rnd3 to its intracellular domain disrupts the interaction between Rnd3 and p190RhoGAP in a competitive manner. In this way, PlexinB2 blocks Rnd3-mediated RhoA inhibition and it has been proposed that this step is required for full RhoA activation in specific cellular compartments (Azzarelli et al., 2014) (**Figure 4D**). Therefore, through competitive Rnd3 binding, p190RhoGAP and PlexinB2 fine-tune the level of RhoA activity appropriate for cortical neuron migration.

Rnd2 has also been found associated with Plexin members like PlexinB1 and PlexinD1. However, in contrast to Rnd3, which co-localizes with PlexinB2 at the plasma membrane in primary cortical neurons, Rnd2 is not found at the plasma membrane (Pacary et al., 2011), therefore making it unlikely that Rnd2 plays a part in plexin downstream signaling that is activated in this subcellular compartment. Instead, Rnd2 is expressed in early endosomes (Pacary et al., 2011), where it interacts with Fbp17/Rapostlin and Vps4, two molecules involved in the formation and the trafficking of endocytic vesicles (**Table 3**) (Fujita

et al., 2002; Tanaka et al., 2006). Therefore, Rnd2 potential interaction with plexins may be an important strategy to control the surface expression of these receptors through the stimulation of their endocytic recycling.

Altogether, these studies suggest that Rnd2 and Rnd3 promote cortical neuron migration by distinct mechanisms that may involve selective interactions with different members of the plexin family of transmembrane receptors in different subcellular compartments.

CONCLUDING REMARKS

In the last decade, the introduction and constant refinement of new technologies, such as *in utero* electroporation of the murine embryonic cerebral cortex, have greatly advanced our understanding of the molecular pathways operating in migrating neurons (Loturco et al., 2009). Through the control of cytoskeleton remodeling, Rho and Rnd proteins have been shown to play crucial roles during neuronal migration in the developing cortex. However, whereas the cellular and molecular functions of Rnd proteins have been thoroughly described in cortical projection neuron development, very little is known about their role in tangentially migrating cortical interneurons. This would be a fertile territory for future research. Nonetheless, the critical function of Rnd proteins in the control of neuronal migration has been further highlighted by a recent study showing the requirement of Rnd3 for the tangential migration of newborn olfactory neurons from the SVZ to the olfactory bulb in the post-natal brain (Ballester-Lurbe et al., 2014).

Rnd2 and *Rnd3* expression in the cortex is under the transcriptional control of the proneural factors Neurog2 and Ascl1, respectively. These factors are well known master regulators of neuronal differentiation and activate a transcriptional program of neurogenesis in neural progenitors (Bertrand et al., 2002). Since Rnd proteins also control other aspects of cortical development, such as progenitor proliferation and neurite extension, it is possible that different transcriptional factors exclusively regulate the expression of different Rnd members to couple specific neuronal migration phases with other neurogenic events.

At the molecular level, Rnd2 and Rnd3 control distinct steps of radial migration, by interfering with RhoA activity in different subcellular compartments. The bHLH transcriptional factors Ascl1 and Neurog2 induce the expression of Rnd proteins as a strategy to repress RhoA during radial migration (Hand et al., 2005; Pacary et al., 2011). A recent model proposes that the bHLH-Rnd pathways are responsible to maintain a low level of background RhoA activity, which is essential to promote neuronal migration, but at the same time RhoA activation may still be required for example downstream of plexin receptors to stimulate actin-based contractility in defined compartments of migrating neurons (Govek et al., 2011; Azzarelli et al., 2014). Indeed, RhoA downstream effectors, such as myosinIIB and mDia1/3, have been found enriched in the proximal region of the leading process and at the cell rear, just before nucleokinesis (Tsai et al., 2007; Solecki et al., 2009; Shinohara et al., 2012). Therefore, Rnd proteins finely orchestrate RhoA levels in migrating neurons, by directing its inactivation to specific subcellular compartments and by being also involved in the signaling that promotes its activation, as in the case of Rnd3.

These studies performed in neuronal cells will contribute to a better understanding of the regulatory function of Rnd proteins in the migration of other cell types. Indeed, Rnd proteins, especially Rnd3, have been shown to also control the migration of non-neuronal cell types, such as epithelial cells (Guasch et al., 1998) or cancer cells (Riou et al., 2010). Rnd3 seems to regulate cancer cell invasion mainly through its effects on RhoA/ROCK activity. However, the specific contribution of Rnd3 to cancer cell invasion is controversial, since it has been shown to both promote and inhibit invasion (Gadea et al., 2007; Klein and Aplin, 2009), suggesting that Rnd3 may act via more than one molecular mechanism (Riou et al., 2010). Further investigation of Rnd functions in cancer cell migration will thus be crucial to a better understanding of the metastatic and invasive behavior of cancer cells.

In conclusion, it is becoming evident that Rnd proteins play important roles in cell migration during mammalian cortical development and in particular, considering their relatively recent evolution, it is possible that they might be involved in mechanisms of brain developmental and neuronal plasticity that are exclusive to vertebrate organisms.

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Neuronal migration and protein kinases

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The formation of the six-layered structure of the mammalian cortex via the inside-out pattern of neuronal migration is fundamental to neocortical functions. Extracellular cues such as Reelin induce intracellular signaling cascades through the protein phosphorylation. Migrating neurons also have intrinsic machineries to regulate cytoskeletal proteins and adhesion properties. Protein phosphorylation regulates these processes. Moreover, the balance between phosphorylation and dephosphorylation is modified by extracellular cues. Multipolar-bipolar transition, radial glia-guided locomotion and terminal translocation are critical steps of radial migration of cortical pyramidal neurons. Protein kinases such as Cyclin-dependent kinase 5 (Cdk5) and c-Jun N-terminal kinases (JNKs) involve these steps. In this review, I shall give an overview the roles of protein kinases in neuronal migration.

Keywords: protein phosphorylation, kinase, phosphatase, migration, cerebral cortex

CYTOSKELETON DYNAMICS DURING NEURONAL MIGRATION

During brain development, the extensive migratory movements of neurons from their birth place to final location are essential for neural circuit formation and proper brain function. The six-layered structure of the mammalian cerebral cortex is formed by coordinated neuronal migration *via* inside-out patterning. While early-born neurons are located in the deep layer, late-born neurons pass through the existing cortical layers to reach the superficial layer to form the six-layered structure. Three coordinated migration modes are observed in radially migrating neurons in the developing cerebral cortex: multipolar migration, glial-guided locomotion, and somal translocation (Nadarajah et al., 2001; Tabata and Nakajima, 2003; Noctor et al., 2004). During these processes, neurons change their morphology and adhesive properties. During the development of the cerebral cortex, radial migrating neurons change their morphology from multipolar to bipolar in the intermediate zone (IZ) (Tabata and Nakajima, 2003). This requires the function of cytoskeletal regulators and is inhibited by many gene mutations and experimental manipulations. These facts imply the importance of the regulation of cytoskeletal dynamics during this morphological transition. Following this, bipolar cells migrate by locomotion along the radially oriented processes of radial glia (Nadarajah et al., 2001; Noctor et al., 2004). During the mode of locomotion in migrating neurons, the nucleus is surrounded by microtubule-enriched arrays, fork-like in the front and cage-like behind (Tsai and Gleeson, 2005). Asynchronous movements of the centrosome (C) and the nucleus (N) are observed in locomotion (Tsai and Gleeson, 2005). The centrosome moves first into a cytoplasmic dilation/swelling in the leading process and then the nucleus follows (nucleokinesis) due to a pulling force from microtubules and dynein motors located at the centrosome. Cytoplasmic dilation/swelling is a structure specific to migrating neurons, at the proximal region of the leading process during the locomotion mode of migration (Nishimura et al., 2014). This coordinated relationship is called nucleus-centrosomal (N-C) coupling (Tsai and Gleeson, 2005). Retraction of trailing process occurs due to actomyosin-dependent motor functions (Bellion et al., 2005). This microtubule-actin remodeling is regulated dynamically during the locomotion mode of radial neuronal migration (Schaar and McConnell, 2005). Finally, migrating neurons along radial glial fibers change their migration mode to terminal translocation (Nadarajah et al., 2001), which is similar to somal translocation.

LESSONS FROM THE HUMAN DISORDER LISSENCEPHALY

Failure of neuronal migration causes severe developmental abnormalities in the layering of the cerebral cortex and results in the human disorder lissencephaly, which means "smooth brain." Microtubule- and actin-associated proteins regulate the dynamics of microtubule and actin cytoskeletons during neuronal migration; therefore, deletions and mutations of crucial genes involved in cytoskeletal processes lead to human lissencephaly (Dobyns, 1987) and mouse mutants with a neuronal migration phenotype.

Mutations in *doublecortin* (*DCX*) are the most common genetic cause of X-linked lissencephaly (des Portes et al., 1998; Gleeson et al., 1998). Male mice with a *Dcx* gene mutation exhibit mild histological defects only in hippocampus (Corbo et al., 2002) due to redundant compensation from *doublecortin-like kinase* (*DCLK*). This notion is supported by phenotypic analysis of *Dcx/Dclk* double-knockout (DKO) mice, which display severe abnormalities in cortical lamination due to neuronal migration defects (Deuel et al., 2006; Koizumi et al., 2006b).

DCX is a microtubule-associated protein (MAP) that has two microtubule-binding domains (Gleeson et al., 1999; Horesh et al., 1999; Taylor et al., 2000). DCX stabilizes microtubules and enhances microtubule polymerization (Francis et al., 1999; Gleeson et al., 1999; Horesh et al., 1999; Taylor et al., 2000; Moores

et al., 2006). *Dcx*-deficient neurons exhibit delayed centrosomal and nuclear movements and weakened N-C coupling, indicating the involvement of DCX in these processes (Corbo et al., 2002; Koizumi et al., 2006a). DCX function is modulated by its phosphorylation by several kinases in site specific manner, including Microtubule affinity-regulating kinase 2 (MARK2), Protein kinase A (PKA), Cyclin-dependent kinase 5 (Cdk5), and c-Jun N-terminal kinases (JNKs) (**Figure 1**). MARK2 and PKA phosphorylate DCX at Ser47 and reduce its microtubule-binding activity (Tanaka et al., 2004a; Toriyama et al., 2012). Phosphorylation of DCX at Ser47 is also required for its proper localization to the leading process of migrating neurons (Schaar et al., 2004). Cdk5 phosphorylates DCX at Sr297 and enhances its microtubule-binding activity (Tanaka et al., 2004b). JNK phosphorylates DCX at Thr321, Thr331, and Ser334, which correspond to Thr326, Thr336, and Ser339 in mouse Dcx (Gdalyahu et al., 2004). We reported that Ser332 is also a JNK phosphorylation site of mouse Dcx (Jin et al., 2010). Phosphorylation at these sites is required for DCX localization in leading process. The importance of the balance between phosphorylation/unphosphorylation is emphasized by the requirement of a dephophorylated state of DCX at neurite tips during neuronal migration (Schaar et al., 2004).

The regulation of DCX function by phosphorylation at specific sites implicates the importance of kinase function in neuronal migration. Phosphorylation is a post-translated modification of proteins. Phosphorylation sites are categorized into two types, Tyr residues and Ser/Thr residues, which are phosphorylated by tyrosine kinases and serine/threonine kinases, respectively. The activation of Src-family tyrosine kinases by Reelin and their roles in neuronal migration will be discussed in other chapters. Thus, I will discuss the major Ser/Thr kinases that regulate neuronal migration.

Cdk5

Cdk5 is serine/threonine kinase and its high activity is detected in post-mitotic neurons. Cdk5 forms heterodimer with its activating subunits, p35 or p39. The involvement of Cdk5 in neuronal migration was revealed by the analyses of Cdk5KO mice (Ohshima et al., 1996; Gilmore et al., 1998). Cdk5KO mice lack the laminar structure of the cerebral cortex (Ohshima et al., 1996). Birth-date labeling of the embryonic brain showed profound migration defects in cortical neurons (Gilmore et al., 1998). p35KO mice have milder abnormalities in neuronal migration (Chae et al., 1997). The identical phenotype of double-knockout p35/p39 mice and Cdk5KO mice indicates the redundant function of p35 and p39 (Ko et al., 2001). Conditional Cdk5KO mice showed an inverted cortical layer structure in layers II–VI (Ohshima et al., 2007). Cdk5 regulates multiple steps of radial migration of cortical neurons during the locomotion mode of migration (**Figure 2**). These include the transition from multipolar to bipolar morphology in the IZ (Ohshima et al., 2007), formation of leading processes (Kawauchi et al., 2006), and formation of a cytoplasmic dilation/swelling, which is a structure specific to migrating neurons, at the proximal region of the leading process (Nishimura et al., 2014).

Inhibition of Cdk5 activity leads to the over-stabilization of microtubules, resulting in the dysregulation of microtubule dynamics in migrating neurons (Kawauchi et al., 2005). Cdk5 phosphorylates a number of microtubule-associated proteins: DCX (Tanaka et al., 2004b), Ndel1 (Lis1-binding protein, also called Nudel) (Niethammer et al., 2000; Sasaki et al., 2000), FAK (Xie et al., 2003), and CRMP2 (Uchida et al., 2005). Ndel1 was originally identified as a novel Lis1-interacting protein and was found to be enriched at centrosomes (Niethammer et al., 2000; Sasaki et al., 2000). Ndel1 is phosphorylated by Cdk5 (Niethammer et al., 2000; Sasaki et al., 2000). Phosphorylated-Ndel1 (p-Ndel1) binds to cytoplasmic dynein heavy chain (CDHC) and katanin; its binding is required for the localization of katanin in the centrosome (Toyo-Oka et al., 2005). 14-3-3epsilon (YWHAE) binds to p-Ndel1 and protects p-Ndel1 from phosphatase attack (Toyo-Oka et al., 2003). Lis1 and 14-3-3epsilon (YWHAE) are important for neuronal migration and their deletions have been found in lissencephaly patients (Hirotsune et al., 1998; Toyo-Oka et al., 2003). These protein localizations in the centrosome, with the Lis1-Ndel1-dynein complex, regulate nucleokinesis by promoting N-C coupling during the locomotion mode of neuronal migration (Shu et al., 2004; Tsai and Gleeson, 2005). FAK phosphorylation by Cdk5 is also required for nucleokinesis (Xie et al., 2003; Xie and Tsai, 2004).

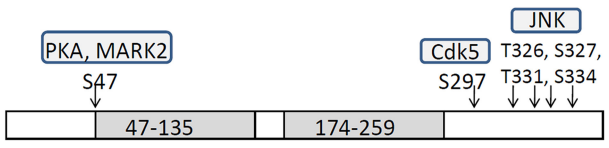

FIGURE 1 | Schematic structure of DCX and phosphorylation sites by each protein kinase. Doublecortin (DCX) has two tubulin-binding domains, 47–135 and 174–259, and patient mutations cluster in these domains (Sapir et al., 2000; Taylor et al., 2000). DCX has S/T-P rich domain and Cdk5 and JNK phosphorylate specific sites in this domain.

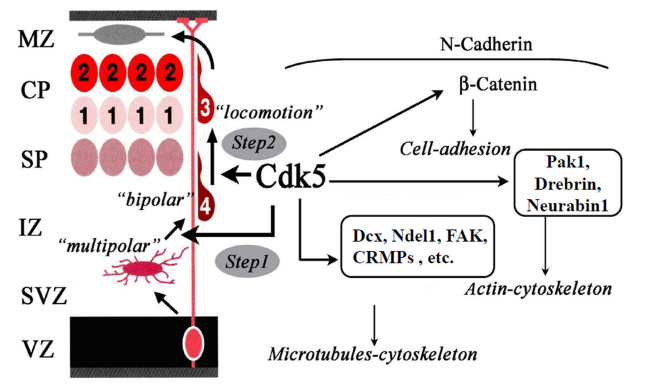

FIGURE 2 | Functions of Cdk5 in neuronal migration. Cdk5 is required for the radial migration of later-generated neurons in the cerebral cortex. Cdk5 is necessary for multipolar-to-bipolar transition (Step 1) and locomotion through the regulation of nucleokinesis of migrating neurons (Step 2). For these steps, Cdk5 regulates the dynamics of microtubules-cytoskeleton, actin-cytoskeleton and cell-adhesion through the phosphorylation of its substrate proteins.

CRMP2 was originally identified as an intracellular mediator of Sema3A signaling (Goshima et al., 1995). We have identified CRMP2 as a Cdk5 substrate by using Cdk5KO mouse brains (Uchida et al., 2005). Interestingly, Cdk5 phosphorylates CRMP2 at Ser522 and its phosphorylation is required for further phosphorylation of CRMP2 by GSK3β at Ser518, Thr514, and Thr509 (Uchida et al., 2005; Yoshimura et al., 2005). CRMP2 binds to the tubulin heterodimer (Fukata et al., 2002) and their binding is regulated by Cdk5/Gsk3β phosphorylation (Uchida et al., 2005; Yoshimura et al., 2005; Yamashita and Goshima, 2012). Involvement of CRMP2 and its phosphorylation in neuronal migration will be tested in CRMP2 mutant mice (Yamashita et al., 2012).

Recently, Nishimura et al. demonstrated that $p27^{kip1}$ that is phosphorylated and stabilized by Cdk5 is required for the formation of a cytoplasmic dilation/swelling (Nishimura et al., 2014). Stabilization of p27kip1 by Cdk5 is also involved in the regulation of the actin cytoskeleton during neuronal migration (Kawauchi et al., 2006). Cdk5 phosphorylates the actin-binding proteins, Drebrin and Neurabin-I, and may regulate neuronal migration (Causeret et al., 2007; Tanabe et al., 2014).

Rap1 signaling is involved in neuronal migration and is regulated by Cdk5 (Utreras et al., 2013). Rap1 activation promotes the cell-surface localization of N-cadherin (Jossin and Cooper, 2011). The N-cadherin-mediated adhesion complex is required for multipolar-bipolar transition (Jossin and Cooper, 2011) and radial fiber-dependent neuronal migration (Kawauchi et al., 2010). A previous study has shown that pharmacological inhibition of Cdk5 activity enhances N-cadherin-mediated cell-cell adhesion (Kwon et al., 2000). Rap1 activation depends upon Rap1-GEFs, including Rap1GEF1 (also known as C3G) and Rap1GEF2. RapGEF1 activation of Rap1 controls somal/terminal translocation triggered by Reelin (Franco et al., 2011; Jossin and Cooper, 2011; Sekine et al., 2012) *via* the stabilization of leading processes toward the marginal zone (Franco et al., 2011; Sekine et al., 2012). Interestingly, RapGEF2 KO mice showed a neuronal migration defect phenotype in the subcortical area, which indicated the involvement of RapGEF2 in multipolar-bipolar transition (Bilasy et al., 2009). Recently, Ye et al. have shown that Cdk5 phosphorylates RapGEF2 at Ser1124 and its phosphorylation is required for Rap1 activation (Ye et al., 2014). Previous studies have shown that RapGEF1-dependent Rap1 activation is dispensable in multipolar-bipolar transition (Sekine et al., 2012); therefore, Cdk5 mediated Rap1 activation *via* RapGEF2 phosphorylation is important for this transition. As proposed by Ye et al. (2014), the two pathways of Reelin and Cdk5 are not simply parallel, but rather act on successive phases of neuronal migration *via* Rap1 activation. Cdk5-mediated RapGEF2 phosphorylation controls multipolar-bipolar transition and Reelin-mediated RapGEF1 activation promotes terminal translocation (**Figure 3**). This idea fits well with our previous observations in mutant mice that lack Cdk5/p35 and Reelin/Dab1 (Ohshima et al., 2001, 2002; Ohshima and Mikoshiba, 2002).

Cdk5 is also required for the radial migration of hippocampal neurons (Ohshima et al., 1996, 2007) and Purkinje cells in the developing cerebellum (Ohshima et al., 1999; Kumazawa et al., 2013). Inward migration of granule cells and migration in the rostral migratory stream is also Cdk5-dependent (Ohshima et al., 1999; Hirota et al., 2012; Kumazawa et al., 2013; Umeshima and Kengaku, 2013). Compared with the analysis of the molecular mechanisms of neuronal migration in radial migration in the cerebral cortex, the mechanisms of neuronal migration in hippocampal and cerebellar neurons remain to be elucidated.

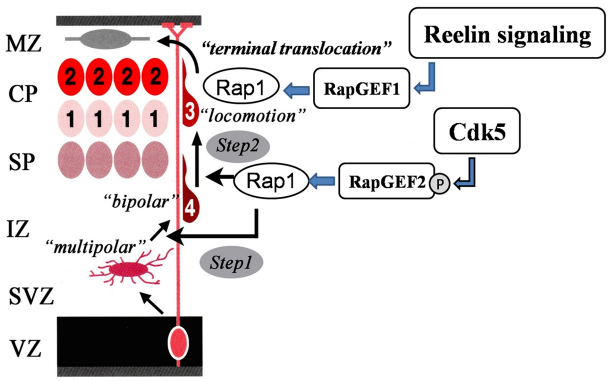

FIGURE 3 | Sequential Rap1 activation by Cdk5 and Reelin signaling. Cdk5 and Reelin signaling activate Rap1 through the activation of different Rap1GEFs in the control of the radial migration of cortical neurons in the cerebral cortex in a sequential manner.

GSK3β

Two members of the GSK-3 family in mammals, GSK3α and GSK3β, show 98% amino acid sequence identity within their kinase domains and overall share 85% identity (Doble and Woodgett, 2003). Both isoforms are highly expressed in the developing brain. GSK3-signaling is a strong regulator of neuronal progenitor proliferation in the developing cerebral cortex (Chenn and Walsh, 2002; Kim et al., 2009). To study the role of GSK3 in neuronal migration, Morgan-Smith et al. produced *Gsk3a*$^{-/-}$*Gsk3b*$^{loxP/loxP}$*; Neurod6-Cre* (*Gsk3:Neurod6*) mice and analyzed neuronal positioning after birth. The *Nuerod6-Cre* mice induce recombination in post-mitotic cortical excitatory neurons after E11 (Goebbels et al., 2006). *Gsk3*-deleted neurons expressing the upper layer marker exhibited migration failure in the cerebral cortex. Radial migration in the hippocampus was also affected (Morgan-Smith et al., 2014). Hypophosphorylation of CRMP2 at Thr514 (Yoshimura et al., 2005) and Dcx at Ser327 (Bilimoria et al., 2010) was observed in the cortex of *Gsk3:Neurod6* mice (Morgan-Smith et al., 2014).

JNK

JNKs are members of MAPK signaling pathway. There are three related genes in mammals: *Jnk1, Jnk2, and Jnk3.* All three *Jnk* genes are expressed in the developing mouse brain. JNKs act as the final effector kinases within a classical cascade consisting of MAPKKKs (MAP3Ks), MAPKKs (MAP2Ks), and MAPKs. Like other MAPKs, JNKs are activated by MAP2K-mediated phosphorylation. MKK4 and MKK7 are the MAP2Ks that phosphorylate JNKs.

Genetic deletion studies of *Jnk1* and the MAP3K and MAP2Ks for Jnk1, *Dlk1, Mkk4,* and *Mkk7*, in mice suggest their involvements in the migration of cortical projection neurons (Hirai et al., 2006; Wang et al., 2007; Westerlund et al., 2011; Yamasaki et al., 2011). Deletion of the upstream activators of JNKs, *Dlk1* (Hirai et al., 2006), *Mkk4* (Wang et al., 2007), and *Mkk7* (Yamasaki et al., 2011) inhibits radial migration. On the other hand, deletion of *Jnk1* results in accelerating radial migration (Westerlund et al., 2011). These results could be explained by *Jnk2* and/or *Jnk3* playing opposing roles to *Jnk1* in radial migration. Double deletion of *Jnk1* and *Jnk2* causes embryonic lethality (Kwon et al., 2000); therefore, further study using the conditional deletion of genes will be necessary to resolve this issue. Pharmacological inhibition of JNK activity using SP600125 inhibits the radial migration of cortical neurons (Kawauchi et al., 2003; Hirai et al., 2006). However, a recent study has shown that SP600125 inhibits 74 kinases (out of 353 tested) at 10 μM, including MEK1, MEK2, MKK3, MKK4, and MKK6 (KINOMEscan LINCS data base). Thus, the results obtained using SP600125 are difficult to interpret because of its low specificity for JNK.

JNKs phosphorylate the microtubule regulatory proteins, DCX, MAP2, MAP1b, and SCG10 (Chang et al., 2003; Kawauchi et al., 2003; Gdalyahu et al., 2004; Tararuk et al., 2006; Jin et al., 2010; Björkblom et al., 2012). We have shown that phosphorylation of DCX at Ser332 by JNK disrupts its microtubule binding (Jin et al., 2010). SCG10 is a tubulin interacting protein, which is phosphorylated by JNK SCG10 at Ser62 and Ser73 (Tararuk et al., 2006). Phosphorylation of SCG10 at Ser73 is reduced in *Jnk1*$^{-/-}$ brains (Tararuk et al., 2006). Knockdown of SCG10 increases the rate of radial migration (Westerlund et al., 2011), suggesting a role for SCG10 in neuronal migration. The involvement of JNK in the regulation of the tangential migration of inhibitory neurons from ganglionic eminence is also reported (Myers et al., 2014).

MARK2

MARK2/Par-1 was originally identified as a regulator of cell polarity in *C. elegans* (Par-1). In parallel it was also identified as a protein kinase that regulates microtubule stability, microtubule affinity-regulating kinase 2 (MARK2) (Drewes et al., 1997). *In vivo* overexpression of MARK2/Par-1 results in a loss of neuronal polarity (Sapir et al., 2008). A reduction in MARK2/Par-1 causes neuronal migration arrest with more stable microtubules (Sapir et al., 2008). MARK2/Par-1 phosphorylates tau, MAP2, MAP4, and DCX (Biernat et al., 1993; Drewes et al., 1997; Schaar et al., 2004). Phosphorylation of these microtubule-associated proteins (MAPs) causes the removal of MAPs and DCX from microtubules.

shRNA-MEDIATED OFF-TARGET TOXICITY CAUSES NEURONAL MIGRATION DEFECTS

Acute inactivation of gene function by shRNA, together with in utero electroporation, is a widely used method to study neuronal migration. In some cases, such as DCX, neuronal migration phenotypes caused by shRNA knockdown or knockout by gene deletion show a discrepancy (Corbo et al., 2002; Bai et al., 2003). Recently, Baek et al. have shown that shRNAs cause neuronal migration defects *via* an off-target effect (Baek et al., 2014). They have demonstrated that shRNA alters endogenous miRNA pathways and leads to reduced let7 miRNA expression. This disruption of let7 causes neuronal migration defects. They have designed scrambled shRNAs of *Dcx* and found half cause neuronal migration defects. These results offer a warning for the interpretation of neuronal migration studies using shRNAs. They have also shown that switching from shRNA to a shmiRNA construct can avoid these toxic effects. Therefore, studies of neuronal migration using the shRNA method need to be re-evaluated by knockdown studies using shmiRNA or genetic deletion.

FUTURE PROSPECTS OF RESEARCH

The activation of protein kinases are regulated by intrinsic and extrinsic factors. For example, Cdk5 activity is regulated by the amount of its activating subunits, p35 and p39. p35, and p39 are expressed in post-mitotic neurons; therefore, they are regulated by the degree of neuronal maturation. Cdk5 activity is also regulated by several extracellular factors (Sasaki et al., 2002; Cheung et al., 2007; Fu and Ip, 2007; Fu et al., 2007). Gsk3β activity is regulated by Wnt signaling and JNK activity is regulated by extracellular stimuli. Therefore, coordinated neuronal migration is regulated by multiple signaling pathways external to migrating neurons through the balanced activation of protein kinases as discussed above. One direction for future studies will be to examine the molecular mechanisms that regulate protein kinase activity by extracellular factors. For example, Sema3A is shown to regulate radial migration (Chen et al., 2008); however, its regulation of intracellular protein kinase activity remains to be elucidated. For this purpose, the development of a method to monitor kinase activity *in vivo* will be valuable for the future research. Studies on the identification of the downstream effectors (substrates) of protein kinases are important to understand the mechanisms by which each protein kinase is involved in neuronal migration. In this regard, comparative phosphoproteomics using brain samples from kinase-null mutant mice will be useful (Uchida et al., 2005; Contreras-Vallejos et al., 2014).

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REVIEW
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Molecular Pathways Underlying Projection Neuron Production and Migration during Cerebral Cortical Development

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Glutamatergic neurons of the mammalian cerebral cortex originate from radial glia (RG) progenitors in the ventricular zone (VZ). During corticogenesis, neuroblasts migrate toward the pial surface using two different migration modes. One is multipolar (MP) migration with random directional movement, and the other is locomotion, which is a unidirectional movement guided by the RG fiber. After reaching their final destination, the neurons finalize their migration by terminal translocation, which is followed by maturation via dendrite extension to initiate synaptogenesis and thereby complete neural circuit formation. This switching of migration modes during cortical development is unique in mammals, which suggests that the RG-guided locomotion mode may contribute to the evolution of the mammalian neocortical 6-layer structure. Many factors have been reported to be involved in the regulation of this radial neuronal migration process. In general, the radial migration can be largely divided into four steps; (1) maintenance and departure from the VZ of neural progenitor cells, (2) MP migration and transition to bipolar cells, (3) RG-guided locomotion, and (4) terminal translocation and dendrite maturation. Among these, many different gene mutations or knockdown effects have resulted in failure of the MP to bipolar transition (step 2), suggesting that it is a critical step, particularly in radial migration. Moreover, this transition occurs at the subplate layer. In this review, we summarize recent advances in our understanding of the molecular mechanisms underlying each of these steps. Finally, we discuss the evolutionary aspects of neuronal migration in corticogenesis.

Keywords: cerebral cortex, neuronal differentiation, radial migration, subplate, cortical evolution

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INTRODUCTION

The mammalian neocortex is a highly organized structure underlying higher brain functions such as cognition, learning, and memory. It consists of a six-layer structure with an inside-out pattern, which is formed by radial migration of neuroblasts that continuously bypass the preceding differentiated and migrated neurons. Because neurons are born in the deeper part of the developing brain and migrate toward the pial surface, proper regulation is crucial, and impairment of this process results in various disorders such as brain malformation or psychiatric diseases. Our understanding of how this mammalian-specific complex structure is organized has advanced

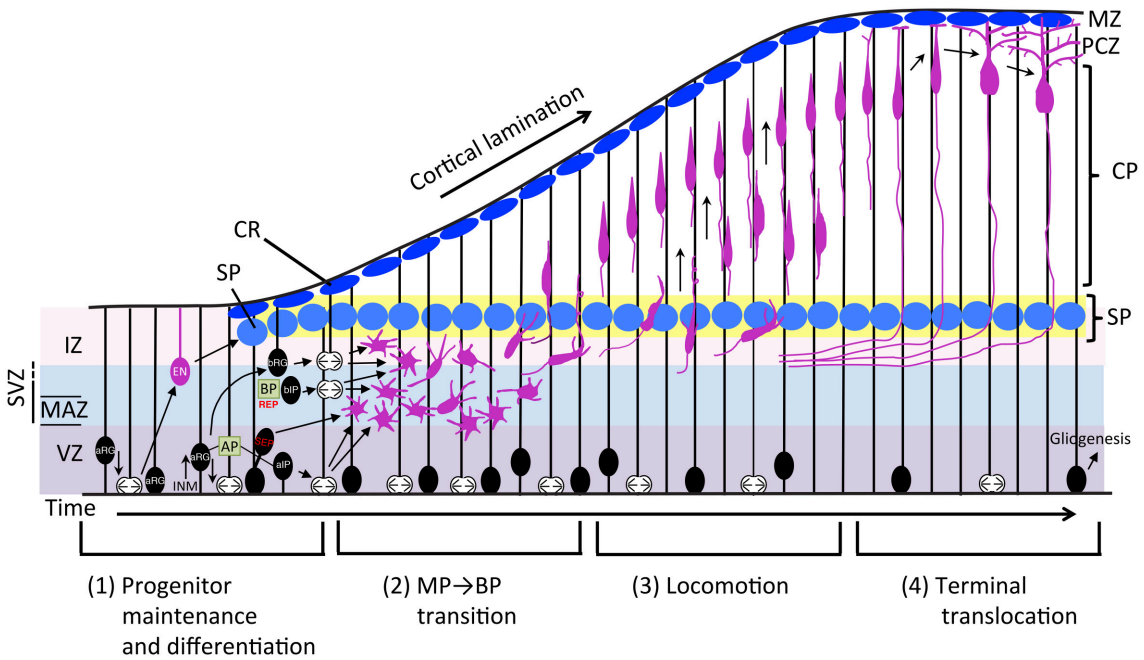

FIGURE 1 | Schematic representation of the neuronal differentiation and migration process. The radial migration of glutamatergic neurons in the developing neocortex can be divided into four steps. (1) Neurons born from RG cells, first exhibit a MP shape and move toward the SP via MP migration where they (2) convert to BP cells. (3) After entering the CP, newborn neurons migrate toward the pial surface in locomotion mode. (4) Finally, neurons complete their radial migration by execution of terminal translocation and the initiation of maturation. aIP, apical intermediate progenitor; AP, apical progenitor; aRG, apical radial glial progenitors; bIP, basal intermediate progenitor; BP, basal progenitor; bRG, basal radial glial progenitors; CP, cortical plate; CR, Cajal-Retzius cell; EN, early born neuron; INM, interkinetic nuclear migration; IZ, intermediate zone; MAZ, multipolar cell accumulation zone; MZ, marginal zone; PCZ, primitive cortical zone; REP, rapidly exiting population; SEP, slowly exiting population; SP, subplate neuron; SVZ, subventricular zone; VZ, ventricular zone.

substantially in the last 20 years. In this review, we summarize the molecular pathways underlying how newly developed neurons travel from their birth to the terminus by dividing the process into four parts, as shown in **Figure 1**. Finally, we discuss evolutionary aspects of the neuronal migration mode.

Abbreviations: aIP, apical intermediate progenitor; AMPK, AMP-activated kinase; AP, apical progenitors; APP, amyloid-b precursor protein; aRG, apical radial glia; ASD, autism spectrum disorder; bIP,basal intermediate progenitor; BM, basement membrane; BMP, bone morphogenetic protein; BP, basal progenitors or bipolar; bRG, basal radial glia; CDK, cyclin dependent kinase; CNS, central nervous system; CP, cortical plate; CR, Cajal Retzius; CSF-1, colony stimulating factor-1; E, embryonic day; ECD, extracellular domain; FGF, fibroblast growth factor, FGFR, fibroblast growth factor receptor; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GPCR, G-protein coupled receptor; IL, interleikin; INM, interkinetic nuclear migration; IP, intermediate progenitor; IUE, *in utero* electroporation; IZ, intermediate zone; JNK, c-Jun N-terminal kinase; MADM, mosaic analysis with double markers; MAZ, multipolar cell accumulation zone; miRNA, microRNA; MST, mitotic somal translocation; MTOC, microtubule organizing center; MP, multipolar; MT, microtubule; mTOR, mammalian target of rapamycin; N-cad, N-cadherin; NPC, neural progenitor cell; OSVZ, outer subventricular zone; PBM, pial basement membrane; PcG, polycomb group; PCM, pericentriolar material; PH, periventricular heterotopia; PMSE, polyhydramnios, megalencephaly, and symptom epilepsy syndrome; PSB, pallial-subpallial boundary; RA, retinoic acid; RAR, retinoic acid receptor; REP; rapidly exiting population; RG,radial glia; RGC, radial glial cell; SEP, slowly exiting population; shRNA, short hairpin RNA; SP, subplate; SVZ, subventricular zone; TACC, transforming acidic coiled coil proteins; TSC, tuberous sclerosis complex; VZ, ventricular zone; WT, wild-type.

PROLIFERATION AND DIFFERENTIATION OF NEURAL PROGENITOR CELLS

Neural progenitor cells (NPCs) of the glutamatergic neurons of the mammalian neocortex proliferate via symmetrical division of neuroepithelial cells in the early developmental stage. During development, neuroepithelial cells become radial glia (RG) cells by expressing marker proteins that are characteristic of astrocytes, including glial fibrillary acidic protein (GFAP), astrocyte-specific glutamate transporter (GLAST), the brain lipid binding protein (BLBP), and tenascin C (TNC) around the onset of neurogenesis. RG cells have a long basal process that extends to the pial surface, and they start producing neurons by asymmetrical division while maintaining symmetrical division (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001). NPCs are classified into two subtypes based on the location of mitosis: apical progenitors (AP) and basal progenitors (BP) (**Figure 1**). APs are located in the ventricular zone (VZ) and include neuroepithelial cells, apical radial glia (aRG), and apical intermediate progenitors (aIPs) (Gal et al., 2006; Kawaguchi et al., 2008; **Figure 2**). aIPs are also called short neural precursors (SNP) which express Pax6 and divide apically (Tyler and Haydar, 2013). Basal progenitors (BPs) include basal radial glia (bRG) and basal intermediate progenitors (bIPs) which are Tbr2-positive and located mainly in the subventricular zone (SVZ). bRG are Pax6-positive RG

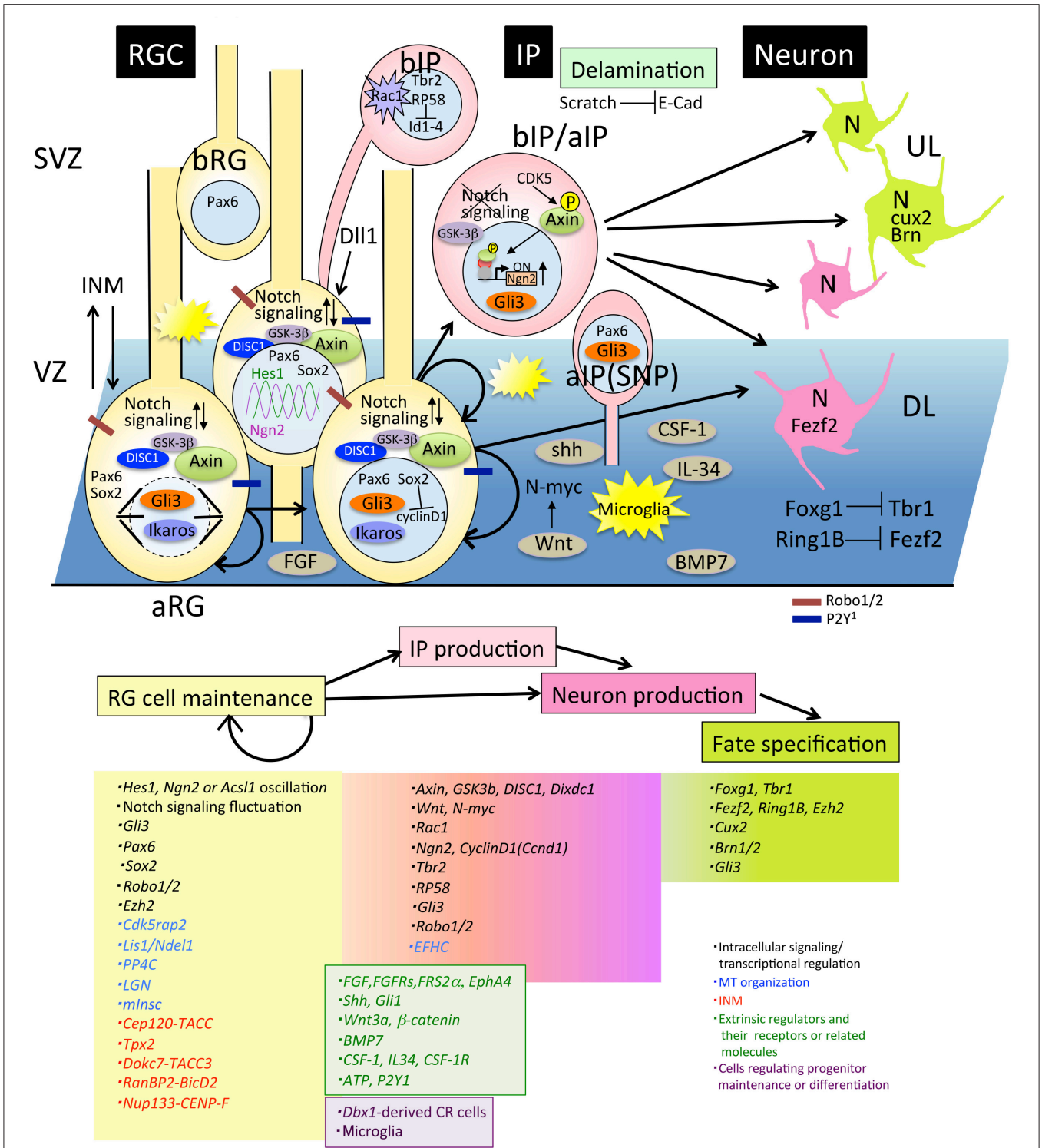

FIGURE 2 | Molecules involved in progenitor maintenance and neuronal differentiation. Cells and molecules that regulate RG cell maintenance and IP or neuron production, fate specification, and delamination are described in Section Proliferation and Differentiation of Neural Progenitor Cells. aIP, apical intermediate progenitor; aRG, apical radial glial progenitors; bIP, basal intermediate progenitors; bRG, basal radial glial progenitors; DL, deep layer neurons; E-cad, E-cadherin; INM, interkinetic nuclear migration; IP, intermediate progenitor; RGC, radial glial cell; SNP, short neural precursor; SVZ, subventricular zone; UL, upper layer neurons.

cells preferentially located in the basal part of the SVZ called the outer SVZ (OSVZ: Smart et al., 2002) and are prominently found in gyrencephalic mammals (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011). Although bRG (also called oRG: outer radial glial cells or OSVZ progenitors) were also found in the developing mouse cortex as a minor population compared with those in humans and ferrets (Shitamukai et al., 2011; Wang et al., 2011b), it is thought that the diverse behaviors of bRG contribute to variations in the cortical structure between mammalian species (Gertz et al., 2014). Compared with neuroepithelial cells that divide only symmetrically in the proliferative state, aRG produce two progenitors by symmetrical division, or one progenitor and one neuron or intermediate progenitor (IP) by asymmetrical division in the neurogenic stage. bIPs are Tbr2-positive and neuron-committed progenitors that divide in the majority of cases once, or in a minority of cells twice, to produce neurons suggesting its role in the amplification of the progenitor pool (Haubensak et al., 2004; Noctor et al., 2004; Kowalczyk et al., 2009). bRG, another population of transient-amplifying cells, are more prominent in the gyrencephalic cortex compared with that in rodents, suggesting that IPs and bRG may contribute to amplifying neuron numbers, the expansion of cortical area, and gyrification during evolution (Lui et al., 2011). In addition, novel progenitor cells, called subapical progenitors (SAP) were described recently (Pilz et al., 2013).

Thus, a variety of progenitors proliferate and differentiate into neurons during the cortical developmental stage, suggesting that an accurate balance between the proliferation state and differentiation into neurons is critical for determination of the final number of cortical neurons (Caviness et al., 1995). In this regard, experiments showed that a disturbance of the balance between self-renewal and differentiation of mouse NPCs promotes cortical expansion (Chenn and Walsh, 2002). In this study, transgenic mice expressing a stabilized β-catenin in NPCs develop enlarged brains with increased cerebral cortical surface area and folds resembling sulci and gyri of higher mammals (Chenn and Walsh, 2002), suggesting that the precise regulation of the proliferative state of either NPC maintenance or NPC differentiation maybe a critical factor for regulating cerebral cortical size during evolution. How this regulation is orchestrated has been a topic of interest, and many researchers have been investigating it from various standpoints. We will summarize the mechanisms of maintenance and differentiation of the NPCs from the intracellular and extracellular standpoints (**Figure 2**).

Regulation of Intracellular Signaling of Neural Progenitor Cells

Regarding intracellular signaling of NPCs (**Figure 2**), studies have revealed that Notch signaling fluctuation plays a critical role in the maintenance of the progenitor state (Kageyama et al., 2008; Shimojo et al., 2008). Their findings originated from the discovery that the expression of the bHLH factor *Hes1* oscillates with a duration of about 2-3 h in many cell types (Hirata et al., 2002). In NPCs, *Hes1* oscillation drives the oscillatory expression of *Neurogenin 2 (Ngn2)*, *Ascl1*, and *Dll1,* a key ligand for activating Notch signaling (Shimojo et al., 2008; Imayoshi et al., 2013). The oscillatory expression between *Ngn2* or *Ascl1* and *Dll1* in the complementary phase leads to mutual activation of Notch signaling within neighboring progenitors, and enables the progenitors to maintain the proliferative state of NPCs. Once this oscillation is diminished by sustained expression of proneural factors, progenitor cells differentiate into different neuronal and glial subtypes based on proneural factor that shows sustained expression levels (Imayoshi et al., 2013). How oscillatory expression levels of *Hes1*, *Ascl1*, *Ngn2,* and *Dll1* influence the fate of progenitor cells in relation to developmental time is still unknown. Although epigenetic modification of the NPC genome could be considered as a candidate, identification of the downstream genes of these oscillation will reveal the molecular mechanisms of cell fate specification in future studies.

Notch signaling fluctuation in NPCs is also supported by the gene expression profiles of a large number of single progenitor cells at the mid-embryonic stage (Kawaguchi et al., 2008). They classified progenitors in three subclasses according to their gene expression profiles and found that APs exhibit highly variable expression patterns of Notch signaling related genes. Attenuation of Notch signaling in APs immediately led to differentiation of APs into nascent IPs. Interestingly, a recent report revealed that IPs provide feedback to the RG progenitors by serving as a source of Dll1 via dynamic and transient processes that directly interact with RG (Nelson et al., 2013). This feedback regulation may be involved in maintaining the RG progenitor pool by activating Notch signaling in RG cells (**Figure 2**). As for the regulators for Notch signaling, Robo1, and Robo2 receptors that are known axon guidance regulators, have been reported to modulate the transition between RG cells and IPs through activation of the Notch effector *Hes1* (Borrell et al., 2012). This study suggested that some regulators have multiple functions in cortical development including neurogenesis and neural circuit formation.

Sox2, a member of the high-mobility group box transcription factors, is highly expressed in RG cells and is essential for maintaining their self-renewal state (Hutton and Pevny, 2011). Recently, the molecular mechanism has been revealed by which Sox2 negatively regulates genes promoting NPC proliferation including *Cyclin D1* using single cell RNA-seq experiment of *in utero* electroporation (IUE) cortex. Upon differentiation to IP cells, upregulated Ngn2 repressed Sox2 followed by cyclin D1 de-repression, and promote IP proliferation (Hagey and Muhr, 2014).

Other factors that are involved in progenitor pool maintenance include Axin (Fang et al., 2013), Disrupted in Schizophrenia 1(DISC1) (Mao et al., 2009), and Rac1 (Leone et al., 2010). Axin is a scaffold protein for many signaling proteins, including GSK-3. An increase in the expression level of Axin in progenitor cells leads to the transient amplification of IPs without affecting the RG pool. In this state, Axin localized in the cytoplasm with GSK-3 as a binding partner contributes to the self-renewal and IP amplification of aRG. As the neurogenic stage proceeds, Axin is phosphorylated by cyclin dependent kinase 5 (CDK5) and translocated into the nucleus with β-catenin as a binding partner, which is followed by a shift to neuronal differentiation. This function of Axin is independent of the canonical Wnt signaling pathway. These results suggested

a novel role of Axin in IP expansion during evolution (Fang et al., 2013). Regarding GSK-3, it has been reported that the deletion of GSK-3 signaling by genetic elimination of all isoforms resulted in massive hyperproliferation of neural progenitors and markedly suppressed generation of both IPs and postmitotic neurons (Kim et al., 2009). DISC1, originally identified as a Schizophrenia- related gene (Blackwood et al., 2001) plays an important role in many aspects of neural development. For progenitor maintenance, DISC1 regulates RG cell proliferation via inhibition of GSK-3 by directly binding and modulating the canonical Wnt pathway together with its binding protein Dixdc1 (Mao et al., 2009; Singh et al., 2010). Taken together, this evidence indicates that GSK-3 signaling is essential for maintenance of the neural progenitor pool during cortical development. Meanwhile, as for Wnt-signaling, it has also been reported that N-myc is a downstream target that promotes IP production (Kuwahara et al., 2010).

Rac1, a small G-protein that is a member of the Rho-GTPase family, has been implicated in regulating the proliferation and differentiation of stem cells of various tissues (Benitah et al., 2005; Chrostek et al., 2006). A forebrain-specific loss of Rac1 leads to reduction in proliferation in a SVZ-progenitor (bIP)-specific fashion, a concomitant increase in cell cycle exit and premature differentiation (Leone et al., 2010), which suggests that Rac1 activity is crucial for maintenance of the progenitor state of bIPs.

Fate Specification of Neural Progenitor Cells

The neuron production stage includes another important step of neuronal subtype specification for which many transcription factors are involved. FoxG1 is a transcriptional repressor that is strongly expressed in progenitors and functions as a repressor of Cajal Retzius (CR) cell competency (Hanashima et al., 2004) as well as a regulator of NPC self-renewal, IP expansion, and the timing of neurogenesis (Shen et al., 2006; Siegenthaler et al., 2008; Fasano et al., 2009). It has been reported that *Foxg1* is necessary and sufficient for inducing deep-layer neurogenesis and that it switches the transcriptional program to acquire upper layer neuron identity through direct repression of transcription expression of T-box brain 1 (Tbr1) transcription factor, an early born postmitotic neuronal marker (Kumamoto et al., 2013; Toma et al., 2014). This suggests that expression of a single transcription factor may enable neural progenitors to alter their intrinsic character.

The transcription factors Fezf2 and Cux2 are neuronal subtype markers expressed in deep and superficial layer neurons, respectively, and they are critical for fate specification (Nieto et al., 2004; Chen et al., 2005). They are also expressed in NPCs in VZ, which has led to a debate about the existence of fate-restricted progenitors in Cux2- or Fezf2-Cre driver mouse lines (Franco et al., 2012; Guo et al., 2013). Although a recent report using the mosaic analysis with double marker (MADM) system (Zong et al., 2005; Hippenmeyer et al., 2010) demonstrated unitary production of deep and superficial layer neurons by individual NPCs (Gao et al., 2014), this dispute remains unresolved.

Other transcription factors involved in neurogenesis regulation include Pou3fs (Brn1, Brn2) and Gli3. Brn1/2 is a crucial regulator of the production of upper-layer neurons, and its expression in VZ progenitors is essential for the transition from early to mid-neurogenesis (Sugitani et al., 2002; Dominguez et al., 2013). Gli3 is a transcription factor in the Hedgehog (Hh) pathway, the loss of which in RG cells results in decreased production of IPs and prolongs the production of deeper cortical neurons, suggesting that Gli3 is required for both the generation and maintenance of IPs and fate specification of IP-originating superficial neurons (Wang et al., 2011a).

Besides transcription factors, it has been shown that chromatin regulators are also critical for the fate specification of NPCs (a review, see Tyssowski et al., 2014). Ring1B is a component of the polycomb group (PcG) complex 1(PCR1) proteins and functions as a repressor of transcription via trimethylation of residue Lys27 of histone H3 (H3K27me3). Reports from the Gotoh research group have shown that Ring1B is essential not only for shifting the neurogenic state to an astrogenic fate (Hirabayashi et al., 2009) but also for terminating the production of deep-layer neurons through direct repression of *Fezf2* promoter activity (Morimoto-Suzki et al., 2014). They have also shown that depletion of *Ezh2*, which is a component of the polycomb group (PcG) complex 2(PCR2), exhibited the same phenotype of prolonged neurogenic phase of NPCs and delayed onset of the astrogenic phase, as depletion of Ring1B at the same stage (E12.5) (Hirabayashi et al., 2009). However, depletion of *Ezh2* before the onset of neurogenesis results in the opposite effects, that is, accelerate differentiation and early onset of astrocyte production (Pereira et al., 2010). These results suggest that Ezh2 may independently regulate the major developmental transitions in cortical progenitor cells: expanding neuroepithelial cell by self-renewing, producing neurons of different laminar fates, and switching from neurogenesis to gliogenesis. Ikaros, another modulator of the chromatin-remodeling complex, is expressed in NPCs at highest levels during the early stage of neurogenesis, and its expression decreases as development proceeds. Sustained expression of Ikaros results in prolonged production of deep-layer neurons, supporting its role in fate determination of deep-layer neurons via chromatin regulation (Alsiö et al., 2013).

Progenitor Maintenance and Microtubule (MT) Organization

Microtubules (MTs) are an important component of cytoskeletons and are vital for the organization of the centrosome and mitotic spindle, which are also crucial for the maintenance of NPCs. EFHC1 is a protein containing a single EF-hand motif, a Ca^{2+} binding domain, which directly interacts with α-tubulin. Mutation in the gene encoding this protein causes juvenile myoclonic epilepsy (Suzuki et al., 2004). Functional analysis of EFHC1 using rat developing neocortex revealed that it is essential for cell cycle exit of NPCs via the assembly and function of mitotic spindle. Impairment of this gene affects mitotic spindle formation and M-phase progression by microtubule bundling defects and increased apoptosis (de Nijs et al., 2009).

Cdk5rap2 is localized at the centrosome of neural progenitors, and loss of this protein causes a failure in the maintenance of the neural progenitor pool by increased cell cycle exit followed by premature neuronal differentiation (Buchman et al., 2010). The microtubule-binding protein Hook3 is recruited to pericentriolar satellites through an interaction with Pericentriolar Material 1 (PCM1). Disruption of the Hook3-PCM1 interaction impairs maintenance of the neural progenitor pool (Ge et al., 2010). This suggests that regulators of centrosome dynamics are also important for progenitor maintenance. In addition, the regulation of mitotic spindle orientation is also important for symmetric division and thereby, progenitor maintenance (Yingling et al., 2008). It has been reported that the mitotic spindle of RG cells orients almost parallel to the ventricular surface in both proliferative and neurogenic stages. Only a fraction of RG cells that adopt divisions with oblique and vertical spindle orientations preferentially generate bIPs and bRG during the neurogenic stage (Konno et al., 2008; Shitamukai et al., 2011). A disturbance of mitotic spindle orientation by knocking out *LGN* (G protein regulator) gene or manipulation of the mouse *Inscuteable*(*mInsc*) gene expression level leads to a disruption of the balance between proliferation and differentiation of NPCs (Konno et al., 2008; Postiglione et al., 2011). Lis1, its binding partner Ndel1, and dynein form a complex that is also required for maintaining spindle orientation perpendicular to the ventricular surface and NPC proliferation (Yingling et al., 2008). Recently, it has been reported that the protein phosphatase PP4c regulates spindle orientation in early cortical progenitor cells by dephosphorylating Ndel1, thereby enabling complex formation with Lis1 to form a functional spindle orientation complex (Xie et al., 2013). These lines of evidence demonstrate that regulation of mitotic spindle orientation is one of the key molecular mechanisms for progenitor maintenance and the transition between symmetric and asymmetric cell division.

The Behavior of Neural Progenitor Cells in the Ventricular Zone

Now we turn our attention to the motion of NPCs. It has been long known that nuclei of progenitor cells exhibit cell-cycle dependent oscillatory movement known as interkinetic nuclear migration (INM), also called elevator movement (Sauer and Walker, 1959; Fujita, 1962, 1963). Although the molecular mechanisms and the biological meaning of this movement are not well understood, recent studies provide insights into the molecular mechanisms of INM. The functional roles of both microtubule and actomyosin motor proteins in INM were identified first (Tsai et al., 2005, 2010; Norden et al., 2009; Schenk et al., 2009). Next, the involvement of other proteins in INM regulation was reported. Centrosomal protein of 120 kD (Cep120) is a centrosomal protein expressed in NPCs and knockdown of Cep120 results in impairment of INM through interactions with transforming acidic coiled-coil proteins (TACCs) (Xie et al., 2007). Hook3, mentioned above, is involved in regulation of INM, suggesting that INM is an important behavior of NPCs for proper neurogenesis of the mammalian neocortex, and it has been reported in other neurogenic systems (Murciano et al., 2002; Del Bene et al., 2008). Tpx2, a microtubule-associated protein, has been identified as an essential protein for apical nuclear migration during G2 phase (Kosodo et al., 2011). Dock7, a member of the DOCK180 superfamily of a distinct class of Rac/Cdc42 GTPase guanine nucleotide exchange factors (GEFs), regulates INM by interacting with TACC3 (Yang et al., 2012b). Meanwhile, it has been shown that dynein recruitment to the nuclear pore is required for apical nuclear migration through "RanBP2-BicD2" and "Nup133-CENP-F" pathways (Hu et al., 2013).

Moreover, a recent study revealed the biological role of INM. Using TAG-1 knockdown, which leads to loss of the basal processes of RG cells and *in toto* imaging, it was shown that proper INM is critical for preventing overcrowding of progenitor cells and for facilitating the smooth departure of the differentiated cells from the VZ (Okamoto et al., 2013).

Extrinsic Regulators of Neurogenesis

Besides the cell-autonomous regulatory mechanisms described above, extrinsic factors are also involved in the regulation of NPC maintenance. Several growth factor signaling pathways including for fibroblast growth factor (FGF), sonic hedgehog (shh), Wnt, bone morphogenetic proteins (BMPs), colony stimulating factor-1 (CSF-1), and interleukin (IL) 34 are involved in the regulation of progenitor self-renewal and differentiation. Targeted disruption of the docking protein FRS2α, a major mediator of FGF signaling, leads to severe impairment of cerebral cortical development with thinner cerebral cortices than wild-type (WT) cells, reduced proliferation and differentiation of Tbr2- positive bIPs (Yamamoto et al., 2005). Genetic disruption of all three FGF receptors (FgfRs) leads to attenuation of Notch signaling and precocious production of bIPs followed by premature termination of neurogenesis (Rash et al., 2011). Recently, it has been reported that this function in progenitor maintenance of FGF occurs in cooperation with EphA4, a member of the receptor tyrosine kinase superfamily (Chen et al., 2015). Shh, known as a regulator of early central nervous system (CNS) development, also regulates progenitor proliferation through upregulation of Gli1, which is a zinc finger transcriptional factor and a mediator of Shh signaling (Dahmane et al., 2001). Targeted disruption of *shh* in the mouse dorsal pallium leads to small cerebral cortices at embryonic day (E)18.5, which was caused by impairment of cell cycle exit and reduced proliferation of NPCs (Komada et al., 2008). This regulatory function of shh in neurogenesis is cooperative with Notch signaling (Dave et al., 2011). As mentioned in Section Regulation of Intracellular Signaling of NPCs, Wnt also regulates IP production. Ectopic Wnt3a expression in the developing cortex causes cortical dysplasia and neuronal heterotopias (Munji et al., 2011). The authors found that Wnt3a promotes expansion of RG and differentiation of IPs. These results suggested that the Wnt-β–catenin pathway regulates both RG self-renewal and IP differentiation (Munji et al., 2011). Bmp7 null embryos exhibited microcephaly by reduced cortical plate thickness. It has been revealed that Bmp7 is required for the proliferation potential of NPCs (Segklia et al., 2012). The CSF-1 receptor (CSF-1R), known in CNS microglial development, has been revealed as another regulator in progenitor maintenance. *Csfr-/-*

mice displayed increased proliferation, apoptosis of NPCs, and reduced differentiation of specific excitatory neuronal subtypes (Nandi et al., 2012). This result suggested that CSF-1 and IL-34, ligands of CSF-1R, suppress self-renewal potential of RG cells and production of IPs to maintain the balance between proliferation and differentiation.

Cells and meninges also function as signaling centers regulating NPC proliferation. The distribution of a subtype of CR cells (*Dbx1*-derived CR cells) influences the proliferation and differentiation of progenitor cells in the VZ (Griveau et al., 2010). Thus, CR cells provide certain information to NPCs for their proliferative state via secretion of signaling molecules. Loss of meninges in the forebrain by *Foxc1* mutation results in the reduction of retinoic acid (RA) secretion and impairment of switching from symmetric to asymmetric division, thus leading to a decrease in neuron and IP production (Siegenthaler et al., 2009). Additionally, tangentially migrating transient glutamatergic neurons that are generated by *Dbx1* positive progenitors at the pallial/subpallial boundary (PSB) at E12.5 contribute to maintain the neocortical progenitor pool (Teissier et al., 2010, 2012). These studies highlight the major involvement of such extrinsic regulators in NPC maintenance and differentiation.

ATP signaling and calcium waves are also involved in the regulation of NPCs. Spontaneous calcium waves that are dependent on connexin hemichannels and $P2Y_1$ ATP receptors propagate through RG cells in the VZ and regulate neuron production (Weissman et al., 2004) and are essential for migration of IPs to the SVZ (Liu et al., 2008). Another class of novel participants among the regulators of progenitor cell maintenance is microglia, resident macrophages in the brain (Cunningham et al., 2013). Activated microglia colonize the proliferative zones of the developing rat and primate forebrains, and the manipulation of microglia cell numbers significantly affects the number of NPCs. Microglial surveying and its crucial role in eliminating injured neurons in adult brains has been known; excessive microglial activation was observed in autism spectrum disorder (ASD) (Nimmerjahn et al., 2005; Tetreault et al., 2012; Suzuki et al., 2013). However, Cunningham et al. showed that microglia also play an important role in normal cortical development during embryogenesis by eliminating NPCs at the end of cortical neurogenesis and, therefore, they may contribute to terminate neurogenesis (Cunningham et al., 2013).

MULTIPOLAR CELL TO BIPOLAR CELL TRANSITION

Newly born late-born neurons finally depart the VZ and start the journey to their final destinations. During radial migration, cell shape and migration mode change markedly. After differentiation, newborn neurons exhibit a multipolar (MP) shape with multiple neurites and migrate in a MP migration mode in random directions (Tabata and Nakajima, 2003). For MP cell migration, it has been reported that two distinct populations in terms of their migrating behaviors exist (Tabata et al., 2009). One is the slowly exiting population (SEP), in which postmitotic MP migrating cells stay in the lower part of the SVZ called the MP cell accumulation zone (MAZ). The other is the "rapidly exiting population (REP)," which migrate rapidly into the SVZ/intermediate zone (IZ) and undergoes further cell division, then converts to MP cells. REP includes bIPs, Olig2-positive glial progenitors, and probably bRG. Whereas, the SEP stays in the MAZ but enters the cortical plate (CP) faster than the REP and contributes to the production of superficial neurons as well as the REP (Tabata et al., 2009; **Figure 1**). This study indicated that the migration behavior of the direct progeny of asymmetric division and IPs are different although both of them exhibit MP shapes. For delamination of differentiated cells from the apical surface of the VZ, Scratch 1, and 2, members of the snail super-family of transcription factors, are involved in this regulation through the suppression of E-cadherin (Itoh et al., 2013). After delamination, MP cells convert to bipolar (BP) cells for locomotion. Neuronal polarization of newborn neurons (neuroblasts) *in vivo* occurs during this step, starting with extension of a thin axon, and one selected neurite became a thick leading process. Time-lapse imaging of cultured slices of electroporated brains showed that axon extension occurs prior to the formation of the leading processes for majority of MP cells (Hatanaka and Yamauchi, 2013). Additionally, this axon specification is dependent on TAG-1-mediated contact between immature neurites and axons of early born neurons (Namba et al., 2014). Neuronal polarization in dissociated primary cultured neurons is defined as axon specification from multiple neuritis. In contrast, leading process formation to convert a BP cell is a critical polarization step *in vivo* (Takano et al., 2015). Many gene knockouts or knockdowns of cortical development exhibit phenotypes of either delay or failure of the MP-BP transition, suggesting that this regulatory mechanism is crucial for the radial migration process. Many genes have been reported to be involved in this regulation. These can be classified roughly into five categories (**Figure 3**): (1) transcriptional regulators, (2) small GTP-binding proteins, (3) proteins related to MT dynamics, (4) receptors and other membrane proteins, and (5) kinases. Nevertheless, the downstream effectors are mostly involved in cytoskeletal regulation. We will summarize recent findings for each category (**Figure 3**).

Transcriptional Regulators Involved in the Multipolar-bipolar Transition

Neurogenin 2 (Ngn2), a proneural transcription factor responsible for glutamatergic neuronal differentiation from NPCs, has been found to also play an important role in the MP-BP transition via direct transcriptional activation of the small GTP-binding protein Rnd2, an atypical Rho-GTPase protein that inhibits RhoA activity and regulates actin cytoskeleton (Heng et al., 2008). Transcriptional repressor RP58 (also known as zfp238, znf238, and zbtb18) is not only another downstream target gene of Ngn2 (Xiang et al., 2012) but also an upstream regulator repressing Ngn2 transcription by negative feedback regulation (Ohtaka-Maruyama et al., 2013). Meanwhile, Rnd2 transcription is directly repressed by RP58 (Heng et al., 2015) and CoupTF-I, a nuclear orphan receptor (Alfano et al., 2011), suggesting that the expression level of Rnd2 is critical for the

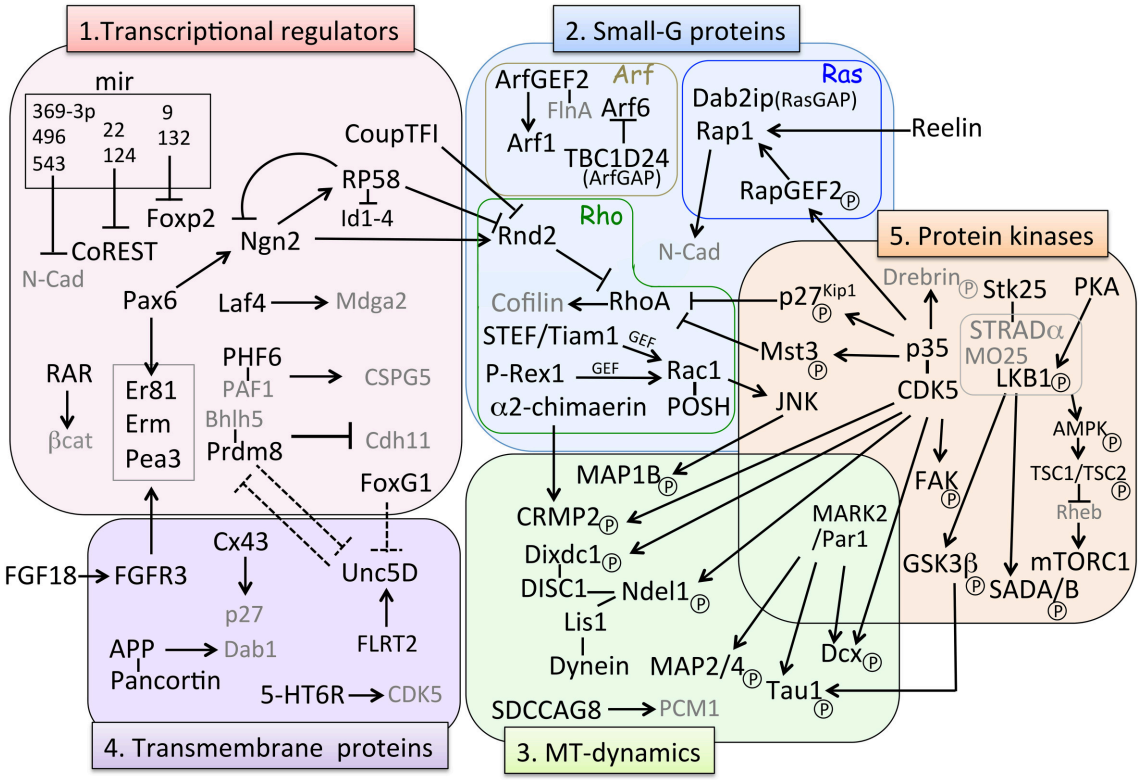

FIGURE 3 | Molecular pathways involved in MP-BP transition. Factors involved in the MP-BP transition can be categorized in the following five groups based on their molecular function: (1) transcriptional regulators, (2) small-G proteins, (3) microtubule (MT)-dynamics, (4) transmembrane proteins, and (5) protein kinases. Black arrows (→) represents positive regulation of either "activated," "stabilized," or "phosphorylated" target factors; ⊣ indicates either "repression of transcription" or "inhibition of activity" of target factors. Dashed lines indicate putative relationships inferred from the experimental data. Factors in gray characters are effectors of their partner molecules but they do not belong to each category. BP, bipolar; MP, multipolar; MT, microtubule.

MP-BP transition (**Table 1**, **Figure 3**). We have been studying the functional roles of RP58 and have found that this transcriptional repressor plays critical roles in neuronal migration as well as neuronal differentiation (Okado et al., 2009; Ohtaka-Maruyama et al., 2012, 2013).We summarize the transcriptional regulators involved in the MP-BP transition in **Table 1**.

RP58 is a Multifunctional Regulator of Cortical Development

Recently, we found that the transcriptional repressor RP58, belonging to the POK/ZBTB proteins, which contain C-terminal zinc fingers and N-terminal BTB/POZ domains, has multiple roles in cortical development. RP58-gene- deficient mice die at birth and exhibit severe phenotypes associated with the proliferative state of NPCs and radial migration (Okado et al., 2009; Hirai et al., 2012; Ohtaka-Maruyama et al., 2013). A detailed analysis revealed that RP58 regulates cell cycle exit and neuronal migration by repressing its downstream targets. The RP58 gene was isolated originally from a screening for translin-associated molecules from a human spleen cDNA library (Aoki et al., 1998). It binds Dnmt3a and may be involved in transcriptional repression via chromatin remodeling (Fuks et al., 2001). Analyses of the spatial and temporal expression patterns during mouse brain development revealed that RP58 is weakly expressed in NPCs in the germinal zones of both the pallium and subpallium in the early developmental stage (Ohtaka-Maruyama et al., 2007). As development proceeds, RP58 is expressed strongly, via- Ngn2 activation in glutamatergic - neurons in the dorsal pallium. The peak expression of RP58 is at E15-16, when neurogenesis occurs most actively in the cortex by prominent promoter activity (Ohtaka-Maruyama et al., 2007, 2012). We demonstrated that RP58 enhances cell-cycle exit, resulting in neurogenesis via the transcriptional repression of Id1- 4 genes (Hirai et al., 2012). In RP58 deficient mice, cell cycle exit is impaired and the Pax 6- and PCNA-positive progenitor population is increased (Okado et al., 2009). Regarding the determination of laminar identity of differentiated neurons in RP58-deficient mouse cortex, neurons expressing the markers for layers II-V and SP neurons decreased remarkably, suggesting that RP58 plays a critical role in the maturation of cortical neurons (Okado et al., 2009). We also showed that RP58 controls MP-BP transition by regulating the Ngn2-Rnd2 pathway independent of its activity in the regulation of cell cycle exit (Ohtaka-Maruyama et al., 2013). Moreover, it has been revealed that RP58 represses Rnd2 transcription directly (Heng et al., 2015). These results suggested that RP58 enables transient

TABLE 1 | A summary of transcriptional regulators involved in the MP-BP transition.

Transcriptional regulator	Gene manipulation method	Examined developmental stage	Migration defect	Related gene, protein	References
Neurogenin2 (*Ngn2*)	*Ngn2(–/–)* (LOF) *Ngn2*$^{Flox/Flox}$ + Cre plasmid (LOF) *Ex vivo* electroporation Slice culture	E14.5→4 DIV	Impairment of CP entering	RhoA *Rnd2*	Hand et al., 2005 Heng et al., 2008
RP58	*RP58*$^{(-/-)}$(LOF) *RP58*$^{Flox/Flox}$ + Cre plasmid(LOF) IUE	E14.5→E17.5, E19.5	Impairment of CP entering Accumulation of MP cells	*Ngn2* *Rnd2*	Ohtaka-Maruyama et al., 2013 Heng et al., 2015
Coup-TFI	*Coup-TFI(–/–)* (LOF) *Coup-TFI*$^{Flox/Flox}$ + Cre plasmid (LOF) IUE, *Ex vivo* electroporation Slice culture	E14.5→4DIV, E18.5, P8	Impairment of CP entering Abnormal MP cell morphology Defective axonal elongation of CPNs	*Rnd2*	Alfano et al., 2011
FoxG1	*CAG-FoxG1-IRES-EGFP(GOF)*	E13.5→E16.5, E19.5	Delay of the radial migration (GOF) Altering the lamina fate (GOF)	Unc5D	Miyoshi and Fishell, 2012
	Ngn2 CreER, FoxG1-C:Flpe/-, R26R-CAG-FRTstop-EGFP reporter + Tmx (LOF)	Tmx E11.5→E14.5 Tmx E13.5→E16.5 Tmx E15.5→E18.5	Impairment of CP entering (LOF)		
	NeuroD1-mCherry IRES CreER (Late LOF) IUE	E12.5 Tmx→E16.5 →E19.5	Late LOF does not affect postmigratory populations		
Laf4/Aff3	sh-RNA (LOF) *Laf4-HA-IRES-GFP* (GOF) IUE, Slice culture	E14.5→6 DIV	Impairment of CP entering	*Mdga2*	Moore et al., 2014
PHF6	sh-RNA (LOF) IUE	E14.5→E17.5, 19.5, P6	Delay of the radial migration Accumulation of MP cells White matter heterotopia	PAF1 NGC/CSPG5	Zhang et al., 2013a
Prdm8	*CAG-Prdm8* plasmid (GOF) sh-RNA (LOF) IUE	E12.5→P5 E14.5→E17, E17.5	Impairment of CP entering (GOF) Accumulation of MP cells (GOF) Premature BP transition (LOF) Altering the laminar fate (LOF)	Unc5D	Inoue et al., 2014
Erm, Er81 (Pea3-Ets family)	DN-plasmid (LOF) IUE	E13.5→E16.5	Impairment of CP entering	FGF18, FFGFR	Hasegawa et al., 2004
miR-9, 132 (Target: FoxP2)	*FoxP2-3′*UTR *FoxP2-* Δ *-3′*UTR (GOF) FoxP2-3′UTR-MT1+2+3 (GOF) IUE	E13.5→E18.5, P15	Delay of the radial migration White matter heterotopia	*FoxP2*	Clovis et al., 2012
miR-379-410 cluster (Target: N-cadherin)	NeuroD1-miRNA plasmid (LOF) NeuroD1-anti-miR LNAs plasmid (GOF) IUE	E13.5→E17.5	Impairment of CP entering (LOF) Enhancement of the radial migration (GOF)	*N-cadherin*	Rago et al., 2014
miR-22,124 (Target: CoREST)	*Dicer* $^{Flox/Flox}$ + *NeuroD:Cre-GFP* plasmid miR expression plasmid (LOF) antagomiR (GOF) IUE, Slice culture	E14.5→3DIV, E17.5 →E18.5, P2 E14.5→E16.5→1DIV →time-lapse	Delay of the radial migration (GOF) Accumulation of MP cells (GOF)	CoREST Dcx	Volvert et al., 2014
Retinoic acid receptor (RAR)	*RARE.hsp68LacZ* + DN-plasmid (LOF)	E12.5→E15.5, P5 E13.5→E16.5, P5 E14.5→E17.5, P5 E15.5→E18.5, P5	Impairment of CP entering Altering the laminar fate Late-born neurons are affected	β-catenin	Choi et al., 2014

BP, bipolar; CP, cortical plate; DIV, day in vitro; E, embryonic day; GOF, gain of function; IUE, in utero electroporation; LOF, loss of function; MP, multipolar; Tmx, Tamoxifen.

expression of Ngn2 and restricts the Ngn2-Rnd2 signaling pathway both directly and indirectly. This regulation is essential for fine-tuning the Ngn2-Rnd2 signaling pathway to achieve proper radial migration. Furthermore, RP58 is involved in the regulation of neurite outgrowth (Ohtaka-Maruyama et al., 2013). Thus, RP58 is a multifunctional repressor for cortical development. Further functional analyses of RP58 will provide new insights into the molecular mechanisms of cortical development.

Other Transcriptional Regulators Involved in the Multipolar-bipolar Transition

FoxG1, a fate determinant factor as mentioned earlier, also plays an important role in radial migration (Miyoshi and Fishell, 2012; **Table 1**, **Figure 3**). Its transient downregulation in the IZ is critical for the MP-BP transition, suggesting that dose-dependent regulation of downstream targets is important for subsequent morphological and migration mode changes. *Laf4*/*Aff3*, a member of the AFF (AF4/FMR2) family, is known as a putative transcription factor and silencing of this gene is associated with neurodevelopmental disorders and intellectual disability (ID) (Steichen-Gersdorf et al., 2008; Metsu et al., 2014). However, its function in normal brain development is unclear. A recent study reported that *Laf4* is strongly expressed in the developing cortex and is required for the MP-BP transition via the transcriptional activation of *Mdga2*, a gene coding for a cell adhesion molecule (Moore et al., 2014; **Table 1**, **Figure 3**). Another intellectual disability-related gene, the X-linked intellectual disability protein PHF6 has also been reported to be associated with the PAF1 transcription elongation complex and to regulate MP-BP transition (Zhang et al., 2013a). The study showed that Neuroglycan C/Chondroitin sulfate proteoglycan 5(NGC/CSPG5), which is a potential schizophrenia-susceptibility gene, is a critical downstream target of PHF6 in this regulation (**Table 1**, **Figure 3**)

Prdm8 is a member of the proto-oncogene transcription family and has intrinsic histone methyltransferase activity (Hayashi et al., 2005; Eom et al., 2009). Prdm8 forms a repressor complex with Bhlhb5 and regulates neuronal circuit assembly through Cadherin-11 repression (Ross et al., 2012). Recently, it has been reported that Prdm8 regulates the MP-BP transition by maintaining the MP state and inducing morphological changes, and it controls genes including those encoding guidance molecules (Inoue et al., 2014; **Table 1**, **Figure 3**). Transcription factors Erm, Er81, and Pea3 belong to the Pea3 subfamily members of Ets (Pea3-Ets) and have also been reported to be involved in the regulation of the MP-BP transition (**Table 1**, **Figure 3**). It has been shown that the expression of these transcription factors is induced by FGF18-FGFR3 signaling in the developing cortex (Hasegawa et al., 2004). Knockdown of Erm, Er81, and Pea3 disrupt the entrance of migrating neurons into the CP, suggesting that Pea3-Ets transcription factors act as key mediators that interpret FGF signaling to confer proper migratory behavior on young MP neurons (Hasegawa et al., 2004). The molecular marker ER81 is expressed in a subset of pyramidal cells of layer V and also in NPCs at the mid- to-late stages of cortical development. Besides its association with the FGF signaling pathway, Pax6 has also been identified as an upstream activator of ER81, binding directly to the ER81 gene promoter (Tuoc and Stoykova, 2008).

In addition to transcription factors, it has recently been reported that microRNAs (miRNAs; miR) expressed in the developing cerebral wall are also involved in the regulation of radial migration (Clovis et al., 2012; Rago et al., 2014; Volvert et al., 2014; **Table 1**, **Figure 3**). miR369-3p, miR496, and miR543, all bind to the 3′-untranslated region of the N-cadherin (N-cad) transcript and regulate neurogenesis and neuronal migration by fine-tuning of N-cad levels (Rago et al., 2014). miR-9 and miR-132 target the 3′-untranslated region of the *Foxp2* transcript and regulate radial migration by controlling the *Foxp2* expression levels (Clovis et al., 2012). Meanwhile, the transcriptional repressor CoREST is also a miRNA target. miR-22 and miR-124 regulate proper expressional levels of CoREST, thereby regulating doublecortin transcription and promoting the MP-BP transition (Volvert et al., 2014). These lines of study suggest that minute transcriptional regulation of target genes including transcriptional factors and other proteins related to cortical development is critical for proper neuronal migration. Although RA signaling has long been known as an important regulator for neuronal development (Sockanathan et al., 2003; Fu et al., 2010), and activated RA receptor (RAR) is present in the developing dorsal and medial pallium (Luo et al., 2004), little is known about the function of RA in corticogenesis. RAR is a nuclear receptor that can act as a transcription factor. A recent study showed that inhibition of RAR function delays late-born neuron migration and leads to failure in maintaining their fate via β-catenin signaling (Choi et al., 2014). This suggests that RA signaling is critical for neuronal positioning as well as maintenance of their neuronal fate.

Small GTP Binding Proteins in the Multipolar-bipolar Transition

Small GTP binding proteins (small GTPases, small G proteins), also known as the Ras superfamily, comprise more than 150 small G proteins that can be divided into five subfamilies: Ras, Rho, Rab, Arf, and Ran (Raimondi et al., 2010). They function as molecular switches in many cellular processes including cell proliferation, cytoskeletal organization, and cell migration. Although the Ras protein was originally recognized as an oncogene, it was recently revealed that small G proteins are indispensable for normal cellular functions including neuronal migration in the developing cerebral cortex (Kawauchi, 2011; Shah and Puschel, 2014). Among these, proteins belonging to the Rho, Ras, and Arf families or their regulator proteins have been reported to be involved in the MP-BP transition required for dynamic morphological changes (**Figure 3**). Rac1, regulator of maintenance of progenitor state is also involved in the regulation of the MP-BP transition. Functional repression of Rac1, its activators STEF/Tiam1, or its downstream molecule, c-Jun N-terminal kinase (JNK) resulted in defective MP-BP transitions of newborn cortical neurons (Kawauchi et al., 2003). This study revealed that Rac1 is essential for the MP-BP transition via regulating microtubule dynamics by activating JNK, followed by phosphorylation of MAP1B. P-Rex1, another activator of

Rac (Rac-GEF), shows more restricted expression in neurons located at the lower part of the IZ of the mid-embryonic cortex, and participates in the regulation of radial neuronal migration via extracellular cues such as neurotrophins (Yoshizawa et al., 2005). Moreover, the Rac1-interacting scaffold protein POSH is required for the proper localization of activated Rac1 in the basal part of leading processes and regulates neuronal migration especially from the IZ into the CP (Yang et al., 2012a). These reports suggested that regulation of Rac1 is essential for proper MP-BP transition (**Figure 3**). α2-chimaerin is a Rac GTPase-activating protein (GAP) and was reported to be essential for neurite extension and axon pathfinding in the locomotor circuit and ocular system (Beg et al., 2007; Iwasato et al., 2007; Miyake et al., 2008). It has also been demonstrated that α2-chimaerin is essential for the MP-BP transition during radial migration (Ip et al., 2012). However, the function of this Rac-GAP protein is not dependent on its GAP activity, but rather via modulation of the activity of the microtubule-associated protein CRMP-2 (Ip et al., 2012; **Figure 3**). This result suggests that Rac regulator proteins have multiple functions mediated through different effectors. Rnd proteins are also important and unique Rho family members that lack intrinsic GTPase activity and are constitutively active. They regulate the actin cytoskeleton through inhibition of Rho-A signaling. As mentioned above with respect to Rnd2 function, fine-tuning of the Rnd2 level via transcriptional regulation is critical for the MP-BP transition of cortical migrating neurons (Heng et al., 2008, 2015; Alfano et al., 2011; Ohtaka-Maruyama et al., 2013; **Figure 3**). In contrast to Rnd2, Rnd3 regulates the early and late steps of radial migration, as described in Section Locomotion below. Some Ras family related proteins have been reported to be involved in MP-BP regulation, including Rap1 (Jossin and Cooper, 2011), RapGEF2 (Ye et al., 2014), and Dab2ip (Lee et al., 2012). Functional inhibition of Rap1 by IUE of Rap1GAP resulted in impairment of the MP-BP transition. Further analysis revealed that reelin-mediated activation of Rap1 in MP cells near the middle of the IZ increased the levels of cell- surface-localized N-cad, possibly by regulating vesicle trafficking of N-cad to ensure a proper morphological change to BP cells (Jossin and Cooper, 2011; **Figure 3**). The same signaling pathway, reelin-Rap1-N-cad, has also been reported to be essential for somal translocation of early born neurons and proper lamination of late-born neurons (Franco et al., 2011). The Rap1 activator RapGEF2 is expressed in the migrating neurons located in the upper IZ and CP. Short hairpin RNA (shRNA)-mediated knockdown of RacGEF2 prevents the MP-BP transition as well as N-cad recruitment to the cell membrane. RapGEF2 is activated by CDK5-dependent phosphorylation, suggesting that the CDK5-Rap1-N-cad signaling pathway is critical for exit from the MP phase (Ye et al., 2014). Furthermore, the Ras-GAP protein Dab2ip, which was initially identified as a tumor suppressor, is essential for the MP-BP transition *in vivo* and neurite outgrowth *in vitro* (Lee et al., 2012).

Periventricular heterotopia (PH) is a human cortical malformation disease associated with mutations in the *ArfGEF2* gene and the actin-binding protein Filamin A (FlnA) (Fox et al., 1998; Sheen et al., 2004). It has been shown that FlnA and its binding partner Filamin A-interacting protein (FILIP) are essential for the MP-BP transition by regulating the actin cytoskeleton (Nagano et al., 2002, 2004; **Figure 3**). Arf1 is a member of the Arf family and is involved in the regulation of vesicle trafficking, and *ArfGEF2* gene products (Big2 proteins) are GEFs for Arf1. *ArfGEF2* null mice develop PH and exhibit neuronal migration defects of the developing cortex (Zhang et al., 2012). Recently, it was revealed that Big2 and FlnA interact directly and regulate neuronal migration and cell adhesion through modulation of Arf1 activity and localization of Big2 to the cell membrane from the Golgi (Zhang et al., 2013b). Arf6 is another Arf family member regulating the MP-BP transition. TBC1 domain family member 24 (TBC1D24) is an Arf6-interacting protein, and mutations in the TBC1D24 gene are associated with cortical malformation, intellectual disability, and epilepsy (Corbett et al., 2010; Falace et al., 2010). Recent finding revealed that TBC1D24 is essential for the MP-BP transition and dendritic arborization through Arf-GAP activity, which prevents Arf6 activation (Falace et al., 2014; **Figure 3**).

As described above, small G-proteins involved in the regulations of membrane trafficking, cytoskeletal organization and cell adhesion play critical roles in dynamic morphological changes during the MP-BP transition during radial migration of neuroblasts. It is undeniable that many causative genes of neurodevelopmental diseases belong to small G-protein family.

Regulation of Microtubule (MT) Dynamics in the Multipolar-bipolar Transition

Lissencephaly-1 (LIS1) is the first identified gene responsible for type I lissencephaly (Reiner et al., 1993). Since then, many lines of evidence have revealed that LIS1 regulates neuronal migration in a dose-dependent manner (Youn et al., 2009; for a review, see Reiner and Sapir, 2013). LIS1 is an MT or microtubule organizing center (MTOC)-associated protein that forms a protein complex with NDE1/NDEL1 and cytoplasmic dynein (Feng et al., 2000; Sasaki et al., 2000). LIS1 is essential for INM, axon extension, and the MP-BP transition by functioning with NDE1/NDEL1 (Tsai et al., 2005; Youn et al., 2009). For the nuclear migration of radially migrating cells, coordinated coupling between translocation of the centrosome and subsequent nuclear movement via dynamic MT organization is essential, and disruption of this coordination leads to failure to enter the CP. In this context, using the MADM system, the distinct and cell-autonomous functions of LIS1 and NDEL1 in neuronal migration have been revealed. LIS1 regulates this step in a dose-dependent manner, whereas NDEL1 is indispensable for entering the CP (Hippenmeyer et al., 2010). It has been reported that NDEL1 also forms a protein complex with DISC1 and Dixdc1 to regulate radial migration; Cdk5 phosphorylation of Dixdc1 is essential for this regulation (Singh et al., 2010; **Figure 3**).

The centrosome is composed of two orthogonally arranged centrioles surrounded by proteinaceous materials called pericentriolar materials called PCM, which contain many proteins required for MTOC activity, including γ-tubulin, PCM-1, pericentrin and ninein (Dammermann and Merdes, 2002; for a review, see Bornens and Gonczy, 2014). Centrosome positioning has been shown to be important for neuronal

polarity establishment (de Anda et al., 2010). A recent time-lapse imaging study of centrosome positioning during the MP-BP transition revealed that centrosomes exhibit the motion feature that targets the basal part of the dominant growing process in MP-migrating neurons (Sakakibara et al., 2014). Another study revealed that the centrosomal protein SDCCAG8 regulates the MP-BP transition through interaction with PCM1 and centrosomal recruitment of PCM (Insolera et al., 2014; **Figure 3**). Knockdown of *SDCCAG8* impairs coordinated coupling of movements of the centrosome and nucleus (Insolera et al., 2014). Mutations of *SDCCAG8* are known to be associated with the human diseases such as nephronophthisis (Otto et al., 2010) and Bardet-Biedl syndrome (BBS) (Schaefer et al., 2011) a rare autosomal recessive ciliopathy with various symptoms, including renal and retinal abnormalities (Forsythe and Beales, 2013). Patients with mutations in *SDCCAG8* often exhibit neurodevelopmental disorders, including mental retardation, cognitive impairment, and seizures, suggesting a roles for SDCCAG8 in brain development (Otto et al., 2010). Taking this into account, neuronal migration defects occurring by knockdown of this centrosomal protein in the developing mouse cortex could contribute to be explained by the neurodevelopmental symptoms of Bardet-Biedle syndrome.

Receptors and Other Membrane Proteins Involved in the MP-BP Transition

Membrane proteins located on the cell surface are important for translating extracellular signals into intracellular signal transduction. Neuroblasts should receive signals from the extracellular space to change their morphology and to acquire their neuronal properties. Actually, a proportion of the reported genes regulating the MP-BP transition are localized in the cell membrane and play critical roles in this regulation including receptors, gap junction protein, and transmembrane glycoprotein.

Unc5D is known as a receptor for the guidance molecule Netrin, and is expressed in MP cells in the SVZ during cortical development (Sasaki et al., 2008). *Svet1* RNA (Tarabykin et al., 2001), which is a known SVZ marker, has been shown to be derived from an intronic region of the *Unc5d* gene locus (Sasaki et al., 2008). Yamagishi et al. (2011) reported that fibronectin and leucine-rich transmembrane protein-2 (FLRT2) are novel repulsive guidance molecules for Unc5D receptors in radial neuronal migration. They further found that the extracellular domains (ECDs) of FLRT2 proteins are shed by proteolytic cleavage and soluble FLRT2 ECDs regulate MP cells in entering the CP. A recent analysis of the crystal structure of the FLRT2-Unc5D-complex confirmed the ligand-receptor-binding site and three-dimensional structure (Seiradake et al., 2014; **Figure 3**). As mentioned previously, downregulation of the transcriptional repressor FoxG1 at the beginning of the MP cell phase contributes to induction of Unc5D expression (Miyoshi and Fishell, 2012). Whereas, for reduction of Unc5D expression, PRDM8 has been suggested to contribute to this regulation of the MP-BP transition (Inoue et al., 2014).

Connexin 43(Cx43) is a gap junction protein that assembles a hemichannel of large- diameter channels in gap junctions. It has been reported that Cx43 is necessary for neuronal migration, especially the MP-BP transition independent of its channel-forming activity (Fushiki et al., 2003; Elias et al., 2007; **Figure 3**). Cx43 plays an important role in the adhesion of migrating neurons to RG fibers to stabilize their leading processes (Elias et al., 2007). A recent study revealed that Cx43 controls the MP phase via $p27^{kip1}$ upregulation; this Cx43-$p27^{kip1}$ signaling is mainly dependent on the adhesive function of Cx43, although there is an auxiliary role of the Cx43 C-terminus (Cina et al., 2009; Liu et al., 2012), which can interact with variety of proteins related to the cytoskeleton (Herve et al., 2012). These lines of evidence suggest that the membrane proteins of migrating MP neurons play crucial roles in translating the extrinsic signal into intracellular information required for cytoskeletal reorganization of leading process formation and stabilization.

Amyloid-β precursor protein (APP) is a type I transmembrane glycoprotein, and its proteolysis product Aβ accumulates in neurons in Alzheimer's disease. However, the cellular function of APP in normal neuronal development remains unknown. Young-Pearse et al. (2007) found that knockdown of APP using IUE resulted in impairment of entering the CP during radial migration of the developing cortex. In this study, the authors also revealed that full-length APP is required for this function, which is regulated by the downstream adaptor protein Disabled-1 (Dab1). Recently, a secreted glycoprotein pancortin was identified as an extracellular binding partner of APP (Rice et al., 2012; **Figure 3**). Pancortin is expressed at high levels in the developing and mature mouse cortex, and the pancortin gene encodes four isoforms. Rice et al. (2012) further revealed that although all four isoforms of pancortin biochemically interact with APP, each isoform regulates the MP-BP transition in a different manner together with APP.

Recently, it was reported that serotonin 6 receptor (5-HT6R), a G protein-coupled receptor (GPCR), is critical for the MP-BP transition and locomotion (Jacobshagen et al., 2014). This function was also found to depend on CDK5 by binding to the intracellular region of 5-HT6R, but was independent of serotonin activation. This suggests that 5-HT6R is an upstream membrane regulator for CDK5 function in neuronal migration (Jacobshagen et al., 2014; **Figure 3**).

Protein Kinases Involved in the Multipolar-bipolar Transition

Protein kinases are a key class of regulatory proteins for many cellular functions. Protein phosphorylation is broadly known as a molecular switch for downstream pathways. It has been reported that protein kinases play a critical roles in the MP-BP transition.

CDK5 is a serine/threonine kinase that plays crucial roles in brain development. CDK5 knockdown in migrating neurons leads to impairment of leading process formation and the MP-BP transition (Kawauchi et al., 2006; Ohshima et al., 2007), suggesting its critical roles in regulating MT or actin cytoskeletal organization. Accumulating evidence has uncovered the molecular pathways and identified its downstream substrates, including the CDK inhibitor $p27^{kip1}$ (Kawauchi et al., 2006), the kinase Mst3 (Tang et al., 2014), the scaffold protein axin (Fang et al., 2013), the actin-binding protein drebrin (Tanabe et al.,

2014), and MT-associated proteins, including DCX (Tanaka et al., 2004), FAK (Xie et al., 2003), NDEL1 (Niethammer et al., 2000; Sasaki et al., 2000), and CRMP2 (Uchida et al., 2005). CDK5 functions as a master kinase for neural development (**Figure 3**). For details of CDK5 pathways in neuronal migration, please see Ohshima's (2014) review in this Research Topic issue.

LKB1, originally identified as an ortholog of the Par4 serine/threonine kinase of *Caenorhabditis. elegans*, has been reported to be involved in axon specification *in vivo* (Asada et al., 2007; Barnes et al., 2007; Shelly et al., 2007). A pseudokinase Ste20-related kinase adaptor α (STRADα can stabilize LKB1 (Veleva-Rotse et al., 2014), and LKB1 is activated by protein kinase A-dependent local phosphorylation on S431 in the trailing processes of newborn migrating neurons, followed by activation of the downstream kinases SAD-A and SAD-B, both of which are known to be essential for axon formation by phosphorylating Tau-1 (Kishi et al., 2005; Barnes et al., 2007). *STRADα* has been identified as the gene responsible for the autosomal recessive neurodevelopmental disorder polyhydramnios, megalencephaly, and symptom epilepsy syndrome (PMSE), which is characterized by macrocephaly, craniofacial dismorphism, hypotonica, cognitive disability, and intrac epilepsy (Puffenberger et al., 2007). STRADα binds LKB1 together with the scaffold protein MO25 and facilitates nuclear export of LKB1 to the cytoplasm (Boudeau et al., 2003; Zeqiraj et al., 2009). It has been reported that human PMSE cortex exhibited abnormal nuclear localization of LKB1 (Orlova et al., 2010). The STRAD/ LKB1 complex inhibits mammalian target of rapamycin (mTOR) signaling via AMP-activated kinase (AMPK) and tuberous sclerosis complex 1 and 2 (TSC1, TSC2) (Inoki et al., 2003; Corradetti et al., 2004; Lizcano et al., 2004). Knockdown of STRADα in mouse NPCs *in vitro* resulted in aberrant mTORC1 activation and abnormal nuclear localization of LKB1. Moreover, Knockdown of STRADα *in vivo* also leads to aberrant mTORC1 activation and impairment of radial neuronal migration (Orlova et al., 2010). Acute inactivation of the STE family serine/threonine kinase Stk25, which is another binding partner of STRADα, causes impairment of the MP-BP transition. These results suggests that STRADα-Stk25-LKB1- mTORC1 signaling, may regulate radial neuronal migration in addition to its role in polarity formation (Matsuki et al., 2010, 2013) and also suggests that hyperactivation of mTORC1 signaling by STRADα gene mutation affect the cortical development at an early stage in human PMSE (**Figure 3**). Through sh-RNA-mediated knockdown of LKB1 by IUE, it has been revealed that LKB1 actually regulates the transition of MP to BP in addition to its role in axon formation (Asada et al., 2007). The authors further revealed that this migration defect is correlated with a defect in centrosomal movement, and that LKB1 mediated inactivation of GSK3β by Ser9 phosphorylation at the leading process tip to stabilize the MT plus-end-binding protein APC for proper forward movement of centrosomes (Asada et al., 2007; Asada and Sanada, 2010). By contrast, other studies did not observe any migration defect by silencing LKB1 (Barnes et al., 2007; Shelly et al., 2007). Although this discrepancy remains to be resolved, all of these experiments of LKB1 knockdown or knockout were performed at different time periods and in different conditions. Moreover, overexpression of LKB1 exhibits a migration defect (Shelly et al., 2007), suggesting the possibility that the requirement of LKB1 for neuronal migration may critically depend on its protein level.

MAP/microtubule affinity- regulating kinase 2 (MARK2/Par-1) is another polarity kinase involved in the regulation of centrosome dynamics (Sapir et al., 2008a,b). MARK2 regulates MT dynamics by phosphorylating the MAPs tau, MAP2/4, and Dcx (Drewes et al., 1997; Schaar et al., 2004). Sapir et al. (2008a), have revealed that reduction of MARK2 in migrating neurons impairs centrosomal movement by centrosome-nucleus decoupling, which results in a defect in the morphological change from MP to BP (Sapir et al., 2008a). They also showed that tight regulation of MARK2 activity, followed by phosphorylation of Dcx and destabilization of MTs is essential for proper neuronal migration (Sapir et al., 2008b; **Figure 3**).

Mammalian Ste2-like kinase 3 (Mst3) is known to regulate axogenesis of dissociated cultured neurons (Irwin et al., 2006; Lorber et al., 2009). A recent study revealed that the novel signal pathway of CDK5-Mst3-RhoA is involved in regulating the MP-BP transition (Tang et al., 2014). Knockdown of Mst3 expression by delivery of shRNA constructs using IUE results in a migration defect of MP cells to convert into BP cells and enter the CP. This function of Mst3 is dependent on S79 phosphorylation by CDK5 as an upstream regulator, and modulation of RhoA activity for regulating the actin cytoskeleton as a downstream effector (Tang et al., 2014; **Figure 3**).

LOCOMOTION

After converting to a BP shape, neurons execute RG-guided locomotion toward the pial surface (**Figure 4**). This migrating movement is completely different from MP migration, suggesting that a distinct set of genes are upregulated or downregulated, and reorganization of signaling pathways may occur using the same protein members in locomoting cells.

In the locomotion step, coupled movement of the centrosome and nucleus by cytoskeletal coordination is essential for nuclear translocation, and endocytosis and neuronal adhesion are involved in the forward movement of the cell body (Kawauchi et al., 2010). The centrosomes (also called MTOCs) are located in the proximal part of the leading processes, and MTs project from the MTOC anteriorly toward the leading process tip and posteriorly toward the nucleus. MTs that surround the nucleus form a fork-like structure that may contribute to pulling the nucleus forward (Xie et al., 2003). The dynein/LIS1/NDEL1 complex plays an essential role in this regulation (Tsai et al., 2007), as described above. Myosin II is another motor protein that produces force for nuclear movement (**Figure 4A**; Schaar and McConnell, 2005). Activated myosin II localized at the rear of the cell generates a pushing force on the nucleus, which is normally coordinated with a centrosome-MT-dynein-mediated pulling force of the nucleus, suggesting that cytoskeletal coordination is essential for proper nucleokinesis during locomotion (Schaar and McConnell, 2005).

With respect to the molecular mechanisms for locomotion, Kawauchi et al. (2010) revealed that Rab-dependent membrane

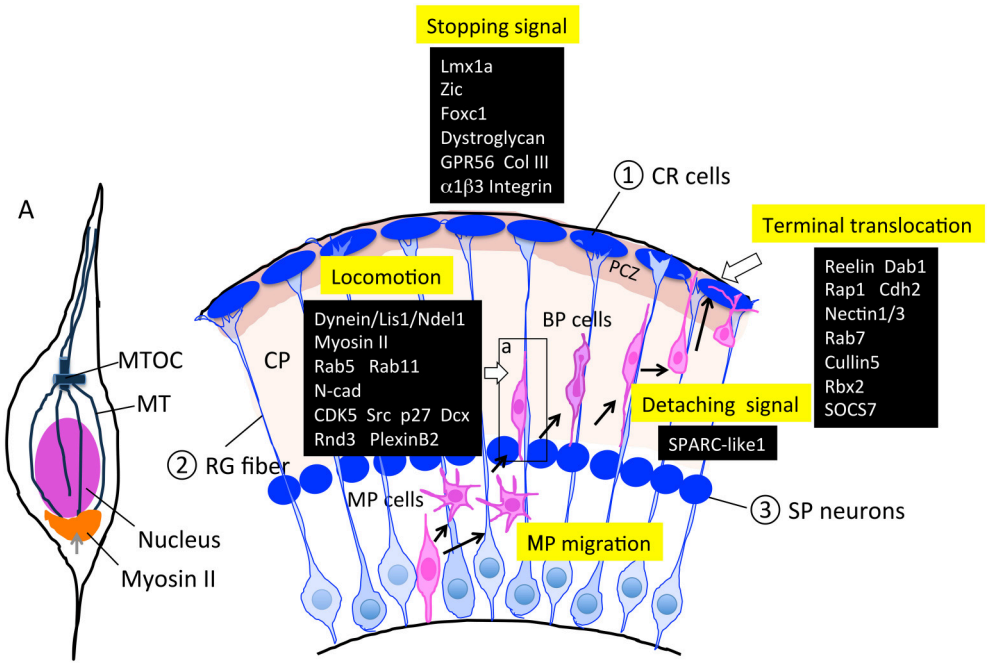

FIGURE 4 | Molecules and structures involved in the locomotion and termination of radial migration. Cajal-Retzius (CR) cells, radial glial (RG) fibers, and subplate (SP) neurons are transient cell structures that mostly disappear after birth. As described in Sections Locomotion and Termination of Radial Migration, various factors are involved in this regulation. PCZ, primitive cortical zone (Sekine et al., 2011) A; an enlarged neuron during locomotion. The microtubule organizing center (MTOC) extends the MT toward the tip of the leading process and toward the trailing process that forms a cage-like structure surrounding the nucleus. Myosin II has been shown to localize to the peri-nuclear region (bottom portion), and contributes to moving the nucleus up to the MTOC during locomotion. BP, bipolar; CP, cortical plate; CR, Cajal-Retzius; MP, multipolar; MT, microtubule; MTOC, microtubule organizing center; RG, radial glia; SP, subplate.

trafficking pathway is essential for locomotion as well as terminal translocation. Using the IUE technique, they further clarified the *in vivo* roles of endocytotic pathways in neuronal migration. In particular, they revealed that the Rab5-dependent endocytosis and Rab11-dependent recycling pathways are essential for the locomotion step, and that N-cad may be one of the major target molecules of this pathway. Whereas, Rab7 knockdown affected terminal translocation, suggesting that Rab7-dependent lysosomal degradation pathways including N-cad as one of these substrates, contribute to the final phase of radial migration.

Using *ex vivo* chemical inhibitor screening and time-lapse imaging of cultured slices of the electroporated embryonic cortex, Nishimura et al. (2010) found that roscovitine and PP2, inhibitors of CDK5 and Src-family kinases, respectively, reduce locomotion speed, suggesting the involvement of the activities of these kinases in the locomotion mode. During locomotion, migrating neurons continue to repeat the morphological change; following extension of the leading process, dilation is formed at the forward part of the nucleus, thereby moving the centrosome into the dilation, which is followed by nuclear elongation and translocation. Recently, Nishimura et al. (2014) reported that CDK5 and its downstream substrates Dcx and $p27^{kip1}$ regulate these cytoplasmic dilation and nuclear translocation steps. Moreover, dynamin and Rab-5-dependent regulation of endocytosis regulated by CDK5 is involved in this pathway (Nishimura et al., 2014).

Rnd3, another member of the Rnd protein family, has been reported to be involved in the regulation of neuronal migration via a mechanism distinct from that of Rnd2 (Pacary et al., 2011). In contrast to Rnd2, in which transcription is directly by Ngn2, Rnd3 is a direct target of Ascl1. Although both Rnd2 and Rnd3 inhibit Rho-A activity, compared with Rnd2, which is localized in the soma and regulates the MP-BP transition, Rnd3 is associated with the plasma membrane, where it inhibits and regulates locomotion by repressing F-actin polymerization. It was reported recently that the semaphorin receptor, plexin B2 interacts with Rnd3 and antagonizes the binding of Rnd3 to the Rho-A suppressor p190 RhoGAP, whereas, plexinB2 activates RhoA through recruiting Rho-GEFs (Azzarelli et al., 2014). This suggests that antagonizing regulation by an extrinsic semaphorin signal and an intrinsic Ascl1 signal is critical for maintaining appropriate RhoA activity required for locomotion. In contrast to these studies, another study using *Emx::Cre/RhoA fl/fl* (cKO) mice showed that RhoA activity is dispensable for migrating neurons, but is required for proper formation of the RG scaffold (Cappello et al., 2012). This discrepancy could be explained by compensative activities for Rho-A in the knockout neurons, but the mechanism is still up for debate.

In summary, some proteins such as CDK5, Lis1, Ndel, and N-cad play important functions in both steps of the MP-BP transition and locomotion. Others, like Rnd proteins (Rnd2 and Rnd3), as mentioned above, have distinct roles in each step although they belong to the same protein family. This suggests that a distinct set of genes participate in regulation of locomotion to fulfill the switching of migration mode during cortical evolution, as we discuss later.

TERMINATION OF RADIAL MIGRATION

Finally, when migrating neurons arrive at their final destinations, they terminate migration and shift to terminal translocation and maturation by extending their axons and dendrites. This termination step should be executed by at least three distinct behaviors of locomoting neurons: locomotion termination, detachment from the RG fiber, and anchoring to the MZ for terminal translocation (**Figure 4**).

Impairment of the stopping signal results in neuronal over-migration into the meninges, leading to neocortical dysplasia resembling cobblestone (type II) lissencephaly with a defect in basement membrane (BM) integrity. This includes over-migration of the early born neuronal population as well as late-born locomoting neurons. Many factors have been reported to be involved in this over-migration defect, including the LIM homeobox gene *Lmx1a* (Costa et al., 2001), the zinc finger transcription factor Zic (Inoue et al., 2008), the forkhead box transcription factor Foxc1 (Hecht et al., 2010), dystroglycan protein (Myshrall et al., 2012), and the GPCR GPR56 (Singer et al., 2013). The brains of mice with mutations in any of these exhibit defects in the interaction between pial basement membrane (PBM) and RG processes and PBM integrity. These defects bring about over-migration of neuroblasts into the meningeal space, followed by disorganization of the laminar structure. Among these factors, the molecular mechanism of the defects is most well studied with respect to GPR56. The gene encoding this orphan GPCR was originally identified to be responsible for the human recessively inherited genetic disorder bilateral frontoparietal polymicroglia (BFPP), which is a cobblestone-like brain malformation (Piao et al., 2004; Bahi-Buisson et al., 2010). Luo et al. (2011), identified collagen III as the ligand for GPR56, and the ligand binding triggers RhoA activation via coupling to $G\alpha_{12/13}$. Mutations in the ligand-binding domain of GPR56 have been found in BFPP patients, suggesting the importance of Collagen III (ColIII)-GPR56 signaling for PBM integrity to prevent neuronal over-migration of preplate neurons. The same research group found that α3β1 integrin functions together with GPR56 to produce a proper stopping signal (Jeong et al., 2013; **Figure 4**).

SPARC-like 1 (SC1), a member of the SPARC family of ECM proteins, is involved in detaching locomoting neurons from the RG fibers, and is expressed at the top and bottom of the RG cell surface. SC1 was identified via antigen screening for radial glial immunoreactive monoclonal antibodies. SC1 possesses an anti-adhesive activity that may contribute to detaching the locomoting neurons from the RG fibers at their final step of locomotion. The absence of SC1 results in defective termination of locomotion and the final positioning of neurons, suggesting that anti-adhesive signaling at the termination phase is essential (Gongidi et al., 2004; **Figure 4**).

Reelin plays an essential role in terminal translocation. Reelin is an extracellular protein secreted from CR cells in the MZ, and extensive analyses have revealed the molecular mechanisms of reelin signaling in neuronal migration (for a review, please see Sekine et al., 2014). Nakajima's group has found a novel thin layer at the outermost region of the mouse cortical plate that is histologically distinct and characterized by densely packed and NeuN-negative immature neurons called the primitive cortical zone (PCZ) (Sekine et al., 2011). This group also revealed that locomoting neurons must enter the PCZ in order to switch their migration mode to terminal translocation. This step is dependent on the reelin-Dab1 signaling pathway and is critical for completion of inside-out lamination at the final stage of radial migration (Kubo et al., 2010; Sekine et al., 2011).

Muller's group has revealed that the reelin-Dab1-Rap1-cadherin signaling pathway is essential for terminal translocation as well as for inside-out lamination (Franco et al., 2011). They also found that adhesion molecules Nectin1 and 3 are indispensable for terminal translocation via Rap1-mediated stabilization of cell surface Cdh2 (Gil-Sanz et al., 2013).

Furthermore, Cooper and colleagues revealed that the regulation of Dab1 degradation by the ubiquitin-proteasome system is critical for producing the stopping signal of migrating neurons (Arnaud et al., 2003). They further uncovered the molecular mechanism for this regulation, in which the E3 ubiquitin ligase complex, including Cullin 5, Rbx2, and their adaptor protein SOCS7, are critical for inhibiting over-migration (Simo et al., 2010; Simo and Cooper, 2013; **Figure 4**).

CORTICAL EVOLUTION AND RADIAL MIGRATION

So far, we have examined each step in radial migration of the developing cortex from the viewpoint of the molecular pathways involved. Now we focus on neuronal migration from an evolutionary perspective.

The six-layered laminar structure of the neocortex is unique to mammals (Nieuwenhuys, 1994). Pyramidal neurons are born sequentially in the VZ and migrate toward the pial surface. In this process, late-born neurons pass early born neurons and form a neuronal layer on top of the older layer in an inside-out manner. In contrast, non-mammalian, amniote brains, such as those of reptiles and birds, do not have this inside-out type layer structure (Northcutt and Kaas, 1995; Molnar et al., 2006; Nomura et al., 2009, 2013). The reptilian cortex is organized in a three-layered structure, but not in an inside-out manner, and the avian cortex contains a nucleus structure instead of a laminar structure. This evolutionary transition raises the question of what is the advantage of the mammalian-specific laminae of the neocortex? A simple analogy that can be used to answer this question is that of a bookshelf. The volume of information and accessibility of the content is markedly different between a highly organized six-shelf bookcase and disorganized stacks of books on the floor in the same space. One can easily find a book of interest on the organized bookshelf much faster than from the stacks of books. For corticogenesis, axon pathfinding of organized neurons in the six-layer structure may easier to reach the targets and more neuronal connections could be formed compared with neurons in a nuclear structure. Among the ancestral amniotes, primitive mammals acquired the layered structure of the cerebral cortex during evolution. Moreover, primate brains evolved remarkable expansion of the neocortex area based on this structure (Molnar

et al., 2006; Lui et al., 2011). During development of the mammalian neocortex, there are at least three transient cell structures that mainly appear in the developing stage to assist with completion of radial neuronal migration: CR cells, RG fibers, and SP neurons (Voigt, 1989; Kostovic and Rakic, 1990; Misson et al., 1991; Arias et al., 2002; Kirischuk et al., 2014; **Figure 4**). These cell structures contribute to execution of mammalian-type neuronal migration (Xie et al., 2002; Nomura et al., 2008); newborn neurons convert from MP cells to BP cells and migrate toward the pial surface in locomotion mode, followed by terminal translocation and maturation. Furthermore, OSVZ progenitor cells (bRG), a third population of neural progenitors, have been identified in the mammalian developing cortex (Fietz et al., 2010; Hansen et al., 2010; Shitamukai et al., 2011; Wang et al., 2011b). This progenitor population is observed more frequently in the primate cortex and is thought to contribute to cortical expansion and gyrification in the process of neocortical evolution. The bRG cells undergo mitotic somal translocation (MST), and the cell soma rapidly ascends along the basal fiber before cytokinesis (Hansen et al., 2010; Wang et al., 2011b). Although the specific migration mode of somal translocation in the non-mammalian developing cortex has not yet been reported, the somal translocation-type migration mode, including terminal translocation and MST, might have been acquired during the neocortical evolution of mammals as well as the locomotion mode. It is intriguing to examine the migration types of the non-mammalian amniote embryonic cortex using time-lapse imaging. Taken together, the switching of migration modes during radial migration of newborn pyramidal neurons may have been essential for evolution of the mammalian-type neocortex.

The Subplate Layer and Switching of Migration Mode

Recently, we revealed that migrating neurons that lack RP58 can migrate to the subplate (SP) layer, but are stacked just below the SP (**Figure 5**; Ohtaka-Maruyama et al., 2013). However, we noticed that knockdown or knockout of the expression of many other genes in migrating neurons resulted in a remarkably similar phenotype, including CDK5, the chondroitin sulfate modifying enzyme GalNAc4S-6ST, the Rac inhibitor α2-chimaerin, the Rap1 activator RapGEF2, and others (Ohshima et al., 2007; Ishii and Maeda, 2008; Ip et al., 2012; Ye et al., 2014). This suggests that the SP layer acts as a specific barrier for migrating neurons to cross over. As described in Section Multipolar Cell to Bipolar Cell Transition, it has been reported that various molecular pathways are involved in regulation of the MP-BP transition. When we carefully observed the cortical sections prepared from the electroporated cortex, we could detect that the SP layer resides in a boundary of the MP-BP transition. MP cells or the transit type to BP cells with multiple neurites were observed below the SP, whereas migrating cells that crossed over the SP possessed thick leading processes and migrate in locomotion mode. Accordingly, a cue to start the signaling pathway of the MP-BP transition may be received by the MP migrating cells when they reach the SP layer. It is conceivable that impairment of this cue or any of the downstream signals would lead to stacking of the MP cells just below the SP layer owing to failure in the transition to BP cells. We hypothesized that this signaling pathway at the SP played a critical role in the evolution of migration mode from the avian-type MP migration to mammalian-type locomotion and terminal translocation (**Figure 6**). The SP layer of the embryonic cortex is rich in ECM, including fibronectin, proteoglycan (CSPG; phosphacan, versican, neurocan, aggrecan), and collagen (collagen11a1) (Sheppard et al., 1991; Maeda et al., 1995; Meyer-Puttlitz et al., 1996; Popp et al., 2003; Hoerder-Suabedissen et al., 2013). Therefore, many signaling molecules could be held in the SP layer during corticogenesis. By elucidating the functional roles of the SP layer in radial neuronal migration, it is anticipated that we can start to resolve the question of how the mammalian neocortex evolved to the present six-layered inside-out structure.

PERSPECTIVE

Our review of recent progress in understanding the molecular pathways involved in radial neuronal migration of glutamatergic

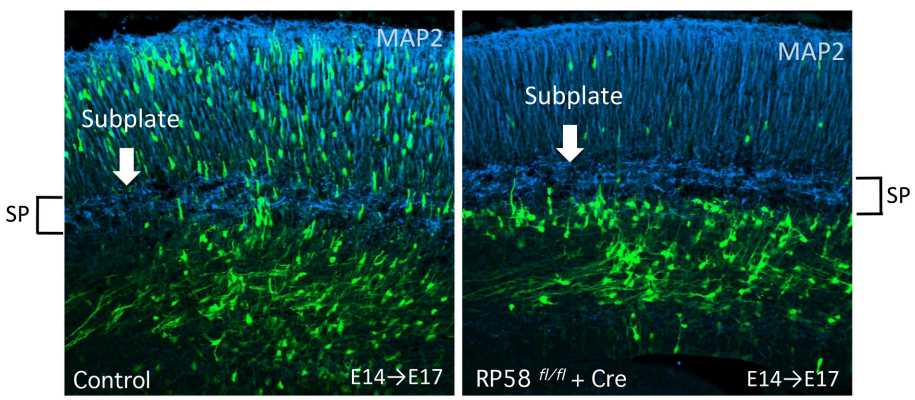

FIGURE 5 | Acute deletion of RP58 results in MP-BP transition. Green fluorescent protein (GFP)-positive cells labeled by IUE at E14 were analyzed at E17. The figure shows Cre-mediated RP58 knockout cells are stacked just under the SP that can be recognized distinctly by MAP2 immunostaining. (The data is Figure 3D from Ohtaka-Maruyama et al., 2013) E, embryonic day; IUE, *in utero* electroporation; SP, subplate.

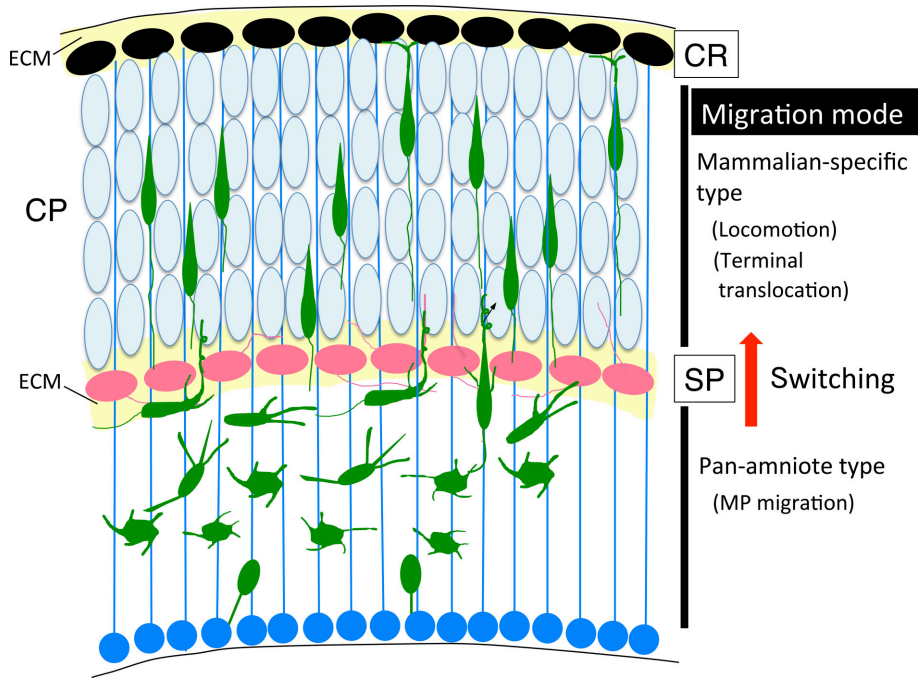

FIGURE 6 | Cortical evolution and neuronal migration. The switching of migration modes, from MP migration (pan-amniote type) to locomotion (mammalian-type) might occur at the SP layer during cortical evolution. BP, bipolar; CP, cortical plate; CR, Cajal-Retzius; ECM, extracellular matrix; MP, multipolar; SP, subplate.

neurons during corticogenesis revealed that proper regulation of the various signaling pathways operating inside or outside of newborn neurons (neuroblasts) is critical for each step of neuronal migration. In general, accurate control of developmental timing for gene expression, translational control, and modification of protein activity is required for embryonic morphogenesis. To understand the regulatory mechanisms, it is important to determine how extrinsic signaling cues translate into intracellular signals. Newborn cortical neurons execute a dynamic morphological change from MP cells to BP cells. During this step, axons are first determined, followed by formation of thick leading processes, before making the transition to locomotion mode. Although the many factors involved in regulation of polarity formation *in vitro* were identified for axon determination, more signaling pathways are required for leading process formation, suggesting the importance of *in vivo* signaling from the extracellular environment.

So far, explorations of gene function have been most commonly performed using knockdown or overexpression experiments, by delivering the DNA constructs with the IUE technique or via genetic modification of the gene locus to establish conventional and conditional knockout mice. However, to achieve more comprehensive understanding of these complex processes beyond determination of individual gene function, we could apply these conventional techniques in combination with more advanced techniques. For example, the MADM system or CLoNe (Garcia-Moreno et al., 2014) allows for clonal analysis, and double electroporation with two different colors in different stages is useful for analyzing the interaction between two cell populations. Moreover, time-lapse imaging of cultured slices of the cortex using calcium indicator-encoding plasmids or a channel rhodopsin system could reveal *in vivo* neuronal activities and the effects of activity manipulation. To investigate protein-protein interactions, or protease activity on the ECM, the use of fluorescent probes such as the proximity ligation assay and protease imaging could be effective techniques.

In conclusion, by using these imaging techniques in combination with conventional genetic manipulation, we could advance our understanding of the molecular mechanisms underlying neuronal migration and brain development *in vivo*. Finally, we would like to emphasize that it is critical to consider an evolutionary perspective in order to understand brain development as a whole system.

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Corrigendum: Molecular Pathways Underlying Projection Neuron Production and Migration during Cerebral Cortical Development

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There are two words missing in the legend of Figure 5 on page 16.

"Acute deletion of RP58 results in MP-BP transition." should be "Acute deletion of RP58 results in impairment of MP-BP transition."

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Secretory function in subplate neurons during cortical development

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Subplate cells are among the first generated neurons in the mammalian cerebral cortex and have been implicated in the establishment of cortical wiring. In rodents some subplate neurons persist into adulthood. Here we would like to highlight several converging findings which suggest a novel secretory function of subplate neurons during cortical development. Throughout the postnatal period in rodents, subplate neurons have highly developed rough endoplasmic reticulum (ER) and are under an ER stress condition. By comparing gene expression between subplate and layer 6, we found that several genes encoding secreted proteins are highly expressed in subplate neurons. One of these secreted proteins, neuroserpin, encoded by the *serpini1* gene, is localized to the ER in subplate cells. We propose that subplate might influence cortical circuit formation through a transient secretory function.

Keywords: subplate neurons, rough endoplasmic reticulum, ultrastructural analysis, ER stress condition, cerebral cortex, neuroserpin, *serpini1*

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Introduction

All eukaryotic cells contain a discernible amount of rough endoplasmic reticulum (ER) because it is needed for the synthesis of plasma membrane proteins and proteins of the extracellular matrix (Depierre and Dallner, 1975). Rough ER is particularly abundant in cells that are specialized to produce secreted proteins. For example, plasma cells produce antibodies, which circulate in the bloodstream, and pancreatic acinar cells synthesize digestive enzymes, which are transported to the intestine. In both types of cells, a large part of the cytosol is filled with rough ER. When cells synthesize secretory proteins in amounts that exceed the capacity of the folding apparatus, unfolded proteins accumulate in the rough ER. To alleviate such an overstretched functional state, eukaryotic cells activate a series of self-defense mechanisms referred to collectively as the ER stress response (also called the unfolded protein response) (Schroder and Kaufman, 2005). ER stress response is especially observed physiologically for dedicated secretory cells, such as plasma cells, pancreatic acinar cells, and pancreatic beta cells, where high levels of secreted protein synthesis require a highly evolved mechanism to properly fold, process and secrete them (Wu and Kaufman, 2006; Kondo et al., 2011).

In neurons, when stained with basic aniline dyes (toluidine blue, thionine, or cresyl violet), rough ER appears under the light microscope as a basophilic granular area called Nissl substance. The amount of Nissl substance varies according to neuronal type and functional state. It is particularly abundant in large nerve cells, especially motor neurons (Einarson, 1935). Under different

Abbreviations: ER, endoplasmic reticulum; BiP, immunoglobulin heavy chain-binding protein; GRP78, 78 kDa glucose-regulated protein; CTGF, connective tissue growth factor; neuroserpin, neuron-specific serine protease inhibitor; Nptx1, Neuronal pentraxin 1; IGFBP-5, Insulin-like growth factor binding protein-5.

physiological conditions, and in certain pathological states, Nissl substance changes it's appearance. However, the mechanism underlying this change remains unclear. Interestingly, it has been reported that malfunction of the ER stress response can result in neurodegenerative disorders (Paschen and Mengesdorf, 2005), but it remains unclear whether ER stress response occurs physiologically in neurons *in vivo*.

Subplate neurons are among the first generated neurons in the mammalian cerebral cortex and are important in establishing correct intra- and extra-cortical connectivity. Transient neurons of the subplate are considered to be instrumental in the development of the cortex and in the establisment of corticothalamic and thalamocortical connections (Kostovic and Rakic, 1990; Allendoerfer and Shatz, 1994; Kanold and Luhmann, 2010; Hoerder-Suabedissen and Molnár, 2015). While, in most mammalian species including primates, the majority of subplate neurons are lost in the development of the cortex (Kostovic and Rakic, 1980), a large proportion of the subplate persists into adulthood in rodents (Woo et al., 1991). Although there has been a huge progress in understanding the role of subplate neurons in establishing cortical circuits, additional functions of subplate neurons have not been clarified.

In this report, we would like to propose a novel secretory function for subplate neurons. We performed morphological analysis with special reference to the rough ER. To examine the functional state, we used Nissl stain and immunohistochemistry for ER stress proteins [Binding immunoglobulin protein (BiP) also known as 78 kDa glucose-regulated protein (GRP-78) or heat shock 70 kDa protein 5 (HSPA5)], and electron microscopic analysis. We also analyzed published subplate gene expression profiles (Hoerder-Suabedissen et al., 2009, 2013; Oeschger et al., 2011) for genes encoding secreted proteins and validated the expression of candidate genes by immunohistochemistry.

Materials and Methods

Animals and Tissue Preparation

All animal experiments were approved by a local ethical review committee and conducted in accordance with personal and project licenses under the UK Animals (Scientific Procedures) Act (1986). For light microscopy analysis, three P8 and three adult C57BL/6 mice were anesthetized using pentobarbitone (Euthatal 150 mg/kg intraperitoneally; Merial Animal Health Ltd, Harlow, UK) and perfused through the heart with 4% paraformaldehyde (PFA; TAAB, Reading, UK) in phosphate-buffered saline (PBS, 0.1 M; pH 7.4). The brains were removed, dissected and fixed in the same fixative for 24 h at 4°C. For electron microscopy analysis, three Wistar rats at P8 were anesthetized using pentobarbitone and perfused through the heart with 4% PFA with 1% glutaraldehyde in 0.1M-phosphate buffer (PB; pH7.4). The brains were removed, dissected, and fixed in the same fixative for 2 days at 4°C.

Histological Processing

Fixed mouse brains were embedded in paraffin. Serial coronal sections were cut at a thickness of 8 μm and divided into two series. One set was used for Nissl staining, and another was prepared for immunohistochemistry. For Nissl staining, sections were stained with 0.1% cresyl violet solution. For immunohistochemistry, the section were incubated in 2% normal goat serum (NGS) diluted in Tris-buffered saline (TBS; 50 mM Tris buffer, 0.09% NaCl, pH 7.4) for blocking, and then incubated for 2 h at room temperature (RT) with mouse anti-KDEL antibody (1:500, Abcam) as anti-BiP (Okiyoneda et al., 2004) and rabbit anti-neuroserpin antibody (1:200, Abcam) in 1% NGS diluted in TBS. Following several washes, anti-mouse-AlexaFluor488 antibody (1:500, Molecular Probes) and anti-rabbit-AlexaFluor546 antibody (1:500, Molecular Probes) diluted in 1% NGS in TBS were applied for 2 h at RT. The sections were imaged using an epifluorescent microscope (DMR; Leica Microsystems). We selected P8 for our analysis based on the data obtained from our microarray-based gene expression analysis (Hoerder-Suabedissen et al., 2013; https://dpag.cloudant.com/subplate-atlas/_design/subplate-atlas/index.htmlindex.html).

Electron Microscopy Processing

Fixed rat brains were rinsed in 0.1M-PB (pH7.4) and post-fixed with osmium tetraoxide. Once the tissue was osmicated it was then rinsed with 0.1M PB followed by dehydration through graded alcohols and placed in propylene oxide. The tissue was prepared for sectioning by placeing it in propylene oxide:Epon Araldite 1:1 overnight, followed by Epon Araldite for a further night, before being embedded in fresh Araldite and placed at 60°C for 48 h to harden fully. Semi-thin (1 μm) sections were stained with 1% toluidine blue in order to select suitable areas for transmission electron microscopy. Sections were mounted on copper grids, stained with uranyl acetate (5% UA in 50% alcohol) and Reynolds lead citrate, and examined in a JEOL EM15007 electron microscope.

Subplate Dissection and RNA Isolation

For detailed description of the microarray experiments identifying subplate enriched genes please see Hoerder-Suabedissen et al. (2009). Briefly, P8 mouse brains were sectioned into 150 μm parasagittal sections and thin strips of anterior subplate and adjacent layer 6 and posterior subplate and layer 6 were dissected out under visual guidance using transillumination on a dissecting microscope. 8 fragments of each tissue type for each brain were included and pooled the fragments of 4 littermates per replicate. A total of 4 biological replicates were collected for each location. Total RNA was isolated using the RNeasy Micro kit (Qiagen, Crawley, UK) following the manufacturer's instructions. The quality and RNA integrity were assessed on a BioAnalyzer; all samples had a RNA Integrity Number 8 (Agilent Laboratories, Stockport, UK). Labeled cRNA for hybridization was generated with the Affymetrix "2 Cycle Target Labeling and Control" kit (Affymetrix, High Wycombe, UK) and MEGAscript T7 polymerase (Ambion) according to the manufacturer's instructions. Labeled anti-sense cRNA was fragmented and the distribution of fragment lengths was measured using a BioAnalyzer (Agilent). Labeled and fragmented cRNA was hybridized to the Affymetrix 430 2.0 whole mouse genome microarray (Affymetrix). A total of 16 chips were used, all from the same batch. Chips were processed on an Affymetrix GeneChip Fluidics Station 450 and Scanner 3000.

Microarray Analysis

For detailed description of the normalization, clustering, statistical analysis on the microarray data please see Hoerder-Suabedissen et al. (2009). Briefly: arrays were Robust Multi-Array (RMA) normalized, and differentially expressed genes were identified using a paired *t*-test with a cut off *p*-value < 0.05 (no multiple testing correction) and a >1.5 fold-change difference between any 2 comparisons. Longer lists of differentially expressed genes (>1.5-fold difference, $p < 0.05$) were generated from RMA taking GC content into account (GCRMA) normalized data.

Computational Gene Ontology Analysis

To classify cellular distribution of the proteins, gene ontology (GO) analysis was performed using GO_Full ontology (http://www.geneontology.org). The list of P8 subplate enriched genes was examined specifically for secretory genes. A list of genes with products localized in the extracellular space was generated and the examples were selected because they had the highest (first 4 on the list) expression levels in absolute mRNA volume (**Table 1** and https://molnar.dpag.ox.ac.uk/subplate/).

Results

Subplate Neurons in P8 Mouse Brain have Extensive Nissl substance

To characterize the morphology and the functional state of subplate neurons in postnatal and adult rodents, we analyzed Nissl stained coronal sections of P8 mouse brains (**Figures 1A,B**) and adult mouse brains (**Figures 1E,F**). At P8, extensive Nissl substance was detected in the large pyramidal cells of layer 5. Plentiful Nissl substance was also observed in layer 2/3 and subplate neurons (**Figure 1A**), suggesting that these neurons produce a large amount of proteins at P8. The morphological features of subplate neurons (**Figure 1B**) are surprisingly similar to those of plasma cells, which also have a large, ovoid cell body with non-central distribution of nucleus and basophilic cytoplasm due to their richness in rough ER (Bloom and Fawcett, 1968).

In the adult mouse brain, abundant Nissl substance was detected in pyramidal cells of layer 2/3 and layer 5 (**Figure 1E**), while the cell bodies of subplate neurons appear small and weakly stained (**Figure 1F**).

TABLE 1 | This table lists some of the genes expressed at a high level in subplate neurons which also localize to the extracellular space.

Gene name	Cellular localization	Probe set	Anterior fold change	Posterior fold change
Connective tissue growth factor (CTGF)	extracellular space	1416953_at	11.2	14.9
Neuron-specific serine protease inhibitor (neuroserpin)	extracellular space	1448443_at	2.4	1.7
Neuronal pentraxin 1(Nptx1)	extracellular space	1434877_at	2.2	2.7
Insulin-like growth factor binding protein 5 (IGFBP-5)	extracellular space	1452114_s_at	2.5	2.1

Affymetrix probe set IDs are given in the probe set column. Fold-changes reflect the difference in gene expression levels between subplate and layer 6a at P8 in anterior (S1) and posterior (V1) regions, and are calculated as mean fold-changes across all four replicates. Data from Hoerder-Suabedissen et al. (2009) and (2013). Additional data can be found at https://molnar.dpag.ox.ac.uk/subplate/.

Endoplasmic Reticulum Stress Occurs in Mouse Subplate Neurons at P8

To investigate whether the amount of Nissl substance correlates with ER stress, we next examined the expression level of the ER stress marker protein BiP (also called GRP78) (Okiyoneda et al., 2004; Kondo et al., 2005, 2012; Schroder and Kaufman, 2005; Wu and Kaufman, 2006). The induction of the ER chaperone protein BiP, which is required for the proper folding and assembly of secretory proteins, is a major ER stress response. BiP is up-regulated under stress conditions, such as glucose deprivation, hypoxia, or the presence of toxic agents (Lee, 2001). Immunohistochemical analysis using the anti-KDEL antibody, which recognizes BiP (Okiyoneda et al., 2004), showed strong expression of the BiP protein in pyramidal cells of layer 2/3 and layer 5 and subplate neurons in the P8 mouse brain (**Figures 1C,D**). In adult brains, although pyramidal cells in layer 2/3 and 5 continue to express massive amounts of BiP protein, we could not detect BiP expression in subplate/layer 6b neurons (**Figures 1G,H**). These results suggest that ER stress occurs in subplate neurons at early postnatal, but not or much less in adult ages.

Subplate Neurons have Highly Developed Rough ER during Development

To confirm directly whether subplate neurons in postnatal rodents have well developed rough ER, we carried out ultrastructural analysis of P8 rat brains using electron microscopy (**Figure 2**). Electron micrographs of subplate neurons showed an abundance of rough ER (**Figures 2A,B**) compared to either neurons in the striatum (**Figure 2C**) or pyramidal cells in layer 5 during postnatal period (Miller and Peters, 1981) or in adult (Parnavelas and Lieberman, 1979). The chromatin in the nucleus of subplate neurons is not strongly aggregated at P8, suggesting that high levels of mRNAs are being produced. The presence of a well-developed rough ER in subplate neurons during the postnatal period suggests an active protein production function for these cells.

Subplate Neurons in P8 Mouse Brain Express Secreted Proteins

The plasma cell has a well-developed rough ER to be able to synthetise and secret massive amounts of antibodies. Because of their very similar subcellular morphology (Bloom and Fawcett, 1968), we postulate that subplate neurons also a secretory function. To elucidate this possibility we analyzed the gene expression profile for P8 mouse subplate generated from a microarray comparison on subplate and layer 6a tissue samples (Hoerder-Suabedissen et al., 2009, 2013). Comparing gene expression in the subplate with the adjacent layer 6a in somatosensory and visual cortices, we identified 601 probe sets (corresponding to 383 genes and hypothetical genes) that were expressed at a

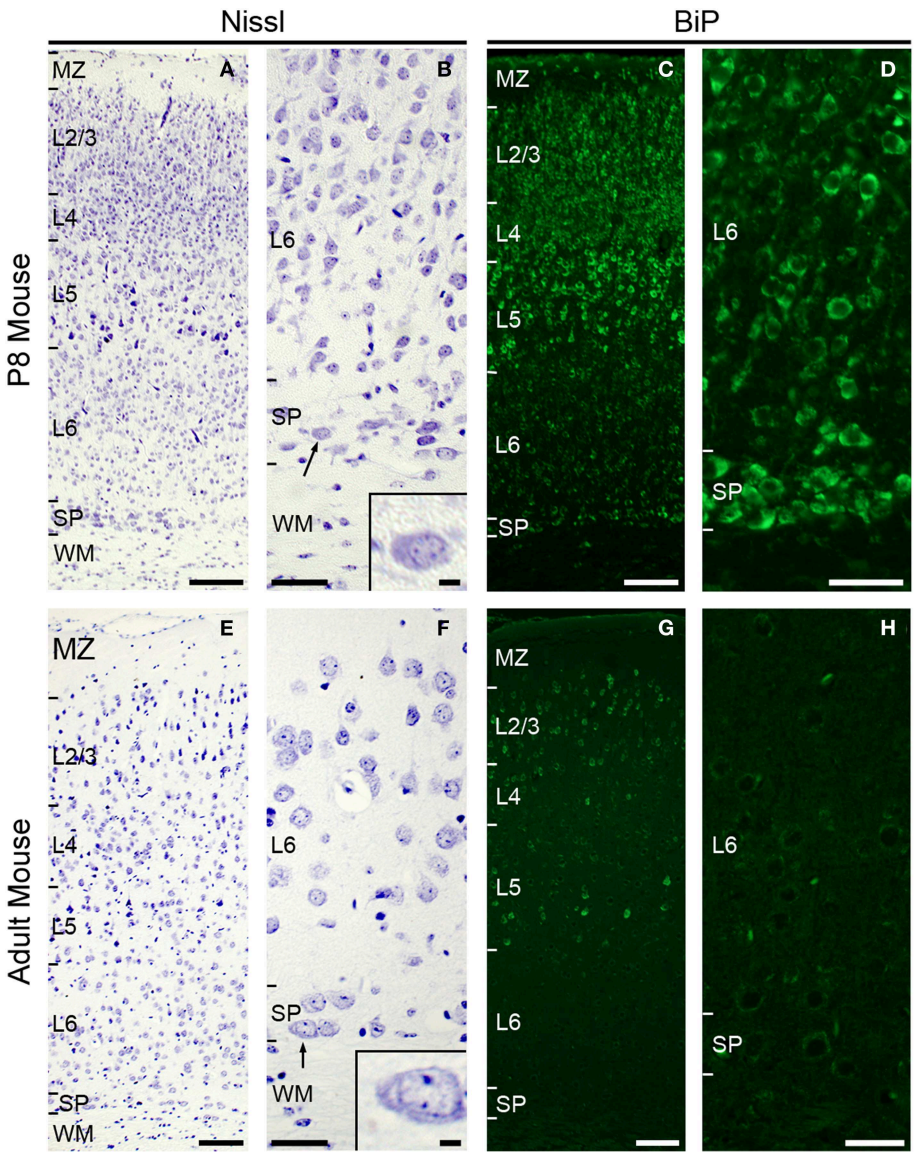

FIGURE 1 | Subplate neurons have large cytoplasm with large amounts of Nissl substance and are under ER stress condition at P8. Nissl staining in coronal section of P8 **(A,B)** and adult **(E,F)** mouse brain. Note, subplate neurons (and some layer 5 and 2–3 neurons) have voluminous cytoplasm with large amounts of Nissl substance (arrow and inset, **B**) at P8. Subplate neurons in adult mouse have relatively small cytoplasm (arrow and inset, **F**). Immunohistochemistry for anti-KDEL antibody, which recognizes BiP/GRP78 (Okiyoneda et al., 2004), in coronal section of P8 mouse **(C,D)**. Layer 5, layer 2–3, some layer 6, and subplate neurons express strong BiP immunoreactivity. Immunohistochemistry for anti-KDEL in coronal section of adult mouse **(G,H)**. Scale bars: 200 μm **(A,C,E,G)**, 50 μm **(B,D,F,H)**, 10 μm (inset in **B** and **F**).

higher (at least 1.5-fold) level in the subplate compared with layer 6 in both comparisons (Hoerder-Suabedissen et al., 2009). Gene ontology (GO) analysis for cellular localization was performed on this list. **Table 1** shows some selected examples of genes that encode extracellular proteins and have the highest four expression levels in absolute mRNA volume. Gene expression of these four genes at P7 was confirmed in the GENSAT Database (**Supplementary Figure 1**). Of these genes, we focussed on the neuron-specific serine protease inhibitor (neuroserpin), which was initially identified as an axonally secreted protein from neuronal cultures of chicken dorsal root ganglia and belongs to a serine protease inhibitor (serpin) gene family (Osterwalder et al., 1996). To analyze the expression pattern of neuroserpin protein in postnatal and adult mouse brain, we performed immunohistochemical analysis (**Figure 3**). In the P8 mouse brain, neuroserpin was detected in layer 5 pyramidal cells and subplate neurons (**Figure 3A**) and co-localized with the ER stress marker BiP (**Figures 3B–F**). The co-localization of neuroserpin and BiP in these neurons suggests that the production and secretion of neuroserpin contributes to the ER stress condition during the postnatal period. In adult, on the other hand, we could not detect neuroserpin expression in subplate neurons. A selected

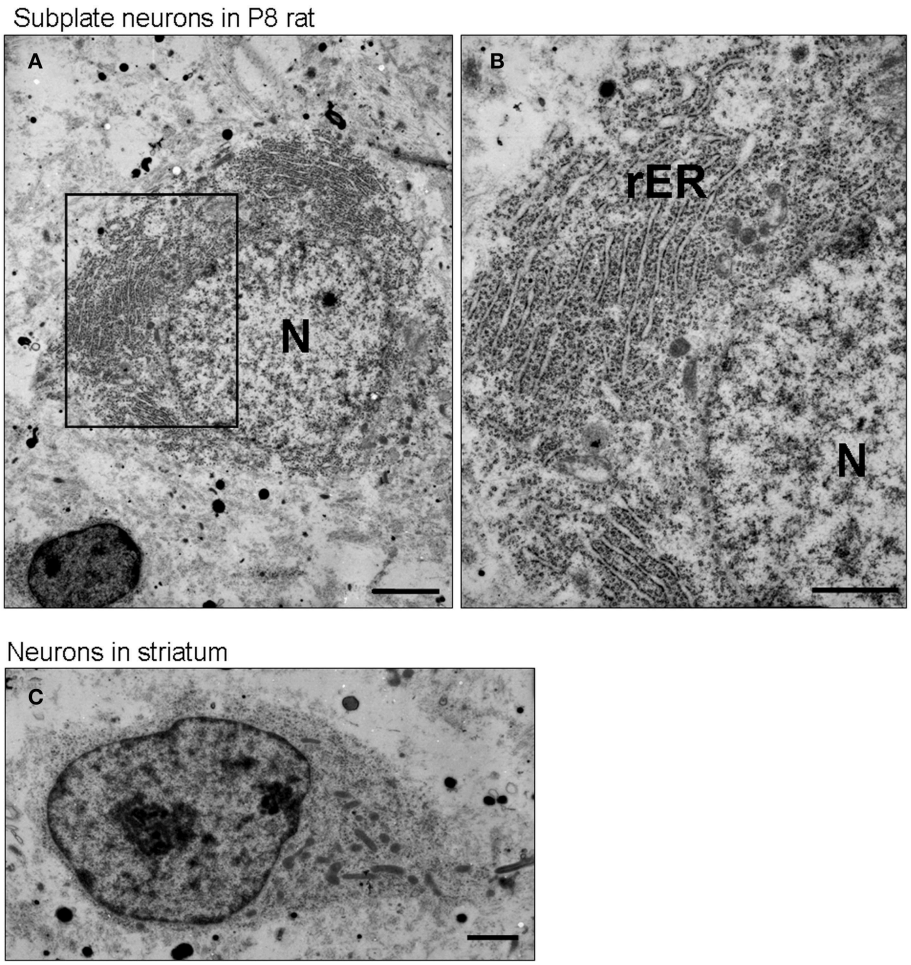

FIGURE 2 | Subplate neurons have a well-developed rough endoplasmic reticulum. Transmission electron microscopic image of a subplate neuron of P8 rat brains **(A,B)**. Note, the large amounts of rough ER (rER) in the subplate neurons. The chromatin in the nucleus (N) is not strongly condensed. For comparison, see the transmission electron microscopic image of a neuron in striatum of P8 rat brains **(C)**, in which cells display much less rER. Scale bars: 2 μm **(A)**, 1 μm **(B,C)**.

population of pyramidal cells in layer 5 expresses large levels of neuroserpin also in the adult. This is further supported by our layer-specific transcriptomic analysis in the adult (Belgard et al., 2011; Hoerder-Suabedissen et al., 2013). Similarly, BiP was absent from the subplate but present in layer 5 pyramidal cells in adult brains (**Figures 3G–I**). These results strongly suggest that subplate neurons have a protein secretion function during the postnatal period, but not or much reduced in adulthood.

Discussion

In this study, we present several lines of evidence that rodent subplate neurons have a protein secretion function in the early postnatal period: firstly, subplate neurons in P8 mouse brain have very rich Nissl substance with ovoid cell shape and a non-central distribution of the nucleus, similar to other cells of known secretion function. Secondly, signs of ER stress are present in subplate neurons at P8, similar to other dedicated secretory cells. Thirdly, our ultrastructural examination of P8 rat subplate neurons revealed highly developed rough ER, which filled a large part of the cytosol. Fourthly, some genes, whose products are known to be secreted into the extracellular space, are expressed at high levels in subplate neurons of the P8 mouse brain (**Table 1**) but not necessarily in adult brains. Finally, neuroserpin, one such secreted protein, is likely to be located in ER of subplate neurons at P8 in the mouse brain (**Figure 3**).

We have shown that rodent subplate neurons (during the early postnatal period) and plasma cells have three common features; non-central distribution of the nucleus, highly developed rough ER filling a large part of the cytosol, and signs of ER stress condition. The characteristic morphological features of plasma cells at the light and electron microscopic levels have been described in details in the literature (Bloom and Fawcett, 1968). These three common features prompt us to suggest that subplate neurons have a protein secretory function.

During the early postnatal period, subplate neurons are the only cortical cell type with these three properties. In contrast, layer 5, some layer 6 and 2–3 pyramidal cells fit just one

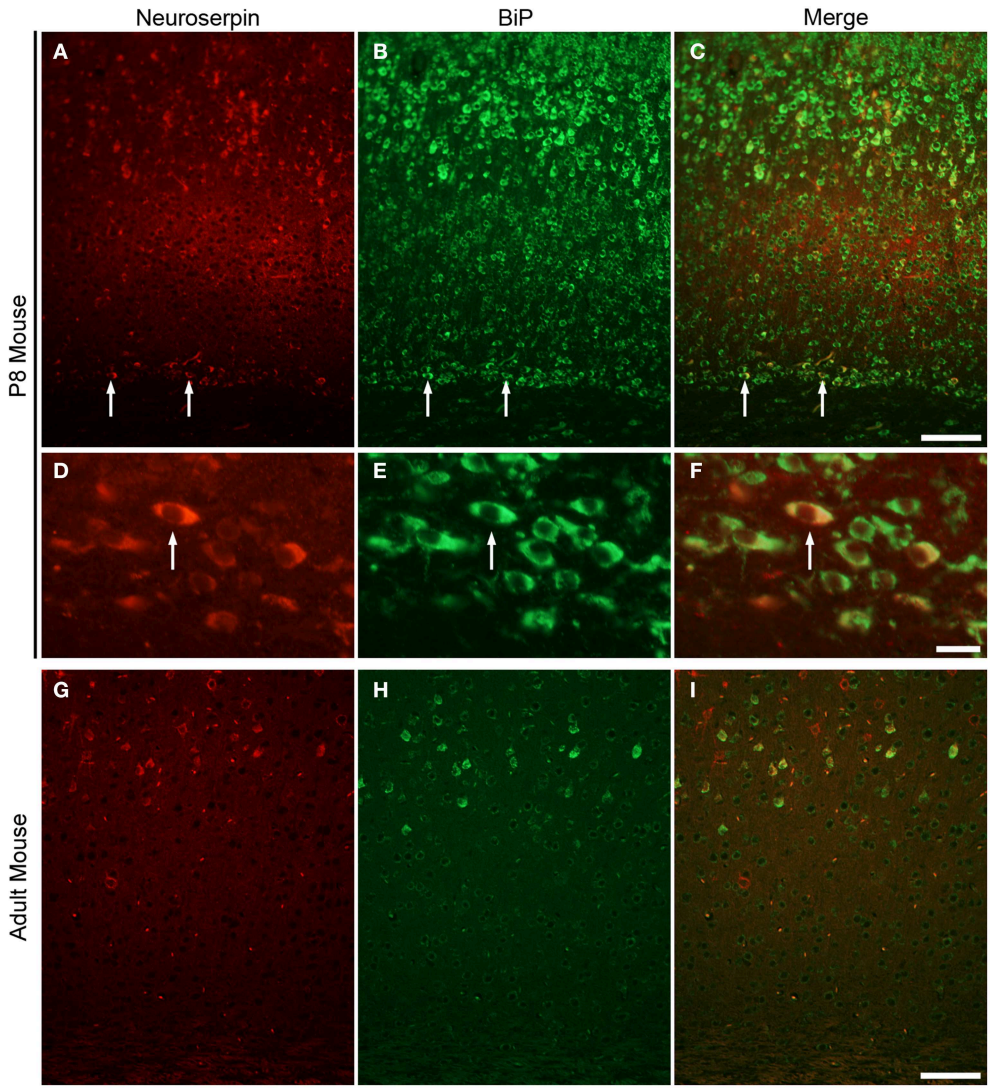

FIGURE 3 | Subplate neurons in P8 mouse brain strongly express neuroserpin. Immunohistochemistry for anti-neuroserpin **(A)** and anti-KDEL **(B)** and their correlation **(C)** in coronal section of P8 mouse. Note, co-localization of neuroserpin and BiP in subplate neurons (arrows, **C**; cell in **D–F**). Immunohistochemistry for anti-neuroserpin **(D)** and anti-KDEL **(H)** in coronal section of adult mouse. Neither neuroserpin nor BiP is strongly expressed in the adult subplate. Scale bars: 100 μm (**A–C**, **G–I**), 10 μm (**D–F**).

condition—exhibiting ER stress hallmarks (**Figures 1C,D,G,H**). The rough ER of layer 5 pyramidal cells is restricted to the cytosol and the nuclei are located centrally within the cytosol (Parnavelas et al., 1978; Miller and Peters, 1981) in contrast with subplate (present study). Furthermore, the proportion of cytosol occupied by rough ER is much higher in subplate than in orther cortical neurons (data not presented). These results suggest that although several cell types may have secretory properties, subplate neurons may be more specialized to protein secretion than other cells. Interestingly, subplate neurons in adult stage have relatively small cytoplasm and are no longer under ER stress conditions (**Figures 1F,H**). This suggests that the secretory function of subplate neurons is transient.

Some genes, whose products are known to localize in the extracellular space, are very strongly expressed in subplate neurons in the P8 mouse brain (**Table 1**). Connective tissue growth factor (CTGF) belongs to a family of secreted, extracellular matrix-associated proteins that are involved in the regulation of cellular functions such as adhesion, migration, mitogenesis, differentiation and survival (Brigstock, 1999) as well as maturation of oligodendrocytes and progression of myelination (Stritt et al., 2009). We have previously reported that CTGF expression is detectable in the subplate region at E18 and increases in the number of cells and the intensity of labeling at P3 and P8 (Hoerder-Suabedissen et al., 2009, 2013). Neuroserpin is an inhibitor of tissue plasminogen activator (tPA) that is expressed in developing and adult nervous systems (Hastings et al., 1997; Krueger et al., 1997). Mutations in neuroserpin result in its misfolding and accumulation in the ER (Miranda et al., 2004). In this study, immunohistochemical analysis demonstrated that

subplate neurons in P8 mouse brain express neuroserpin, which may be released by secretion. Neuronal pentraxin 1 (Nptx1), predicted to be a secreted protein, is selectively expressed in the nervous system and has been suggested to be involved in synaptic functions (Schlimgen et al., 1995; Dodds et al., 1997). Insulin-like growth factor binding protein 5 (IGFBP-5), which is an extracellular modulator of Insulin-like growth factor (IGF) signaling, has been highlighted as a focal regulatory factor during the development of several key cell lineages, e.g., myoblasts and neural cells (Clemmons, 1997; Cheng et al., 1999; Pera et al., 2001). GENSAT Database shows that the genes encoding these secreted proteins are expressed in the subplate region at P7 mouse brain (**Supplementary Figure 1**; Hoerder-Suabedissen et al., 2013). This period coincides with the major changes in somatodendritic morphology and death of subplate cell populations (Hoerder-Suabedissen and Molnár, 2012, 2013, 2015). To elucidate the function of subplate neurons during postnatal period, it may be useful to investigate the functions of these secreted proteins during normal development and in pathological conditions, including after perinatal hypoxic ischaemic brain damage (Okusa et al., 2014).

Conclusion

Our work shows that during the postnatal period subplate neurons in rodents have highly developed rough ER, transiently express neuroserpin, a secreted protein and show signs of ER stress. Taken together, these results suggest a transient protein secretory function of rodent subplate neurons during the postnatal period.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnins.2015.00100/abstract

Supplementary Figure1 | Expression patterns of secretory protein genes in P7 sagittal mouse brain. *In situ* hybridization images from the GENSAT NCBI website (http://www.ncbi.nlm.nih.gov/sites/entrez), numbers indicate references at the time of the download. **(A)** CTGF: (GENSAT Image 60754), **(B)** Neuroserpin/SERPINI1: (GENSAT Image 51784), **(C)** Nptx1: (GENSAT Image 18886), **(D)** IGFBP-5: (GENSAT Image 17034).

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Non-cell autonomous and non-catalytic activities of ATX in the developing brain

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The intricate formation of the cerebral cortex requires a well-coordinated series of events, which are regulated at the level of cell-autonomous and non-cell autonomous mechanisms. Whereas cell-autonomous mechanisms that regulate cortical development are well-studied, the non-cell autonomous mechanisms remain poorly understood. A non-biased screen allowed us to identify Autotaxin (ATX) as a non-cell autonomous regulator of neural stem cells. ATX (also known as ENPP2) is best known to catalyze lysophosphatidic acid (LPA) production. Our results demonstrate that ATX affects the localization and adhesion of neuronal progenitors in a cell autonomous and non-cell autonomous manner, and strikingly, this activity is independent from its catalytic activity in producing LPA.

Keywords: cortical development, radial glia, autotaxin, LPA, neuronal stem cell, *in utero* electroporation

INTRODUCTION

How excitatory neurons reach their proper position in the developing brain has been the focus of intense research, since perturbations in this process have been shown to result in a wide spectrum of brain diseases, ranging from severe brain malformations, to diseases such as cognitive impairment and autism. Most of the molecular mechanisms known to control radial neuronal migration are cell autonomous and include for example proteins, which are involved in regulation of the cytoskeleton and cytoskeleton-associated motor proteins (reviews Ayala et al., 2007; Rakic et al., 2007; Jaglin and Chelly, 2009; Valiente and Marin, 2010; Reiner, 2013). Key examples of such proteins are LIS1 and DCX, where mutations of the corresponding genes in humans result in a brain malformation known as lissencephaly (Reiner et al., 1993; Des Portes et al., 1998; Gleeson et al., 1998, reviews Jaglin and Chelly, 2009; Valiente and Marin, 2010; Reiner, 2013; Reiner and Sapir, 2013). LIS1 is involved in regulation of microtubules and the microtubule associated molecular motor, cytoplasmic dynein, as well as regulation of the actin cytoskeleton through the activity of small GTPases (Faulkner et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000; Smith et al., 2000; Kholmanskikh et al., 2003; Yamada et al., 2013) (review Reiner and Sapir, 2013). DCX is a microtubule and actin-associated protein, which interacts with cytoplasmic dynein and a member of the kinesin superfamily of proteins (Gleeson et al., 1999; Caspi et al., 2000; Kim et al., 2003; Tsukada et al., 2003, 2006; Gdalyahu et al., 2004; Schaar et al., 2004; Tanaka et al., 2004b; Bielas et al., 2007; Bechstedt and Brouhard, 2012; Liu et al., 2012). Despite these so-called cell autonomous functions, experimental evidence suggests that LIS1 (Hippenmeyer et al., 2010) and DCX (Bai et al., 2003) may also affect neighboring cells in a non-cell autonomous fashion.

To better understand the non-cell autonomous aspects of radial neuronal migration, we developed an *in vivo* assay in which migration defective cells, following treatment with either *Dcx* or *Dclk* shRNA, were isolated and subjected to microarray analysis. We identified mRNA encoding for secreted and transmembrane proteins, which were differentially expressed in the area where the impaired neurons clustered in the brain. While both shRNA treatments exhibited non-cell autonomous inhibition of neuronal migration, the morphology of the stalled cells differed between treatments. Comparison of the gene expression profile in both treatments revealed several differentially expressed genes, among which we detected autotaxin (ATX, also known as ENPP2, PD-Iα or lysoPLD).

Autotaxin is a secreted enzyme of 99 kDa, thus may fit to act in a non-cell autonomous way. It was originally identified as an autocrine factor, which stimulates tumor cell motility (Stracke et al., 1992). ATX becomes active and is secreted to

the extracellular space following glycosylation and proteolytic cleavage of its N-terminal signal peptide (Jansen et al., 2005, 2007). ATX is a member of the ENPPs (ectonucleotide pyrophosphatase/phosphodiesterases) family. Each of the ENPPs contains a conserved catalytic domain, which hydrolyzes phosphodiester bonds of different nucleotides and phospholipids (Stefan et al., 2005). ATX is unique, as it is the sole member of the ENPPs that utilizes this catalytic domain for lysophospholipase D (lysoPLD) activity. ATX catalyzes lysophosphatidic acid (LPA) production from lysophosphatidylcholine (LPC) (Tokumura et al., 2002; Umezu-Goto et al., 2002). ATX is considered as the major producer of LPA, and deletion of one allele reduces LPA concentration in the plasma by half (Tanaka et al., 2006; Van Meeteren et al., 2006). Thus, it is thought that ATX acts predominantly through LPA production. LPA is a potent molecule, which acts through binding to its cognate receptors (LPAR1-5) thus instigating several downstream signaling pathways. Nevertheless, single LPAR knockout mice develop normally. LPA influences multiple events during cortical development including polarity establishment in hippocampal neurons (Yamane et al., 2010). In addition, LPA regulates proliferation, survival and differentiation in sundry cell populations. Heuristically, physiological concentrations of LPA ($0.1 \sim 1\,\mu M$) promote proliferation of several neuronal progenitors and stem cells and enhance cortical growth (Kingsbury et al., 2003; Fukushima, 2004; Svetlov et al., 2004; Cui and Qiao, 2006; Estivill-Torrus et al., 2008; Hurst et al., 2008), while higher concentrations of LPA evoke necrosis and apoptosis (Holtsberg et al., 1998; Steiner et al., 2000). LPA has been shown to be a survival factor of neuroblasts (Kingsbury et al., 2003) and post-mitotic neurons (Fujiwara et al., 2003; Zheng et al., 2005; Estivill-Torrus et al., 2008). LPA has been shown to stimulate both neuronal differentiation, possibly through LPAR1 (Cui and Qiao, 2006; Fukushima et al., 2007; Spohr et al., 2008), and glial differentiation (Cui and Qiao, 2007), yet other studies suggest that LPA inhibits neuronal differentiation (Dottori et al., 2008). In mice, ATX knockout is lethal and embryos die around E9–E10 (Tanaka et al., 2006; Van Meeteren et al., 2006; Fotopoulou et al., 2010). These mice display vascular defects in embryo and yolk sac, allantois malformation, neural tube defects, asymmetric headfolds, increased cell death, decreased proliferation and neurite outgrowth deficits. Neurite outgrowth was rescued by addition of LPA (Fotopoulou et al., 2010). Heterozygous-knockout mice, exhibiting half of the lysoPLD activity and LPA levels, showed attenuated nerve injury-induced neuropathic pain (Inoue et al., 2008). High ATX expression, on the other hand, is associated to and found in many pathophysiological conditions, including several cancer types (Okudaira et al., 2010), neuropathic pain (Inoue et al., 2004, 2008; Ueda, 2008), Alzheimer-type dementia (Umemura et al., 2006), multiple sclerosis (Hammack et al., 2004) and following brain lesion (Savaskan et al., 2007). During embryonic development, ATX expression is first detected at the floor plate of the neural tube, and later in the choroid plexus, cerebrospinal fluid and the ventricular area of the embryonic brain (Abramova et al., 2005; Ohuchi et al., 2007; Savaskan et al., 2007; Zappaterra et al., 2007). Following birth, ATX is detected in leptomeningeal cells, oligodendrocytes and astrocytes, but not in neurons. ATX induces neurite retraction of differentiated PC12 via LPA production (Sato et al., 2005). In oligodendrocytes ATX is upregulated during maturation and is temporally correlated with the process of myelination. ATX facilitates morphological changes of oligodendrocytes, decreases their adhesion to the ECM and promotes complex process network (Fox et al., 2004; Dennis et al., 2008, 2011). Little is known about the role of ATX during cortical development. Our studies show cell-autonomous and non-cell autonomous roles of ATX in regulation of cell position and adhesion in progenitors of the developing cortex. Markedly, these activities did not require ATX catalytic activity.

MATERIALS AND METHODS

ANIMALS

ICR were purchased from Harlan laboratories. Mice in which the first two exons of *ATX* gene are flanked by two loxP sites were obtained from Vassilis Aidinis (Fotopoulou et al., 2010) and were bred with mice which express the recombinase Cre under the control of the *EMX1* promoter (Jackson). Genotyping for the Atx^{flox} and Atx^{-} alleles was described previously (Fotopoulou et al., 2010). Briefly, four primers were used: A1, B1, C1, and B2. A1: 5′-CGCATTTGACAGGAATTCTT; B1: 5′-ATTTGTCACGTCCTGCACGA; C1: 5′-ATCAAAATACT GGGGCTGCC; B2: 5′-TACACAACACAGCCGTCTCA. Primer combination A1 and C1 was used to detect wild type (WT) alleles. Primer combination A1 and B1 was used to detect the floxed (neo) allele. Primer combination A1 and B2 was used to detect the deleted allele. The primers used for detecting the *EMX1-Cre* transgene were 5′-AACATGCTTCATCGTCGG and 5′-TTCGGATCATCAGCTACCACC. Embryonic day 0 (E0) was defined as the day of confirmation of the vaginal plug. Mice were raised in the Weizmann Institute of Science transgenic facility. All animal procedures were approved by IACUC.

IMMUNOHISTOCHEMISTRY

Antibodies used were as follows: mouse anti-5-iodo-2′-deoxyuridine(IdU)/5-bromo-2′-deoxyuridine(BrdU) (1:50; BD Biosciences), rat anti-BrdU (1:100; Becton Dickinson), rabbit anti-phosphorylated histone H3 (pH3) (1:100; Upstate Biotechnology), goat anti-GFP (1:400; Abcam), chicken anti-GFP (1:500; Abcam), rat anti-ATX [1:40; kindly provided by J. Aoki (Tanaka et al., 2004a)], chicken anti-Tbr2 (1:400; Millipore), chicken anti-Tbr1 (1:400; Millipore), mouse anti-Tuj1 (1:300; Covance), goat anti-Par-3 (1:50; Santa Cruz Biotechnology), rabbit anti-β-Catenin(1:300; Sigma), rat anti-ZO-1(1:70; Developmental Studies Hybridoma Bank), mouse anti-Numb (1:300; Developmental Studies Hybridoma Bank), goat anti-Par-6 (1:100; Santa Cruz Biotechnology), rabbit anti-Pax6 (1:300; Covance), pFAK 925 (1:100; Cell Signaling).

Floating sections or cryosections were permeabilized using 0.1% Triton X-100 and blocked in blocking solution (PBS, 0.1% Triton X-100, 10% HS; or PBS, 0.1% Triton X-100, 2% HS for ATX staining) for 60 min. Antibodies were incubated in blocking solution over night at 4°C. After washing, appropriate secondary antibodies (Jackson ImmunoResearch) were diluted in blocking solution, and incubated for 2–3 h at room temperature. Slices were mounted onto glass slides using Aqua Polymount (Polysciences).

Cover slips containing fixed cells were permeabilized using 0.1% Triton X-100 and blocked three times in PBS supplemented with 0.1% BSA (Sigma). Coverslips were incubated with antibodies, stained with DAPI and mounted onto glass slides using Aqua Polymount (Polysciences). To visualize ATX, sections were first incubated with 10 mM citrate buffer for 30 min in 80°C, then cooled at RT for 30 min. After washing, sections were immunostained as described above.

ANALYSIS OF NEURONAL MORPHOLOGY

The z-stack images from the slices of the *in utero* electroporated (E14.5–E18.5) brains with either *Dcx* or *Dclk* shRNA were acquired with confocal microscope (LSM480, Zeiss, x40). Each fluorescent cell in the resulted images was classified as either bipolar, cells with 3–4 processes or multipolar. Slices from four different brains for each condition were used for the analysis. In total 188 and 212 cells from *Dcx* and *Dclk* shRNA condition respectively were analyzed.

SAMPLE PREPARATION AND MICROARRAY ANALYSIS

In utero electroporation was performed on E14.5 mouse brains with *Dcx* or *Dclk* shRNA together with GFP in 3:1 ratio. On E17.5 the mice were sacrificed and the embryos collected in L-15 (Biological Industries) supplemented with gentamycin, glucose (0.6%) and saturated with oxygen in RNase-free environment. The fluorescent area of the cortex was cut out with a razor under the fluorescent binocular and homogenized in TRI Reagent (Sigma, Israel). After addition of 0.2 ml of chloroform per 1 ml of TRI Reagent used, the samples were mixed and centrifuged at 13000 rpm for 15 min at 4°C. The upper aqueous phase was precipitated with 0.5 ml Isopropanol. The precipitated RNA was washed with 70% Ethanol, dissolved in water, and cleaned with RNeasy Mini Kit (Qiagen). The Mouse Gene 1.0 ST Array was used for Affymetrix analysis. The experiments were repeated twice, and each repeat was composed of a RNA pool derived from 4 to 6 electroporated brains. The correlation between the repeats was very high ($R^2 = 0.9955$ and 0.9925 for *Dcx* and *Dclk* shRNA conditions, respectively). Only genes that showed at least 1.9 fold-difference of expression were selected for further analysis.

PLASMIDS AND RNAi CONSTRUCTS

ATX shRNA1 and shRNA2 are pLKO.1 lentiviral shRNA constructs purchased from Open BioSystems (TRCN0000080829 and TRCN0000080830, respectively). The experiments shown in the figures are corresponding to shRNA2, but most experiments were conducted using both shRNA sequences in parallel and no differences were noted. Control shRNA was previously described (Sapir et al., 2012).

The full-length human ATX (hATX) was provided from Prof. Junken Aoki (Hashimoto et al., 2012). The full-length rat ATX (rATX) was provided from Prof. Mathieu Bollen (Jansen et al., 2005), and subcloned into pCAGGS vector using the NheI and NotI restriction sites. Site-directed mutagenesis of the catalytic domain (T210A) in hATX and rATX were performed using the primers 5′-TCCCTACATGAGGCCGGTGTACCCAA CTAAAgCCTTTCC and 5′-GCCTCTGGTGAAGAGCTCAG for hATX, and 5′-CTGTGTACCCCACAAAAgCCTTCCCTAATC and 5′-GATTAGGGAAGGcTTTTGTGGGGTACACAG for rATX. The PCR product of the mutant hATX was subcloned into full-length hATX using the EcoRI and EcoNI restriction sites. The mutant hATX contained additional mutations T241S, V279S, T294S, H298N; all of which are conserved in mouse and rat. The mutant rATX contained an additional mutation at the linker region (L581F). Plasmids were co-electroporated with a fluorescent protein. Co-electroporation of pCAGGS-GFP, pCAGGS-mCherry was performed for the *in utero* and *ex utero* electroporation experiments. Co-electroporation of pCAGGS-GFP, EF-LPL-lynGFP, Tα-LPL-GAP43-Strawberry, Tα-Cre (provided from Prof. Akira Sakakibara) and PGK-Cre was performed for the lattice culture and flow cytometry experiments. FUCCI cell cycle reporters (Sakaue-Sawano et al., 2008) were subcloned into pCAGGS.

IN UTERO ELECTROPORATION

Plasmids were transfected by *in utero* electroporation using previously described methods (Sapir et al., 2008). Briefly, E14 or E13 pregnant female ICR mice were anesthetized by intraperitoneal injection of 10% ketamine/20 mg/ml xylazine (1/10 mixture, 0.01 μl/g of body weight, i.p.), alternatively isoflurane anesthesia was utilized. The uterine horns were exposed, and plasmids (0.5–1 μl) mixed with Fast Green (2 μg/μl; Sigma) were microinjected by mouth pipette through the uterus into the lateral ventricles of embryos by pulled glass capillaries (Sutter Instruments). Electroporation was accomplished by delivering five electrical pulses (50 ms duration) at intervals of 950 ms with a square-pulse electroporator (Nepa Gene), using a platinum-plated tweezer electrodes (Protech International).

For knockdown or overexpression, a GFP expression vector with either shRNA, ATX, or mutant ATX expression vector (3:1 ratio) were used. For rescue experiments, equal amounts of ATX shRNA2 and either hATX or mutant hATX were used. Cell cycle analysis was performed by *in utero* electroporation with FUCCI reporters.

For the analysis of cell location, morphology and type, embryos were intracardially perfused using 4% paraformaldehyde–phosphate buffered saline (PFA-PBS). Brains were post-fixed overnight and sectioned (60 μm; vibrotome, Leica).

EX UTERO ELECTROPORATION

E14 embryos were removed from pregnant dams. DNA mixtures (equal concentrations as used for the *in utero* electroporation) were injected to the ventricles and electroporation was conducted by delivering five electrical pulses (50 ms duration) at intervals of 950 ms with a square-pulse electroporator (Nepa Gene), using 5-mm-diameter platinum-plated tweezer electrodes (Protech International). Brains were removed in cold L-15 (Biological Industries) supplemented with gentamycin, glucose (0.6%) and saturated with oxygen. Freshly isolated whole brains were cut into 250 μm coronal slices and then transferred onto inserts (MilliCell-CM; 0.4 μm; Millipore) floating on 1 ml of either serum-free medium (Neurobasal medium supplemented with B27, N2, GlutaMax, glucose, and gentamicin) or condition-medium (described below in cell-culture and condition media). Brain slices were cultured at 37°C and 5% CO_2 for 2 days. Half

of the media was replaced with fresh media after 24 h. Slices were fixated with 4% PFA-PBS overnight, incubated at 4°C in PBS/30% sucrose, frozen on dry ice with OCT compound and cryosectioned (10 μm; Leica CM3050S).

CELL-CULTURE AND CONDITIONED MEDIA

Conditioned media for *ex utero* experiments were prepared using HEK293 cell line overexpressing either GFP and ATX or mutant ATX. Cells were grown at 37°C and 5% CO_2 in MEM (Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 5% horse serum, B27, Glucose, GlutaMax and Gentamicin). Media was collected 2 days following calcium-phosphate transfection (Graham and Van Der Eb, 1973) of 2 μg DNA. Collected media was diluted 1:3 in fresh MEM and kept in 4°C for 2–5 days.

Microscopy

Images were taken either with wide-field microscopy with the DeltaVision system package (Applied Precision, Issaquah, WA, USA), Pannoramic MIDI scanner (3DHisthech) or by confocal microscopy (LSM510, Zeiss, LSM 780).

Analysis

Cell counts were analyzed using the spots module of Imaris software (Bitplane, Zurich, Switzerland). Intensity and circularity were measured using the ImageJ software (NIH).

Statistical analysis was conducted using Prism 5 for Macintosh (GraphPad Software, Inc.).

RESULTS

SCREENING FOR NON-CELL AUTONOMOUS FACTORS INVOLVED IN RADIAL MIGRATION

Knockdown of *Dcx* and *Dclk* was reported to impair radial neuronal migration (Bai et al., 2003; Koizumi et al., 2006; Ramos et al., 2006). In line, we could show that *Dcx* or *Dclk* knockdown impaired cell migration (**Figures 1A–F**). Although reduction of DCX induced cells to arrest with multipolar morphology (Bai et al., 2003) (**Figures 1B,E,G**), cells treated with *Dclk* shRNA exhibited bipolar morphology (**Figures 1C,F,G**). To investigate cell autonomous and non-cell autonomous effects of a particular intervention we modified a previously described approach (Bai et al., 2003). The experimental design included labeling and monitoring two distinct populations in the developing embryonic brain by consecutive electroporation. The first population was treated with shRNA (at day E13) and labeled with GFP. We have confirmed that no plasmid that is injected in the early timepoint lingered in the ventricle (data not shown). The position of the first population reflected cell autonomous effects. The second cell population was electroporated with a red fluorescent protein expression construct only a day later (E14) and reflected non-cell autonomous effects emanating from the first (green) population. *Dcx* shRNA treatment inhibited neuronal migration in a cell autonomous way (Bai et al., 2003) (control shRNA treated green cells in **Figures 1H,J** in comparison with *Dcx* shRNA treated **Figures 1K,M** quantified in **Figures 1H′,K′** respectively) as well as in a non-cell autonomous fashion (Bai et al., 2003) (dsRed labeled cells in **Figures 1I,J** in comparison with **Figures 1L,M** quantified in **Figures 1I′,L′** respectively). Likewise, *Dclk* shRNA treatment affected neuronal migration in a cell autonomous and non-cell autonomous fashion (**Figures 1N–P**). Therefore, we conclude that both *Dcx* and *Dclk* shRNA treatments affect the position of the transfected cells themselves in a cell autonomous and in addition, the transfected cells affect neighboring cells, born a day later, in a non-cell autonomous way. The distribution of the Golgi within the cell was used as a marker for its polarization. Control cells showed compact Golgi either at E17 (where more cells can be detected at the SVZ/IZ border) or E18 (**Figures 2A–F** respectively). However, cells treated with *Dcx* shRNA but also their neighboring cells displayed dispersed Golgi (**Figures 2G–I**, higher magnification **Figures 2J–L**, quantified in **Figure 2Y**), yet the Golgi appeared compact in cells treated with *Dclk* shRNA and their neighbors (**Figures 2M–O**, higher magnification **Figures 2P–R**, quantified in **Figure 2Y**). In addition, to better visualize cell autonomous effects on the Golgi, brains were co-electroporated with the corresponding shRNA, GFP and a Golgi marker. In case of *Dcx* shRNA the Golgi was dispersed (**Figures 2S–U**) and in case of *Dclk* shRNA the Golgi was compact (**Figures 2V–X**). Therefore, it was possible to visualize that *Dcx* shRNA treated cells exhibit abnormal polarity, as revealed by dispersed Golgi staining, and also the untreated neighboring cells exhibited abnormal polarity. Since both shRNA treatments affected cell migration in a non-cell autonomous fashion, while the stalled cells exhibited different states of cell polarization, we set out to identify differentially expressed genes in the cells residing in the stalling area. Areas enriched with stalled fluorescent cells were dissected out at day E17 from brains, which had been electroporated *in utero* at day E14. The extracted mRNA was converted to cDNA and subjected to Affymetrix chip analysis (scheme in **Figure 2Z**). This approach identified a few distinct genes that differed in their expression levels between the *Dcx* and *Dclk* shRNA-treated cells (**Table 1**).

Results identified 14 novel genes, most of which encode secreted or extracellular proteins, suggesting an involvement of non-cell autonomous mechanisms. Of particular interest, *Enpp2*, Ectonucleotide Pyrophosphatase/Phosphodiesterase 2, PD-Iα or lysoPLD, also known as *Autotaxin (Atx)* had a twofold expression in the bipolar *Dclk* shRNA treated neurons. This result was reconfirmed using real-time qPCR (**Figure 2AA**) and Western blot analysis (data not shown). We therefore focused on the cell autonomous and non-cell autonomous roles of *Atx* in the developing brain.

ATX IS EXPRESSED IN THE DEVELOPING BRAIN

Previous studies indicate that *Atx* mRNA is expressed in the choroid plexus and the ventricular zone (VZ) during cortical embryonic development (Ohuchi et al., 2007; Savaskan et al., 2007) (**Figure 3A**, from http://www.genepaint.org/). Immunostaining of mouse E14 brain sections using a previously characterized monoclonal antibody (Tanaka et al., 2004a), revealed strong expression in the VZ, but also in the cortical plate (CP) (**Figure 3B**). ATX protein is expressed throughout cortical development, as demonstrated by Western blot analysis (**Figure 3C**). The subcellular localization of ATX was analyzed using E15 dissociated cortical neurons. ATX was expressed by all neurons. Notably, most of the protein was localized perinuclear in vesicular structures (**Figures 3D–F,D′–F′**), and colocalized with

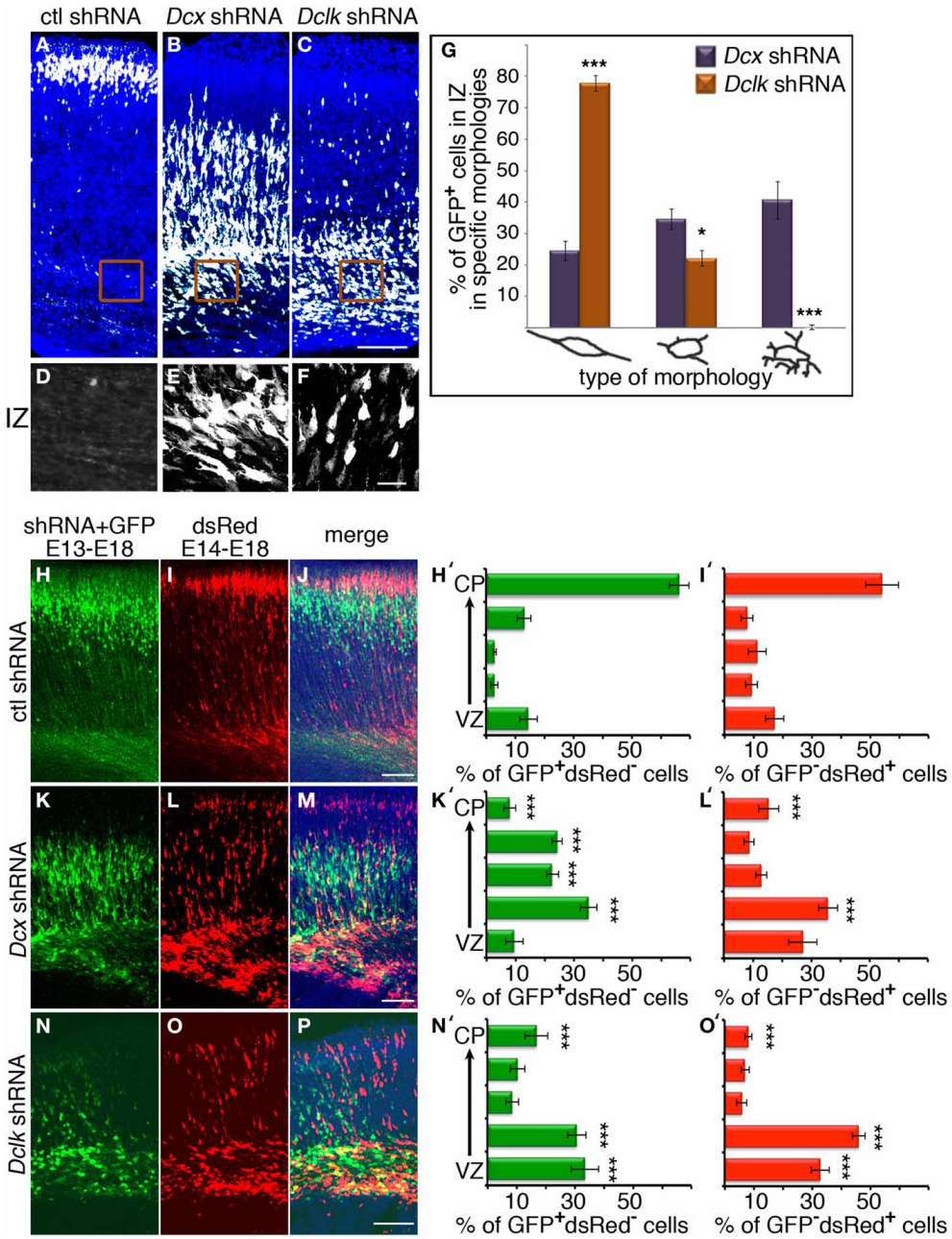

FIGURE 1 | Cell autonomous and non-cell autonomous effects of *Dcx* and *Dclk* on neuronal migration. (A–C) *Dcx* and *Dclk* impair radial neuronal migration. Brains electroporated *in utero* on E14 with control **(A)**, *Dcx* **(B)** or *Dclk* **(C)** shRNA constructs together with GFP, were analyzed on E18. Scale bar, 100 μm. **(D–F)** The morphology of cells arrested in the IZ at **(E)** with reduced DCX **(E)** or DCLK **(F)**. No cells in the IZ were observed in control shRNA. Scale bar, 20 μm. **(G)** Quantification of cells arrested in the IZ exhibiting bipolar, multipolar and highly branched multipolar morphologies from sections from four different brains of each treatment (*Dcx* and *Dclk* shRNA). *Student t-test* $^{*}p < 0.05$; $^{***}p < 0.001$. **(H–P)** Non-cell autonomous effect on migration of *Dcx* and *Dclk* shRNA. Brains were *in utero* electroporated with control shRNA **(H–J)**, *Dcx* shRNA **(K–M)** or *Dclk* shRNA **(N–P)** together with GFP on E13, followed by electroporation with dsRed on E14. The analysis was performed on E18. GFP-positive cells **(H,K,N)**, dsRed-positive cells **(I,L,O)** and merged images **(J,M,P)** are shown. Only single positive cells were counted and used for quantifications and statistical analysis. In each section the total number of cells were considered 100% and the relative % of green and red cells were calculated in relation to the same population. In the histograms the % of GFP positive dsRed negative or % of GFP negative dsRed positive cells positioned in the different bins are indicated. The statistical analysis is based on number of cell bodies that were counted in five arbitrary bins spanning the width of the cortex. The analysis was done using the Imaris©software **(H′,I′,K′,L′,N′,O′)**. Scale bar, 100 μm.

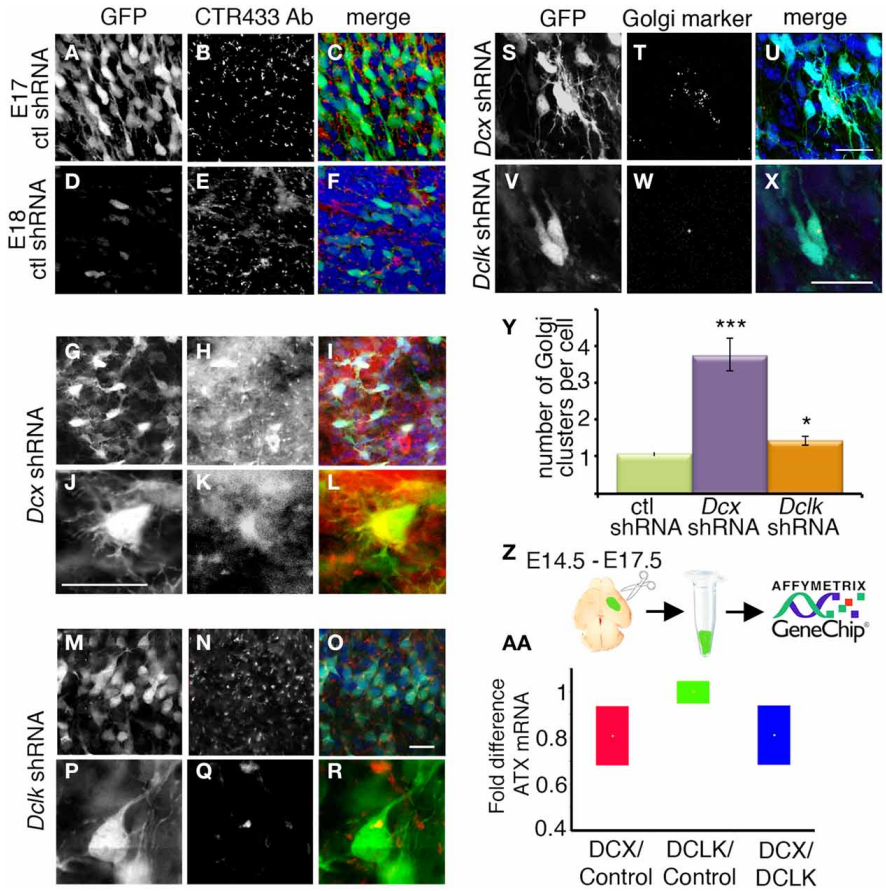

FIGURE 2 | (A–X) The organization of the Golgi apparatus in *Dcx* and *Dclk* shRNA transfected neurons stalled in the IZ. **(A–R)** Golgi organization is shown by Golgi specific immunostaining. Control **(A–F)**, *Dcx* **(G–L)** or *Dclk* **(M–R)** shRNA were electroporated brains (E14–E18) were immunostained with CTR433 Golgi antibodies. Since there are practically no cells in the area of interest (IZ) in the control experiment, an additional control was used, in which the control shRNA was electroporated from E14 to E17, when some control cells still reside in the IZ **(A–C)**. GFP **(A,D,G,J,M,P)** serves as a marker for electroporated cells. **(S–X)** Verification of Golgi organization shown by coelectroporation with a plasmid expressing a Golgi marker. *Dcx* **(S–U)** or *Dclk* **(V–X)** shRNA were coelectroporated with a Golgi marker plasmid, 82 amino acids of β1,4-Galactosyltransferase fused to mCherry. GFP **(S,V)** serves as a marker for electroporated cells. **(Y)** Quantifications of Golgi clusters per cell was performed on control (E17), *Dcx* or *Dclk* shRNA treated cells (an average of 20 cells was used for quantifications and statistical analysis) $^{*}p < 0.05$, $^{***}p < 0.001$. **(Z)** Experimental design of the Affymetrix GeneChip experiment. Embryos were electroporated *in utero* on E14; on E17 the areas where stalled electroporated cells were dissected. RNA or protein was extracted from 5 embryos per experiment. **(AA)** Real-Time PCR validation of Affymetrix Gene Chip experiment. The *Atx* mRNA levels in the *Dcx* shRNA treated brain were reduced by 40% in comparison to *Dclk* shRNA or in comparison to control shRNA in concordance with Affymetrix results. Scale bars: panels 20 μm.

the Golgi apparatus, immunostained with CTR433 antibodies (**Figures 3E,E′**). Part of the protein was noticed in the growing neurites.

ATX AFFECTS CELL ADHESION IN THE VENTRICULAR ZONE

To examine the effect of ATX reduction in the developing brain, we *in utero* electroporated brains with either *Atx* shRNA or control shRNA at E13 and examined them at E14 (**Figures 4A–F**). Real-time PCR indicated that the shRNA reduced *Atx* mRNA levels to 30.8 ± 5.2% ($n = 3$ ±S.D.) in comparison with control. Cells with reduced ATX levels demonstrated distorted morphology, most of the cells were round, and in some cases the endfeet were not tethered to the apical aspect of the ventricular zone (**Figures 4E,F**). These results were recapitulated when brains were *in utero* electroporated at E14 and analyzed at E15 (**Figures 4G–V**). To gain additional information regarding the observed phenotype, the brain sections were immunostained with the apical markers Numb, ZO-1 and Par6. As expected, these proteins were apical in control brain sections, however, they displayed abnormal positioning in shRNA treated sections. Immunostaining of treated brain sections with β-catenin and Par3 antibodies did not reveal any changes in the localization of these proteins in the ATX knockdown brains (data not shown). Cellular morphology and the proper positioning of Numb, ZO-1 and Par6 were largely restored following the addition of human ATX expression construct, which is resistant to the shRNA (**Figures 4M,Q,U**). Surprisingly, introduction of the catalytically inactive human ATX expression construct was able to restore these observed phenotypes (**Figures 4N,R,V**). The measured circularity index statistically differed from control

Table 1 | The analyzed results of the Affymetrix experiment.

Gene name	Fold difference	
(A) UNKNOWN GENES: HIGHER TRANSCRIPTION LEVEL IN *Dclk* shRNA TREATED EMBRYOS		
ENSMUSG00000074558	4.42	Predicted gene encoding protein with 5 TM domains, a member of ENSFM00360000113264 gene family
ENSMUSG00000074562	9.51	Predicted gene encoding protein with 5 TM domains, a member of ENSFM00360000113264 gene family
ENSMUSG00000074566	9.15	Predicted gene encoding protein with 5 TM domains, a member of ENSFM00360000113264 gene family
ENSMUSG00000075014	4.31	Predicted gene encoding protein with 5 TM domains, a member of ENSFM00360000113264 gene family
ENSMUSG00000058736	3.29	Putative gene encoding secreted peptide
(B) GENES WITH HIGHER TRANSCRIPTION LEVEL IN *Dclk* shRNA TREATED EMBRYOS		
Ctsc	2.02	Cathepsin C
Enpp2	1.95	Ectonucleotide pyrophosphatase/phosphodiesterase 2
Ifitm3	2.70	Interferon induced transmembrane protein 3
Serping1	2.10	Serine (or cysteine) peptidase inhibitor, clade G, member 1
Ttr	7.02	Transthyretin
(C) GENES WITH HIGHER TRANSCRIPTION LEVEL IN *Dcx* shRNA TREATED EMBRYOS		
Gcg	2.04	Glucagon
Penk	2.05	Preproenkephalin
Tcfap2d	2.30	Transcription factor AP-2, delta
Zfp125	2.47	ZT2 gene encoding zinc finger protein 125

The genes with different transcription level between Dcx and Dclk shRNA electroporated brain regions are divided into 3 categories: unknown genes (A), known genes with higher transcription level in Dclk shRNA condition (B), and known genes with higher transcription level in Dcx shRNA condition (C). Averages for fold differences of 2 biological repeats are shown.

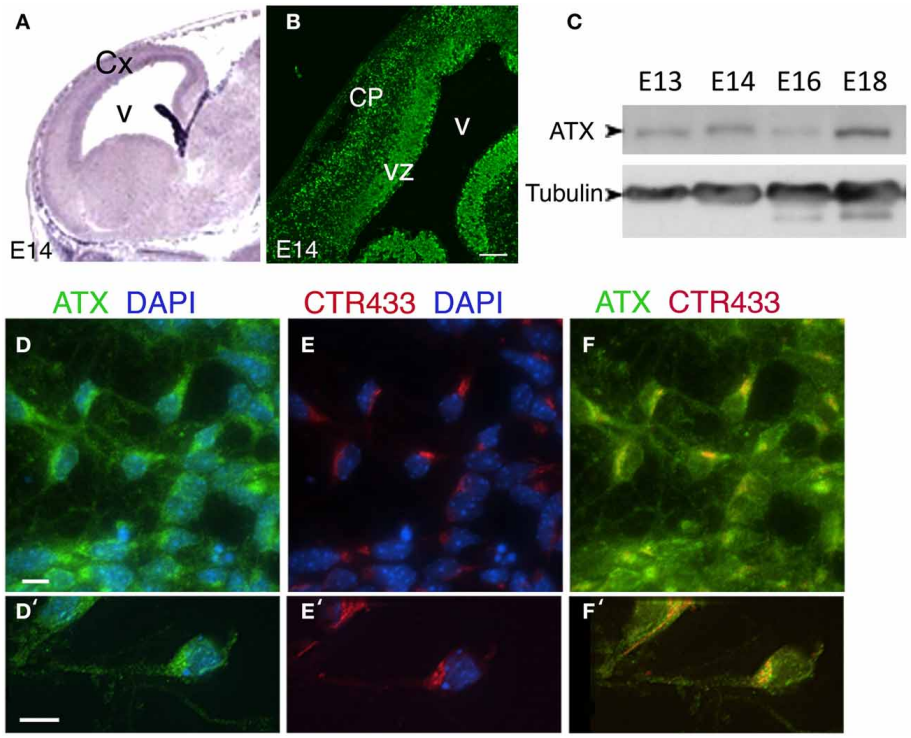

FIGURE 3 | Expression and localization of ATX in the developing mouse cortex. (A) *In situ* hybridization of *Atx* in the developing mouse cortex at E14 (from http://www.genepaint.org/). **(B)** Coronal cryosections of the mouse embryonic cortex at E14 immunostained for ATX. **(C)** ATX levels in cortical lysates at different developmental stages. α -tubulin was used as a loading control. **(D–F′)** Cultured cortical neurons isolated from E14 mouse cortices were grown 3 DIV, immunostained for ATX (green) **(D–F, D′–F′)** and the Golgi marker CTR433 (red) **(E,E′)**, and counterstained with DAPI. Scale bars: **(A,B)** 100 μm, **(D′–E)** 10 μm. CP, cortical plate; Cx, cortex; V, ventricle; VZ, ventricular zone.

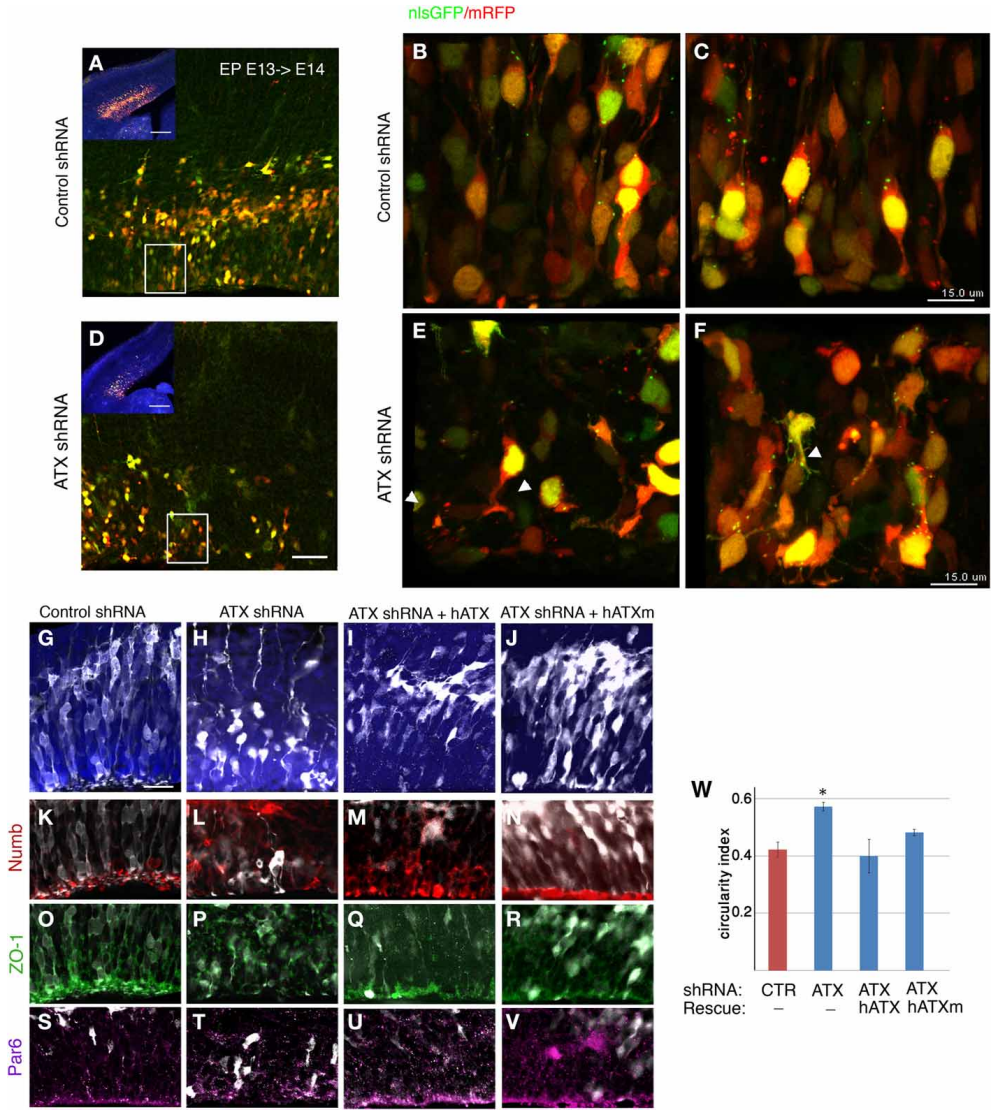

FIGURE 4 | ATX knockdown affects the radial progenitors adhesion and VZ polarity. (A–E) coronal sections from E14 electroporated brains. E13 embryos co-electorporated with control shRNA, dsRed and NLS-GFP **(A–C)** or with *Atx* shRNA **(D–F)**. Lower magnification is shown in **(A,D)**. White rectangle in panels **(A,D)** indicate area magnified in **(B)** and **(E)** respectively. White arrowheads point at endfeet that are not tethered to the apical surface of the VZ following ATX shRNA introduction. **(C–H)** ATX knockdown impairs polarity at the VZ and affects organization and morphology of cells. **(G–V)** E15 brains that had been electroporated at E14 are depicted. Whereas in the control brains **(G)** most of the cells (white, GFP) exited the VZ, upon ATX knockdown **(H)** cells were generally reside within the VZ. ATX knockdown distrups the structure of the VZ. ATX knocked-down cells exhibited a long and crooked radial process, and are much rounded than control cells. These knockdown effects were rescued with the co-electroporation of the *Atx* shRNA and human ATX cDNA (hATX), which is resistant to the Atx shRNA **(I)**. Partial rescuing effect was seen with the co-electroporation of a mutated non-catalytic human ATX (hATXm, **J**). Immunolabeling of the apical polarity markers Numb **(K–N)**, ZO-1 **(O–R)** and Par6 **(S–V)**, demonstrated a loss of polarized localization of these protein in the ATX knockdown brains. The localization was restored upon introduction of hATX as well as non-catalytic ATX. **(W)** Changes in cellular roundness were measured and analyzed using the circularity index. Data are presented as mean±SEM; $n = 3$ brains for each condition. $^*p < 0.05$ (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test). Scale bars: **(A,D)** (insert) 200 μm, **(A,D)** 50 μm, **(C,F)** 15 μm **(G)** 25 μm, **X**(upper panel) 50 μm, **X**(lower panel), 25 μm.

only with the sole addition of *Atx* shRNA (**Figure 4W**), while the addition of ATX or mutant ATX resulted in an elongated morphology and the recurrence to control circularity levels. To confirm the results obtained by knockdown experiments we used a genetic model. We therefore examined the cellular localization of several proteins in floxed *Atx* mice deleted with *Emx1*-drived Cre. Adherens junctions were immunostained using phosphorylated FAK antibodies (**Figures 5A–B′**). Images were acquired from thick sections show that adherence junctions form from the ventral side of the ventricular zone in control brains, while in ATX depleted brains adherens junctions were somewhat distorted, recapitulating our findings in

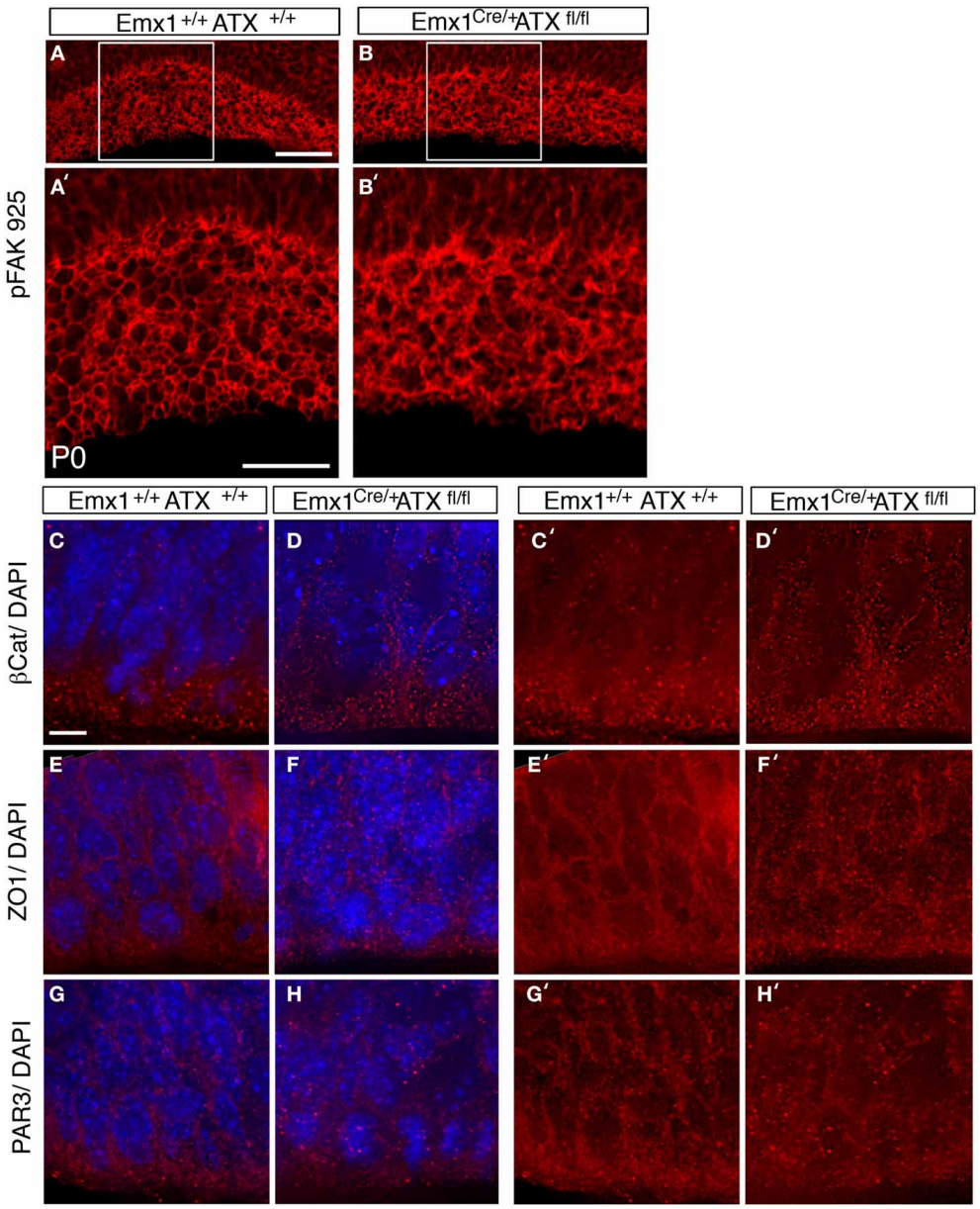

FIGURE 5 | ATX knockout have subtle effects on adherence junctions in the apical VZ. (A–B′) Disruption in pFAK 925 immunostaining is observed in ATX knockout mouse. **(B,B′)** when compared to floxed allele carrier, non-deleted littermate at P0 **(A,A′)**. **(C–H′)** Staining with adherence junction markers, βCatenin **(C–D′)** ZO1 **(E–F′)** and with the apical polarity marker Par3 **(G–H′)** in brains of E14 wt **(C,C′,E, E′,G,G′)** and mutant littermates **(D,D′,F,F′,H, H′)**. All three antigens accumulated at the cell–cell contact sites in both wt and mutant brains sections. Size markers: **(A)** 50 μm, **(A′)** 25 μm **(C)** 3 μm.

the acute knock down experiments. Additional immunostainings with β-catenin (**Figures 5C–D′**), ZO1 (**Figures 5E–F′**) and Par3 (**Figures 5G–H′**) did not reveal very striking differences. To further explore the adhesion junctions following *in utero* electroporation, we conducted electron microscopy analysis on sections from treated brains (**Figures 6A–C**). The presence of adherens junctions is obvious in the control shRNA and *Atx* shRNA sections (**Figures 6A,B**, marked with red arrowheads). Quantitative analysis revealed a slight reduction in the density of the adhrens junctions in the *Atx* shRNA treated sections (**Figure 6C**). A possible effect on cellular adhesion may involve proteins such as N-cadherin and E-cadherin that are normally accumulated at the ventricular surface. N-cadherin is known to regulate neuronal migration as well as ventricular structures (Kawauchi et al., 2010; Jossin and Cooper, 2011). Therefore, we examined the possibility that Atx knockout neurons exhibit neuronal migration deficits (**Figures 6D–I**). *In utero* electroporation of a GFP expression plasmid at E14 and analysis at E18 of wildtype embryos (Emx1-Cre negative) (**Figure 6D**), heterozygote for the floxed allele (Emx1-Cre positive Atx1 fl/+), and homozygote for the floxed allele (Emx1-Cre positive Atx1 fl/fl), exhibited no obvious differences (quantified in **Figure 6G**). The position of CTIP2 positive cells,

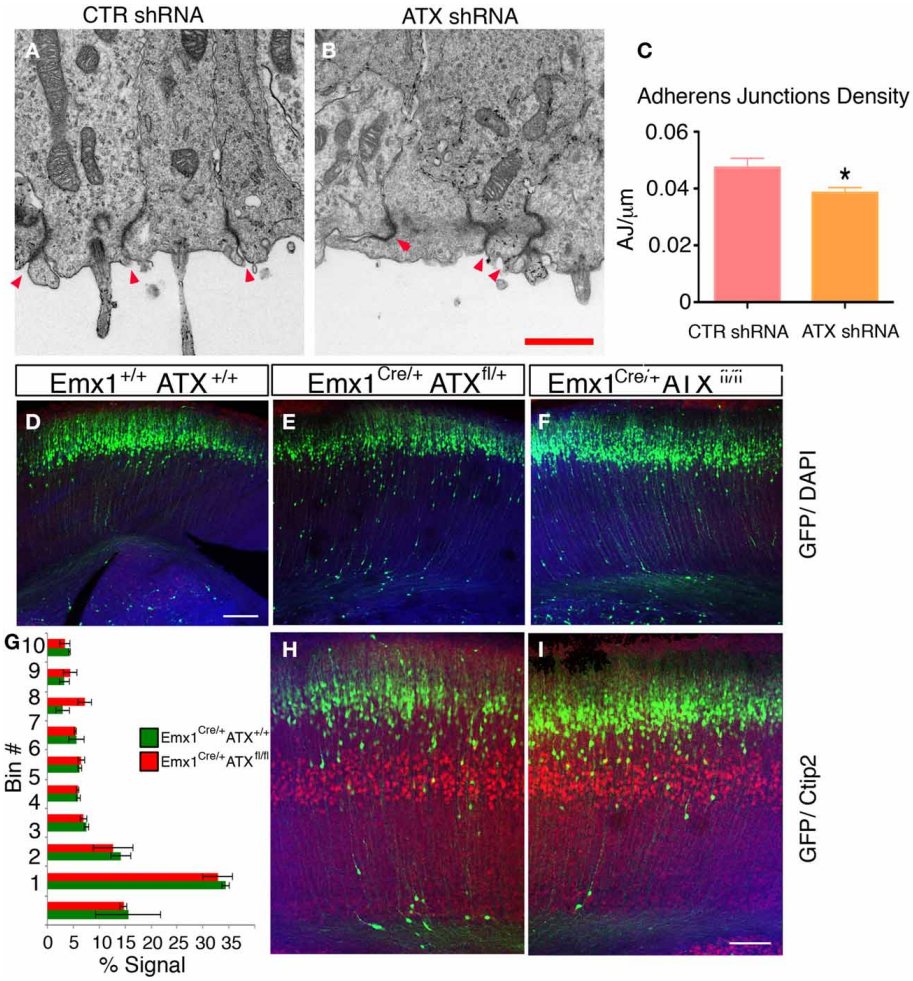

FIGURE 6 | (A–C) ATX Knockdown has minor effect on adherence junctions density in the apical aspect of the ventricular zone. **(A,B)** Representative electron micrographs of ventricular zones of Control shRNA **(A)** or ATX shRNA **(B)** treated brains. The sections were obtained from E14 brains, electroporated a day earlier. The areas imaged were identified as electroporated by a GFP signal (not shown), prior to preparation for imaging. Adherence junctions are the dense areas decorating the border between adjacent cells, red arrowheads **(C)** Adherence Junctions density as measured from (7 to 15) electron micrographs recorded from two brains per treatment, $^*p < 0.05$. **(D–I)** ATX Knock out embryos do not display a neuronal migration phenotype. **(D–F)** littermates of indicated genotypes were electroporated *in utero* at E14 with GFP expressing plasmid and analyzed 4 days later. The location of the GFP+ cells did not differ in WT **(D)** heterozygous **(E)** or ATX knockout embryos **(F,G)** The distribution of GFP+ cells along 10 arbitrary bins spanning the width of the cortex in mutant (Red) and WT (green) embryos are presented. The shown results are averaged from 3 to 4 brains. **(H,I)** Deeper layers neurons (Ctip2+, layer V) are normally layered in WT **(G)** and Knockout **(I)** E18 embryos. Size markers **(F)** 100 μm, **(H)** 1 μm.

which label layer 5, did not differ between the heterozygotes and the mutant mice (**Figures 6H–I**). Overall, our results suggest that ATX affects cell adhesion in the ventricular zone and this activity is in part not dependent upon its enzymatic activity.

ATX AFFECTS CELL POSITIONING IN THE VENTRICULAR ZONE IN A CELL AUTONOMOUS AND NON-CELL AUTONOMOUS WAY

During the analysis of brain sections of *Atx* shRNA treated brains, we noted that the position of the knocked down cells differed from the control (**Figure 4**). This observation was strengthened using both *ex utero* and *in utero* electroporations (**Figure 7**). We questioned whether the positioning of the cells can be rescued in a non-cell autonomous way. To answer this question *ex vivo,* mouse brain were electroporated *ex utero*, the brain were sectioned, and conditioned media collected from HEK293 cells transfected with either wild type ATX expression construct, or a catalytically inactive mutant, was added to the sectioned brains. The position of *Atx* shRNA treated cells differed from the control in a significant manner (**Figures 7B,B′**) vs. (**Figures 7A,A′**), (2/10 bins $p < 0.01$, 1/10 bins $p < 0.05$; $n \geq 3$ brains for each condition. ANOVA analysis followed by Dunnett's multiple comparison test). Cell positioning was significantly restored using either wild-type ATX (**Figure 7C**) or mutant ATX (**Figure 7D**). The mutant protein was somewhat less effective than the wild-type, but the two treatments did not differ in a statistically significant manner. Both proteins were expressed at similar levels (**Figure 7E**), and the protein was not degraded during the 24 h incubation with the brain slice (**Figure 7E′**). Next, we questioned whether expression of ATX in

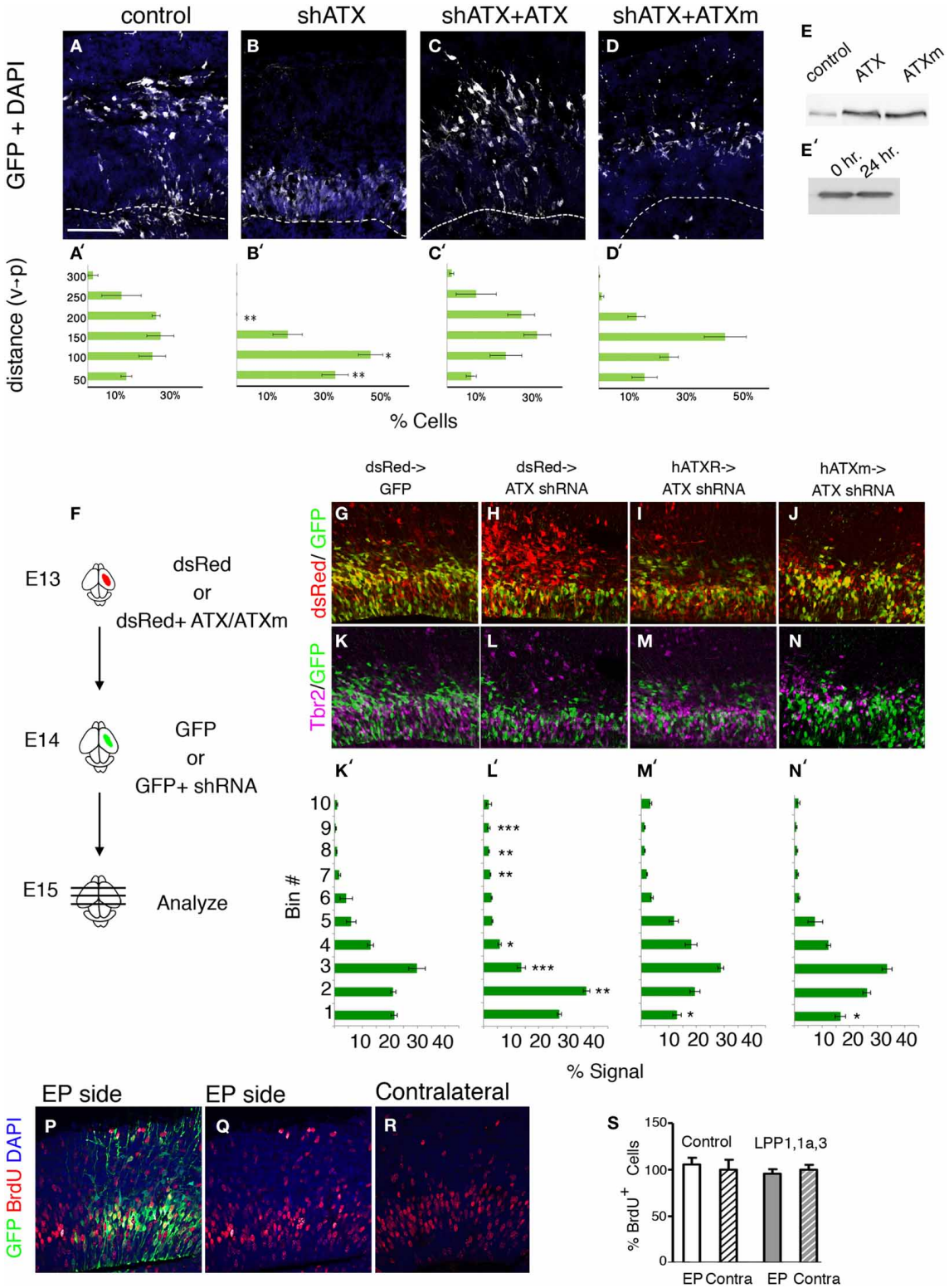

FIGURE 7 | ATX non-cell autonomous, non-catalytic activity. (A–D′) E14 mouse embryos were subjected to *ex utero* electroporation with a GFP expression plasmid together with either a control shRNA or *Atx* shRNA. Coronal sections of brains were kept for 2 DIV with condition media. **(B)** Ectopic positioning of cells within the VZ/sVZ can be rescued by addition of external ATX **(C,D)**. **(C–D′)** External addition of ATX **(C,C′)** or mutated ATX (ATXm, **D,D′**) restores normal distribution of knocked-down cells **(B,B′)**. **(E)** ATX constructs were expressed in HEK 293 cells, and media were collected. Albeit ATX is expressed endogenously and secreted (control transfection),

(Continued)

FIGURE 7 | Continued
transfection of rat ATX or a mutated non-catalytic rat ATX (ATXm) lead to distinctly higher levels of ATX in the media. **(E′)** Western blot of media containing secreted ATX showing that ATX is stable when incubated with brain slices for 24 h. **(F–N) (F)** Experimental design: consecutive electroporation done at E13 and E14. dsRed expressing plasmid was electroporated with or without cDNA encoding human ATX (hATX) or calytically inactive ATX (hATXm) at E13. One day later, GFP alone or GFP and shRNA targeting ATX were injected to the same embryos. **(G–N′)** 24 h after the second injection the brains were collected for analysis. Representative sections from control brains **(G,K)** ATX shRNA treated brains **(H,L)** and brains pretreated with hATX **(I,M)** or mutated hATX **(J,N)** were stained with Tbr2 **(K–N)** and DAPI. **(K′–N′)** Signal recorded along the width of the cortex showing the dispersion of cells along the radial aspect of the cortex in arbitrary bin. Smaller numbered bins are apical. ($n = 3$). **(P–S)** LPP overexpression do not affect progenitors postionining during S phase. LPP1/LPP1a/LPP3 expressing or control vectors where coelectroporated with GFP plasmid *in utero* at E14 and analyzed 24 h later. BrdU labeling was done 1 h prior to the analysis. **(Q–R)** The position of BrdU positive cells in the electroporated (EP side, **Q**) BrdU positive cells on the electroporated side as well as in the non electroporated hemisphere (contralateral, **R**) are shown. **(S)** The relative percentage of BrdU positive, GFP positive cells in the electroporated and contralateral cortical hemispheres is plotted. Size markers: **(A)** 100 μm. Statistical analysis, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

earlier born cells can rescue the position of later born cells in which ATX levels were reduced. We have again preformed a consecutive electroporation in which ATX cDNA (as well as mutated ATX or dsRed alone) was introduced to the ventricular zone 1 day prior to the injection of the shRNA. We have presumably allowed the cells to express and secrete the protein prior to the reduction of the mRNA levels in the next wave of proliferating neuroblasts (a scheme is shown in **Figure 7F**, representative images in **Figures 5G–M**). We later quantified the location of both populations and found that both ATX expression constructs had similar rescue effects regardless of their catalytic activity (**Figures 7M′,N′** in comparison with **Figure 7L′**). The position of the cells in the ATX shRNA treated brains differed in a statistically significant manner from control in 6 out of 10 arbitrary bins along the width of the cortex (One-Way ANOVA, Dunn's multiple comparison test, $p < 0.05$). The non-cell autonomous rescue experiments did not differ from the control in 9 out of 10 bins, suggesting that the rescue was almost complete. Collectively, these data suggest that ATX regulates cell positioning in the ventricular zone in a non-cell autonomous manner.

Since re-expression of enzymatic deficient ATX was able to rescue cell positioning in VZ, we validated this finding by analyzing the effect of decreased LPA (the synthesis product of enzymatic ATX activity) on VZ neurogenesis. Local LPA concentrations are on the one side controlled via the synthetizing enzyme ATX and on the other side via dephosphorylating enzymes like the LPPs. We therefore electroporated LPP1/LPP1a/LPP3 expressing or control vectors in the VZ of the lateral ventricle wall at E14 and analyzed the pups after 24 h (**Figures 7P–S**). Neurogenesis was assessed using BrdU 1 h prior to dissection. Quantitative assessment of BrdU -positive cells on the electroporated side as well as in the non electroporated hemisphere revealed no significant difference after electroporation of the control or the LPP1/LPP1a/LPP3 expressing vectors, respectively, corresponding to the catalytic-independent functions of ATX. In addition, there was no obvious difference in the position of transfected cells and/or BrdU labeled cells in the transfected or non-transfected side of the brain.

In the ventricular zone, the position of the cell nucleus is tightly linked with cell cycle progression. Furthermore, disruption of the VZ polarity may result in cell cycle defects and interference with neuronal differentiation. Based on our finding of abnormal VZ polarity following knockdown of ATX, we reasoned that ATX might influence cell cycle and proliferation of neuronal progenitors. The effect of *Atx* knockdown on neuronal proliferation in the developing cortex of the mouse, was examined using modified fluorescence ubiquitination cell cycle indicators (FUCCI) (Sakaue-Sawano et al., 2008) (**Figures 8A–D**). The short-lived fluorescent proteins allow visualizing G1 (red), G1 to S transition (yellow, simultaneous expression of the red and the green fluorescent proteins) and S,G2,M (green) (**Figures 8E,F**). FUCCI cell cycle reporter plasmids were introduced into E13 developing brains together with either control or *Atx* shRNA. Analysis at E14 revealed that *Atx1* knockdown did not change the percentage of cells in the different stages of the cell cycle in a significant manner (green cells 29 ± 3.1 vs. 34 ± 4.1, red cells 63.1 ± 3.3 vs. 56.7 ± 4.0, and yellow cells 7.8 ± 1.6 vs. 9.2 ± 1.8, in control and *Atx* shRNA treatments respectively, $N = 8$, *Student t-test*). Nevertheless, the position of the different colored cells differed significantly, as can be observed in the representative images (**Figures 8A,C**). Quantification detected a statistical significant difference in the basal position of cells in G1 (red cells) ($p < 0.001$, $N = 8$, One-Way ANOVA). Polarity at the VZ, which is known to regulate differentiation, was disorganized following ATX depletion. In addition, *Atx* shRNA treated cells were localized within the VZ and displayed a long radial process, a feature of radial glial progenitors. Therefore, we hypothesized that ATX knockdown influences the decision of radial glial to switch from a self-renewing proliferative mode to a differentiation mode. We analyzed the above described using *ex utero* experiments and immunostaining with the postmitotic neuronal marker β-III tubulin (Tuj1). In comparison with control treated cells (**Figure 8G**), ATX knocked-down cells were more abundant in the VZ and were rarely noted in the IZ (**Figure 8H**). The percentage of post mitotic Tuj1+GFP+ cells was significantly lower in the *Atx* shRNA treated cells in comparison with control shRNA treated cells (**Figures 8G′–J′** quantified in **Figure 8K**) ($p < 0.05$; $n \geq 3$ brains for each condition. ANOVA followed by Tukey's HSD test). External addition of either catalytic ATX (**Figures 8F,H**) or non-catalytic ATX (**Figures 8I,J**) restored both the localization of the cells as well as the relative percentage of Tuj1+ transfected cells. Compared to the addition of either catalytic or non-catalytic ATX, the percentage of Tuj1+ transfected cells was significantly lower in the ATX knocked-down cells (**Figures 8H,K**) ($p < 0.05$; $n \geq 3$ brains for each condition. ANOVA followed by Tukey's HSD test). Collectively, these experiments demonstrated that ATX affects cell positioning and neuronal differentiation in the ventricular zone.

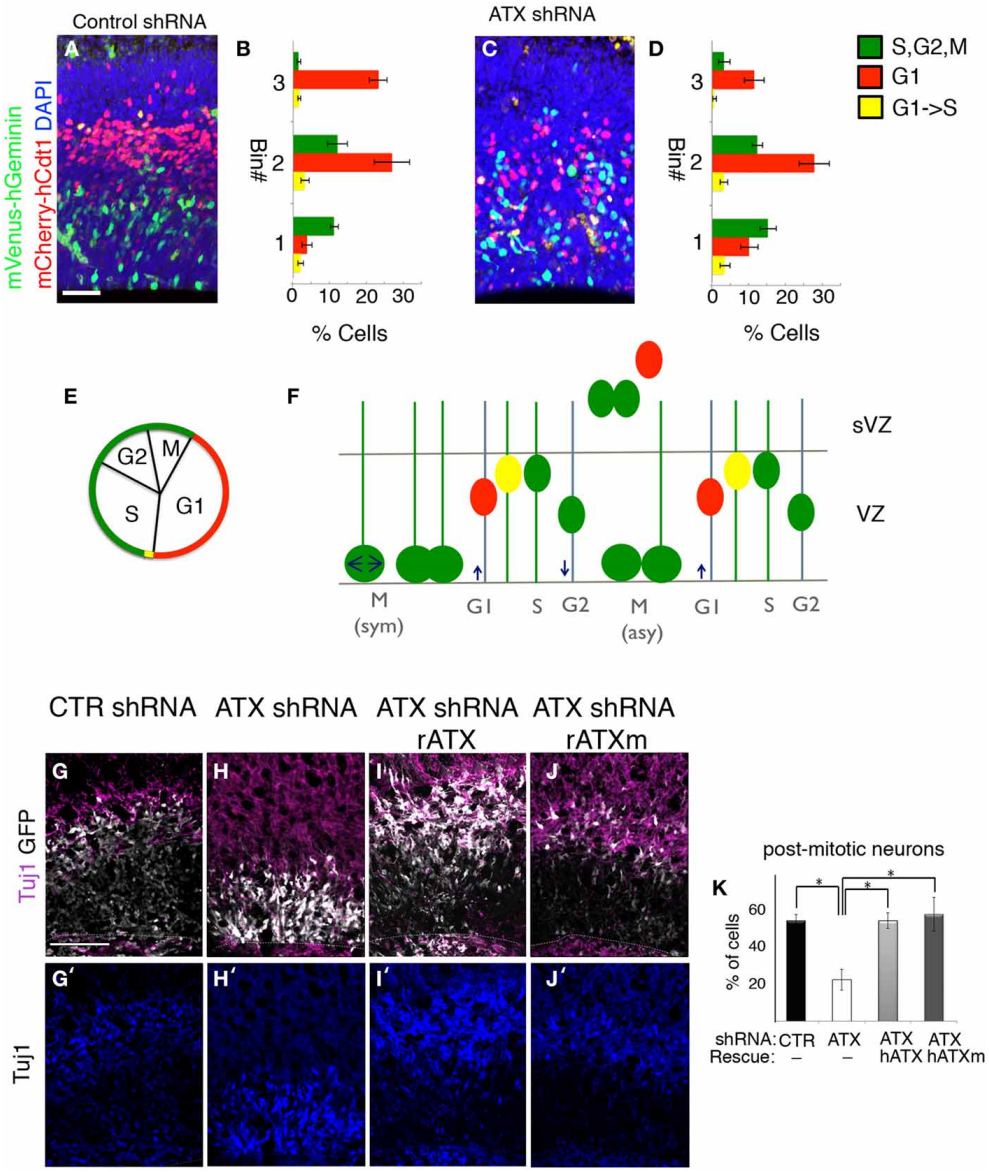

FIGURE 8 | ATX knock down interferes with progenitors differentiation. **(A–D)** Fucci markers (mCherry-hCtd1, mVenus-hGeminin) were electroprated at E13 with control **(A,B)** or ATX shRNA **(C,D)**. Brains were analyzed 24 h later. The location of red cells (G1) Green cells (S,G2,M) and yellow cells (G1->S) was recorded in three arbitrary bins along the width of the cortex. **(E)** Cell cycle correlates with FUCCI markers expression. **(F)** Interkinetic nuclear movement in the ventricular zone (VZ), and cell exiting to the subventricular zone (sVZ) would accumulate the FUCCI markers and alternate colors in correlation with cell cycle. **(G–K)** E14 mouse embryos were subjected to *ex utero* electroporation following organotypic slice culture in condition media, as described earlier. Differentiated post-mitotic neurons with Tuj1 (purple) shows higher colocalization of treated cells (GFP, white) and Tuj1 incontrol cells **(G,G′)** compared with ATX knocked-down cells **(H,H′)**. External addition of both rat catalytic and non-catalytic ATX (rATX, rATXm) restored colocalization of Tuj1 and treated cells **(I–K′)**. **(K)** Statistical analysis, $^*p < 0.05$.

DISCUSSION

Our unbiased screen for molecules, which participate in non-cell autonomous regulation of neuronal migration revealed *Atx* as a molecule, which is differentially expressed and allowed us to uncover unexpected roles of this molecule in the developing brain. Our results depict a role for ATX in regulation of cell adhesion and cell positioning in neuronal progenitors located in the ventricular zone of the cerebral cortex. We have shown that these activities are (1). cell-autonomous, since the knockdown of *Atx* affects the target cells, but also (2). non-cell autonomous, since knocked down cells could be rescued by addition of external ATX, or by ATX produced by neighboring cells. Remarkably, we observed that the enzymatic activity of ATX was not required to rescue the observed phenotypes. These findings were unexpected since studies in knockout mice revealed that ATX is the major LPA-producing enzyme *in vivo*

(Van Meeteren et al., 2006). LPA has been found to affect neural stem cell viability, differentiation and proliferation (Kingsbury et al., 2003; Dottori et al., 2008; Frisca et al., 2013). Nevertheless, previous studies have implicated possible non-catalytic functions for ATX. ATX has been found to induce lung epithelial cell migration *in vitro* through both catalytic-dependent and -independent pathways (Zhao et al., 2011). In addition, ATX promotes changes in cellular adhesion to the extracellular matrix, thereby inducing morphological remodeling in differentiating cultured oligodendrocytes and in CHO-K1 cells which express the P2Y(12) receptor (Fox et al., 2004; Dennis et al., 2008, 2011). Even though our findings emphasize that the role of ATX in neuronal progenitors is predominantly catalytic-independent, a catalytic role of ATX should not be excluded. LPA has essential roles in cortical development, therefore reduced LPA production, due to ATX depletion in neuronal progenitors, might be compensated by other genes involved in LPA homeostasis. Several LPA-regulating genes are expressed in the developing cortex, including phosphatases that degrade LPA such as LPP1 and LPP3 (Giraldi-Guimaraes et al., 2004; Escalante-Alcalde et al., 2007, 2009). Notably, we have shown that increased expression of LPP1, 1a or LPP3 did not affect the proliferation or position of neuroblasts in the ventricular zone. In addition, there are enzymes that produce LPA from different precursors (such as secreted PLA2, Yoshihara et al., 1992; Forlenza et al., 2002; Kurusu et al., 2008). Knockdown of ATX might alter the activity of these genes, thus maintaining normal LPA concentrations. Alternatively, LPA could be supplied from a non-cortical source. In the *ex vivo* experiment, LPA was provided from the cell culture medium. *In vivo,* ATX is highly expressed in the choroid plexus and secreted to the cerebral spinal fluid (CSF) (Sato et al., 2005; Zappaterra et al., 2007). Knockdown of neuronal progenitor-driven ATX could impair both catalytic-dependent and -independent functions. Nevertheless, it should be noted that in our experimental system, CSF-driven ATX did not compensate for the catalytic-independent activities, and therefore effects were observed. However, we cannot exclude the possibility that the LPA derived from the CSF diffuses into the cortex and is sufficient to compensate for the lack of catalytic activity of progenitor-driven ATX.

We uncovered a role for ATX in the regulation of neuronal progenitors. Depletion of ATX disrupted VZ adhesion and polarity establishment. This was documented by the non-polarized expression of several apically-localized proteins and impaired rounded morphology of cells. In addition, we observed proliferation defects and alteration of the cell cycle. Precisely how ATX participates in neuronal progenitor regulation remains to be clarified. We propose that the principal role of ATX is in regulating cellular polarity and attachment to the apical membrane. Changes in proliferation and neurogenesis may stem from altered VZ polarity. Normal adhesion to the apical membrane results in proper cell cycle of progenitors, and the proliferative or neurogenic divisions ensue. Following reduction in ATX levels, adhesion to the apical membrane is diminished. Several lines of evidence established a link between polarity at the VZ, cell cycle progression and cell fate decisions. Adherens junctions act as a self-supporting stem cell niche that maintains cells in a proliferative state (Song et al., 2002; Lien et al., 2006; Stocker and Chenn, 2009; Zhang et al., 2010). Disrupting the maintenance of adherens junctions impairs the Wnt pathway, shortens cell cycle and induces early neuronal differentiation. Both the apically localized Numb and β-catenin are negatively correlated with neuronal differentiation; that is, their constitutive expression results in decreased differentiation, and their reduction leads to decreased cell proliferation (Reiner et al., 2012). Likewise, the PAR complex is positively associated with maintaining a proliferative fate (Cappello et al., 2006; Costa et al., 2008; Bultje et al., 2009). Interkinetic nuclear movement is regulated by the VZ polarity, tightly associated with cell cycle control, and could couple polarity and cell fate decisions (Reiner et al., 2012). We propose that the role of ATX in neuronal progenitors relies on this coordination between polarity at the VZ and cell cycle progress. How is ATX involved in establishment of cellular polarization? Generation of polarity usually requires a signal, which is mediated by a gradient. However, we added external ATX to *Atx* shRNA transfected brains, where the external ATX was distributed equally in the medium. Therefore, ATX might function as a permissive regulator of polarity. Alternatively, ATX might bind to proteins that have polarized distribution and thereby regulate polarity in an instructive manner. Recent studies uncovered that ATX localizes to specific areas in the cell, through binding to either purinergic receptors (Dennis et al., 2011; Zhao et al., 2011) or cell surface integrins (Fulkerson et al., 2011; Hausmann et al., 2011). The interaction with integrins is mediated through the N-terminal somatomedin B-like domain of ATX, while interaction with the P2Y(12) ADP receptor is mediated through the C-terminal part of ATX. These interactions would allow ATX to function in a polarized fashion. In conclusion, this study presents ATX as a crucial regulator of neuronal progenitors. We suggest that ATX regulates polarity, mainly through a catalytic-independent mechanism, and thus influences cell adhesion, positioning and differentiation. ATX was scarcely studied in cortical development, and we hope that future studies will shed light on the underlying mechanisms through which ATX regulates development of the cortex.

AUTHOR CONTRIBUTIONS

RG, AG, TS, JB, VA, JA, RN, JV and OR were involved in design of the study and writing of the manuscript.

RG, AG, TS, SL, JB, VZ and MS, participated in conducting the experiments, collecting and analysing the data and writing of the experimental results.

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Proteoglycans and neuronal migration in the cerebral cortex during development and disease

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Chondroitin sulfate proteoglycans and heparan sulfate proteoglycans are major constituents of the extracellular matrix and the cell surface in the brain. Proteoglycans bind with many proteins including growth factors, chemokines, axon guidance molecules, and cell adhesion molecules through both the glycosaminoglycan and the core protein portions. The functions of proteoglycans are flexibly regulated due to the structural variability of glycosaminoglycans, which are generated by multiple glycosaminoglycan synthesis and modifying enzymes. Neuronal cell surface proteoglycans such as PTPζ, neuroglycan C and syndecan-3 function as direct receptors for heparin-binding growth factors that induce neuronal migration. The lectican family, secreted chondroitin sulfate proteoglycans, forms large aggregates with hyaluronic acid and tenascins, in which many signaling molecules and enzymes including matrix proteases are preserved. In the developing cerebrum, secreted chondroitin sulfate proteoglycans such as neurocan, versican and phosphacan are richly expressed in the areas that are strategically important for neuronal migration such as the striatum, marginal zone, subplate and subventricular zone in the neocortex. These proteoglycans may anchor various attractive and/or repulsive cues, regulating the migration routes of inhibitory neurons. Recent studies demonstrated that the genes encoding proteoglycan core proteins and glycosaminoglycan synthesis and modifying enzymes are associated with various psychiatric and intellectual disorders, which may be related to the defects of neuronal migration.

Keywords: chondroitin sulfate, extracellular matrix, heparan sulfate, neuronal migration, proteoglycan

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Abbreviations: BDNF, brain-derived neurotrophic factor; C4-ST, chondroitin 4-*O*-sulfotransferase; C6-ST, chondroitin 6-*O*-sulfotransferase; CHPF, chondroitin polymerization factor; CHSY, chondroitin synthase; CSGalNAcT, chondroitin sulfate *N*-acetylgalactosaminyltransferase; E, embryonic day; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GalNAc 4S-6ST, *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase; GlcA, glucuronic acid; GDNF, glial cell line-derived neurotrophic factor; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine: GPI, glycosylphosphatidylinositol; ECM, extracellular matrix; HAS, hyaluronan synthase; Hh, hedgehog; HS, heparan sulfate; H2ST, heparan sulfate 2-*O*-sulfotransferase; H3ST, heparan sulfate 3-*O*-sulfotransferase; H6ST, heparan sulfate 6-*O*-sulfotransferase; HS C5-EP, heparan sulfate C5-epimerase; IdoA, iduronic acid; LAR, leukocyte common antigen-related phosphatase; NDST, *N*-deacetylase/*N*-sulfotransferase; NGF, nerve growth factor; NT, neurotrophin; ptc, patched; PHF, plant homeodomain finger; PHD, plant homeodomain; Sema, semaphorin; UST, uronyl 2-*O*-sulfotransferase; Xyl, xylose.

Introduction

The extracellular matrix (ECM) is a complex network of molecules composed of proteoglycans, hyaluronic acid, fibrous proteins, and various glycoproteins, which fills up the extracellular space within all tissues and organs (Mouw et al., 2014). The ECM also retains water and ions, and constitutes the direct environment surrounding cells, in which multiple types of molecules are cross-linked to each other through protein-protein and protein-carbohydrate interactions, forming the three-dimensional meshworks (**Figure 1**). The ECM serves not only as a physical scaffold for tissue construction, but also as a dynamic field of signaling that regulates the behavior of cells. In the meshwork of ECM, various signal molecules such as growth factors and chemokines are stored, and the concentration gradients of morphogens such as BMPs and Wnts are also formed. The ECM also serves as an adhesive substrate for the cells, regulating their motility and shape. The structures of ECM are not fixed and static, but are dynamically reorganized by the biosynthesis of its components and their degradation by various proteases and glycanases. Thus, the dynamics of the ECM is quite important in the regulation of cell growth, differentiation, migration, adhesion and tissue morphogenesis.

Unlike other organs, the brain does not normally contain fibrillar collagens except for the basal lamina surrounding the blood vessels and surface of the brain. Instead, major components of brain ECM are proteoglycans. Until recently, many neuroscientists had believed that the brain contains almost no ECM, in spite of the early biochemical and histochemical pioneering work by Margolis et al. (1975, 1976) and Nakanishi (1983), showing the presence of a large amount of glycosaminoglycans and proteoglycans in the developing brain. In the early 1990s, the brain-specific chondroitin sulfate proteoglycans were biochemically characterized (Rauch et al., 1991; Maeda et al., 1992; Oohira et al., 1994), and then their cDNAs were cloned (Rauch et al., 1992; Maeda et al., 1994; Maurel et al., 1994; Watanabe et al., 1995). Then, many investigations revealed the importance of proteoglycans and ECM in the development and disorders of the brain (Franco and Muller, 2011; Maeda et al., 2011; Berretta, 2012; Soleman et al., 2013). However, even now, it seems that the significance of ECM molecules is underestimated in the field of neuroscience, except for reelin. It is desirable that more and more neuroscientists pay attention to the brain ECM molecules.

In this review, I will introduce the structure, binding partners and assembly of proteoglycans and glycosaminoglycans, and discuss their roles in the neuronal migration in the cerebral cortex and their emerging significance in human intellectual disability and psychiatric disorders.

Assembly of Extracellular Matrix Components

In the developing cerebral cortex, very high levels of ECM molecules are expressed, the major components of which are

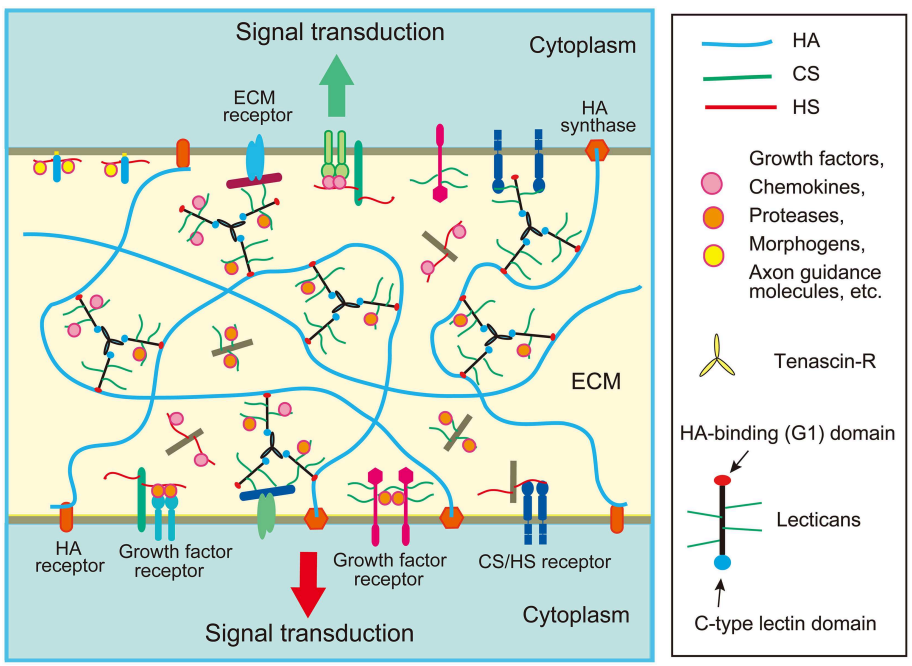

FIGURE 1 | Schematic structure of extracellular matrix (ECM) in the brain. The ECM of the brain is mainly composed of chondroitin sulfate (CS) and heparan sulfate (HS) proteoglycans, hyaluronic acid (HA), and glycoproteins such as tenascins. Lectican family CS proteoglycans form large aggregates with HA and tenascins, which store various proteins such as chemokines, growth factors and axon guidance molecules. The ECM proteoglycans may bind with a CS/HS receptor on the cell surface such as RPTPσ. Cell surface proteoglycans may function as receptors or co-receptors for growth factors.

chondroitin sulfate proteoglycans. Neurocan, versican, aggrecan, and brevican are lectican family chondroitin sulfate proteoglycans expressed in the brain. Lecticans are secreted proteoglycans that bind with hyaluronic acid through the N-terminal G1 domain that contains an immunoglobulin-like loop and two link modules (**Figure 2**). These proteoglycans also have a C-type lectin domain at the C-terminal, and chondroitin sulfate chains are covalently attached to the region between the N- and C-terminal domains. The C-type lectin domain of lecticans binds with tenascin family proteins by protein-protein interaction independent of the carbohydrate moiety (Aspberg et al., 1997). Tenascin family proteins are oligomeric glycoproteins with EGF-like repeats and fibronectin-III domains. The brain contains hexameric tenascin-C and trimeric tenascin-R, which promote the assembly of lecticans by protein-protein interaction. On the other hand, hyaluronic acid is a very long polysaccharide consisting of repeating disaccharides of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc), which are polymerized at the plasma membrane by hyaluronan synthases (HASs). Hyaluronic acids are anchored in the plasma membrane through HASs, or bound to hyaluronan receptors on the cell surface, such as CD44 and RHAMM (**Figure 1**). The tenascin-lectican complexes bind to the hyaluronic acids through the G1 domains of lecticans, which is stabilized by link proteins (Haplns), forming huge aggregates surrounding cells (**Figure 1**). It is considered that these huge aggregates serve as a basic framework to construct the ECM in the brain.

Besides secreted chondroitin sulfate proteoglycans, there are also cell surface proteoglycans. PTPζ/RPTPβ and neuroglycan C are major cell surface chondroitin sulfate proteoglycans with a membrane-spanning region. PTPζ is a receptor-type protein tyrosine phosphatase that is synthesized as a chondroitin sulfate proteoglycan (Krueger and Saito, 1992; Maeda et al., 1994). PTPζ has an N-terminal carbonic anhydrase-like domain, a fibronectin-III domain, a membrane-spanning region and two C-terminal tyrosine phosphatase domains. The extracellular domain of this receptor generated by alternative splicing is secreted as a major soluble chondroitin sulfate proteoglycan in the developing brain, phosphacan (Maurel et al., 1994). Phosphacan binds with multiple proteins including pleiotrophin, midkine, tenascins, contactin, and NCAM (Peles et al., 1998). Neuroglycan C is a transmembrane chondroitin sulfate proteoglycan with an EGF module at the juxtamembrane region of the extracellular domain (Watanabe et al., 1995). Chondroitin sulfate-modification of neuroglycan C is developmentally and regionally regulated, and the expression of the non-proteoglycan form increases with development (Aono et al., 2004).

Another major group of proteoglycans in the developing nervous system is heparan sulfate proteoglycans: syndecans and glypicans (**Figure 2**). The syndecan family is composed of four members, syndecan-1 to -4, each of which has an extracellular domain, a transmembrane region and a conserved short C-terminal cytoplasmic domain (Lambaerts et al., 2009). The N-terminal portion of the extracellular domain of syndecans is

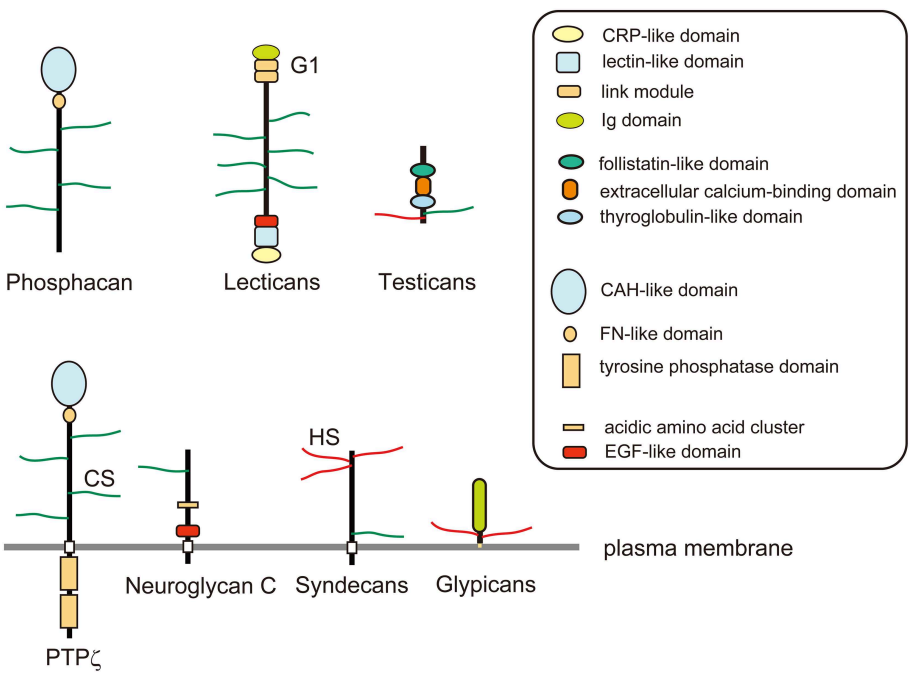

FIGURE 2 | Schematic structures of proteoglycans. Phosphacan and lectican family chondroitin sulfate (CS) proteoglycans are major constituents of brain extracellular matrix. PTPζ is a CS proteoglycan-type protein tyrosine phosphatase, which is a splice variant of phosphacan. Neuroglycan C is a transmembrane CS proteoglycan that is classified as Neuregulin-6. Glypicans are a family of glycosyl-phosphatidylinositol (GPI)-anchored heparan sulfate (HS) proteoglycans. Syndecans are a family of transmembrane HS proteoglycans, some members of which may also be modified by CS chains. Testicans are a family of secreted CS/HS proteoglycans.

modified with heparan sulfate chains. The extracellular domains of syndecan-1 and -4 may be additionally decorated with chondroitin sulfate chains near the transmembrane region (Deepa et al., 2004). It has been considered that most of the extracellular ligand molecules interact with syndecans through binding with heparan sulfate portions. The transmembrane domains of syndecans play important roles in their ligand-induced multimerization and the subsequent signaling (Choi et al., 2005). The intracellular domains of syndecans are divided into two conserved regions (C1 and C2) and a variable region (V), which interact with various kinases and intracellular cytoplasmic components such as src family kinases, CASK, and syntenin (Lambaerts et al., 2009).

Glypicans are glycosyl-phosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans, composed of six family members (glypican-1 to -6) carrying 2–5 heparan sulfate chains (Filmus et al., 2008; Filmus and Capurro, 2014). Glypican-5 was reported also to be modified with chondroitin sulfate in rhabdomyosarcoma cells (Li et al., 2011). The core proteins of glypicans consist of an α-helical domain containing 14 evolutionarily conserved Cys residues, a heparan sulfate-attachment region near the C-terminus, and the C-terminal GPI-anchor attachment signal sequence. Biochemical and genetic studies demonstrated that glypicans bind and regulate Hedgehogs, Wnt, bone morphogenetic proteins and fibroblast growth factors (FGFs) (Filmus and Capurro, 2014). In particular, genetic studies using *Drosophila* demonstrated that glypicans (Dally and Dally-like) play critical roles in the gradient formation of morphogens during wing development (Wu et al., 2010; Raftery and Umulis, 2012). Although it has long been believed that the core proteins of glypicans adopt a globular shape, a recent X-ray crystallographic study indicated that the structure of glypican-1 is actually cylindrical (Svensson et al., 2012).

Testicans are extracellular chondroitin/heparan sulfate proteoglycans, which have been poorly characterized to date. They are composed of three family members (testican-1 to -3), characterized by an N-terminal testican-specific domain, a follistatin-like domain, an extracellular calcium-binding domain, a thyroglobulin-like domain, and a domain with glycosaminoglycan-attachment sites (Schnepp et al., 2005).

Structure and Biosynthesis of Chondroitin Sulfate and Heparan Sulfate

Chondroitin sulfate and heparan sulfate are unbranched sulfated polysaccharides covalently attached to the serine residues in proteoglycan core proteins via common linkage tetrasaccharides, GlcAβ1-3galactose(Gal)β1-3Galβ1-4xylose(Xyl)β1-O-Ser (**Figure 3**). Chondroitin sulfate is composed of repeating disaccharide units of *N*-acetylgalactosamine (GalNAc) and GlcA, (GlcAβ1-3GalNAcβ1-4)n, whereas heparan sulfate is composed of repeating disaccharide units of GlcNAc and GlcA, (GlcAβ1-4GlcNAcα1-4)n. Biosynthesis of these polysaccharides is initiated by the addition of Xyl residues to the specific serine residues in

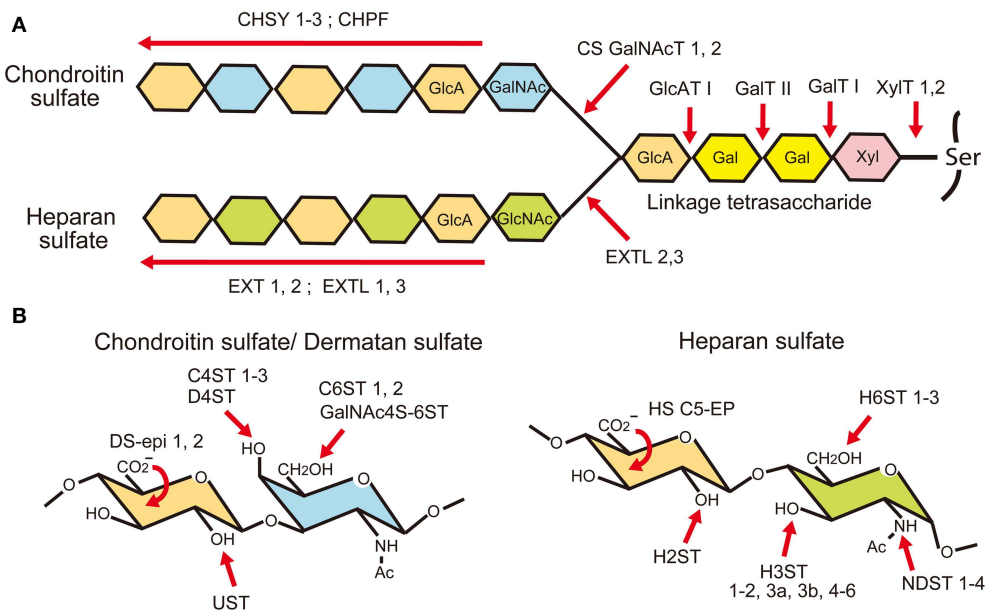

FIGURE 3 | Biosynthesis of glycosaminoglycans. (A) Chondroitin sulfate (CS) and heparan sulfate (HS) chains are covalently attached to the proteoglycan core proteins through a common linkage tetrasaccharide. It is considered that the biosynthesis of a chondroitin chain starts with the addition of an *N*-acetylgalactosamine (GalNAc) residue to the linkage tetrasaccharide by CS *N*-acetylgalactosaminyltransferases (CSGalNAcT1, 2). After that, glucuronic acid (GlcA) and GalNAc residues are co-polymerized by chondroitin sulfate synthases (CHSY-1, -2, -3) and chondroitin polymerization factor (CHPF). When an *N*-acetylglucosamine (GlcNAc) residue is added to the linkage tetrasaccharide instead of GalNAc by EXTL 2 or 3, a heparan chain is polymerized by EXT family members. **(B)** After polymerization, they are modified by sulfation and epimerization reactions by many glycosaminoglycan modifying enzymes.

core proteins by xylosyl transferases (XYLT1, 2) (Nadanaka and Kitagawa, 2008; Mikami and Kitagawa, 2013; Mizumoto et al., 2013a). This is followed by the addition of two Gal residues and a GlcA residue by galactosyltransferase-I (B4GALT7), galactosyltransferase-II (B3GALT6) and glucuronyltransferase-I (B3GAT3), respectively. After that, repeating disaccharide units of chondroitin or heparan sulfate are polymerized in the Golgi apparatus. Chondroitin sulfates are polymerized by chondroitin sulfate synthases (CHSY-1 to -3), chondroitin polymerization factor (CHPF) and chondroitin sulfate *N*-acetylgalactosaminyltransferases (CSGalNAcT-1, -2), whereas heparan sulfates are polymerized by EXT family members (EXT1, EXT2, EXTL1, EXTL2, EXTL3). Since these glycosaminoglycans use a common linkage tetrasaccharide, the chain initiation step determines whether a chondroitin or a heparan chain elongates. If the first GalNAc residue is added to the linkage tetrasaccharide possibly by CSGalNAcT-1 or -2, a chondroitin chain is elongated by the CHSY/CHPF complex. If a GlcNAc residue is alternatively added to the linkage tetrasaccharide possibly by EXTL 2 or 3, a heparan chain is polymerized by the EXT1/EXT2 complex. Although the genes encoding these biosynthetic enzymes have been identified, almost nothing is known about the mechanism for the selection of glycosaminoglycan types at the chain initiation step.

After the polymerization of repeating disaccharides, they are heavily modified by the C5 epimerization of GlcA residues and sulfation reactions (Nadanaka and Kitagawa, 2008; Mikami and Kitagawa, 2013; Mizumoto et al., 2013a) (**Figure 3**). The majority of the GalNAc residues in chondroitin sulfate are 4-*O*-sulfated by chondroitin 4-*O*-sulfotransferases (C4ST-1 to -3) or 6-*O*-sulfated by 6-*O*-sulfotransferases (C6ST-1 and -2). Although many of the disaccharide units in chondroitin sulfate are mono-sulfated A units [GlcAβ1-3GalNAc(4-SO_4)] or C units [GlcAβ1-3GalNAc(6-SO_4)], they may be further sulfated by GalNAc 4-sulfate 6-*O*-sulfotransferase (GalNAc 4S-6ST) or chondroitin uronyl 2-*O*-sulfotransferase (UST), generating di-sulfated disaccharides, E units [GlcAβ1-3GalNAc(4, 6-bis-SO_4)] or D units [GlcA(2-SO_4)β1-3GalNAc(6-SO_4)], respectively. Furthermore, some of the GlcA residues are C5-epimerized to iduronic acid (IdoA) by dermatan sulfate epimerases 1 and 2 (encoded by *DSE* and *DSEL*, respectively). The resulting IdoAα1-3GalNAc units are sulfated by dermatan 4-*O*-sulfotransferase (D4ST), generating an iA unit [IdoAα1-3GalNAc(4-SO_4)], which may be further sulfated by UST, generating an iB unit [IdoA(2-SO_4)α1-3GalNAc(4-SO_4)]. Chondroitin sulfate with a high content of IdoA is often called dermatan sulfate.

The modification of heparan sulfate begins with the *N*-sulfation reaction by *N*-deacetylase/*N*-sulfotransferase (NDST-1 to -4), which removes acetyl groups from some of the GlcNAc residues in the heparan sulfate chain and replaces them with sulfate groups (**Figure 3**). Then, some of the GlcA residues are C5 epimerized to IdoA by heparan sulfate glucuronyl C5-epimerase (HS C5-EP), followed by *O*-sulfation reactions. The *O*-sulfation includes 2-*O*-sulfation of GlcA/IdoA residues by heparan sulfate 2-*O*-sulfotransferase (H2ST), 3-*O*-sulfation of glucosamine (GlcN) units by heparan sulfate 3-*O*-sulfotransferases (H3ST-1, -2, -3A, -3B, -4, -5, and -6), and 6-*O*-sulfation of GlcN units by heparan sulfate 6-*O*-sulfotransferases (H6ST-1 to -3). Because *N*-sulfation of GlcN units by NDSTs generates substrates for the subsequent modification enzymes, highly *N*-sulfated regions in heparan sulfate are also highly modified by C5-EP and various *O*-sulfotransferases. Thus, heparan sulfate chains display domain structures: highly modified NS-domains, poorly modified NA-domains characterized by stretches of *N*-acetylated disaccharide units, and the interspacing NA/NS-domains composed of both *N*-acetylated and *N*-sulfated disaccharide units.

Binding Partners of Glycosaminoglycans

Recent studies using microarrays and surface plasmon resonance revealed that chondroitin sulfate and heparan sulfate chains bind with many proteins that play important roles in brain development, especially neuronal migration (Deepa et al., 2002; Kawashima et al., 2002; Maeda et al., 2006; Shipp and Hsieh-Wilson, 2007; Conrad et al., 2010; Rogers et al., 2011; Mizumoto et al., 2013b). Both heparan sulfate and chondroitin sulfate chains bind with various axon guidance molecules in a sulfation pattern-dependent manner (Shipp and Hsieh-Wilson, 2007). While Slit2 shows a preference for heparan sulfate sequences that contain 6-*O*-sulfation and *N*-sulfation, netrin 1 requires sulfation at the 2-*O*-, 6-*O*-, and *N*-positions. Semaphorin5B (Sema5B), ephrinA1 and ephrinA5 prefer 2-O- and *N*-sulfation. On the other hand, all of these axon guidance molecules bind strongly with E unit-rich highly sulfated chondroitin sulfate E from squid cartilage (CS-E) (Shipp and Hsieh-Wilson, 2007). Sema5B also binds moderately with D unit-rich shark cartilage chondroitin sulfate D (CS-D), and weakly with A unit-rich whale cartilage chondroitin sulfate A (CS-A), pig skin dermatan sulfate (CS-B) and C unit-rich shark cartilage chondroitin sulfate C (CS-C). EphrinA1 binds moderately with CS-C, and weakly with CS-B and CS-D. Slit2, netrin1 and ephrinA5 bind only weakly with CS-A, -C, -D, and -B. In addition, it has been reported that Sema3A binds strongly with CS-E (Dick et al., 2013). EphrinA3 also binds with heparan sulfate and chondroitin sulfate, although the structural requirement is unknown (Irie et al., 2008; Conrad et al., 2010).

Neurotrophin family growth factors [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4)] bind strongly with CS-E (Rogers et al., 2011). BDNF, NT-3, and NT-4, but not NGF bind moderately with CS-A, whereas CS-C shows almost no binding to these proteins. PC12 cells express E unit-rich chondroitin sulfate, and its removal upon chondroitinase ABC treatment significantly attenuated TrkA activation by NGF or NT-4, suggesting that endogenous chondroitin sulfate plays important roles in neurotrophin signaling (Rogers et al., 2011). Glial cell line-derived neurotrophic factor (GDNF) associates with heparan sulfate in a 2-*O*-sulfation-dependent manner, promoting the binding of this protein to its receptor component GFRα1 (Rickard et al., 2003).

Chemokines are a family of small proteins that induce chemotaxis of various cells including cortical interneurons (Marin, 2013). Glycosaminoglycans interact with chemokines such as CCL2 (MCP-1), CCL5 (RANTES), and CXCL12 (SDF-1) in a

chain length- and sulfation pattern-dependent manner (Kuschert et al., 1999; Hirose et al., 2001). Among the chemokines, CXCL12 plays important roles in the tangential migration of cortical interneurons (see below). CXCL12 binds strongly with CS-E and the highly sulfated S domains in heparan sulfate, and also interacts weakly with CS-A, -B, -C, and -D (Murphy et al., 2004; Mizumoto et al., 2013b). In addition, it was revealed that versican interacts with CXCL12 through chondroitin sulfate chains (Hirose et al., 2001). It has been considered that these interactions in the ECM and cell surface contribute to the formation of immobilized or haptotactic gradients of chemokines (Kuschert et al., 1999).

Pleiotrophin and midkine are a family of multifunctional heparin-binding growth factors that bind with both heparan sulfate and chondroitin sulfate (Perez-Pinera et al., 2007; Muramatsu, 2014). These growth factors bind strongly with highly sulfated heparan sulfate and CS-E, moderately to CS-B and CS-D, and very weakly to CS-A (Maeda et al., 2003; Zou et al., 2003; Mizumoto et al., 2013b).

The structures of glycosaminoglycans are determined at least partly by the combinatorial expression of their modifying enzymes. During development of the brain, the sulfotransferases involved in the chondroitin/heparan sulfate synthesis show dynamic spatiotemporal expression patterns (Yabe et al., 2005; Mitsunaga et al., 2006; Ishii and Maeda, 2008b). This suggests that the expression of specific functional domains in these glycosaminoglycan chains is strictly regulated in the developing brain. Conway et al. (2011) showed that the expressions of *HS2ST* and *HS6ST-1* are distinctively regulated at the optic chiasm, and the mutant mice lacking these genes exhibit different axon guidance defects. Interestingly, $HS2ST^{-/-}$ and $HS6ST\text{-}1^{-/-}$ phenotypes closely match those of $Slit1^{-/-}$ and $Slit2^{-/-}$, respectively, suggesting that slit family proteins are regulated by specific sulfation of heparan sulfate.

Ligand Binding to Proteoglycans

As described above, glycosaminoglycans bind with various protein ligands in a structure-dependent manner. However, the ligand binding to proteoglycans is extremely complex because many proteoglycans carry multiple glycosaminoglycan chains that may function cooperatively. Furthermore, cooperation between the core protein and attached glycosaminoglycan chains may also occur. Accordingly, proteoglycans usually exhibit much higher affinity and/or avidity for the ligand proteins than free glycosaminoglycans (Herndon et al., 1999). Thus, degradation of proteoglycan core proteins by extracellular proteases may terminate such cooperativity and release the ligand molecules, leading to the activation or inactivation of the signaling in a context-dependent manner. Cooperation is observed not only between glycosaminoglycans of the same type but also between heparan sulfate and chondroitin sulfate chains. Syndecan-1 and syndecan-4 carry both heparan sulfate and chondroitin sulfate chains, which cooperatively regulate the binding dynamics of pleiotrophin, midkine and FGF-2 to these proteoglycans (Deepa et al., 2004).

PTPζ/phosphacan binds to pleiotrophin and midkine, in which both chondroitin sulfate and core protein portions contribute to the interaction (Maeda et al., 1996, 1999, 2003). While intact phosphacan preparation shows low ($Kd = 3$ nM) and high affinity binding ($Kd = 0.25$ nM) for pleiotrophin, this proteoglycan exhibits only single very low affinity binding after chondroitinase ABC-treatment ($Kd = 13$ nM) (Maeda et al., 1996). This suggests that the binding affinity of phosphacan for pleiotrophin is regulated by the structural variation of chondroitin sulfate. In fact, the structure of chondroitin sulfate on phosphacan changes during rat brain development, and a slight increase in the content of oversulfated D unit drastically strengthens the binding of this proteoglycan to pleiotrophin (Maeda et al., 2003).

Another prominent example of the cooperation between the core protein and glycosaminoglycan chain in the signaling has been reported by the group of Filmus (Li et al., 2011; Filmus and Capurro, 2014). They revealed that glypican-3 and glypican-5 oppositely regulate the Hedgehog (Hh) signaling in rhabdomyosarcoma cell proliferation. Glypican-3 binds to Hh through its core protein, reducing the amount of Hh available to its receptor Patched 1 (Ptc1), with the consequent decrease in signaling. On the other hand, glypican-5 interacts with both Hh and Ptc1 through heparan sulfate and chondroitin sulfate chains, facilitating Hh-Ptc1 binding with the consequent increased signaling. The heparan sulfate chains of glypican-5 show a higher degree of sulfation than those of glypican-3, which may explain why the glycosaminoglycan chains of glypican-5 but not those of glypican-3 interact with Hh/Ptc1.

Sema5A is an axon guidance molecule that can exert both inhibitory and permissive effects on growing axons. Kantor et al. (2004) revealed that Sema5A interacts with the glycosaminoglycan portion of both chondroitin sulfate and heparan sulfate proteoglycans. The axonal heparan sulfate proteoglycans are required for the Sema5A-mediated attraction of growing axons of the fasciculus retroflexus. On the other hand, the extracellular chondroitin sulfate proteoglycans precisely localize Sema5A in a specific area, where Sema5A acts as a repulsive guidance cue for these growing axons. Thus, the bifunctional roles of Sema5A are regulated by chondroitin sulfate and heparan sulfate proteoglycans, demonstrating the cooperation among these two types of proteoglycans during the process of axon pathfinding.

Recently, Coles et al. (2011) reported that receptor protein tyrosine phosphatase σ (RPTPσ) is a receptor for both chondroitin sulfate and heparan sulfate proteoglycans. Heparan sulfate proteoglycans induce oligomerization of RPTPσ on the growth cone, leading to inactivation of the tyrosine phosphatase activity and growth promotion. On the other hand, extracellular chondroitin sulfate proteoglycans inhibit oligomerization of this receptor with consequent suppression of axon growth. It is considered that multiple RPTPσ molecules bind to the islands of high/intermediate sulfation on heparan sulfate chains (NS to NA/NS domains), which stabilize the receptor oligomers. Conversely, the receptor binding sites are considered to be sparsely distributed on chondroitin sulfate chains, and therefore receptor oligomerization cannot occur. If so, the signaling of RPTPσ should be highly dependent on the glycosaminoglycan structures. Proteoglycans bearing low sulfated heparan sulfate chains may

inhibit receptor oligomerization, and conversely proteoglycans bearing highly sulfated chondroitin sulfate may induce receptor oligomerization. It is also reported that another receptor tyrosine phosphatase, leukocyte common antigen-related phosphatase (LAR) is a functional receptor for chondroitin sulfate proteoglycan (Fisher et al., 2011).

Although the structure of glycosaminoglycans is basically determined by the biosynthetic processes, the sulfation pattern of heparan sulfate may be modified extracellularly by the endosulfatases, Sulf1 and Sulf2 (Nagamine et al., 2012). These sulfatases catalyze the desulfation of the 6-*O*-sulfate group from GlcN residues in the trisulfated disaccharides in heparan sulfate. Thus, highly sulfated NS domains in heparan sulfate are preferentially desulfated by these enzymes, leading to the change in the binding affinity of various ligands to heparan sulfate proteoglycans. This results in the activation or suppression of specific signaling molecules such as Wnt, GDNF, and FGF during various developmental processes (Ai et al., 2003, 2007; Wang et al., 2004). Thus, functions of proteoglycans are intricately regulated at multiple levels.

Roles of Proteoglycans in Neuronal Migration

In the developing neocortex, postmitotic pyramidal neurons generated in the ventricular zone show a multipolar shape, and migrate in random directions in the subventricular and intermediate zones (**Figure 4**). After that, they transform into the bipolar shape and attach to the radial glial fibers, upon which they rapidly migrate toward the marginal zone. This multipolar-to-bipolar transition occurs when the neurons reach the subplate, suggesting that this layer contains critical factor(s) regulating neuronal behavior (Ohtaka-Maruyama et al., 2013). The radial migration of pyramidal neurons stops at the interface between the cortical plate and the marginal zone, forming the "inside-out" arrangement of neurons. On the other hand, inhibitory neurons tangentially migrate in the neocortex through the marginal zone, subplate, and lower intermediate/subventricular zones. These migration patterns of both excitatory and inhibitory neurons suggest that specific cortical layers play critical roles in the regulation of neuronal migration. Nakanishi (1983) demonstrated that glycosaminoglycans stained by colloidal iron distributed principally in the marginal zone and subplate in the developing mouse cerebral cortex. In the cortices of reeler mutants, where radial migration of pyramidal neurons is severely disturbed, most glycosaminoglycans are localized in the outer layer of the cortex. From such an expression pattern, it was suggested that glycosaminoglycans are involved in the neuronal migration and/or laminar pattern formation of the neocortex. Then, the brain-specific chondroitin sulfate proteoglycans were identified, and it was revealed that neurocan and phosphacan are richly expressed in the marginal zone and subplate (Oohira et al., 1994; Maeda et al., 1995; Meyer-Puttlitz et al., 1996) (**Figure 5**). Versican was also localized in these layers (Popp et al., 2003), raising the possibility that chondroitin sulfate proteoglycans regulate neuronal migration in the cortex.

As described above, PTPζ/phosphacan binds to pleiotrophin with high affinity (Maeda et al., 1996, 1999). Pleiotrophin induces oligomerization of PTPζ, which leads to the inactivation of its

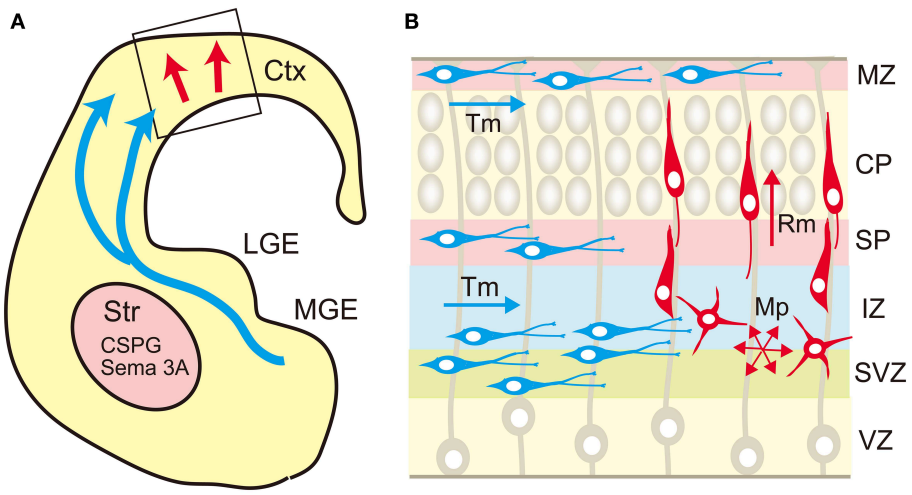

FIGURE 4 | Migration routes of excitatory and inhibitory neurons in the cerebrum. (A) The excitatory neurons are generated in the ventricular zone of the neocortex (Ctx) and migrate radially toward the brain surface (red arrow). The cortical inhibitory neurons are generated mainly in the medial ganglionic eminence (MGE), and migrate tangentially toward the neocortex (blue arrows). The migrating interneurons avoid the striatum (Str) that expresses chondroitin sulfate proteoglycans (CSPG) and semaphorin 3A (Sema 3A). **(B)** In the neocortex, the excitatory neurons (red cells) born in the ventricular zone (VZ) show multipolar morphology and migrate in random directions (Mp) in the subventricular (SVZ) and intermediate (IZ) zones. When the multipolar neurons reach the subplate (SP), they transform into a bipolar shape and migrate radially (Rm) in the cortical plate (CP) toward the marginal zone (MZ). On the other hand, the tangential migration (Tm) of interneurons (blue cells) occurs in a layer-specific manner, in which interneurons prefer MZ, SP, lower IZ, and SVZ.

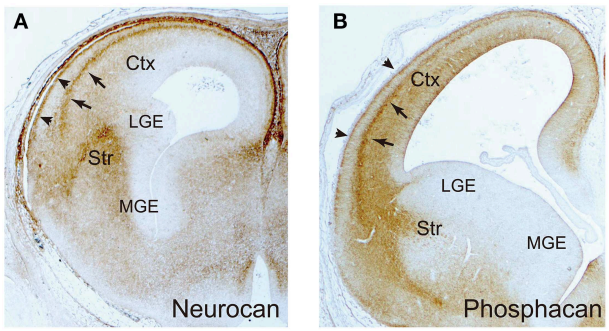

FIGURE 5 | Immunohistochemical localization of neurocan and phosphacan. The frontal sections of embryonic day 16 rat brains were immunohistochemically stained with anti-neurocan **(A)** and anti-phosphacan **(B)** monoclonal antibodies. They are selectively expressed in the marginal zone (arrowheads) and subplate (arrows) in the neocortex, and the striatum (Str). The medial (MGE) and lateral (LGE) ganglionic eminences are negative.

tyrosine phosphatase activity, initiating downstream signaling (Meng et al., 2000; Fukada et al., 2006). Pleiotrophin is deposited along radial glial fibers, and PTPζ is expressed on the migrating pyramidal neurons, raising the possibility that pleiotrophin on radial glial fibers regulates the radial migration of excitatory neurons (Maeda and Noda, 1998). In fact, *in vitro* cell migration assay demonstrated that pleiotrophin-PTPζ signaling induces migration of cortical neurons (Maeda and Noda, 1998). Subsequently, this signaling system has been demonstrated to promote the migration of various types of normal and tumor cell in a chondroitin sulfate-dependent manner (Polykratis et al., 2005; Feng et al., 2010; Koutsioumpa et al., 2013). Phosphacan and free chondroitin sulfate suppress the pleiotrophin-induced neuronal migration by competitive inhibition of the binding between pleiotrophin and cell surface PTPζ (Maeda and Noda, 1998; Maeda et al., 1999). Soluble chondroitin sulfate proteoglycans such as phosphacan, neurocan and versican expressed in the subplate and marginal zone may regulate the migratory behavior of neurons by inhibiting pleiotrophin- PTPζ signaling.

Recently, it was found that neuroglycan-C is involved in the radial migration of pyramidal neurons from a study of the plant homeodomain (PHD) finger 6 (PHF6) gene (Zhang et al., 2013). *PHF6* is an X-linked gene encoding the protein that has four nuclear localization signals and two PHD-type zinc finger domains, which functions as a transcription repressor. Mutations of PHF6 cause Börjeson-Forssman-Lehmann syndrome, characterized by intellectual disability associated with seizures, short stature, hypogonadism, hypometabolism, marked gynecomastia, truncal obesity, tapered fingers, narrow palpebral fissure, and large ears (Liu et al., 2014). Using *in utero* electroporation, Zhang et al. (2013) demonstrated that knockdown of PHF6 severely impaired the radial migration of cortical neurons. They also identified neuroglycan-C as a downstream target of PHF6. Knockdown of neuroglycan-C phenocopied the neuronal migration phenotype of PHF6 knockdown, suggesting that PHF6 controls the expression level of neuroglycan-C in the cortical neurons, thus regulating the radial neuronal migration. Neuroglycan C binds with pleiotrophin and midkine, in which its chondroitin sulfate portion increases the affinity of the core protein for these growth factors (Ichihara-Tanaka et al., 2006; Nakanishi et al., 2010). There is a possibility that pleiotrophin/midkine-neuroglycan C signaling is involved in the radial migration in the cerebral cortex.

Pleiotrophin also binds with syndecan-3 in a heparan sulfate-dependent manner (Raulo et al., 1994; Kinnunen et al., 1996). Like PTPζ, syndecan-3 is required for pleiotrophin-induced neuronal migration, suggesting that PTPζ and syndecan-3 are redundant pleiotrophin receptors on cortical neurons (Hienola et al., 2006). Syndecan-3 knockout mice showed delayed radial neuronal migration in the cortex, and this delay was partially caught up at ∼10 days after birth (Hienola et al., 2006). The binding of pleiotrophin to syndecan-3 triggers the phosphorylation of src, which then activates cortactin and modulates the assembly of the actin cytoskeleton. Syndecan-3 knockout mice also show migration defects of interneurons (Bespalov et al., 2011). At embryonic day 15, calbindin-positive precursors of interneurons were accumulated in the ganglionic eminence of syndecan-3 knockout mice, and the density of GABA-immunoreactive cells was lower in the dorsomedial cortex of adult knockout mice than in that of control mice. It has been suggested that GDNF binds to the heparan sulfate portion of syndecan-3 on interneurons, promoting their migration. On the other hand, syndecan-1 is expressed by the neural progenitor cells in the cerebral cortex (Wang et al., 2012). Knockdown of syndecan-1 using *in utero* electroporation resulted in the reduction of neural progenitor cells and the promotion of neuronal differentiation in the cortex (Wang et al., 2012). In these cortices, there were fewer cells in the ventricular/subventricular zone, and more neurons moved into the intermediate zone and cortical plate compared with the cortices electroporated with control plasmid. These findings suggest that syndecan-1 and syndecan-3 are differentially expressed and play distinct roles in the developing cortex.

Roles of Glycosaminoglycans in Neuronal Polarization

During development of the mouse cerebrum, the disaccharide composition of chondroitin sulfate changes dynamically (Ishii and Maeda, 2008a). At embryonic day 16 (E16), the major components are A and C units, and then the content of A unit increases and that of C unit decreases until maturation. A small but significant amount of E unit is detected at E16 to 18, and the content decreases thereafter. D unit is a minor component, but is constantly detected during embryonic and postnatal development. Since D and E units contribute significantly to the binding of various ligand molecules as described above, we investigated the roles of these oversulfated structures in neuronal migration (Ishii and Maeda, 2008a). Using *in utero* electroporation, we introduced shRNA constructs for GalNAc 4S-6ST and UST into neural progenitor cells in the ventricular zone of the E14 cortex. At E18, the embryos were dissected out, and the migration of the cortical neurons was examined. Knockdown of both sulfotransferases severely disrupted the radial migration of

cortical neurons. The neurons knocked down for these enzymes were accumulated in the subventricular zone and in the intermediate zone, and showed multipolar morphology. This suggested that oversulfated chondroitin sulfate is required for the multipolar-to-bipolar transition of pyramidal neurons.

Neuronal polarization of dissociated hippocampal pyramidal cells is a well-established *in vitro* model of multipolar-to-bipolar transition of newborn neurons (Dotti et al., 1988). Dissociated hippocampal pyramidal neurons extend several morphologically indistinguishable minor processes several hours after plating. Then, one of these minor processes extends rapidly and becomes an axon, and the other processes differentiate into dendrites. In contrast, the hippocampal neurons cultured in the presence of chondroitinase ABC extended multiple axon-like processes that were highly unstable and repeatedly extended and retracted (Nishimura et al., 2010). The morphology and behavior of the chondroitinase ABC-treated neurons were similar to those of multipolar neurons in the developing cortex. Furthermore, knockdown of GalNAc 4S-6ST and UST also disturbed the neuronal polarization of cultured hippocampal neurons, suggesting the importance of oversulfated chondroitin sulfate in this process. In the cultured hippocampal neurons, the oversulfated chondroitin sulfate was accumulated in the focal contacts in the cell bodies and axons. Chondroitinase ABC-treatment suppressed the tyrosine phosphorylation of FAK at the focal contacts, suggesting that the proteoglycans bearing oversulfated chondroitin sulfate strengthen the adhesion of axons and cell bodies to the substrate, leading to the stabilization of neuronal morphology. In contrast to the chondroitinase ABC-treatment, the axons extended steadily and showed almost no retraction when hippocampal neurons were treated with heparitinases that specifically degrade heparan sulfate. This suggests that heparan sulfate proteoglycans destabilize the neuronal morphology, inducing retraction of axons. In fact, it has been reported that heparan sulfate proteoglycans on growth cones are essential for the repulsive activities of Slit2 and ephrin-A3 (Hu, 2001; Irie et al., 2008). Thus, chondroitin sulfate and heparan sulfate proteoglycans expressed on hippocampal neurons play opposing roles during neuronal polarization.

Roles of Glycosaminoglycans in Tangential Neuronal Migration

Cortical interneurons are born in the medial ganglionic eminence (MGE), caudal ganglionic eminence and preoptic area in the ventral telencephalon, and migrate tangentially toward the cortex (Evsyukova et al., 2013; Marin, 2013) (**Figure 4**). The newborn interneurons exiting the ganglionic eminence avoid entering the striatum, and migrate into the neocortex through the marginal zone, subplate, or lower intermediate/subventricular zones, suggesting that complex interplay of repulsive and attractive cues regulates the migration route of these neurons. Neuregulin-1, NT-4, and GDNF were shown to be chemoattractive factors for cortical interneurons, whereas Slit 1, Sema 3A, ephrin a3, and ephrin a5 act as chemorepulsive factors (Zhu et al., 1999; Polleux et al., 2002; Flames et al., 2004; Rudolph et al., 2010; Bespalov et al., 2011; Marin, 2013; Steinecke et al., 2014). As described above, biochemical studies revealed that these factors bind with chondroitin and/or heparan sulfate in a sulfation pattern-dependent manner. Thus, there is a possibility that chondroitin/heparan sulfate proteoglycans regulate the spatial distribution and/or activity of these factors. In fact, a recent report demonstrated that chondroitin sulfate plays an important role in the tangential migration of interneurons (Zimmer et al., 2010). Chondroitin sulfate proteoglycans are highly expressed in the striatal mantle zone, which is avoided by tangentially migrating interneurons (**Figure 5**). *In vitro* Boyden chamber cell migration and stripe assays demonstrated that chondroitin sulfate proteoglycans exert repulsive effects on cortical interneurons. These repulsive effects were suppressed by chondroitinase ABC-treatment, suggesting that chondroitin sulfate directly acts as a repellent for these neurons. Furthermore, in the chondroitinase ABC-treated brain slices, cortical interneurons actively invaded the striatum, although they avoided this region in the control slices. Sema3A is retained in the striatum by binding to the chondroitin sulfate chains, and repels migrating interneurons that express Sema3A receptor, neuropilin 1. Thus, it was shown that chondroitin sulfate proteoglycans exert not only direct, but also indirect repulsive effects on interneurons by anchoring repulsive factors in the striatum (**Figure 4**).

After the interneurons enter the neocortex, they avoid the cortical plate, where chondroitin sulfate proteoglycans are poorly expressed (**Figure 4**). Instead, they migrate through the chondroitin sulfate proteoglycan-rich marginal zone and subplate. They also prefer the subventricular zone and the lower intermediate zone, where the content of chondroitin sulfate proteoglycans is relatively low. Thus, it seems that chondroitin sulfate proteoglycans do not act as a repellent for interneurons in the neocortex. Tangential migration of interneurons in the neocortex is induced by the chemokine CXCL12 (SDF1), which is concentrated in the marginal zone, subplate, and lower intermediate/subventricular zones (Li et al., 2008; Lopez-Bendito et al., 2008). It has been reported that CXCL12 binds with both chondroitin sulfate and heparan sulfate (Mbemba et al., 2000). The marginal zone and subplate are highly enriched with phosphacan, neurocan and versican (Oohira et al., 1994; Maeda et al., 1995; Meyer-Puttlitz et al., 1996) (**Figure 5**), and thus CXCL12 may be anchored to the chondroitin sulfate chains of these proteoglycans. On the other hand, syndecan-1 is highly expressed in the ventricular/subventricular zone and in the lower intermediate zone (Wang et al., 2012), and may concentrate CXCL12 in these layers through heparan and/or chondroitin sulfate moieties. Thus, it seems that proteoglycans can be either attractive or repulsive substrates depending on the proteins bound to their glycosaminoglycan chains.

As described above, syndecan-3 functions as a GDNF receptor expressed on migrating interneuron (Bespalov et al., 2011). In this case, only the matrix-bound form of GDNF acts as a ligand of syndecan-3, and the soluble form is not active. It may be that GDNF bound to the chondroitin/heparan sulfate proteoglycans in the extracellular matrix activates the syndecan-3 signaling in the interneurons.

TABLE 1 | Human disorders caused by mutations of proteoglycan-related genes.

Genes (coded proteins)	Clinical features	References
XylT1 (Xylosyltransferase 1)	Autosomal recessive short stature syndrome; distinct facial features, alteration of fat distribution, intellectual disability	Schreml et al., 2014
B3GALT6 (Galactosyltransferase II)	Pleiotropic Ehlers-Danlos-syndrome-like connective tissue disorder; skin fragility, delayed wound healing, joint hyperlaxity, and contractures, muscle hypotonia, spondyloepimetaphyseal dysplasia, intellectual disability	Malfait et al., 2013.
CHSY1 (Chondroitin synthase 1)	Temtamy preaxial brachydactyly syndrome; bilateral preaxial brachydactyly and hyperphalangism of digits, facial dysmorphism, dental anomalies, sensorineural hearing loss, intellectual disability	Li et al., 2010.
NDST1 (NDST1)	Intellectual disability, muscular hypotonia, epilepsy, postnatal growth deficiency	Reuter et al., 2014.
SPOCK1 (Testican-1)	Intellectual disability, partial agenesis of corpus callosum, prenatal-onset microcephaly, artrial septal defects	Dhamija et al., 2014.
GPC3 (Glypican 3)	Simpson-Golabi-Behmel syndrome type I; pre/postnatal overgrowth, distinctive craniofacial features, macrocephaly, organomegaly. Intellectual disability and epilepsy in some cases	Tenorio et al., 2014.

Proteoglycans, Psychiatric Disorders, and Intellectual Disabilities

Since proteoglycans are major components of the connective tissue, mutations in the proteoglycan-related genes cause various skeletal and connective tissue disorders (Huegel et al., 2013; Mizumoto et al., 2013a). Recently, the involvement of these genes in intellectual and psychiatric disorders has also begun to be revealed (**Tables 1**, **2**). A hypofunctional mutation of *XYLT1* encoding xylosyltransferase 1 causes an autosomal recessive short stature syndrome associated with intellectual disability (Schreml et al., 2014). Mutations of *B3GALT6* encoding galactosyltransferase II cause a pleiotropic Ehlers-Danlos-syndrome-like connective tissue disorder, which is also associated with intellectual disability (Malfait et al., 2013). As described above, these two enzymes are involved in the biosynthesis of the linkage tetrasaccharides that are used commonly for the chain initiation of chondroitin and heparan sulfates, implying that these glycosaminoglycans are essential for the development of higher intellectual function of the brain. In fact, an earlier study suggested that *EXT1*, encoding a heparan sulfate co-polymerase, is associated with autism (Li et al., 2002). Loss-of-function mutations in *CHSY1* encoding chondroitin synthase 1 cause Temtamy preaxial brachydactyly syndrome, which is characterized by delayed motor and mental development as well as bilateral, symmetric preaxial brachydactyly and hyperphalangism of digits, facial dysmorphism, and dental anomalies (Li et al., 2010). Furthermore, it was found that missense mutations of *NDST1* cause intellectual disability, muscular hypotonia, and epilepsy, suggesting that normal modification of heparan sulfate is essential for the development of functional neuronal circuits (Reuter et al., 2014).

In addition to glycosaminoglycan biosynthetic enzymes, genes encoding proteoglycan core proteins are also associated with intellectual disability. Missense mutation in *SPOCK1* encoding testican-1 causes intellectual disability with dyspraxia, dysarthria, partial agenesis of corpus callosum, and prenatal-onset microcephaly (Dhamija et al., 2014).

TABLE 2 | Proteoglycan-related genes proposed to be associated with mental disorders.

Genes (coded proteins)	Mental disorders	References
DSEL (DS epimerase 2)	Bipolar disorder, depressive disorder	Goossens et al., 2003; Shi et al., 2011
UST (UST)	Job-related exhaustion	Sulkava et al., 2013
NDST3 (NDST3)	Schizophrenia, bipolar disorder	Lencz et al., 2013
EXT1 (EXT1)	Autism	Li et al., 2002
NCAN (Neurocan)	Schizophrenia, bipolar disorder	Muhleisen et al., 2012; Schultz et al., 2014
PTPRZ1 (Phosphacan/PTPζ)	Schizophrenia	Buxbaum et al., 2008; Takahashi et al., 2011
CSPG5 (Neuroglycan C)	Schizophrenia	So et al., 2010

Simpson-Golabi-Behmel syndrome is an overgrowth/multiple congenital anomalies syndrome caused by mutations in *glypican 3*. This disease shows high clinical variability, and in some cases, intellectual disability is present (Tenorio et al., 2014). It will be important to examine whether these intellectual disabilities are caused by abnormal neuronal migration in the cortex.

It has recently been revealed that early developmental defects of neural network formation including abnormal neuronal migration and myelination can cause various psychiatric diseases such as schizophrenia (Stolp et al., 2012). Muhleisen et al. (2012) identified variation in the neurocan gene (rs1064395) as a common risk factor for bipolar disorder and schizophrenia. In schizophrenia patients, *neurocan* risk status was found to be associated with higher folding in the right lateral occipital cortex and left dorsolateral prefrontal cortex (Schultz et al., 2014). Neurocan may play important roles in the neuronal migration and/or formation of axonal fibers in the cerebral cortex, and the deficits in these processes may influence the folding of the occipital and prefrontal lobes, leading to an increased risk of schizophrenia.

It is well known that several members of the Neuregulin/ErbB signaling system are susceptibility genes of schizophrenia, bipolar disorders and depression (Mei and Nave, 2014). Neuregulins constitute a family of EGF-like signaling molecules that stimulate ErbB receptor family tyrosine kinases, the signaling of which regulates neuronal migration, myelination, neurotransmission and synaptic plasticity (Mei and Nave, 2014). Recent studies suggested that proteoglycans regulate Neuregulin/ErbB signaling, and thus are related to psychiatric disorders. Buxbaum et al. (2008) reported that *PTPRZ1*, which encodes both PTPζ and phosphacan, is associated with schizophrenia in a Caucasian population, although no association was found in the Japanese population (Ito et al., 2008). They demonstrated that PTPζ binds with ErbB4 through the scaffolding protein, MAGI, and inhibits the Neuregulin-1/ErbB4 signaling. Furthermore, Takahashi et al. (2011) found that the expression of PTPζ is increased in the brains of schizophrenia patients, and also demonstrated that transgenic mice overexpressing PTPζ showed reduced Neuregulin-1 signaling, and abnormal glutamatergic, GABAergic and dopaminergic activity as well as delayed oligodendrocyte development. In particular, it is remarkable that the number of parvalbumin-positive interneurons is decreased in the cortex of this transgenic mouse. Flames et al. (2004) demonstrated that loss of Neuregulin-1/ErbB4 signaling causes an alteration in the tangential migration of cortical interneurons and reduction in the number of GABAergic interneurons in the postnatal cortex. Therefore, PTPζ/phosphacan may negatively regulate Neuregulin-1/ErbB4 signaling, and inhibit the tangential migration of cortical interneurons.

Neuroglycan C was identified as a potential susceptibility gene for schizophrenia in a Southern Chinese population (So et al., 2010). As described above, neuroglycan C is involved in the radial neuronal migration in the neocortex (Zhang et al., 2013), and thus defects in this process may be involved in the etiology of schizophrenia. In addition, neuroglycan C has an EGF-like domain, acts as a direct ligand for ErbB3, and thus is classified as Neuregulin-6 (Kinugasa et al., 2004). It has been reported that ErbB3 is associated with schizophrenia in a Caucasian population (Li et al., 2009), and thus there is a possibility that neuroglycan C-ErbB3 signaling is involved in the pathophysiology of schizophrenia. ErbB3 plays important roles in oligodendrocyte differentiation and myelination (Mei and Nave, 2014), and neuroglycan C may regulate these processes. In this context, it is interesting to note that midkine-neuroglycan C signaling promotes process elongation of the oligodendrocyte precursor-like cell line, CG-4 (Ichihara-Tanaka et al., 2006).

In addition to proteoglycan core proteins, glycosaminoglycan-modifying enzymes have also been associated with psychiatric disorders. A genome-wide association study revealed that *NDST3* is associated with schizophrenia and bipolar disorder, suggesting that the sulfation pattern of heparan sulfate plays an important role in the pathophysiology of these disorders (Lencz et al., 2013). Neuregulin-1 binds with heparan sulfate in a sulfation pattern-dependent manner, in which the *N*-sulfate group is the most important (Pankonin et al., 2005). Thus, the *N*-sulfated region in heparan sulfate may be important for normal Neuregulin-1/ErbB4 signaling.

The genomic region containing *DSEL* encoding dermatan sulfate epimerase 2 has been found to be associated with bipolar disorder (Goossens et al., 2003) and depressive disorder (Shi et al., 2011). In addition, it was reported that *UST* is associated with job-related exhaustion and response to antidepressant (Uher et al., 2010; Sulkava et al., 2013). Thus, chondroitin sulfate proteoglycans bearing oversulfated dermatan/chondroitin sulfate may play important roles in the etiology of mood disorders.

Perspective

As described above, brain proteoglycans regulate the migration of both excitatory and inhibitory neurons by binding with various proteins. Besides neuronal migration, proteoglycans play important roles in the proliferation and differentiation of neural progenitor cells, axon pathfinding, myelination, axon regeneration, and maturation and plasticity of synapses (Maeda et al., 2011; Soleman et al., 2013; Silver and Silver, 2014; Theocharidis et al., 2014), the defects of which may be related to the pathogenesis of various brain disorders. More mechanistic studies are necessary to elucidate the relationship between proteoglycans and these diseases. In this context, it should be noted that many extracellular matrix proteins that interact with proteoglycans are overlooked in the field of developmental neuroscience. These include extracellular matrix proteases and their inhibitors such as MMP, ADAMTS, ADAM, and Timp family members. Proteoglycans turn over very rapidly in the developing brain, and their degradation would lead to drastic change in the distribution and activity of growth factors, chemokines, axon-guidance molecules, and so on. Thus, it is likely that degradation of proteoglycans would profoundly influence the behavior of neurons. Future study is necessary to shed light on this issue.

Finally, I would like to emphasize that the functions of proteoglycans are regulated in a context-dependent manner. It is often said that chondroitin sulfate proteoglycans are repulsive molecules. However, this over-simplified view has been challenged by the finding that chondroitin sulfate proteoglycans such as PTPζ and neuroglycan C promote the radial migration of cortical neurons. Furthermore, chondroitin sulfate proteoglycans may function as either a repulsive or an attractive substrate depending on the factors attached to the chondroitin sulfate chains. In addition, it should be noted that even though the proteoglycan core protein is the same, the structures of the attached glycosaminoglycan chains may be highly variable leading to the diversification of proteoglycan functions. I expect that careful experimental design and interpretation of the results would uncover the important functions of brain proteoglycans.

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REVIEW
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Neuronal and microglial regulators of cortical wiring: usual and novel guideposts

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Neocortex functioning relies on the formation of complex networks that begin to be assembled during embryogenesis by highly stereotyped processes of cell migration and axonal navigation. The guidance of cells and axons is driven by extracellular cues, released along by final targets or intermediate targets located along specific pathways. In particular, guidepost cells, originally described in the grasshopper, are considered discrete, specialized cell populations located at crucial decision points along axonal trajectories that regulate tract formation. These cells are usually early-born, transient and act at short-range or via cell-cell contact. The vast majority of guidepost cells initially identified were glial cells, which play a role in the formation of important axonal tracts in the forebrain, such as the corpus callosum, anterior, and post-optic commissures as well as optic chiasm. In the last decades, tangential migrating neurons have also been found to participate in the guidance of principal axonal tracts in the forebrain. This is the case for several examples such as guideposts for the lateral olfactory tract (LOT), corridor cells, which open an internal path for thalamo-cortical axons and Cajal-Retzius cells that have been involved in the formation of the entorhino-hippocampal connections. More recently, microglia, the resident macrophages of the brain, were specifically observed at the crossroads of important neuronal migratory routes and axonal tract pathways during forebrain development. We furthermore found that microglia participate to the shaping of prenatal forebrain circuits, thereby opening novel perspectives on forebrain development and wiring. Here we will review the last findings on already known guidepost cell populations and will discuss the role of microglia as a potentially new class of atypical guidepost cells.

Keywords: guidance molecules, glial cells, Cajal-Retzius cells, microglia, axon guidance

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Introduction

Functioning of the mammalian cerebral cortex relies on complex networks of axonal connections between neurons located in specific positions. The initial building of these exquisite circuits occurs during embryonic development and early post-natal days. During this "critical" period, neurons are first generated from spatially restricted proliferative niches and after or while reaching their final destination throughout active migration, extend oriented axons to form synaptic connections with their targets. Cellular migration is hence essential in the first part of brain wiring, because it allocates cells to specific positions and their subsequent settlement and differentiation, leading

to the emergence of a functional system. In particular, cells can undertake radial or tangential migratory trajectories. During these processes, neurons can migrate locally or far away from their production sites as well as extend local axons or form long-range connections.

Because neurons are generated over a long-time period in the mammalian brain, neuronal migration and axonal navigation occur concomitantly during the constant process of brain development. How are these processes coordinated spatially and temporally to ensure the proper wiring of neural circuits? Over the last decades, this intriguing question has begun to receive answers in a developmental context in which cellular migration and axonal navigation take prominent places, namely the development of the embryonic mammalian forebrain (Borrell and Marin, 2006; Griveau et al., 2010; Villar-Cervino et al., 2013). In distinct regions of the embryonic mammalian forebrain, such as the dorsal cerebral cortex and the ventrally located subpallium, extensive events of radial and tangential migration reallocate neuronal populations and orient axonal navigation. For example, early-born neurons such as Cajal-Retzius cells spread out from different regions of origin to cover all the surface of the cortical primordium; inhibitory interneurons originate from the basal ganglia from which they tangentially migrate to populate the telencephalon; corticothalamic and thalamocortical axons traverse intermediate targets to reach their respective final targets. These events are fundamental to assemble cortical circuits and build the intricate circuitry essential for its functioning. Since defects in migratory processes during embryogenesis have been correlated with the onset of several neurologic and psychiatric diseases, it is crucial to decipher how they are regulated. Besides cells redistribution and morphogenesis, past and more recent studies showed that, throughout neuronal migration, an additional prominent event occurs during forebrain development that is the positioning of molecular cues, which instructs the trajectories of other migrating cells and growing axons. The cells that show these driving properties have different origins, but they share some common characteristics, for which they have been defined as guidepost cells. The purpose of this review is first to provide a definition of the usual concept of guidepost cells, giving an overview of already well-known examples. Moreover, we propose to extend the classical concept of guidepost cells, by speculating on recent findings concerning novel roles of microglia, the macrophages of the brain, in embryonic forebrain wiring.

Toward a "Modern" Definition of Guidepost Cells

The concept of guidepost cells emerged from the studies on the developing limb bud of the grasshopper embryo (Borrell and Marin, 2006; Griveau et al., 2010; Kwon et al., 2011; Villar-Cervino et al., 2013). Bate and others described how pioneer projecting axons rely on some intermediate targets positioned along the future axonal path to follow a highly stereotyped pathway (Kwon et al., 2011). These intermediate targets consist of immature neuronal cells that show high affinity for the pioneer growth cones, and that are able, upon direct contact, to stabilize their filopodia and reorient the axonal growth cones on the pathway (Kwon et al., 2011). These important findings laid the foundation of the term "guidepost cells," as located discontinuously along the future axonal trajectory providing short-range cues thereby precisely controlling axonal navigation.

Since these seminal studies, several other cases of guidepost cells have been reported in different organisms and developmental systems (Borrell and Marin, 2006; Griveau et al., 2010; Kwon et al., 2011; Villar-Cervino et al., 2013). To date, the vast majority of the identified guidepost cells in vertebrates belongs to the class of glial cells, such as the radial glia of the optic chiasma (Misson et al., 1988; Guillery et al., 1995; Marcus et al., 1995; Marcus and Mason, 1995; Wang et al., 1995), glial bridges of anterior and postoptic commissures (Silver et al., 1982; Pires-Neto et al., 1998; Barresi et al., 2005; Lent et al., 2005), floor plate cells (Tessier-Lavigne et al., 1988; Bovolenta and Dodd, 1990, 1991; Placzek et al., 1990; Campbell and Peterson, 1993; Kennedy et al., 1994; Serafini et al., 1994, 1996), boundary cap cells (Golding and Cohen, 1997; Fraher et al., 2007), glial cells of the corpus callosum (Silver et al., 1982, 1993; Silver and Ogawa, 1983; Shu and Richards, 2001; Shu et al., 2003a,b). More recently, some populations of tangential migrating neurons have also been discovered to play a guidepost role, with a consequent need to expand the conceptual definition (Sato et al., 1998; Lopez-Bendito et al., 2006; Niquille et al., 2009; Bielle et al., 2011; Hirata et al., 2012). Guidepost cells have been then defined as usually early born, discrete cell populations, with specialized functions that control and regulate axonal navigation, by being located at crucial decision points along the axonal trajectories. These cells can eventually extend an axon along the upcoming path of the tract and, in contrast to other long range-intermediate targets, guideposts act at short range or directly by cell-cell contact. They constitute decisive landmarks for guiding the axons along the correct pathways, which is a fundamental requirement for accurate circuitry assembly. The demonstration of their importance has been highlighted in different systems, by specific cell ablation experiments (Bentley and Caudy, 1983; Sretavan et al., 1995; Del Rio et al., 1997; Sato et al., 1998) and by the use of genetic mutants (Bovolenta and Dodd, 1990; Lopez-Bendito et al., 2006; Bielle et al., 2011), which resulted in aberrant pioneer axonal trajectories (Bielle et al., 2011), eventually with ectopic collateral branches formation (Bentley and Caudy, 1983; Bovolenta and Dodd, 1990) and in failure in axonal progression and in specific axonal innervation (Sretavan et al., 1995; Del Rio et al., 1997; Sato et al., 1998; Lopez-Bendito et al., 2006), respectively. The mechanism by which guidepost cells exert their function in guiding pioneer axonal tracts is throughout the secretion of guidance cues, which can act as attractive or repulsive signals. Both glial cells and tangential migrating guidepost cells have been found to express various families of guidance molecules or adhesion molecules, such as Slits (Erskine et al., 2000; Plump et al., 2002; Shu et al., 2003c), Robos (Bielle et al., 2011), Wnts (Keeble and Cooper, 2006), Neuregulin (Lopez-Bendito et al., 2006), Draxin (Islam et al., 2009), Ephrins (Williams et al., 2003; Mendes et al., 2006) or extracellular matrix proteins (Kuhn et al., 1995; Mandai et al.,

2014). The correct positioning of the same migrating guidepost cells at the intermediate targets along the path is itself instructed by guidance cues (Kawasaki et al., 2006; Nomura et al., 2006; Ito et al., 2008; Bielle et al., 2011). Since these cells act mainly at short range or by cell-cell contact, their proper localisation is fundamental for the subsequent axonal tract development. This has been clearly shown in various guidance molecule mutant models in which altered positioning of guidepost cells led to consequent specific axonal pathfinding defects (Kawasaki et al., 2006; Bielle et al., 2011).

Tangential Migrating Guidepost Cells in the Pathfinding of the Lateral Olfactory Tract

The Lateral Olfactory Tract (LOT) is the main efferent axonal bundle that conveys the olfactory information from the bulb to several higher olfactory centers in the brain, including the anterior olfactory nucleus, the olfactory tubercle, the piriform and entorhinal cortices and the amygdala (Borrell and Marin, 2006; Griveau et al., 2010; Villar-Cervino et al., 2013) (**Figure 1**). Indeed, sensory olfactory neurons, residing in the nasal cavities project onto tufted and mitral cells of the olfactory bulb, which in turn extend their axons into the LOT to reach cortical and associated regions. LOT pioneer axons initiate their outgrowth around embryonic day (E) 11.5, followed by the other mitral cells that collectively form the main axonal bundle around E13. Starting from E14.5, LOT axons extend superficial collaterals toward the olfactory cortices and into the other target regions.

Long-range guiding activities are involved in shaping the pathfinding of the LOT. Diffusible repulsive guidance proteins, such as Slit1 and Slit2 derived from the septum, regulate the lateral pathfinding of the mitral cell axons, throughout their receptors, Robo1 and Robo2 (Pini, 1993; Nguyen Ba-Charvet et al., 1999; Nguyen-Ba-Charvet et al., 2002; Fouquet et al., 2007). Some proteins of the Semaphorin class are involved in the growth of olfactory bulb axons (Sema3B) and repulsion of LOT axons (Sema3F) (De Castro et al., 1999; De Castro, 2009). Besides these diffusible long-range signals, it was shown that a peculiar population of cells supplies short-range permissive guidance activity in the formation of the LOT (Sugisaki et al., 1996). These "lot" cells have been identified by the expression of the lot1 antibody (Sato et al., 1998), recently shown to recognize the beta isoform of the metabotropic glutamate receptor subtype-1 (mGluR1) (Hirata et al., 2012). Lot cells are the first reported example of migrating neuronal guidepost cells involved in axonal pathfinding and are amongst the first generated neurons in the brain, around E9.5 and E11.5. They have been proposed to have a pallial origin and migrate toward the pallial subpallial boundary (PSB) (Tomioka et al., 2000). Once arrived in the PSB, they change their orientation and extend a long process toward the amygdala region (Kawasaki et al., 2006; Hirata et al., 2012). They correspond to previously identified cells horizontally disposed in the developing PSB (Derer et al., 1977) and their positioning occurs way before the arrival of LOT axons (Sato et al., 1998), for which they constitute a growing substrate. Around E12.5, the superficial growing of LOT axons displaces lot cells in the internal border of the path, where they are found subsequently in association with growing collateral axons (Hirata and Fujisawa, 1999). Although initially lot cells where considered as a distinct and unique cell population (Sato et al., 1998), these cells have been recently identified as a subset of Cajal-Retzius (CR) cells, a population of early born cortical neurons, since they share the expression of common molecular markers such as p 73 and Reelin (Dixit et al., 2014).

The role of CR-lot cells as guidepost for the developing LOT tract has been shown throughout toxic ablation experiments by the local use of a neuronal toxin, 6-hydoxydopamine, which

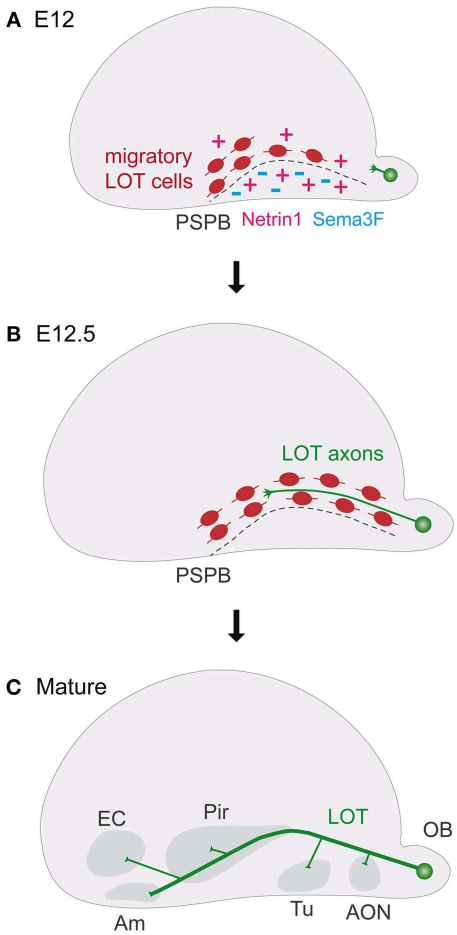

FIGURE 1 | Guidepost cells in lateral olfactory tract (LOT) development. (A–C) The panels represent schematic lateral views of mouse embryonic cerebral vesicles. **(A)** Lot cells are amongst the first generated neurons in the brain. At E12 they are located along the pallial subpallial boundary (PSPB, dashes) before the arrival of LOT axons. Netrin1 attracts Lot cells toward the PSPB and Sema3F limits their migration. **(B)** At E12.5, LOT axons originating from the olfactory bulbs extend superficially in close contact with lot cells. **(C)** In the mature brain, the LOT contains axons projecting from the olfactory bulb to the anterior olfactory nucleus (AON), the olfactory tubercle (Tu), the piriform cortex (Pir), the entorhinal cortex (EC), and the amygdala (Am). Am, amygdala; AON, anterior olfactory nucleus; EC, entorhinal cortex; LOT, lateral olfactory tract; OB, olfactory bulb; Pir, piriform cortex; PSPB, pallial subpallial boundary; Tu, olfactory tubercle.

provokes CR-lot cell death with consequent stall of mitral cell axons in strict proximity (Sato et al., 1998). This role is further highlighted by the analysis of mutant mice that affect this cell population such as Lhx2 (Saha et al., 2007) or Neurog1 and Neurog2 double mutants (Dixit et al., 2014). It has been proposed that lot cells form transient connections with LOT axons, as their final targets in the piriform cortex, amygdala and other higher olfactory centers are not yet mature (Sato et al., 1998; Hirata et al., 2012).

The importance of the proper positioning of CR-lot cells is thus highlighted by their guidepost function to orient LOT axons along their pathway. Still, how these cells act on axons and whether they are required for the progression, channeling or guidance of all axons remains largely to be characterized. By contrast, several guidance cues have been shown to play a role in the positioning of CR-lot cells in the ventral PSB. Netrin1 has been shown to act as an attractant cue for migrating CR-lot cells and participates, in part, to their ventral positioning (Kawasaki et al., 2006). However, in knockout mutant animals for *Netrin1* or its receptor *DCC*, only the location of the most ventral CR-lot cells resulted affected, associated with specific pathfinding defects on the ventral most LOT axons (Kawasaki et al., 2006). In double *Slit1; Slit2* mutants the LOT axonal tract is severely disrupted, with only few axons present in their correct positions. In this context, the proper positioning of CR-lot cells appears to be not drastically affected, thereby revealing that both long-range and local signals cooperate in LOT axonal pathfinding (Fouquet et al., 2007). Another important regulator of the ventral tangential migration of CR-lot cells is the molecule Sema3F that, by the interaction with its specific receptor neuropilin-2 (Nrp-2), confines CR-lot cells on the telencephalic surface (Ito et al., 2008). Sema3F, expressed in the subpallium and cortical plate, acts as a repellent signal, which prevents CR-lot cells to penetrate into deep brain regions, where some are ectopically found in case of *Sema3F* or *Nrp-2* invalidation (Ito et al., 2008). So far, there are not yet reported defects of LOT projections in *Nrp-2* mutants (Chen et al., 2000), raising the possibility that these guidepost cells may act locally. Furthermore, since many of these guidance cues can directly act on the axons, additional eventual effects of these genetic invalidations on the pathfinding of LOT axons deserve further analyses.

Cajal-Retzius Cells: Guideposts in the Formation of Entorhino-Hippocampal Projections

Besides their emerging role in LOT axonal guidance, Cajal-Retzius cells, together with GABAergic interneurons, have been involved in the development of entorhino-hippocampal projections (Borrell and Marin, 2006; Griveau et al., 2010; Villar-Cervino et al., 2013). The major afferent excitatory projections in the hippocampus derive from pyramidal neurons in layers II and III of the entorhinal cortex. In particular, layer II pyramidal neurons form axonal connections with the dendrites of the granule cells of the outer molecular layer (OML) of the dentate gyrus (DG), whereas layer III neurons connect mainly with pyramidal cells in the stratum lacunosum-moleculare (SLM) in the cornu ammonis 1 and 3 (CA1 and CA3) (Borrell and Marin, 2006; Griveau et al., 2010; Villar-Cervino et al., 2013). Notably, during brain formation, the entorhinal axons already reach their final positions in the hippocampal regions, before the definitive development of their targets. Indeed, in mouse brain, entorhinal axons arrive in the hippocampus around E15, then they form arborisations in the SLM around E17 and are detected into the OML starting from the first postnatal day (Super and Soriano, 1994; Super et al., 1998; Deng and Elberger, 2001; Deng et al., 2006) (**Figure 2**). Therefore, even if hippocampal pyramidal neurons and granule cells are generated between E14 and E16, it is only around the second postnatal day that their apical dendrites start to be seen in the SLM, arising as final targets for entorhinal axons (Caviness, 1973; Soriano et al., 1986, 1989; Bayer and Altman, 1987; Super et al., 1998). This process of precise axonal addressing is regulated by Cajal-Retzius cells, which, as in LOT formation, have been reported to regulate axonal outgrowth. Cajal-Retzius (CR) cells are early born neurons, which are produced at E9-11 by focal pallial sources, including cortical hem, septum, PSB, and thalamic eminence (Grove et al., 1998; Meyer et al., 1999, 2002; Meyer and Wahle, 1999; Hevner et al., 2003; Takiguchi-Hayashi et al., 2004; Bielle et al., 2005; Cabrera-Socorro et al., 2007; Tissir et al., 2009; Ceci et al., 2010; Meyer, 2010; Gu et al., 2011; Martinez-Cerdeno and Noctor, 2014). CR cells migrate tangentially from their sources in the marginal zone of the cerebral cortex and rapidly cover the entire sheet. Their marginal localization and migration is regulated by CXCL12 produced by the meninges, which acts through CXCR4 and CXCR7 receptors (Borello and Pierani, 2010; Trousse et al., 2014). The marginal maintenance of CR cells also requires radial glia integrity, as revealed by

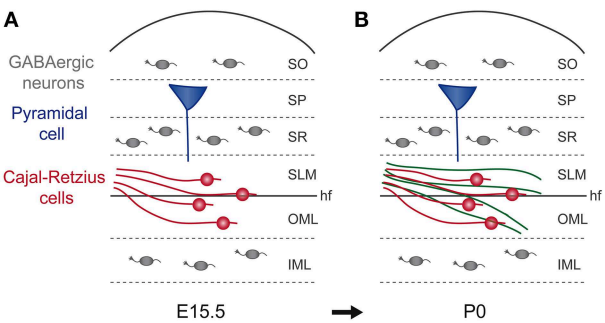

FIGURE 2 | Cajal-Retzius cells are guideposts for enthorhino-hippocampal axons. (A,B) The panels represent schematic coronal sections of mouse hippocampi at E15.5 and P0. **(A)** At E15.5, Cajal–Retzius cells (red) are distributed in SLM and OML and GABAergic neurons (gray) are distributed in stratum oriens (SO), stratum radiatum (SR), and inner molecular layer (IML). **(B)** The major afferent excitatory projections in the hippocampus derive from pyramidal neurons in layers II and III of the entorhinal cortex. Between E16.5 and P0, entorhinal axons (green) invade specifically the SLM and OML in close association with Cajal–Retzius cells even if their future final target, the apical dendrites neurons of the pyramidal layer (SP, blue) develop later. hf, hippocampal fissure; IML, inner molecular layer; OML, outer molecular layer; SLM, stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum.

analysis of β1 integrin conditional knockout (Kwon et al., 2011). In parallel, interactions between CR cells or with surrounding structures have been shown to control their random dispersion and distribution by Eph/ephrin-dependent contact repulsion or PlexinD1 signaling (Villar-Cervino et al., 2013; Bribian et al., 2014). Such migratory behaviors enable CR cells of different sources to preferentially cover cortical regions (Griveau et al., 2010; Gu et al., 2011). Functionally, CR cells have been shown to regulate cortical layering, neuronal and radial glia morphology, and cortical regionalisation, via the production of the secreted glycoprotein Reelin or additional membrane-bound or secreted factors (Borello and Pierani, 2010; Griveau et al., 2010; Gil-Sanz et al., 2013; Trousse et al., 2014). In the hippocampus, as the structure folds during development, CR cells localize in the future SLM and OML (Takiguchi-Hayashi et al., 2004; Bielle et al., 2005; Yoshida et al., 2006). Using electron microscopy, it has been shown that during embryogenesis pioneer entorhinal axons form transient synaptic contacts with Cajal-Retzius cells in SLM and OML, the future regions that will host pyramidal and granule cells apical dendrites (Super et al., 1998). Moreover, in organotypic culture experiments, this interaction has been shown to be fundamental for the growth of entorhinal axons in the hippocampus (Frotscher and Heimrich, 1993; Li et al., 1993; Frotscher et al., 1995; Del Rio et al., 1997). Indeed, eliminating CR cells from cultured slices with the local toxin 6-hydoxydopamine avoided entorhinal axonal growing in the hippocampus (Del Rio et al., 1997). These experiments therefore constituted a strong evidence of the role as placeholders for CR cells in the formation of entorhino-hippocampal connections (Forster et al., 1998). About the molecular cues that could be involved in entorhinal axon guidance by CR cells, the most obvious candidate could be the glycoprotein Reelin, of which CR cells constitute the main source (D'arcangelo et al., 1995, 1997; Hirotsune et al., 1995; Ogawa et al., 1995; Tissir and Goffinet, 2003). It has been previously shown that Reelin controls cortical layering, organization and the orientation of radially migrating neurons (Borello and Pierani, 2010; Frotscher, 2010; Griveau et al., 2010; Martinez-Cerdeno and Noctor, 2014). Nevertheless, antibody-mediated blocking of Reelin in *ex vivo* co-cultures of hippocampal slices and entorhinal tissue, does not lead to dramatic defects in entorhinal axonal pathfinding. However, fewer entorhinal fibers reach the hippocampal layers, developing shorter axonal branches. These findings have been confirmed *in vivo* in *reeler* mice, a natural Reelin mutant, which presents severe defects in cortical lamination. Similarly to co-cultures experiments, the absence of Reelin had no dramatic effects about entorhinal axonal ingrowth or targeting, but entorhino-hippocampal axonal terminations appear thinner than in control animals. Moreover, these defects are transient, since then in *reeler* adult mice a normal branching density is observable (Frotscher and Heimrich, 1993; Li et al., 1993; Frotscher et al., 1995; Del Rio et al., 1997; Borrell et al., 1999; Deller et al., 1999). Altogether, these results confirm the important role of CR as guidepost cells in entorhino-hippocampal innervation and reveal reelin as an important factor for branching, collateral formation and synaptogenesis of entorhinal axons. However, they leave still an open question about additional molecular cues involved in the pathfinding of entorhinal axons in the hippocampus.

En Route to the Cortex: Guidepost Cells Open a Path for Thalamocortical Connections

Mammalian neocortex forms connections with the rest of the brain via the internal capsule, which includes bundles of corticofugal efferent axons and reciprocal afferent thalamocortical projections, which convey sensory and motor information to the neocortex. In the context of axonal pathfinding, the development of the internal capsule has been extensively studied (Molnar et al., 2012; Garel and Lopez-Bendito, 2014). Indeed, this system has a major physiological relevance, but also allows a variety of experimental approaches, due to its important size and extension in the developing brain.

During the years, many findings had contributed to elucidate the routes and the molecular mechanisms that shape thalamocortical and corticofugal connection paths. In mouse development, these important axonal systems start to form during early/mid gestation. Thalamocortical axons (TCAs) originate from neurons located in the thalamus, grouped in distinct nuclei, showing a topographic organization that corresponds to the spatial innervation of different cortical areas (Molnar et al., 2012; Garel and Lopez-Bendito, 2014). From E12 to E15, TCAs extend ventrally, crossing the prethalamus, and traverse the diencephalic/telencephalic boundary, entering the subpallium at the level of the internal capsule. At E14, early TCAs reach the PSB, where they encounter the reciprocal pioneer corticothalamic axons (CTAs). Subsequently, from E14.5 to E18.5, TCAs form transient connections with subplate cells residing in their respective target cortical areas. After this waiting period, TCAs send collaterals into the cortical plate and finally establish thalamocortical connections (**Figure 3**). Meanwhile, corticofugal axons grow along the same path of TCAs and split in CTAs and in corticosubcerebral axons that proceed toward other subcortical regions. This reciprocal wiring has been shown to be tightly controlled, in part by guidepost cells, transient axonal populations and several structures that have been shown to act as milestones along the path.

Chronologically, pioneer cortical subplate neurons have been firstly proposed as guidepost cells in regulating the entering and progression of TCAs into the cortical plate (Garel and Lopez-Bendito, 2014; Hoerder-Suabedissen and Molnar, 2015). These observations have been strongly supported by several experimental evidences. To date, in the visual cortex, subplate neurons ablation avoids the entering of the corresponding thalamic geniculocortical axons (Ghosh et al., 1990; Ghosh and Shatz, 1993). In mutant mice, such as *reeler*, $p35^{-/-}$ and $cdk5^{-/-}$ that present subplate cells in the marginal zone, due to severe defects in preplate splitting, TCAs form abnormal projections to connect with the ectopic subplate in the marginal zone. Since the discovery of subplate cells, other groups of cells have been found to exert a role of guidepost for TCAs. In mouse, early born cells, named perireticular cells, have been identified in the

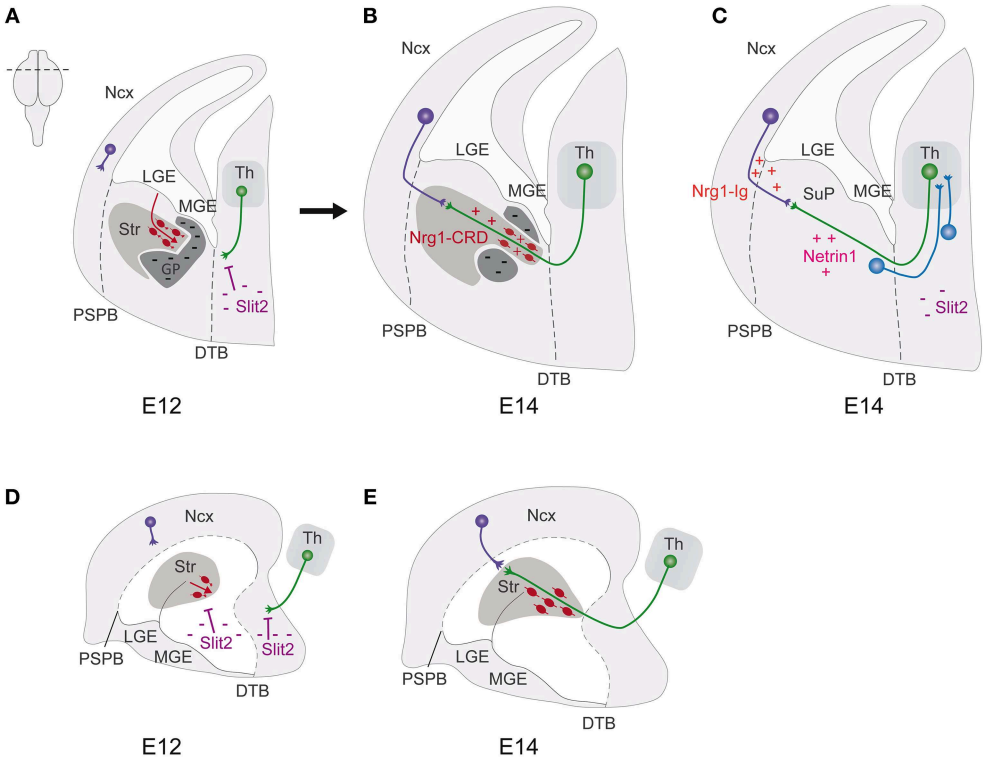

FIGURE 3 | Corridor neurons shape the internal pathfinding of thalamocortical axons. (A–C) Schematic representation of hemicoronal sections of mouse embryonic telencephalons. **(D,E)** Schematic representation of median sagittal views of mouse embryonic telencephalons. **(A,D)** At E12, tangentially migrating LGE-cells (red), repelled by Slit2, form in the MGE a permissive corridor for thalamocortical axons. Thalamocortical axons (green), repelled by hypothalamic Slit2, turn to enter the MGE. **(B,E)** At E14, pioneer thalamocortical axons grow through the permissive corridor (red cells) and the Str, where they encounter the reciprocal pioneer corticothalamic axons (purple). Guidance cues including Slits, Netrin1, Nrg1-IG, Ng1-CRD present along the pathway, orient the axons. **(C)** Thalamocortical axons (green) extend from the dorsal thalamus (Th) toward the neocortex (Ncx), crossing the diencephalic-telencephalic boundary (DTB, black dashes), and enter the subpallium (SuP) at the level of the internal capsule. At E14, thalamocortical axons reach the pallial subpallial boundary (PSPB, black dashes), where they encounter the reciprocal pioneer corticothalamic axons (purple). Thalamocortical axon guidance is regulated by different cellular and molecular actors: prethalamus (blue) and SuP cells sending an axon to the Th; the repellent Slit2 in the hypothalamus, the attractant Netrin1 in the SuP, Nrg1-Ig in the neocortex. DTB, diencephalic-telencephalic boundary; GP, globus pallidus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Ncx, neocortex; PSPB, pallial subpallial boundary; Str, striatum; SuP, subpallium; Th, dorsal thalamus.

future path of the internal capsule, which at E12.5 send a cellular process to the thalamus. The hypothesized role of these cells is to provide a cellular scaffold for the future TCAs and CTAs, which is consistent with several experimental evidences (Mitrofanis, 1992, 1994; Mitrofanis and Baker, 1993; Mitrofanis and Guillery, 1993; Adams and Baker, 1995; Metin and Godement, 1996; Molnar et al., 1998b; Braisted et al., 1999; Molnar and Cordery, 1999). In effect, different defects in TCA pathfinding have been reported in mutant mice presenting absence, reduction or displacement of perireticular cells (Tuttle et al., 1999; Bishop et al., 2000, 2003; Lopez-Bendito et al., 2002; Lakhina et al., 2007). Unfortunately, the current absence of specific molecular markers and the wide distribution of these cells experimentally limit the investigation about their origin and function.

More recently, another population of guidepost cells has been observed in the subpallium, which comprises the lateral and medial ganglionic eminences (LGE and MGE). These cells are GABAergic LGE derived-neurons that tangentially migrate in the MGE, forming a permissive corridor, in an otherwise not permissive territory, for the growth of TCAs along an internal path toward the cortex (Lopez-Bendito et al., 2006). Because of their function, they have been named "corridor" cells; they are located in the MGE, in which they migrate from E11.5 to E14, but express LGE molecular markers, such as Islet1, *Ebf1* and *Meis2*. By gain-of-function experiments in cultured organotypic embryonic brain slices and by the use of full or conditional mutant mice of ErbB4 and Neuregulin1 respectively, it has been revealed that corridor cells, via the expression of Neuregulin1, provide a permissive corridor for TCAs, which express the corresponding ErbB4 receptor (Lopez-Bendito et al., 2006). How are corridor cells positioned? A ventral repulsive activity from the subpallium, mediated by Slit2 and Robo1 and Robo2 respective receptors, has been shown to limit, *in vitro*, *ex vivo,* and *in vivo*, the ventral tangential migration of corridor cells, playing a role in the formation of corridor shape. Indeed, *Slit2* inactivation leads to abnormal ventral migration of corridor cells, with aberrant corridor shaping and consequent defects on TCAs pathfinding (Bielle et al., 2011). These findings constitute a starting point

for further investigations on the origin, specification and fate of corridor cells.

In addition to guidepost cells, several important structures and molecules present in the subpallium or prethalamus have been shown to play critical roles in the guidance of both TCAs and reciprocal CTAs (Metin and Godement, 1996; Braisted et al., 1999; Garel et al., 1999; Sussel et al., 1999; Marin et al., 2002; Marin and Rubenstein, 2002; Yun et al., 2003). For instance, knockout mutation analyses, have highlighted the importance of classical guidance cues and their receptors, such as Slit1, Slit2, Robo1, Robo2, Netrin1, and Sema6A, for the local regulation of TCAs guidance (Braisted et al., 2000, 2009; Leighton et al., 2001; Bagri et al., 2002; Bonnin et al., 2007; Lopez-Bendito et al., 2007; Powell et al., 2008; Little et al., 2009). Other important regulators are the members of the protocadherin family, which have been shown to be important for both TCAs and CTAs progression into the subpallium (Wang et al., 2002, 2006; Tissir et al., 2005; Uemura et al., 2007; Zhou et al., 2008, 2009; Qu et al., 2014). For instance, inactivation of OL-protocadherin was shown to impair the subpallial crossing of TCA associated with defects in striatal axonal outgrowth (Uemura et al., 2007). In addition, mutant mice with constitutive or specific inactivation of *Celsr3* in the prethalamus and subpallium present similar impairments, which consist in the stall of TCAs in the ventral subpallium, across the diencephalic/telencephalic boundary, and in CTAs arrest in the proximal part of the LGE, suggesting a possible cooperation of these two factors in the process (Tissir et al., 2005; Zhou et al., 2008, 2009; Qu et al., 2014). Strikingly, these phenotypes are almost phenocopied by deletion of *Frizzled3*, which is associated with *Celsr* signaling pathway (Wang et al., 2002, 2006). Last, but not least, the expression of the transmembrane protein Linx is required on subplate cells, subpallium and prethalamaus guideposts, for the progression of TCAs and CTAs revealing a role for this molecule in axon/axon interactions and potentially guideposts/axons interactions (Mandai et al., 2014). More generally, it will be essential to precise which of the aforementioned guidance cues regulates the positioning and/or function of guidepost cells located along the internal capsule path.

In addition to delineating an internal trajectory for TCAs, guidepost neurons have been shown to play additional roles in thalamo-cortical wiring. First, there is now solid evidence that TCAs and CTAs interact to form reciprocal connections, as proposed by the handshake hypothesis (Blakemore and Molnar, 1990; Molnar and Blakemore, 1991, 1995; Chen et al., 2012; Molnar et al., 2012; Deck et al., 2013; Garel and Lopez-Bendito, 2014). As such, guideposts that shape TCAs path have an indirect impact on the guidance of reciprocal CTAs. Second, while TCAs originating from principal thalamic nuclei all grow internally in the corridor, they adopt distinct rostrocaudal positions in the capsule, depending on their nucleus of origin and their cortical target (Molnar et al., 1998a, 2012; Garel and Lopez-Bendito, 2014). This topographic ordering has been shown to depend on local subpallial positional information (Dufour et al., 2003; Bonnin et al., 2007; Wright et al., 2007; Powell et al., 2008; Bielle et al., 2011; Demyanenko et al., 2011a,b; Lokmane et al., 2013). Indeed, guidance factors such as Slit1 and Netrin1 (and their combinatorial activity), Sema3A, ephrinAs, as well as L1, CHL1 participate to the topographic ordering of TCAs deriving from different thalamic nuclei, with a dramatic impact on their final cortical addressing (Bonnin et al., 2007; Wright et al., 2007; Powell et al., 2008; Bielle et al., 2011; Demyanenko et al., 2011a,b; Lokmane et al., 2013). Remarkably, positional information has been shown to be present already in the corridor, as TCAs enter the subpallium (Bielle et al., 2011). Accordingly, the aforementioned guidance factors are present in the corridor, especially Slit1, supporting the idea that they act as TCAs grow internally (Bielle et al., 2011). Importantly, altering the ordering of TCAs in the subpallium by genetic manipulation has been shown to impair the fine topography of TCAs in the somatosensory cortex (Lokmane et al., 2013; Lokmane and Garel, 2014). These experiments reveal that in addition to cortical signals, intermediate ordering of axons, in part by corridor cells, is important for fine-grained topography of TCAs. Together, such recent studies highlight additional roles of corridor internal guideposts in reciprocal and topographical wiring.

Microglia Cells: Novel Unusual Guidepost Cells?

Microglia are the resident macrophages of the brain, which control brain homeostasis in physiologic conditions and constitute the first line of defense in case of diseases and against pathological threats. Initially described by Del Rio-Hortega (1932), the physiological functions and the origin of these cells have been remained controversial for a long time. Until recently, most studies focused on the roles of microglia in brain damage and diseases and in their participation to neuro-inflammatory processes via the release of neurotrophic and pro-inflammatory factors as well as the ability to perform phagocytosis. Over last decade, several landmark studies have revealed that, using conserved cellular mechanisms, microglia contribute to normal brain functions. Indeed, microglia have been shown to modulate synaptic transmission, to regulate synaptic formation and elimination, and to shape postnatal and embryonic brain circuits as reviewed in Paolicelli and Gross (2011), Schafer et al. (2013), Bilimoria and Stevens (2014), Katsumoto et al. (2014), Paolicelli et al. (2014), Salter and Beggs (2014) and Casano and Peri (2015). Below, we will focus on specific features of microglia during early brain wiring that bear similarities with those of guideposts cells, such as their capacity to act at short range, their early origin and focal positioning as well as the production of several molecular factors by which they can interact with and condition their surrounding neural environment.

Microglia Survey and Interact with Their Local Environment

In the last decade, technological advancements such as two-photon laser scanning microscopy, allowed the observation of microglia behavior *in vivo*, in normal conditions. Ramified microglia, initially thought to be in a resting state in opposition to the activated, amoeboid morphology observed following brain injury, were found to be extremely active in

surveying their environment. Indeed, very frequent extensions and retractions of microglia ramifications were observed in contact with neighboring neuronal cells, astrocytes and blood vessels; furthermore their extensions were increased by changes in both neuronal activity, blood vessel lesions and ATP variation levels *in vivo* (Davalos et al., 2005; Nimmerjahn et al., 2005). Processes of "resting microglia" were found to interact with synapses in somatosensory and visual neocortex, forming direct appositions with different synaptic elements. In particular, morphological changes of microglia processes, with the appearance of phagocytic structures and modifications of synaptic apposition frequencies were shown to be modulated by variation in visual experience (Wake et al., 2009; Tremblay et al., 2010). Following similar lines, microglia were found to modulate neuronal activity (Li et al., 2012; Pascual et al., 2012). For instance, in zebrafish larvae optic tectum, microglia were shown to contact highly activated neurons for longer time, correlating with a subsequent decreased neuronal activity (Li et al., 2012). Conversely, microglia activation *in vitro* by LPS stimulation was reported to indirectly increase the frequency of spontaneous synaptic AMPAergic post-synaptic currents in hippocampal neurons (Pascual et al., 2012). Such findings have fundamentally changed our conception of microglia by revealing that these cells exert the capacity to act at short-range on their surrounding neural environment. Since then, besides their immune-defensives functions, these cells have started to be considered as active modulators during healthy brain development and maturation as well as actors of pathologic brain wiring and functioning.

Early Origin of Microglia

Originally thought to arise form peripheral bone marrow derived-macrophages that invade the brain after birth, microglia have been show, throughout series of fate-mapping experiments, to originate from yolk sac myeloid progenitors and to be dependent on Pu.1, Irf8 (Kierdorf et al., 2013) and colony-stimulating factor 1 receptor (CSF1R) (Ginhoux et al., 2010, 2013; Erblich et al., 2011; Schulz et al., 2012; Gomez Perdiguero et al., 2013; Kierdorf et al., 2013; Hoeffel et al., 2015). In mice, yolk sac derived-microglia precursors migrate into the neural folds during embryogenesis and, by *in situ* proliferation, generate microglia that populate the adult brain. Under normal conditions, microglia comprise resident cells since the infiltration of peripheral monocytes or macrophages into the CNS is limited by the blood-brain barrier (Mildner et al., 2007; Ginhoux et al., 2010; Schulz et al., 2012; Gomez Perdiguero et al., 2013). Thus, microglia enter the brain from early prenatal stages and form an autonomous, self-sustained population. Remarkably, colonization of embryonic brain tissues by microglia appears to be a highly conserved process across vertebrate species (Perry et al., 1985; Ashwell, 1991; Cuadros and Navascues, 2001; Herbomel et al., 2001; Verney et al., 2010; Schlegelmilch et al., 2011; Swinnen et al., 2013), suggesting that embryological "seeding" of the microglial population may be also conserved.

How is the number or density of microglia regulated? Different embryonic or postnatal methods have been reported for the ablation of microglia *in vivo* or in cultured brain slices (Duffield et al., 2005; Heppner et al., 2005; Varvel et al., 2012; Ueno et al., 2013; Elmore et al., 2014; Squarzoni et al., 2014). Among those methods, pharmacologic depletion models acting on CSF1R signaling, revealed that after birth, a complete microglia repopulation occurs in a 1-week time window (Elmore et al., 2014; Squarzoni et al., 2014). These results show that there is a homeostatic control over the microglial population and raise the questions of the underlying mechanisms. While the origin of these repopulating cells is still debated, it has been shown for the adult repopulation that a local brain pool of nestin-positive cells differentiates into microglia thereby restoring their usual number (Elmore et al., 2014). Collectively, these essential findings match some forward-looking theories formulated by del Rio Hortega, which postulated that microglia enter the brain during embryogenesis (Del Rio-Hortega, 1932); at the same time, they highlight how the constant presence of microglia within the brain is tightly regulated.

Microglia in Defining the Number of Neurons: Neurogenesis and Survival

Microglia have been recently shown to take part to several important events which contribute to shaping of neural circuits, including neurogenesis, neuronal survival, synaptic remodeling and maturation. The role in neurogenesis and survival has been examined in both the adult niche and the developing brain. For instance, in adult murine hippocampus, unchallenged microglia regulate by phagocytosis the number of immature neurons maintained in the subventricular zone, one of the few sites of postnatal neurogenesis (Sierra et al., 2010, 2013). In macaque and rat neocortex, alteration of microglia activity by maternal immune activation through LPS, Doxycycline treatment or microglia elimination by Liposomal clodronate exposition, significantly affects the number of neuronal precursors in the embryonic and postnatal brain (Cunningham et al., 2013).

In parallel, microglia have been also reported to regulate neuronal number by active induction of apoptosis, or oppositely to contribute to neuronal survival, in different regions of the brain. For instance, early postnatal apoptosis in the cerebellar Purkinje cell (PC) population was shown to be induced by superoxide ions generated from microglial respiratory bursts (Marin-Teva et al., 2004). These results provided support to previous studies showing that the depletion of microglia in brain culture slices *in vitro* resulted in increased PC survival (Van Rooijen et al., 1997). Likewise, in perinatal mouse hippocampus, microglia was found to enhance hippocampal neuronal apoptosis by the CD11b/DAP12 integrin signaling-dependent production of reactive oxygen species (Wakselman et al., 2008). Conversely, microglia have been shown to actively sustain postnatal cell survival of layer V cortical neurons in mouse by the production of the trophic factor IGF1 (Ueno et al., 2013). Indeed, postnatal microglia inactivation by minocycline, microglia temporal elimination in CD11b-DTR transgenic models, as well as the use of IGF1R inhibitors and *igf1* siRNA, resulted in increased cell death of layer V cortical neurons (Ueno et al., 2013). Altogether, these findings show that microglia regulate the number of neurons produced and maintained in the brain, through a balanced

activity on progenitors, immature neurons and maturing neurons.

Microglia Shape the Postnatal Brain: Synapse Formation and Synapse Pruning

Besides their roles on neurogenesis and neuronal cell homeostasis, microglia have been found to contribute to synaptogenesis, synaptic remodeling and brain maturation. Similarly to IGF1 for layer V cortical neurons, the production of the neurotrophin BDNF by microglia has been shown to promote synapse formation via signaling to its cognate receptor TrkB. Remarkably, specific microglia or microglia-BDNF depletion, using *CX3CR1*^{CreER} mice, both lead to deficits in multiple learning tasks and learning-induced synaptic remodeling (Parkhurst et al., 2013). These major findings highlight the ability of microglial cells to impact on the building and homeostasis of neural circuits throughout the very local active production of secreted factors.

In addition, microglia were shown to play an active role in postnatal synaptic pruning, contributing to the shaping and maturation of the brain, by their close spatial and temporal contact with synapses (Paolicelli et al., 2011; Schafer et al., 2012, 2013; Kettenmann et al., 2013). In particular, this has been directly observed in the retinogeniculate system, where surrounding microglia participate to the activity-dependent synaptic remodeling, eliminating the weaker presynaptic connections through a C3/CR3 complement-dependent mechanism (Schafer et al., 2012, 2013). Similarly, in the hippocampus microglia were found to contribute to synaptic refinement. Specifically, the CX3CR1/fractalkine signaling pathway plays a central role in microglia/synapses communication, since *Cx3cr1*$^{-/-}$ mice show temporal reduction of hippocampal microglia number, leading to a deficit in synaptic pruning. Consistently, with early defects in synaptic communication, these mice were shown to exhibit reduced functional brain connectivity, together with social interaction and behavioral deficits (Paolicelli et al., 2011; Zhan et al., 2014). In the somatosensory neocortex, reduced density of microglia cells of *Cx3cr1*$^{-/-}$ mice due to a delay in recruitment of these cells, has been shown to impact on the maturation of thalamocortical synapses (Hoshiko et al., 2012). Thus, the density of these cells, their proper functioning, as well as their capacity to specifically perform local phagocytosis or production of secreted factors, constitutes an important factor for sculpting postnatal brain circuits.

Microglia in the Embryonic and Perinatal Brain: the Importance of Spatial and Temporal Positioning

What about a role of microglia during embryogenesis? In contrast to their later homogeneous distribution in the adult brain, embryonic, and perinatal microglia show an uneven distribution in different species (Ashwell, 1991; Verney et al., 2010; Arnoux et al., 2013; Cunningham et al., 2013; Swinnen et al., 2013; Squarzoni et al., 2014). In particular, in the mouse, round or more ramified microglia have been observed in different focal hotspots, which are not particularly related to apoptosis, whereas some zones, such as the cortical plate is largely devoid of microglial cells (Ashwell, 1991; Verney et al., 2010; Cunningham et al., 2013; Swinnen et al., 2013; Squarzoni et al., 2014). More in depth analyses revealed that microglia accumulations correspond to important decision landmarks in axonal paths or cellular migratory routes. In particular, discrete groups of microglia associate with the corpus callosum, the external capsule or establish a contact with incoming dopaminergic axons in the ventral telencephalon. Specific associations with progenitor zones have also been observed in mice and other mammals, potentially regulated by chemokine production by these progenitors (Cunningham et al., 2013; Arno et al., 2014).

Pharmacologic or genetic ablations of microglia have been used to probe the roles of these cells during embryonic brain wiring (**Figure 4**). Together with maternal immune activation (MIA) and genetic microglial impairment (*Cx3cr1*$^{-/-}$), these studies showed that microglia regulate the outgrowth of dopaminergic axons, thereby revealing the importance of the precise spatial-temporal microglia localisation (Squarzoni et al., 2014). In addition, microglia contribute to the development of the Corpus Callosum (CC), the largest commissural structure between the cerebral hemispheres (Pont-Lezica et al., 2014). Indeed, genetic functional impairment of microglia (*Dap12*$^{-/-}$) or developmental functional alteration by MIA, down-regulate the expression of genes related to neuritogenesis in microglia, with a consequent impairment on the CC fasciculation in these mouse models. A similar CC fasciculation phenotype has been equally observed in the genetic model of microglia ablation, *Pu·1*$^{-/-}$ (Pont-Lezica et al., 2014). Together these studies suggest that the spatial and temporal positioning of embryonic microglia modulates the development of specific and important axonal tracts. The underlying cellular and molecular mechanisms still remain to be deciphered.

In addition, microglia, which show a timely invasion of the cortical plate (CP) (Cunningham et al., 2013; Swinnen et al., 2013; Squarzoni et al., 2014) were found to regulate the assembly of cortical circuits. Cortical circuits are formed by an intricate network of a majority of excitatory neurons and a minority of functionally important inhibitory interneurons (Marin and Rubenstein, 2003; Sur and Rubenstein, 2005; Batista-Brito and Fishell, 2009; Cossart, 2011; Fishell and Rudy, 2011; Rico and Marin, 2011; Rubenstein, 2011; Marin and Muller, 2014). Indeed, various classes of interneurons shape the network output and interneuron dysfunction as well as defects in the excitation/inhibition balance have been associated with several neurodevelopmental disorders such as Autism Spectrum Disorders (ASD) or Schizophrenia. As aforementioned, microglia regulate the number of neuronal precursors in the subventricular zones of the neocortex (Cunningham et al., 2013) and are firstly excluded from the CP, which they invade after E16.5, remaining initially confined to the deeper layers (Swinnen et al., 2013; Squarzoni et al., 2014). Absence, immune activation or genetic impairment of microglia were found to impact on the laminar distribution of a specific population of interneurons that express the transcription factor Lhx6. Indeed, in absence of microglia (pharmacological

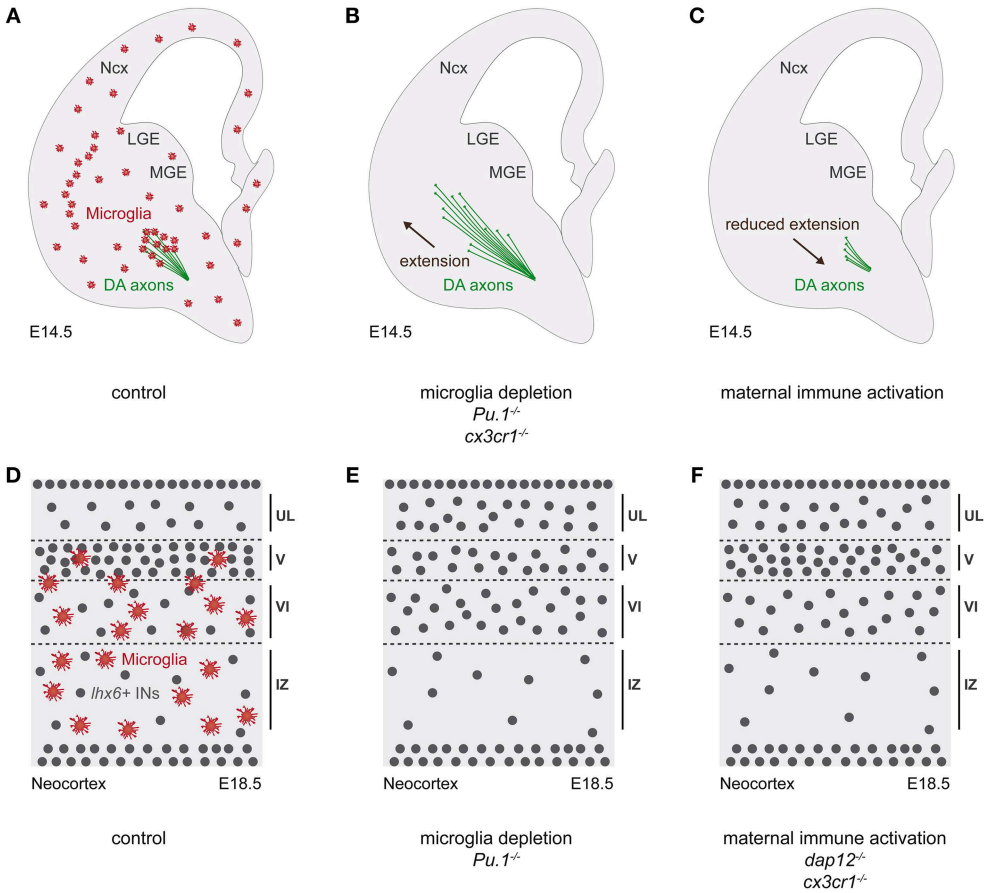

FIGURE 4 | Microglia in the outgrowth of dopaminergic axons and the positioning of cortical interneurons. (A–C) Schematic representations of coronal hemisections of E14.5 mouse embryonic brain. **(D–F)** Schematic representations of E18.5 coronal sections through the somatosensory neocortex of mouse embryonic brain. **(A)** At E14.5, microglia (red) establish a contact with dopaminergic axons (green) entering in the ventral telencephalon. **(B)** Pharmacologic or genetic cellular ablation (*Pu1*$^{-/-}$), as well as functional impairment (*Cx3cr1*$^{-/-}$) of microglia promote dopaminergic axonal outgrowth in the striatum. **(C)** Conversely, microglia functional alteration by maternal immune activation (MIA) leads to reduced dopaminergic axonal outgrowth. **(D)** At E18.5, microglia localize in the deeper layers of the cortical plate, with *lhx6*-expressing interneurons (gray dots) being concentrated in layer V. **(E,F)** In absence of microglia (pharmacological depletion or *Pu1*$^{-/-}$) pharmacological immune activation (MIA) or genetic functional alteration of microglia (*Dap12*$^{-/-}$; *Cx3cr1*$^{-/-}$), *lhx6*-expressing interneurons prematurely entered the cortical plate, followed by an altered laminar distribution. DA, dopaminergic axons; INs, interneurons; IZ, intermediate zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Ncx, neocortex; UL, upper layers.

depletion; *Pu·1*$^{-/-}$), or in case of pharmacological (MIA) or genetic functional alteration (*Dap12*$^{-/-}$; *Cx3cr1*$^{-/-}$), a premature entry of *lhx6*-expressing interneurons in the CP was observed, followed by an altered laminar distribution, with long lasting postnatal effects on a subset of *lhx6*-expressing interneurons, the fast-spiking parvalbumin-positive interneurons (Squarzoni et al., 2014). These specific interneurons have been shown to play a major role in cortical networks as well as to be impaired in ASD and Schizophrenia (Penagarikano et al., 2011; Marin, 2012; Meechan et al., 2012). While these results reveal a surprising role of microglia in cortical circuits assembly, as well as a potential involvement in the etiology of neuropsychiatric diseases, they raise the question of the underlying mechanisms. Besides the requirement of Dap12 and Cx3cr1 signaling, the processes involved deserve further investigation.

These results reveal that microglia modulate brain wiring at various developmental steps, starting from embryonic, post-natal and adult stages. Moreover, they underlie the importance of spatial and temporal positioning of these cells to accomplish their roles as modulators of dopaminergic axonal outgrowth, CC development and neocortical interneuron laminar distribution, which are major events in forebrain wiring.

Conclusions and Perspectives

While the concept of guidepost cell has substantially changed since its first description, including a potential motility as well as a diverse cellular identity (neuronal or glial), there are still some conserved properties: they are usually early born, immature, located at a crucial point along a pathway and able to act at

short-range or by direct cell-cell contact on its target. Along these lines, recent studies have revealed that microglia cells may be to some extent, envisaged as novel guideposts during embryonic forebrain wiring. By their transient specific localization during embryogenesis they may act on restricted neuronal subgroups and modulate forebrain wiring. If a comprehensive knowledge of all microglia functions is still fragmentary, the tremendous potential of these cells in shaping and remodeling circuits, during normal and pathological conditions, opens a novel framework for our understanding of brain wiring.

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Cellular dynamics of neuronal migration in the hippocampus

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A fine structure of the hippocampus is required for proper functions, and disruption of this formation by neuronal migration defects during development may play a role in some psychiatric illnesses. During hippocampal development in rodents, pyramidal neurons in the Ammon's horn are mostly generated in the ventricular zone (VZ), spent as multipolar cells just above the VZ, and then migrate radially toward the pial surface, ultimately settling into the hippocampal plate. Although this process is similar to that of neocortical projection neurons, these are not identical. In addition to numerous histological studies, the development of novel techniques gives a clear picture of the cellular dynamics of hippocampal neurons, as well as neocortical neurons. In this article, we provide an overview of the cellular mechanisms of rodent hippocampal neuronal migration including those of dentate granule cells, especially focusing on the differences of migration modes between hippocampal neurons and neocortical neurons. The unique migration mode of hippocampal pyramidal neurons might enable clonally related cells in the Ammon's horn to distribute in a horizontal fashion.

Keywords: hippocampus, migration, climbing mode, Ammon's horn, dentate gyrus, layer pattern

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Introduction

The hippocampal formation is a unique structure comprising the Ammon's horn (the hippocampus proper), dentate gyrus, entorhinal cortex, parasubiculum, presubiculum, and subicular complex. In the rodent brain, this architecture is located on and around the convexly curved medial lobule of the lateral cortex and is dorsally continuous with the neocortex. The hippocampus is a part of the limbic circuit and is functionally associated with spatial learning, as well as short- and long-term memory. In addition, functional magnetic resonance imaging (fMRI) analyses of some neuropsychiatric disorders have indicated its involvement in various types of mental activities; for example, decreased hippocampal volume was reported in patients with depression or post-traumatic stress disorder (PTSD) (Campbell et al., 2004; Woon et al., 2010). Anatomical abnormalities in the hippocampus are also observed in pathological conditions of some neuropsychiatric disorders, such as epilepsy, lissencephaly, and schizophrenia (Baulac et al., 1998; Harrison, 2004; Donmez et al., 2009). Some of these symptoms are thought to be associated with the migration deficit of hippocampal neurons during development (Barkovich et al., 1991; Dobyns et al., 1996; Montenegro et al., 2006). Neuronal migration in the neocortex is well-studied, and the cellular dynamics and molecular mechanisms involved in neuronal migration are also well-understood. Because the hippocampus and neocortex are included in the cerebral cortex, their neuronal migration was thought to be similar. However, differences in neuronal migratory behavior between these regions exist. Studies on cellular behavior of hippocampal neurons are broadly classified into two categories in terms of their methods, classical cellular labeling and molecular biological approaches. Classical techniques, such as Golgi staining and [3H] thymidine autoradiography labeling, were

used to discover neuronal origins, cellular arrangements, neuronal migration paths, and neuronal morphologies in the hippocampus (Bayer, 1980; Nowakowski and Rakic, 1981; Rakic and Nowakowski, 1981; Altman and Bayer, 1990a,b,c). Furthermore, the development of molecular biological approaches, such as *in utero* electroporation, *in utero* virus transfer, and generation of transgenic mice, has shed light on the cellular dynamics of migrating neurons, successive behavior of neurons, neuronal lineages, and molecular mechanisms of hippocampal development (Nakahira and Yuasa, 2005; Li et al., 2009; Kitazawa et al., 2014; Seki et al., 2014; Xu et al., 2014).

In this review, we describe cellular dynamics and molecular mechanisms of migration of pyramidal neurons in the Ammon's horn and granule cells in the dentate gyrus during hippocampal development. There are three distinct hippocampal neuroepitheliums—the Ammonic neuroepithelium, the primary dentate neuroepithelium, and the fimbrial glioepithelium (Altman and Bayer, 1990a). Pyramidal neurons in the Ammon's horn are mainly generated from the Ammonic neuroepithelium and undergo radial migration to reach their final destination (Altman and Bayer, 1990b; Nakahira and Yuasa, 2005; Kitazawa et al., 2014), whereas cells comprising the dentate gyrus are originally produced from the primary dentate neuroepithelium (dentate notch), move in a migratory stream, and then migrate radially to form the dentate granule cell layer (Altman and Bayer, 1990a,c; Nakahira and Yuasa, 2005; Li et al., 2009; Seki, 2011; Li and Pleasure, 2014; Seki et al., 2014). We also compare neuronal migration between the neocortex and the hippocampus proper during development.

Migration of Neocortical Pyramidal Neurons

Before describing migration of hippocampal neurons, we briefly outline migration of pyramidal neurons during rodent neocortical development (**Figure 1A**) to compare the migration mode between hippocampal pyramidal neurons and neocortical neurons (for a detailed illustration of neocortical neuronal migration, see reviews by Tabata et al., 2012; Evsyukova et al., 2013; Tan and Shi, 2013; Sekine et al., 2014). Pyramidal neurons generated in the neocortical ventricular zone (VZ) undergo morphological transformation before migrating up beneath the marginal zone (MZ), as summarized below.

Neocortical pyramidal neurons are generated from radial glial cells in the VZ (Miyata et al., 2001; Noctor et al., 2001) or from basal progenitors or basal radial glia in or around the subventricular zone (SVZ) (Noctor et al., 2004; Shitamukai et al., 2011; Wang et al., 2011). Neurons produced in the VZ remain there for at least 10 h with an apical process reaching the ventricular surface. The cells then move to just above the VZ (multipolar cell accumulation zone, MAZ), where they assume multipolar morphology and stay for about 1 day (Tabata and Nakajima, 2003; Tabata et al., 2009). Multipolar neurons in the MAZ repeatedly extend and retract multiple thin processes, and slowly wander and move toward the cortical plate (CP) (Tabata and Nakajima, 2003; Tabata et al., 2009). This unique behavior of multipolar neurons is called "multipolar migration." The multipolar neurons then transform into bipolar cells with a leading process extending

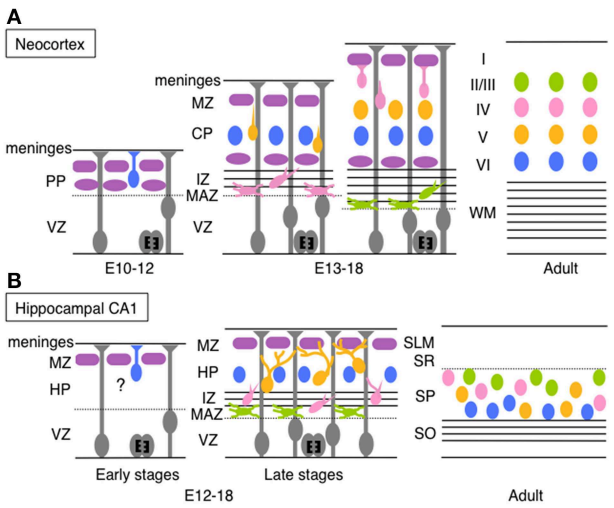

FIGURE 1 | Schematic diagrams of migration and layer arrangement on the neocortex and hippocampal CA1 during cortical development. **(A)** Neocortical neurons born between E10 and E12 radially migrate using the somal translocation mode. In contrast, late-born neurons transform their migration mode sequentially to multipolar migration, locomotion mode, and terminal translocation mode during their radial migration. These neurons form neocortical layers in a birthdate-dependent inside-out manner. **(B)** Hippocampal CA1 neurons born at late developmental stages change the migration mode to multipolar migration and then to the climbing mode. The migration mode used by early-born CA1 neurons remains unknown (somal translocation mode is a candidate). The layer arrangement in the Ammon's horn is thought to occur roughly in a birth-date dependent inside-out manner (another claim was also reported; see text for details). PP, preplate; VZ, ventricular zone; MZ, marginal zone; CP, cortical plate; IZ, intermediated zone; MAZ, multipolar cell accumulation zone; WM, white matter; HP, hippocampal plate; SLM, stratum lacunosum-moleculare; SR, stratum radiatum; SP, stratum pyramidale; SO, stratum oriens.

from a spindle-shaped cell body. These bipolar neurons migrate radially through the intermediated zone (IZ) and the CP along with a radial glial fiber. This migration mode is called "locomotion" (Rakic, 1972; Nadarajah et al., 2001). When the leading process of migratory neurons reaches the MZ, the neurons are thought to anchor the tip of the leading process in the MZ and leave from the radial glial fiber. Then, the neuronal cell body is pulled up while shortening the leading process and the cells stop just beneath the MZ (Nadarajah et al., 2001; Sekine et al., 2011). This final migration mode is termed "terminal translocation." Neurons born in the mouse VZ at E14, for example, take about 4–5 days to complete their migration (Ajioka and Nakajima, 2005). Because newly generated pyramidal neurons pass through earlier-born neurons before reaching beneath the MZ, pyramidal neurons are arranged in a birth-date-dependent inside-out manner, in which earlier-born neurons are positioned in the deep layers and later-born neurons are located in the more superficial layers in the CP (Angevine and Sidman, 1961).

Migration of Hippocampal CA1 Pyramidal Neurons

The Ammon's horn is compartmentalized into the CA1, CA2, and CA3 along with the transverse axis, and horizontally divided

into several ordered layers—stratum oriens, stratum pyramidale (SP or the pyramidal cell layer), stratum radiatum (SR), and stratum lacunosum-moleculare (in only the CA3 region, the stratum lucidum exists between the SP and the SR). Pyramidal neurons in the hippocampal CA1 region are generated in the Ammonic neuroepithelium mainly from E16 to E20 with the peak around E18–19 in rats (Bayer, 1980; Altman and Bayer, 1990a,b), and from E12 to E18 with the peak between E14 and E16 in mice (Angevine, 1965; Caviness and Sidman, 1973; Smart, 1982; Kitazawa et al., 2014). Because the generation of pyramidal neurons and neuronal behaviors during development are different between the hippocampal CA1 and the CA3, we describe migratory behaviors of CA1 pyramidal neurons first and then those of CA3 neurons later (details of CA2 pyramidal neuronal migration are not well-known). Pyramidal neurons in the hippocampal CA1 region are mostly generated in the VZ, while a small population is produced from basal progenitors in the SVZ (Kitazawa et al., 2014). The newly born neurons leave the VZ and stay just above the VZ. These post-mitotic cells transform into multipolar cells with multiple thin processes and slowly move toward the IZ with repeated extension and retraction of these processes (Nakahira and Yuasa, 2005; Kitazawa et al., 2014). Existence of these multipolar neurons in the Ammon's horn has also been identified in the rabbit (Stensaas, 1967a,b) and the monkey (Nowakowski and Rakic, 1979). The time length of assuming multipolar morphology above the VZ (or in the hippocampal MAZ) differs depending on birthdates; neurons born at E12 or E13 stay as multipolar cells within 1 day, whereas those generated at E15 or E16 densely accumulate in the MAZ as multipolar cells for 3–4 days (Kitazawa et al., 2014). When late-born CA1 pyramidal neurons (generated from E14 to E16) move into the IZ, they transform into a bipolar spindle-shaped morphology, with one major leading process and multiple thin processes extending toward various directions (Nakahira and Yuasa, 2005; Kitazawa et al., 2014). These spindle-shaped neurons migrate through the IZ toward the hippocampal plate (HP, future stratum pyramidale), but sometimes transform their morphology back to a multipolar morphology. Upon observation of fixed tissue sections, the neurons seem to migrate along with radial glial fibers in the IZ, even when the fibers bend and curve (Nakahira and Yuasa, 2005). Recent time-lapse imaging has revealed that neurons in the IZ move obliquely at first and gradually migrate radially (Kitazawa et al., 2014), which coincides with the track of radial fibers. Nowakowski and Rakic also identified the apposition of neurons with radial glial fibers in the IZ of the monkey hippocampus in electron microscopic analyses (Nowakowski and Rakic, 1979). Just before neurons enter the HP, they extend one or two major branched leading process(es) with multiple thin processes (Nowakowski and Rakic, 1979; Kitazawa et al., 2014) and touch multiple radial glial fibers at the tip or the middle of the branched processes (Kitazawa et al., 2014). When they migrate through the HP, they dynamically extend and retract branched leading processes as if they were searching for the radial glial fibers. On the other hand, the cell soma of the migrating neuron moves up to the first branching point of the leading process. Subsequently, one of the branches grows and becomes a new leading process, followed by the movement of the cell soma again to the first branching point of the new leading process. In the HP of the CA1, migratory neurons repeat this process, thereby changing their migration scaffold (radial glial fiber) one after another until they reach the top of the HP. Consequently, hippocampal pyramidal neurons move in a zigzag manner, in contrast to the almost straight path of neocortical migrating neurons. Because this hippocampal migration mode is different from the well-known modes of migration, it was termed a "climbing mode" (Kitazawa et al., 2014) (**Figure 1B**).

The Difference between Migration of Hippocampal CA1 Pyramidal Neurons and that of Neocortical Neurons

There are a couple of similarities in the mode of neuronal migration between the hippocampal CA1 and the neocortex. One is the place of neuronal production, which is located in the VZ and SVZ in both structures. Neurons are generated near the ventricle and principally migrate toward the pial surface. The second similarity is the transformation into multipolar morphology when neurons migrate out of the VZ. The neurons reside in the MAZ for some time, and then transform into a bipolar morphology. However, there are also several major differences between the regions, such as migration mode, length of time required for completion of migration, and alignment pattern of the pyramidal neurons.

During development, neocortical neurons migrate in locomotion and terminal translocation modes through the CP (Nadarajah et al., 2001; Sekine et al., 2011; Evsyukova et al., 2013), while pyramidal neurons in the hippocampal CA1 region adopt a climbing migration mode, at least during the late stages of hippocampal development. Neocortical neurons in the locomotion mode migrate almost straight along individual radial glial fibers in the CP. In the outermost region of the CP [primitive cortical zone, PCZ (Sekine et al., 2011)], they take the terminal translocation mode before stopping beneath the MZ. In contrast, hippocampal neurons in the climbing migration mode migrate in a zigzag manner using several scaffold radial glial fibers in the HP (Kitazawa et al., 2014). The migratory speed for each migration mode is also different. The average migrating speeds of hippocampal CA1 neurons in the climbing mode and neocortical neurons in the locomotion mode are 7.1 and 20.5 μm/h, respectively (Kitazawa et al., 2014). The speed of neocortical neurons in the terminal translocation mode is much faster, up to 50 μm/1–2 h (Sekine et al., 2011). Neocortical migrating neurons in a locomotion mode basically use a single radial glial fiber as the scaffold, whereas hippocampal neurons proceed using multiple radial glial fibers. The difference in these processes may bring about the difference in migration speed. Because cell density in the HP in late developmental stages is much greater than in the CP, with exception of the PCZ, it may be difficult for hippocampal CA1 neurons to migrate straight, unlike locomoting neurons in the neocortical CP. Because the Ammon's horn is widely extended during development (Altman and Bayer, 1990b), it is thought that pyramidal neurons born near the ventricle may need to move obliquely using the climbing mode of migration to fill up layers without gaps.

The second difference is the time spent migrating. Hippocampal CA1 pyramidal neurons generated at E15 or E16 spend 7–9 days to reach their final destinations (Tomita et al., 2011; Kitazawa et al., 2014), whereas it takes only 4–5 days for the neocortical late-born neurons to migrate beneath the MZ, although the migratory distance is much longer in the neocortex. This difference is observed not only in mice, but also in rats (Altman and Bayer, 1990b) and monkeys (Nowakowski and Rakic, 1981). Two factors apparently cause this difference. One is the difference in neuronal migration speed as mentioned above. The other is the time period when neurons remain in the MAZ as multipolar cells, termed "sojourning" cells by Altman and Bayer (1990b). Neocortical neurons spend about 1 day in the MAZ (Tabata et al., 2009). In contrast, the hippocampal CA1 neurons generated in late embryonic days spend almost 3 days in the MAZ, while this period varies depending on their birthdates (Nakahira and Yuasa, 2005; Kitazawa et al., 2014). Why do hippocampal neurons generated during late stages stay in the MAZ so long? There are at least two possibilities. One is the existence of alveolar channels, which are cell-free transient extracellular matrices dispersed in the IZ, just above the MAZ (Altman and Bayer, 1990b). Altman and Bayer displayed that this matrix becomes filled with axonal fibers of an unknown origin, and suggested that multipolar cells in the CA1 region might wait for the appearance of this matrix because of the connection to these axons (Altman and Bayer, 1990b). In addition, Deguchi et al. exhibited that neurons born with the same birthdate in the CA1, CA3, and dentate gyrus had similar gene expression patterns and preferentially connected with each other (Deguchi et al., 2011). Multipolar cells in the CA1 region may leave from the MAZ after CA3 neurons extend their axons and connect with them. In contrast, we showed that axon bundles appeared just above the MAZ at E15 and E16. These axon bundles originated from earlier-born neurons in the hippocampal CA1 region, and when they were transfected with GFP at E13.5, for example, the labeled axon bundles were located just above the multipolar cells at E18.5 (Kitazawa et al., 2014). The appearance of these axonal bundles coincided with the accumulation of multipolar cells in the hippocampal MAZ. CA1 neurons born on earlier days spend a much shorter time in a multipolar morphology and reach the pial surface in a short time, whereas axon bundles are not observed above multipolar cells in these earlier days (Kitazawa et al., 2014). Axonal bundles from earlier-born neurons may interfere with the migration of late-born pyramidal neurons. Future studies are needed to better understand the behavior of multipolar cells.

Finally, the pattern of neuronal alignment in the hippocampus may be different from the neocortex. Labeling experiments using [3H] thymidine indicate that hippocampal laminar formation occurs in a birthdate-dependent inside-out pattern in which earlier-born neurons comprise the deep SP region and later-born neurons join the superficial region, which is similar to the neocortical layer formation (Bayer, 1980; Rakic and Nowakowski, 1981; Altman and Bayer, 1990b). Recently, Xu et al. reported that hippocampal clonally related neurons in the CA1 region are distributed in a horizontal manner, not in a vertical column as neocortical neurons, shown using retroviral labeling and transgenic mice to label clonally related cells (Xu et al., 2014). The authors claimed that hippocampal layer formation did not occur in a birthdate-dependent inside-out pattern, contrary to previous reports. In addition to differences in their analytical method, this disparity might be explained as follows. Because neurons born on a certain day are distributed rather widely in the SP with a birthdate-dependent peak position, neurons with different birthdates are mixed with each other, obscuring the inside-out pattern. In addition to the birthdate-dependent positioning in the vertical/radial axis, however, it is also reasonable that the clonally related neurons are horizontally aligned. Because the SP (or HP) in the Ammon's horn region is horizontally expanded during development and is not as thick as the CP in the neocortex, this suggests the difficulty of clonally related neurons in the hippocampus proper to be aligned in a vertical manner like neocortical clonal neurons. Even if neurons in the HP of the Ammon's horn tend to align in an inside-out pattern, they would also move to cover spaces in the layer yielded by the structural expansion, resulting in lateral/horizontal expansion of clonally related neurons. The climbing mode of migration is thought to be suitable to fill up gaps in the layer, because neurons in this mode can move to various directions, enabling clonal sister neurons to distribute broadly within the layer. Bending of radial glial fibers near the HP/SP would also be partly related to horizontal distribution of sister neurons (Xu et al., 2014), but similar bending of radial glial fibers beneath the CP/subplate is also observed in the neocortex, especially in the lateral part (Tabata and Nakajima, 2001), indicating that the bending morphology of radial fibers cannot fully explain the horizontal distribution of sister neurons in the hippocampus. Schematic models of neuronal behaviors in the neocortex and the Ammon's horn during development are illustrated in **Figure 1**.

Migration of Hippocampal CA3 Pyramidal Neurons

The SP in the CA3 region has a unique U-shaped curve that reaches the dentate hilus. In rats, the HP appears from E18 and expands curvilinearly until E21, at which time the layer begins to medially expand and forms a U-shaped structure by E22 (Altman and Bayer, 1990b). The CA3 pyramidal neurons have a neurogenic gradient such that pyramidal neurons near the CA1 region are generated earlier than those near the dentate gyrus (Bayer, 1980). The neurogenesis gradient from ventral to dorsal is also observed in the CA3 region. The neurogenesis of CA3 pyramidal neurons takes place in the VZ between E16 and E20, with a peak between E17 and E18 in rats, which is earlier than the generation of hippocampal CA1 neurons that peak around E18 and E19 in rats (Bayer, 1980; Altman and Bayer, 1990a,b). The generated neurons move to just above the VZ and transform into a multipolar morphology, similar to CA1 pyramidal neurons (Nakahira and Yuasa, 2005). However, CA3 neurons remain longer in the MAZ than CA1 neurons. In mice, neurons generated in the CA3 region at E14 exhibit multipolar morphology even at E18, while CA1 pyramidal neurons born at the

same time have already migrated into the HP (Nakahira and Yuasa, 2005). This phenomenon is also observed in rats using administration of [3H] thymidine (Altman and Bayer, 1990b). Reports have shown that the sojourning period for CA3 pyramidal neurons assuming a multipolar morphology is 1 day longer than for CA1 neurons (Altman and Bayer, 1990b; Nakahira and Yuasa, 2005). Altman and Bayer hypothesized that multipolar cells in the CA3 region might wait for a connection with granule cells of the dentate gyrus, resulting in a longer sojourning time (Altman and Bayer, 1990b). Again, the report by Deguchi et al. showed that neurons generated at the same time in different sub-regions connect preferentially with each other (Deguchi et al., 2011), which may also explain a longer sojourn to wait for a connection with dentate gyrus cells. The CA3 pyramidal neurons accumulate in the MAZ for 4 days then migrate upward. The migration modes of CA3 pyramidal neurons are thought to be similar to CA1 pyramidal neurons, though only a limited number of studies have been reported on the behavior of hippocampal CA3 pyramidal neurons (Nakahira and Yuasa, 2005). Interestingly, Nakahira and Yuasa also found that neurons generated at E16 in the mouse CA3 VZ migrate tangentially toward the subpial area, and that some neurons then detach from the stream and migrate radially, with a unipolar shape, along with radial glial fibers directed to the HP of the CA3 region (Nakahira and Yuasa, 2005). This neuronal behavior is different from CA3 neurons generated at E14. Although the CA3 neurons born at E16 account for only a small portion (Bayer, 1980), multiple migration modes may exist for CA3 neurons depending on their birthdates.

The major difference between the CA1 and the CA3 during hippocampal development is the layer shape. The HP in the CA1 is mildly curved and in parallel with its ventricular surface, whereas the CA3 HP has a U-shape with one end invading the dentate hilus. This end is apart from the VZ. Considering that neurons at this end of the HP are also generated from the VZ in the CA3, the long journey for the migrating cells is thought to be one of the causes of delayed HP formation in the CA3, which occurs 1 day later than HP formation in the CA1 (Altman and Bayer, 1990b).

Although the CA1 and the CA3 are continuous architectures via the CA2, pyramidal cells in each region express specific markers; for example, SCIP, a POU domain transcriptional factor, in CA1 pyramidal neurons (Frantz et al., 1994; Tole et al., 1997), and KA1, a glutamate receptor subunit, in CA3 neurons (Wisden and Seeburg, 1993; Tole et al., 1997). Interestingly, these markers are already expressed in each cell group at E15.5 in mice, when the cells are still localized in the IZ (Tole et al., 1997). Explant culture experiments performed by Tole et al. revealed that this marker is expressed in a cell-autonomous manner (Tole and Grove, 2001). Therefore, the destination of cells comprising the HP in the CA3 is thought to already be determined at, or soon after, the multipolar cell stage. For future CA3 pyramidal cells, especially those in the HP end of the dentate hilus, the climbing migration mode may be adequate for migration and detours to reach their final positions. Eventually, CA3 pyramidal neurons might migrate more horizontally through the HP than CA1 pyramidal neurons.

Migration of Cells Comprising the Dentate Gyrus

Since Altman found postnatal neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (Altman, 1963), it is well-established that the dentate gyrus is one of the two regions where adult neurogenesis occurs. Compared with studies on adult neurogenesis, the development of the dentate gyrus and migratory dynamics of granule cells have been less extensively studied. The generation and migration of dentate cells during development are complex and quite different from pyramidal neurons in the Ammon's horn (**Figure 2**). Dentate cells are generated in the primary dentate neuroepithelium located around the dentate notch, which is ventral to the Ammonic VZ and dorsal to the fimbria at E16 and E17 of rats (Altman and Bayer, 1990c) and at E13.5 and E14 in mice (Li et al., 2009; Seki et al., 2014). By E15.5 in mice (E18 in rats), some cells that are generated in the primary dentate VZ (Altman and Bayer, 1990c; Seki et al., 2014) migrate out to the subpial region through the suprafimbrial region, which is

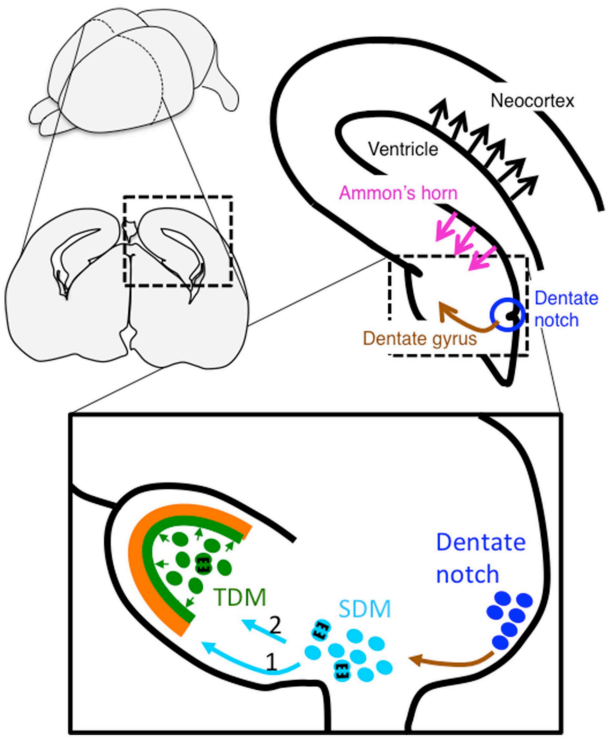

FIGURE 2 | Schema of migration of dentate cells during hippocampal development. Newborn granule cells from the dentate notch migrate to the secondary dentate matrix (SDM) (indicated by a brown arrow). The cells then migrate to the subpial surface to form the outer part of the dentate granule cell layer (light blue arrow 1), followed by the dentate hilus (light blue arrow 2), which is called the tertiary dentate matrix (TDM) at this stage, to later form the inner part of the layer. Cells in the TDM exhibit proliferative activities into adulthood, although the proliferative region becomes restricted to the subgranular zone. In contrast, pyramidal neurons in the hippocampal CA1 and neocortex are generated in the Ammonic ventricular zone and the neocortical ventricular zone, respectively, and migrate in a radial direction (indicated by magenta and black arrows, respectively).

designated as "dentate migration." During this migration, these cells still exhibit proliferative activity and form the secondary dentate matrix (SDM in **Figure 2**) on the migratory route (Altman and Bayer, 1990c; Pleasure et al., 2000). On the migrating stream, the cells extend many processes to various directions (Nakahira and Yuasa, 2005), and the migration route of granule cells and progenitors are separated into two routes. One is the "first dentate migration" in which early-born cells migrate to the crest of the dentate gyrus through the subpial route (route 1 in **Figure 2**) (Altman and Bayer, 1990c). The dentate cells in the subpial region also exhibit highly proliferative activity (Li et al., 2009). The morphologies of cells in the subpial region are diverse; for example, bipolar morphology and multipolar cell morphology (Nakahira and Yuasa, 2005). This diversity is probably owing to the variety of cellular maturation. The cells reaching the subpial region radially migrate toward the supra-granular blade of the dentate gyrus apposed to the radial glial fibers (Nakahira and Yuasa, 2005). At this stage, these cells form a unipolar or bipolar shape with one or two branches (Nakahira and Yuasa, 2005). The dentate granule cells in the subpial region first form the outer shell of the supra-granular blade of the dentate gyrus, and then gradually shift to form the outer shell of the infra-granular blade of the dentate gyrus. Around E17.5 and 18.5 in mice, a prototype of the dentate gyrus can be observed (Seki et al., 2014). In late embryonic and early postnatal days, the "second dentate migration" appears in which the cells migrate toward and reach the future dentate hilus (route 2 in **Figure 2**). Because these cells still exhibit proliferative activity even in the hilus, the zone where these cells exist is called the tertiary dentate matrix (TDM in **Figure 2**). Dentate neurons generated from the TDM form the inner part of the dentate gyrus. As a result, dentate granule cells in the dentate granule cell layer adopt a birthdate-dependent outside-in pattern, in which earlier-born neurons locate to the outer part of this layer and later-born neurons position to the inner region (Rakic and Nowakowski, 1981; Altman and Bayer, 1990c). Although neurogenesis in the TDM continues into adulthood, this zone becomes gradually restricted to the boundary between the dentate granule cell layer and the hilus, called the SGZ (Altman and Bayer, 1990c). Bayer suggests that granule cells in the dentate gyrus are generated from E15 to adulthood in rats, and about 80–85% of total granule cells are generated after birth (Bayer, 1980).

Seki et al. traced the granule cells using a glial fibrillary acidic protein (GFAP)-GFP transgenic mouse line (Seki et al., 2014). GFAP was not thought to be a marker for "embryonic" progenitors of dentate granule cells, although GFAP is a well-known marker for "adult" progenitors. Seki et al. found that migrating progenitors of dentate granule cells, unlike those of neocortical pyramidal neurons, express GFAP from the beginning of dentate development, and these cells could be traced using a GFAP-GFP transgenic mouse line. Immunohistochemical analyses using this transgenic mouse line showed maturation of granule cells during migration. For example, neurogenin-positive proneural cells are mainly localized in the VZ, whereas Tbr2-positive early neural progenitors are principally located in the migratory stream and the developing dentate gyrus. NeuroD-positive immature neurons are mostly located in the migratory stream, the developing dentate gyrus, and the hilus, whereas prox1-positive granule cells are positioned in the developing dentate gyrus and the hilus. Sox2-positive progenitor cells are distributed all over the dentate gyrus at E18 in mice. Accordingly, while these granule cells gradually mature during migration, cells in each region are heterogeneous as to their degree of maturation. How this heterogeneous group could migrate along the same route is not yet known.

Molecular Mechanism of Neuronal Migration in the Hippocampus

A number of mutant mouse lines or the introduction of shRNA-expression vectors for various genes into neurons *in utero* have been used to show abnormal layer formation and mis-positioning of neurons in the Ammon's horn and the dentate gyrus during hippocampal development. Some of the examples are summarized below.

Reelin (and Related Molecules, ApoER2, VLDLR, and Dab1)

Reelin is a giant glycoprotein secreted from Cajal–Retzius cells in the MZ during development (D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995). Reelin is known to be essential for neuronal positioning in the brain and spinal cord (Yip et al., 2000, 2011; Honda et al., 2011; Sekine et al., 2014). For example, in the neocortex, the *reelin*-deficient autosomal recessive mouse, *reeler,* displays disrupted layer formation, including overall approximate inversion of the birthdate-dependent layering (Caviness, 1973). Anatomical analyses, such as [3H] thymidine injection and *in situ* hybridization, disclosed that hippocampal layer formation is also inverted in the *reeler* mouse (Caviness, 1973; Stanfield and Cowan, 1979a,b; Stanfield et al., 1979; Niu et al., 2004; Boyle et al., 2011). Injection of CR-50, a function-blocking antibody against Reelin protein, into the ventricle of mouse embryos also resulted in a similar layer pattern to that of *reeler* mice in the hippocampal Ammon's horn (Nakajima et al., 1997). Additionally, *reeler* mice exhibit a divided SP in the CA1, a meandered SP in the CA3, a less densely packed dentate gyrus, and a reduced number of granule cells (Caviness, 1973; Stanfield and Cowan, 1979a,b; Boyle et al., 2011).

Reelin binds to Apolipoprotein E receptor 2 (ApoER2) and very low-density lipoprotein receptor (VLDLR) (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999), subsequently induces phosphorylation of Disabled-1 (Dab1) by Fyn or Src kinases, and then transduces the signal to several downstream pathways to regulate neuronal migration and cellular positioning (Honda et al., 2011; Sekine et al., 2014). Double KO mice of *apoer2* and *vldlr* show similar phenotypes to those of *reeler* mice. Additionally, KO mice of *apoer2* show slightly more severe SP splitting than the *vldlr* KO mice, while the phenotypes of these single KO mice are milder than those of double KO mice (Trommsdorff et al., 1999; Drakew et al., 2002; Weiss et al., 2003). The *dab1* KO mice also exhibit the same hippocampal abnormality as the *reeler* mice (Howell et al., 1997; Weiss et al., 2003).

Analysis of the migratory stream of dentate cells using nestin-GFP transgenic mice mated with *reeler* mice suggests that Reelin

is not necessary for the migration of dentate precursors (Nestin-GFP positive cells) from the dentate notch to the subpial zone, but is indispensable for later migration of these cells from the subpial zone to the granule cell layer (Li et al., 2009). Prox1-positive granule cells in these mice are arranged abnormally in the dentate area, suggesting the involvement of Reelin in the final radial migration of dentate granule cells. Forster et al. reported that Reelin and Dab1 affect radial glial cell differentiation and branching in the hippocampus and the loss of these genes causes abnormal radial fiber formation, resulting in failed neuronal migration in the hippocampus (Förster et al., 2002). Zhao et al. rescued *reeler* malformation in the orientation of radial glial fibers and dentate cell migration *in vitro* during dentate gyrus development when they adjacently co-cultured *reeler* dentate gyrus with rat wild-type dentate gyrus (Zhao et al., 2004, 2006). These results collectively imply that Reelin controls neuronal migration in the hippocampus in cell-autonomous and non-cell-autonomous manners.

Cyclin-dependent Kinase 5 (Cdk5)/p35

Cdk5 is a serine/threonine kinase and is activated by binding with its regulatory co-factor p35 or p39. Cdk5 activity is rich in brains during development, and p35 or p39 expressions are restricted to the brain. Cdk5 regulates various aspects of neuronal functions such as cell migration, cytoskeletal remodeling, and adult neurogenesis via phosphorylation of various types of molecules (Su and Tsai, 2011). The *cdk5* KO mice are lethal by birth and display disrupted laminar formation in the neocortex and hippocampus (Ohshima et al., 1996). The *p35* KO mice also exhibit mis-positioning of neuronal cells in the Ammon's horn (partly distinct SP is observed in the CA3, but not in the CA1) and the dentate gyrus, though the phenotypes are moderate compared with *cdk5* KO mice (Wenzel et al., 2001; Ohshima et al., 2005). Cdk5 regulates multipolar-to-bipolar transition of migratory neurons in the neocortex via RapGEF2 phosphorylation (Ohshima et al., 2007; Ye et al., 2014). Because hippocampal pyramidal neurons also perform this transition, a similar mechanism may also play a role during Ammon's horn development.

Doublecortin (Dcx)

DCX is a microtubule-associated protein involved in neuronal migration and a causative gene for X-linked lissencephaly (des Portes et al., 1998; Gleeson et al., 1998). Patients with lissencephaly with *DCX* mutations exhibit defects in neocortical and hippocampal lamination (Barkovich et al., 1991). Hemizygous male *dcx* mutant mice are lethal by early postnatal days and display disrupted hippocampal formation, that is, abnormal neuronal positioning/migration in the Ammon's horn and partial dividing of the SP in the CA3, while neocortical laminar formation and the dentate gyrus are quite normal (Corbo et al., 2002). The *dcx* heterozygous female mice display milder malformation in the hippocampus and deficits in learning and memory (Corbo et al., 2002). KO mice of *dclk1 or dclk2*, doublecortin-like kinase 1 or 2, respectively, are anatomically normal in the hippocampus (Deuel et al., 2006; Tanaka et al., 2006; Kerjan et al., 2009), but the double KO mice of *dcx* and *dclk1* show severe abnormalities in hippocampal laminar formation in the Ammon's horn and the dentate gyrus (Deuel et al., 2006; Tanaka et al., 2006). In contrast, double KO mice of *dcx* and *dclk2* exhibit disrupted laminar formation in CA1 and CA3 region and a less-packed dentate granule layer (Kerjan et al., 2009). Considering the majority of mutant mice mentioned in this review also show abnormalities in the neocortex, the hippocampus and neocortex are likely to share molecular pathways during development. Mutant mice that exhibit malformations specifically in the hippocampal region, such as *dcx* KO mice, may become a key tool to better understand the molecular mechanisms underlying the unique process of hippocampal development, such as the climbing mode of migration. Analyses of *dcx* KO mice and double KO mice of *dcx* and *dclk1* or *dclk2* may also provide insight into differences between hippocampal CA1 and CA3 regions.

Pafah1b1 (formerly Lis1)

PAFAH1B1 is another causative gene for lissencephaly (Reiner et al., 1993; Hattori et al., 1994; Lo Nigro et al., 1997). Pafah1b1 regulates microtubule-based transport by binding with Dynein motor proteins and Ndel1 (formerly Nudel) (Sasaki et al., 2000). Heterozygous *Pafah1b1* KO mice exhibit malformations of the hippocampal cytoarchitecture, which results from delayed neuronal migration and abnormal cellular positioning (Hirotsune et al., 1998; Fleck et al., 2000). Consequently, hippocampal layers in the Ammon's horn become discontinuous and multiple in this mouse, whereas granule cells in the dentate gyrus are less concentrated and loosely packed (Fleck et al., 2000). Another lissencephaly-associated protein, tubulin alpha 1A (Tuba1a), is also involved in hippocampal layer formation (Keays et al., 2007). The *tuba1a* S140G mutant mice induced by injection of N-ethyl-N-nitrosourea (ENU) exhibit deficits in neuronal migration, resulting in a double layer of the hippocampal CA1 and CA3 regions, as well as abnormal laminar formation in the neocortex (Keays et al., 2007).

Cxcl12 (SDF-1)/Cxcr4

SDF-1 is another secreted protein that regulates cellular migration (Bleul et al., 1996; Ma et al., 1998; Klein et al., 2001). In the dentate gyrus, SDF-1 is expressed in the meninges and Cajal–Retzius cells, whereas its receptor Cxcr4 is expressed in migratory granule cells in the second dentate matrix and the migratory stream (Bagri et al., 2002). Disruption of the normal SDF-1 gradient by the ectopic SDF-1 expression to the hippocampal field using electroporation into slice culture causes a deficit in granule cell migration, suggesting SDF-1 is a chemoattractant factor for dentate migration (Bagri et al., 2002). The *cxcr4* KO mice exhibit a disrupted dentate gyrus, caused by migration defects of granule cells along the subpial stream and subsequent radial migration (Bagri et al., 2002; Lu et al., 2002; Li et al., 2009).

Nuclear Factor Ib (Nfib)

Nfib is a member of nuclear factors I (Nfia, b, c, and d) and functions as a transcriptional factor. The *nfib* KO mice display abnormal hippocampal formation, including the CA3, dentate gyrus, and fimbria, which may be due to aberrant maturation of radial glial fibers in the Ammon's horn (Steele-Perkins et al., 2005; Barry

et al., 2008). Although the deficient mouse exhibits normal cell proliferation, the dentate cells accumulate in the subpial region during dentate migration. Barry et al. suggest that this abnormal positioning of dentate cells is attributed to failed radial migration (Barry et al., 2008).

Disrupted-in-Schizophrenia-1 (Disc1)

DISC1 is a risk gene for major psychiatric disorders, including schizophrenia. We have previously reported that *Disc1* knockdown by *in utero* electroporation causes abnormal migration of hippocampal CA1 neurons; knockdown cells fail to enter the pyramidal layer (Tomita et al., 2011). Disc1 is also related to migration and positioning/integration of dentate granule cells during development and adulthood (Duan et al., 2007; Kim et al., 2009; Meyer and Morris, 2009). KO mice of *girdin*, a Disc1 interacting molecule, show dispersion of granule cells in the dentate gyrus, mis-positioning of pyramidal neurons in the CA1 and the CA3, and split layer in the CA2 (Enomoto et al., 2009). Knockdown of *girdin* in dentate granule cells results in over-migration, as observed for the *Disc1* knockdown cells. Because other genes implicated in neuropsychiatric diseases, such as *CNTNAP2* (Peñagarikano et al., 2011) and *FMRP* (La Fata et al., 2014), are reported to be involved in neuronal migration during neocortical development, it will be interesting to determine whether these genes also affect hippocampal neuronal migration.

Hippocampal neuronal migration is controlled by extracellular factors, such as Reelin, SDF-1, and radial glial fibers, as well as intracellular molecules as mentioned above. Both the *reeler* mouse and the *nfib* KO mouse exhibit abnormal radial glial fibers and disrupted dentate gyrus formation. However, both KO mice exhibit normal dentate cell migration and cells accumulate in the subpial region; the final migration toward the dentate layer is conducted along radial fibers, whereas dentate migration to the subpial region may be independent of these molecules.

Pafah1b1, Dcx, and Tuba1A regulate microtubule dynamics. Dab1 is reported to bind to Lis1 downstream of Reelin signaling (Assadi et al., 2003). Dcx is phosphorylated by Cdk5 at Ser297, resulting in reduced microtubule polymerization and binding affinity to microtubules (Tanaka et al., 2004). Cdk5 also regulates the Pafah1b1-Ndel1-Dynein complex via Ndel1 phosphorylation (Niethammer et al., 2000; Sasaki et al., 2000). Furthermore, Cdk5 and Reelin signaling synergistically contribute to neuronal positioning (Ohshima et al., 2001, 2007; Beffert et al., 2004). Therefore, microtubule dynamics is critical for migration of the hippocampal cells, similar to neocortical neurons.

A number of mutant mice exhibiting abnormal hippocampal formation also display splitting of the SP in the Ammon's horn, although the extent of splitting is not uniform. This may suggest the existence of multiple migration modes for hippocampal pyramidal neurons. In the rodent hippocampus, deep and superficial sublayers are visibly distinguished by cellular density and morphology in the ventral two-thirds of CA1 (Slomianka et al., 2011). Slomianka et al. also reviewed the distinction of histological, molecular, and connective features between deep and superficial sublayers in the hippocampal Ammon's horn (Slomianka et al., 2011). For example, superficial pyramidal cells express Satb2 during development, Nov and Nr3c2 in the hippocampal CA1 in adulthood, and Kcnq5 in the CA3 (Thompson et al., 2008; Dong et al., 2009). In contrast, deep pyramidal neurons express Sox5 during development, Ndst4 and Astn2 in the CA1 in adulthood, and St18 in the CA3 (Thompson et al., 2008; Dong et al., 2009). Furthermore, Mizuseki et al. showed physiological differences between deep and superficial sublayers in the hippocampal CA1 region of rats, such as theta phase preference during REM sleep and gamma phase preference during behavioral task (Mizuseki et al., 2011). In the hippocampal CA1, the climbing mode of migration is observed for late-born neurons (Kitazawa et al., 2014). In contrast, Morest reported that hippocampal cells extended their leading process through the HP and kept it until they reach the pial surface during the early developmental stages of opossum (Morest, 1970). Rodent hippocampal cells may also use this somal-translocation-like mode, especially during early stages of development. If this is the case, early-born neurons and late-born neurons might use different modes of migration and ultimately settle in their respective positions in the SP. The distinct populations between superficial and deep layers may be reflected by specific gene expressions and functions.

Conclusion

This review discusses the migration of pyramidal neurons in the Ammon's horn and granule cells in the dentate gyrus during hippocampal development. The structure of the hippocampus is dynamically expanded and becomes complicated during development. Because the climbing mode of migration is a flexible migration mode, it may be necessary for hippocampal neurons to accommodate to this hippocampal formation. The migration of dentate cells is well-organized, while cellular maturation is diverse along the migratory stream. Integration of molecular biological studies with histological studies has led to novel discoveries focused on cellular and molecular mechanisms of hippocampal development. Further studies on behaviors of hippocampal neurons are expected in the future to fully understand hippocampal development and functions.

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Genotype-phenotype correlation in neuronal migration disorders and cortical dysplasias

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Neuronal migration disorders are human (or animal) diseases that result from a disruption in the normal movement of neurons from their original birth site to their final destination during early development. As a consequence, the neurons remain somewhere along their migratory route, their location depending on the pathological mechanism and its severity. The neurons form characteristic abnormalities, which are morphologically classified into several types, such as lissencephaly, heterotopia, and cobblestone dysplasia. Polymicrogyria is classified as a group of malformations that appear secondary to post-migration development; however, recent findings of the underlying molecular mechanisms reveal overlapping processes in the neuronal migration and post-migration development stages. Mutations of many genes are involved in neuronal migration disorders, such as *LIS1* and *DCX* in classical lissencephaly spectrum, *TUBA1A* in microlissencephaly with agenesis of the corpus callosum, and *RELN* and *VLDLR* in lissencephaly with cerebellar hypoplasia. *ARX* is of particular interest from basic and clinical perspectives because it is critically involved in tangential migration of GABAergic interneurons in the forebrain and its mutations cause a variety of phenotypes ranging from hydranencephaly or lissencephaly to early-onset epileptic encephalopathies, including Ohtahara syndrome and infantile spasms or intellectual disability with no brain malformations. The recent advances in gene and genome analysis technologies will enable the genetic basis of neuronal migration disorders to be unraveled, which, in turn, will facilitate genotype-phenotype correlations to be determined.

Keywords: lissencephaly, heterotopia, polymicrogyria, tubulinopathy, interneuronopathy, *LIS1, DCX, ARX*

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Introduction

The characteristic six-layered neocortex in the human brain is formed by two types of neuron, projection neurons and interneurons, which migrate from their birth places, such as the ventricular zone and ganglionic eminence, respectively. Neuronal migration disorders are human (or animal) diseases that result from the disruption of normal movement of neurons from their original birth site to their final destination during early development. As a consequence, the neurons remain somewhere along their migratory route, their location depending on the pathological mechanism and its severity. Many genes have been found to be responsible for neuronal migration disorders, such as *LIS1* and *DCX* in classical lissencephaly spectrum, *TUBA1A* in lissencephaly with cerebellar hypoplasia, *ARX* in X-linked lissencephaly with abnormal genitalia (XLAG), *FLNA* and *ARGEF2* in periventricular heterotopia, *FCMD* and glycosylation-related

genes, such as *POMT1*, *POMT2*, *POMGNT1*, *POMGNT2*, *FKRP*, *LARGE*, *TMEM5*, *POMK*, *ISPD*, *GMPPB*, *B3GNT1*, and *B3GALNT2* in cobblestone dysplasias, *GPR56*, *SRPX2*, and some tubulin-related genes, e.g., *TUBA8*, *TUBB2B*, and *TUBB3*, in polymicrogyria (Kato and Dobyns, 2003; Vuillaumier-Barrot et al., 2012; Buysse et al., 2013; Stevens et al., 2013; Fry et al., 2014). Recently, we found that mutations in *COL4A1*, which encodes type IV collagen alpha 1 subunit, cause schizencephaly accompanied by polymicrogyria in the adjacent cortex of the transmantle cleft as well as focal cortical dysplasia (Yoneda et al., 2013). Historically, brain malformations including neuronal migration disorders have been classified based on a postmortem examination. The advancement and spread of neuroimaging techniques, particularly magnetic resonance imaging (MRI), make it easier to find out many types of brain malformations, but make it more complicated to classify them. Moreover, the unveiling of responsible genes for brain malformations has changed the classification scheme and causes most neuroscientists and even physicians trouble to follow it. Here, I review the clinical manifestation of neuronal migration disorders, focusing mainly on lissencephaly, in terms of genotype-phenotype correlations.

Lissencephaly Spectrum: Classical Lissencephaly to Subcortical Band Heterotopia

Lissencephaly is classified as a spectrum of disorders caused by widespread abnormal transmantle migration, ranging from classical lissencephaly (agyria or pachygyria) to subcortical band heterotopia or double-cortex syndrome (Barkovich et al., 2012). Classical lissencephaly is characterized by a smooth (*lissos* in Greek) brain surface with a decreased number of sulci and wide gyri. Mutations in *LIS1*, located on chromosome 17p13.3, or *DCX* on Xq23 are the main cause for classical lissencephaly (**Table 1**) (Kato and Dobyns, 2003). Mutations in *DCX* are causative for classical lissencephaly in male individuals and subcortical band heterotopia in female individuals. A combination of a severity grading scale [the most severe form, Grade 1 (total agyria) to the mildest form, Grade 6 (subcortical band heterotopia) via the intermediate forms comprised of a combination of agyria, pachygyria, and subcortical band heterotopia] and an anterior or posterior gradient scale is useful to predict the causative gene for lissencephaly spectrum (Kato and Dobyns, 2003). For instance, mutations of *LIS1*, *ARX*, or *TUBA1A* result in a posterior more severe than anterior gradient, while mutations of *DCX* or *RELN* lead to an anterior more severe than posterior gradient. LIS1 participates in cytoplasmic dynein-mediated nucleokinesis, somal translocation, and cell motility (Smith et al., 2000) as well as mitosis or neurogenesis and chromosomal segregation (Faulkner et al., 2000). DCX is a microtubule-associated protein and is involved in microtubule polymerization and stabilization (Gleeson et al., 1999). Missense mutations in *DCX* responsible for lissencephaly spectrum are mainly located in two tandem repeats (N-terminal or C-terminal doublecortin domains), which bind to microtubules or free tubulin and other components (Friocourt et al., 2005), respectively.

MRI of the brain is useful to discriminate agyria, pachygyria, and subcortical band heterotopia. Agyria is generally characterized by the disappearance of deep sulci in more than one lobe and the thickness of the cortex is 10–20 mm (**Figure 1**). The gyri in pachygyria are wider than in the normal cortex and the thickness of the cortex is 4–9 mm (**Figure 2**). Brain MRI of subcortical band heterotopia shows bilateral continuous symmetric bands of gray matter underlying an almost normal cortical mantle with relatively shallow sulci (**Figure 3**). More than 90% of patients with subcortical band heterotopia are female and the cause is usually heterozygous *DCX* mutation. Subcortical band heterotopia in male patients is caused by somatic mosaic *DCX* mutations or *LIS1* mutations (Gleeson et al., 2000; Kato et al., 2001; D'agostino et al., 2002; Poolos et al., 2002). Coexistence of agyria and pachygyria or pachygyria and subcortical band heterotopia can be seen in the same patient, suggesting common mechanisms for these phenotypes. Microscopically, agyria and pachygyria present a four-layered cortex with an outer molecular layer, superficial layer, cell sparse layer, and deep cellular layer. In the marginal zone between pachygyria and subcortical band heterotopia, the outer molecular layer corresponds to layer I of the normal six-layered cortex, the superficial layer corresponds to layers II–VI, the cell sparse layer corresponds to subcortical white matter, and the deep cellular layer corresponds to band heterotopia with a mass of unlayered ectopic neurons (**Figure 4**). The primary pathology of lissencephaly due to the *DCX* mutations shows only minor differences compared with that caused by *LIS1* mutations, for example, inferior olivary ectopia is present in *LIS1* mutation brains but is absent in *DCX* mutation brain (Berg et al., 1998); however, Viot et al. report a different cortical architecture for *DCX* lissencephaly (Viot et al., 2004).

The severity of the clinical manifestations of lissencephaly spectrum is correlated with the degree of brain malformation. Patients with agyria show severe muscle hypotonia from infancy (known as floppy infant) and achieve neither head control nor are they able to say meaningful words. A specific form of epileptic seizure, epileptic spasms, occurs in 80% of patients with agyria or pachygyria, although electroencephalography (EEG) may not present with typical hypsarrhythmia, which is characteristically seen in infantile spasms or West syndrome (Guerrini, 2005). However, the main clinical features of subcortical band heterotopia are intellectual disability and epileptic seizures, both of which are milder than those of agyria or pachygyria. Intellectual disability ranges from normal to severe retardation and correlates with the thickness of the band and the degree of pachygyria (Barkovich et al., 1994; Bahi-Buisson et al., 2013). Genetic counseling is particularly important for parents that have a boy with classical lissencephaly or a girl with subcortical band heterotopia because the mother may be a heterozygous carrier of the *DCX* mutation.

Miller-Dieker syndrome is a contiguous gene syndrome caused by a microdeletion in 17p13.3, a region that contains *LIS1* and *YWHAE* (which encodes 14-3-3 protein epsilon). Phenotypes of Miller-Dieker syndrome are more severe than

TABLE 1 | Clinical features of gene mutations causing cortical disruptions.

Gene	Locus	Inheritance mode	LIS	HET	PMG	MIC at birth	ACC	PCH	Brain	Other findings
LIS1~YWHAE	17p13.3	AD	+						Total agyria (Figure-of-8 appearance)	Characteristic face and MCA
LIS1	17p13.3	AD	+	+, rare					Agyria to subcortical band HET, mainly pachygyria in anterior and agyria in posterior	
DCX (male)	Xq23	XL	+	+					LIS. Subcortical band HET due to somatic mosaic mutation.	
DCX (female)	Xq23	XL	+, rare	+					Subcortical band HET	
TUBA1A	12q13.12	AD	+	+, rare	+, rare	+	+	+	MIC, agyria to subcortical band HET, PMG, PCH, ACC	
TUBA8	22q11	AR			+		+		PMG, agenesis or hypogenesis of the corpus callosum, dysmorphic brainstem	Optic nerve hypoplasia
TUBB2A	6p25.2	AD						+	Mild PCH	
TUBB2B	6p25.2	AD			+	+		+	MIC, PMG, dysmorphic basal ganglia, PCH, dysmorphic brainstema	CFEOM
TUBB3	16q24.3	AD			+			+	PMG, gyral disorganization, dysmorphic basal ganglia, PCH	CFEOM
TUBB	6p21.33	AD		+	+	+	+		MIC, focal band HET or PMG, dysmorphic basal ganglia, abnormal corpus callosum	Microophthalmia
TUBG1	17q21.2	AD	+	+					Posterior dominant lissencephaly, dysmorphic corpus callosum	
ARX (male)	Xp22.13	XL	+				+		Posterior dominant LIS with ACC and dysmorphic basal ganglia	Hypoplastic genitalia, diarrhea
ARX (female)	Xp22.13	XL					+		ACC in half of the cases	
RELN	7q22.1	AR	+					+	Anterior dominant diffuse pachygyria with PCH	
VLDLR	9p24.2	AR	+					+	Diffuse pachygyria with PCH	
MCPH1	8p23.1					+			MIC	
WDR62	19q13.12	AR	+	+	+	+	+		MIC, pachygyria, PMG, or subcortical band HET, abnormal corpus callosum	
NDE1	16p13.11	AR				+	+		MIC, simplified gyral pattern, ACC	
COL4A1	13q34	AD, low penetrance							Porencephaly, schizencephaly, focal cortical dysplasia	Myopathy, hematuria, anemia

ACC, Agenesis of the corpus callosum; CFEOM, Congenital fibrosis of the extraocular muscle; HET, heterotopia; LIS, classical lissencephaly or agyria/pachygyria; MCA, multiple congenital anomalies; MIC, microcephaly; PCH, pontocerebellar hypolasia; PMG, polymicrogyria.

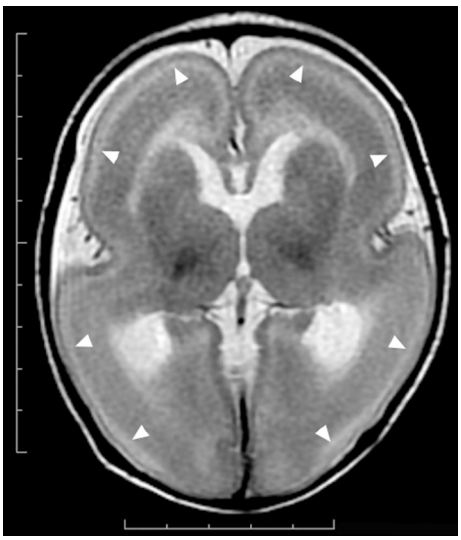

FIGURE 1 | Complete agyria in a *DCX* mutation patient (Grade 1 on the severity scale). T2-weighted axial MRI image. Wide shallow sylvian fissures create a figure-of-eight appearance. The thickness of the cortex is over 10 mm. A high-intensity (white) line (arrow heads) beneath the cerebral surface is consistent with a cell sparse layer of the four-layered cortex.

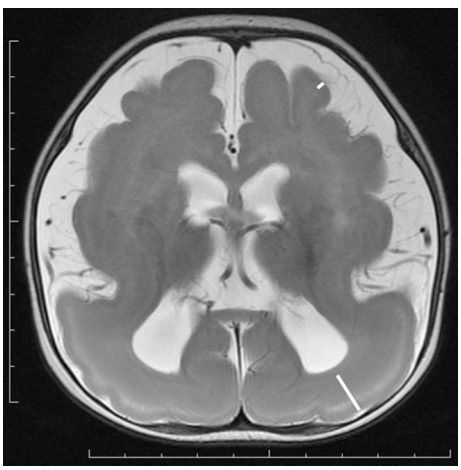

FIGURE 2 | Anterior pachygyria and posterior agyria in a *LIS1* mutation patient (Grade 3 on the severity scale). T2-weighted axial MRI image. Note the difference in the width of gyri, the depth of sulci and the thickness of the cortex (bars) between anterior and posterior regions.

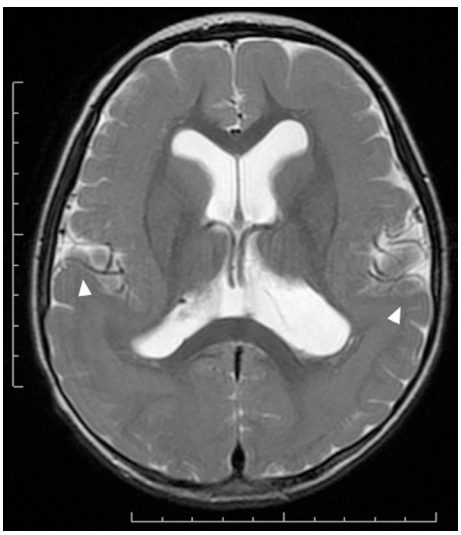

FIGURE 3 | Subcortical band heterotopia or double cortex syndrome in a *DCX* mutation patient (Grade 5 on the severity scale). T2-weighted axial MRI image. Subcortical heterotopic gray matter in the posterior region fuses into the pachygyric cortex in the anterior region (arrowheads).

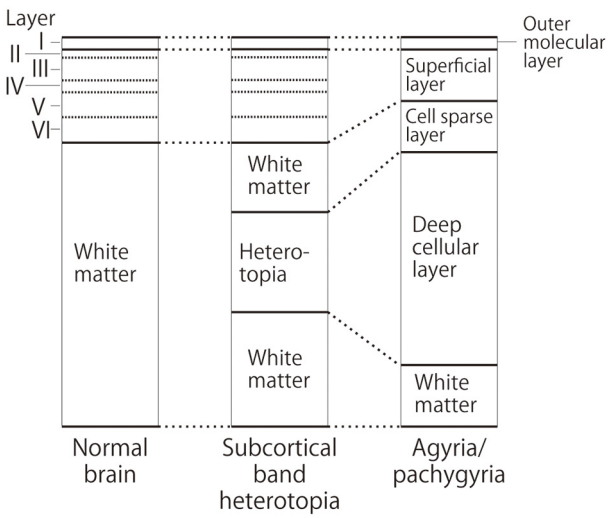

FIGURE 4 | Schematic diagram of cortical layers in the lissencephaly spectrum compared to the normal brain. Deep cellular layer of the pachygyric or agyric cortex fuses with laminar or band heterotopia in the subcortical white matter, but not with normal six-layered cortex.

that of classical lissencephaly because of an isolated *LIS1* mutation. They are characterized by complete agyria and facial abnormalities including prominent forehead, bitemporal hollowing, short nose with upturned nares, prominent upper lip with downturned vermilion border and small jaw, and sometimes other congenital defects involving the heart, kidneys, intestine, or fingers (Kato and Dobyns, 2003). Neurological findings of Miller–Dieker syndrome are similar to those of patients with agyria, such as severe developmental delay with weak muscle tone and profound intellectual disability, intractable seizures, dysphagia, and poor prognosis with recurrent infection of the respiratory system.

Tubulin-Related Disorders, Tubulinopathies

Microtubules provide the main structural framework for the shafts of axons and dendrites, and with actin serve as tracks for intracellular trafficking and to provide the driving force underlying neurite extension and intracellular movement of organelles during mitosis (Flynn et al., 2013). Recently, genes

involved in microtubule function have been identified to be causative for various human diseases, such as lissencephaly (Keays et al., 2007; Poirier et al., 2007), polymicrogyria (Abdollahi et al., 2009; Jaglin et al., 2009; Jansen et al., 2011), simplified gyral patter in which the cortical thickness is normal (Cushion et al., 2014), complex brain malformations (Poirier et al., 2010, 2013; Breuss et al., 2012), abnormal eye movement (Tischfield et al., 2010), torsion dystonia (Hersheson et al., 2013), and hypomyelinating leukodystrophy (Simons et al., 2013). All the above are classified as tubulinopathies (Cushion et al., 2013; Bahi-Buisson et al., 2014). Microtubules are assembled from soluble tubulin heterodimers consisting of alpha- and beta-tubulin. Multiple isoforms of both tubulins are encoded by different genes. Mutations of *TUBA1A*, which encodes alpha tubulin, cause lissencephaly spectrum, particularly diffuse agyria or perisylvian pachygyria, with microcephaly, agenesis of the corpus callosum, and cerebellar hypoplasia (**Figure 5**) (Bahi-Buisson et al., 2008). *TUBA1A* mutations account for only 1% of isolated classical lissencephaly; however, they account for approximately 30% of patients with lissencephaly associated with cerebellar hypoplasia (Kumar et al., 2010). Dysgenesis of the anterior limb of the internal capsule and disorganization of the hippocampus are other neuroimaging features for *TUBA1A* mutation (Poirier et al., 2007). Mutations of *TUBA1A* cause polymicrogyria as well. Interestingly, mutations of *TUBB2B* cause polymicrogyria with or without congenital fibrosis of the external ocular muscles as well as bilateral perisylvian pachygyria(Cederquist et al., 2012; Romaniello et al., 2014). Polymicrogyria is classified as a group of malformations that appear secondary to post-migration development; however, recent findings of the underlying molecular mechanisms reveal overlapping process in neuronal migration and post-migration development stages.

Mutations of *TUBA8* cause polymicrogyria with optic nerve hypoplasia and display autosomal recessive inheritance (Abdollahi et al., 2009). Mutations of *TUBB2A*, which encodes beta-tubulin, cause infantile-onset epilepsy with simplified gyral patterning (Cederquist et al., 2012; Cushion et al., 2014; Romaniello et al., 2014). Mutations of *TUBB3* cause two distinct forms. One is congenital fibrosis of the external ocular muscles or oculomotor nerve hypoplasia and later-onset peripheral axon degeneration with dysgenesis of the corpus callosum, anterior commissure, and internal capsule, but with no cortical dysplasia suggesting migrational defects (Tischfield et al., 2010). Another is cortical dysgenesis including polymicrogyria, pontocerebellar hypoplasia, and abnormal basal ganglia, but with no ocular motility defects (Poirier et al., 2010). The main mechanisms underlying the phenotypes caused by*TUBB3* mutations are impaired axon guidance owing to disrupted microtubule dynamics and kinesin interaction (Tischfield et al., 2010). Tubulinopathies caused by the mutations of the genes encoding alpha- or beta-tubulin demonstrate more extensive phenotypes compared to other gene mutations, such as *LIS1*, *DCX*, or *RELN*. Mutations of *TUBA1A*, which encodes alpha-tubulin 1A, is the most frequently found in patients with brain malformations, while more genes encoding beta-tubulin, such as *TUBB2A*, *TUBB2B*, *TUBB3*, *TUBB4A*, and *TUBB*, are identified in a wide spectrum of disorders besides brain malformations. Pathological mechanisms and discrepancy between alpha- and beta-tubulinopathies should be elucidated.

ARX-Related Disorders, Interneuronopathies

The embryonic cerebral cortex at the stage of neuronal migration contains neuronal cells with two modes of migration; radial migration from the ventricular zone toward the pia and tangential migration from ganglionic eminence along a tangential trajectory into the developing cortex. Radially

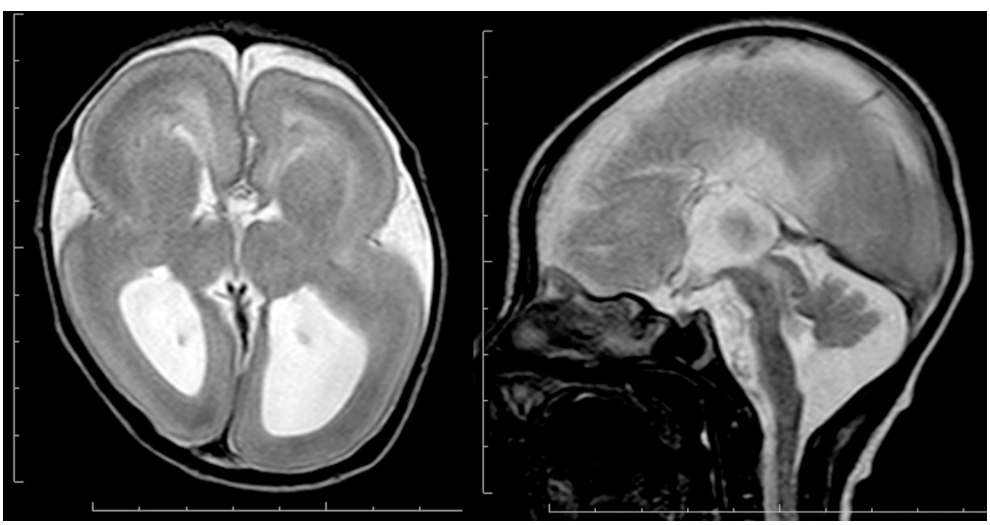

FIGURE 5 | Complete agyria in a *TUBA1A* mutation patient (Grade 1 on the severity scale). T2-weighted axial MRI image (left) and midsagittal image (right). The boundary of the caudate nucleus and lentiform nucleus is obscure. Complete agenesis of the corpus callosum and pontocerebellar hypoplasia are also seen.

migrating neurons in the cortex are mainly excitatory projection neurons expressing glutamate as a neurotransmitter. Tangentially migrating neurons are inhibitory interneurons expressing the neurotransmitter GABA. XLAG is caused by mutation of *ARX*, which is expressed in the embryonic ganglionic eminence, neocortex, and hippocampus and plays important roles in neuronal proliferation, interneuronal migration, and differentiation in the embryonic forebrain, as well as a secondary role in differentiation of the testes (Kitamura et al., 2002). Patients with XLAG present occipital-predominant classical lissencephaly, particularly anterior pachygyria and posterior agyria, or a simplified gyral pattern, agenesis of the corpus callosum, and abnormal basal ganglia (Kato et al., 2004). In the most severe form of XLAG, patients show hydranencephaly with a large occipital cavity. Female carriers of *ARX* mutations causing XLAG have a risk of agenesis of the corpus callosum with no cortical defects. Abnormalities of external genitalia range from hypoplastic penis or undescended testes to complete female appearance, while the karyotype is 46,XY. Neuropathological studies show a complete loss or a decreased number of cortical interneurons in human XLAG and in *Arx*-null mice (Bonneau et al., 2002; Kitamura et al., 2002) and a three-layered cortex in human XLAG (Forman et al., 2005). Patients with XLAG show intractable seizures soon after birth, suggesting a great disparity between excitatory projection neurons and inhibitory interneurons. *ARX* mutations in patients with XLAG are null mutations or non-conservative missense mutations at critical amino acids in the homeodomain, while other missense mutations or expansion mutations in the polyalanine tract result in X-linked intellectual disability with or without dystonia, West syndrome, Ohtahara syndrome, or early infantile epileptic encephalopathy with suppression burst on EEG but with no brain malformation (Bienvenu et al., 2002; Stromme et al., 2002; Guerrini et al., 2007; Kato et al., 2007, 2010). Interestingly, longer polyalanine expansion is correlated with more severe and earlier onset phenotypes. A wide spectrum of ARX-related disorders forms a group of interneuronopathies based on the role of ARX during neurogenesis, as seen in patients and in the *Arx*-null mouse model (Kato and Dobyns, 2005; Marsh et al., 2009).

Classical Lissencephalies Associated with Other Forms of Brain Malformation

Classical lissencephaly caused by *LIS1* or *DCX* mutations usually exist in isolated forms and only show cortical dysplasia on brain MRI. Rare variant forms of lissencephaly are associated with congenital microcephaly, cerebellar hypoplasia, or agenesis of the corpus callosum. Each form demonstrates characteristic radiological findings and some of the causative genes have been identified.

A lissencephaly group with cerebellar hypoplasia can be classified into several types according to brain imaging, additional clinical features, and causative genes (Ross et al., 2001). Among them, frontal predominant mild lissencephaly (diffuse pachygyria) with severe hippocampal and cerebellar hypoplasia or Reelin-type lissencephaly is caused by mutation of either *RELN* or *VLDLR* and shows autosomal recessive inheritance (Hong et al., 2000; Boycott et al., 2005). Dysequilibrium syndrome is an allelic disorder of the *VLDLR* locus (Moheb et al., 2008). Reelin-type lissencephaly has an inverted or no clear pattern of cortical lamination attributable to abnormal migration of the neurons in an outside-in birth order (Cooper, 2008; Dekimoto et al., 2010).

Lissencephaly can be associated with congenital microcephaly, though the head circumference of lissencephaly caused by the *LIS1* or *DCX* mutations is usually within the normal range. Lissencephaly with a head circumference of less than −3 SD at birth is classified as microlissencephaly (Barkovich et al., 2005) or microcephaly with lissencephaly (Barkovich et al., 2012). Although many genes identified to be responsible for primary microcephaly, such as *MCPH1*, *ASPM*, *CENPJ*, *CDK5RAP2*, and *PNKP*, are involved with the cell-cycle phase of mitosis affecting neurogenesis (Barbelanne and Tsang, 2014), the causative genes for microlissencephaly remain unknown in many cases. Mutations of *WDR62*, which encodes a protein localized to centrosomes throughout mitosis and nucleoli during interphase, cause microcephaly with pachygyria or polymicrogyria (Bilguvar et al., 2010). Mutations of *NDE1*, which encodes a protein that binds dynein and functions in centrosome duplication, as well as the *TUBA1A* mutations mentioned above, cause microcephaly with a simplified gyral pattern, agenesis of the corpus callosum, and cerebellar hypoplasia (Alkuraya et al., 2011; Bakircioglu et al., 2011).

Conclusion

Neuronal migration disorders are classified based on causative genes as well as on brain MRI and neuropathological findings. There are strong relationships between clinical manifestations and mutation of a particular gene, in accordance with the expression and functions of that gene. Recent advances in gene and genome analysis technology will enable the genetic basis of neuronal migration disorders to be readily determined, facilitating the elucidation of genotype-phenotype correlations.

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Neuronal migration abnormalities and its possible implications for schizophrenia

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Schizophrenia is a complex mental disorder that displays behavioral deficits such as decreased sensory gating, reduced social interaction and working memory deficits. The neurodevelopmental model is one of the widely accepted hypotheses of the etiology of schizophrenia. Subtle developmental abnormalities of the brain which stated long before the onset of clinical symptoms are thought to lead to the emergence of illness. Schizophrenia has strong genetic components but its underlying molecular pathogenesis is still poorly understood. Genetic linkage and association studies have identified several genes involved in neuronal migrations as candidate susceptibility genes for schizophrenia, although their effect size is small. Recent progress in copy number variation studies also has identified much higher risk loci such as 22q11. Based on these genetic findings, we are now able to utilize genetically-defined animal models. Here we summarize the results of neurodevelopmental and behavioral analysis of genetically-defined animal models. Furthermore, animal model experiments have demonstrated that embryonic and perinatal neurodevelopmental insults in neurogenesis and neuronal migrations cause neuronal functional and behavioral deficits in affected adult animals, which are similar to those of schizophrenic patients. However, these findings do not establish causative relationship. Genetically-defined animal models are a critical approach to explore the relationship between neuronal migration abnormalities and behavioral abnormalities relevant to schizophrenia.

Keywords: schizophrenia, GABAergic interneuron, mouse models, Neuregulin1, DISC1, 22q11 deletion syndrome, Dgcr8, Cxcr4

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Introduction

Schizophrenia is a chronic psychiatric disorder with a strong genetic component. Twin studies indicated that the heritability for schizophrenia is estimated to be 0.81 (Sullivan et al., 2012). Most of genetic linkage studies failed to identify highly-shared risk alleles due to the complexity of genetic architecture of schizophrenia except for DISC1 (Millar et al., 2000). Many combinations of different gene variants cause genetic risk of schizophrenia. Genome-wide association studies have identified many schizophrenia susceptibility candidate genes. Most of such common variants confer only slight increase in risk for schizophrenia (odds ratio < 1.2) (Ripke et al., 2013), and often failed to be replicated. Some of them, Neuregulin1, ErbB4, and Reelin are involved in the regulation of neuronal migration. On the other hand, rare and *de novo* chromosomal microdeletion

or microduplication [copy number variations (CNVs)] have been implicated in schizophrenia (Levinson et al., 2011; Rees et al., 2014). Such CNVs are from thousands to millions nucleotides and contain many genes and their odds ratios are high (2∼20) compared with common variants. 22q11.2 deletion syndrome (22q11DS) is the most frequent known genetic cause of schizophrenia (Pulver et al., 1994). However, it remains to be elucidated how combinations of these genetic variants play pathogenic roles of schizophrenia.

Schizophrenia is believed to result from embryonic developmental abnormalities not from neuronal degenerations (Weinberger, 1987; O'Connell et al., 1997). Cytoarchitectural abnormalities were reported in the entorhinal cortex (Jakob and Beckmann, 1986; Arnold et al., 1991, 1997; Falkai et al., 2000) and the subcortical white matters in schizophrenia (Akbarian et al., 1993a,b, 1996). Decreased neuronal density in the superficial white matter and increased density in the deep white matter suggest neuronal migration defects in schizophrenia. However, other studies failed to replicate these findings (Akil and Lewis, 1997; Krimer et al., 1997; Bernstein et al., 1998; Beasley et al., 2002, 2009), suggesting these abnormalities might be too subtle to be detected without special methods. Prior human brain imaging studies also have indicated reduced cerebral volume, ventricular enlargement, and reduced hippocampal volume in schizophrenia (Shenton et al., 2001).

The development of the human cerebral cortex is similar to that in the mouse (Rakic, 2009; Hansen et al., 2013; Ma et al., 2013), which enabled investigation of the functions of schizophrenia susceptibility candidate genes in neuronal developments. The mammalian cerebral cortex consists mainly of excitatory glutametergic and inhibitory GABAergic neurons. Glutamatergic neurons are generated from neural progenitors in the dorsal forebrain (Glover et al., 2009; Rakic, 2009), whereas most of inhibitory neurons are thought to derive from the ventral pallium: medial, lateral and caudate ganglionic eminence (MGE, LGE, and CGE) (Hansen et al., 2013; Ma et al., 2013). Two types of migration are observed in the cortex. One is radial migration of glutamatergic neurons from the underlying ventricular zone along the radial glial fiber, while the other is tangential migration from the ventral forebrain of GABAergic interneurons (Corbin et al., 2001; Marin and Rubenstein, 2003). However, the differences between the human and mouse cortices are also reported. In the mouse, about 70% of cortical interneurons generate from the MGE and ∼30% are from the CGE (Miyoshi et al., 2010). In contrast, more than half of interneurons derive from the CGE in the human (Hansen et al., 2013). The human cortex showed a much higher diversity in the interneuron types compared with that of the rodents (Feldman and Peters, 1978). In spite of the limitations caused by these differences, animal models are still valuable to elucidate the roles of neuronal migration deficits in the pathogenesis of schizophrenia. Many genetically-modified animal models with construct validity and cell-specific gene modification technique are available. The great advantage of rodent model to study schizophrenia is that we can establish a causal relationship between genetic abnormalities, neuronal developments, and behavioral abnormalities. Here we review a group of studies using rodent models which give insights into the pathogenesis of schizophrenia.

Developmental Neuronal Disruption Model of Schizophrenia

Perinatal insult of neuronal development can cause anatomical and behavioral deficits similar to human schizophrenic patients. One of the examples is a gestational day 17 (GD17) methylazoxymethanol acetate (MAM) administration rat model (Grace and Moore, 1998; Flagstad et al., 2004; Gourevitch et al., 2004; Paredes et al., 2006). MAM is a mitotic toxin and MAM administration specifically disrupts proliferating region. GD17 MAM treatment results in specific subtle reductions in the volume of prefrontal cortex (PFC) and hippocampus (HP), heterotopias in the HP resulting from neuronal migration deficits (Le Pen et al., 2006; Moore et al., 2006), which are characteristics of schizophrenia (Kovelman and Scheibel, 1984; Shenton et al., 2001; Heckers, 2004; Honea et al., 2005). GD17 MAM-treated rats also display decreased density of parvalbumin (PV)-positive interneurons in medial PFC and HP (Lodge et al., 2009). Interestingly, postmortem studies of human schizophrenia have shown decreased expression of PV and the 67 KDa isoform of glutamic acid decarboxylase (GAD67), which is an enzyme responsible for GABA synthesis, in PFC of schizophrenia subjects (Akbarian et al., 1995; Volk et al., 2000; Hashimoto et al., 2003; Fung et al., 2010). However, no difference is observed in the density of PV-positive interneurons in schizophrenia (Hashimoto et al., 2003). PV-positive interneurons are known to be indispensable for synchronized firing of excitatory pyramidal neurons in gamma frequencies (30–80 Hz), which plays essential roles for cognitive functions (Howard et al., 2003). Altered gamma oscillation activity and cognitive deficits have been reported in schizophrenia (Cho et al., 2006; Minzenberg et al., 2010). Consequently, PV-positive interneuron deficits are thought to be the cause of impairments of gamma oscillation and cognition in individuals with schizophrenia (Lewis et al., 2012). MAM-treated rats also show behavioral deficits in prepulse inhibition (PPI), which reflects an inability to filter out irrelevant sensory information, and working memory task (Flagstad et al., 2005; Le Pen et al., 2006; Moore et al., 2006), which are typical symptoms of schizophrenia in humans (Braff and Geyer, 1990; Liddle and Morris, 1991; Goldman-Rakic, 1994; Swerdlow et al., 1994; Nuechterlein et al., 2004). Furthermore, electrophysiological studies have shown that enhanced activity of ventral HP leads to dopaminergic neuronal activation in MAM-treated rats (Lodge and Grace, 2007). Again, these altered hippocampal activities are also observed in human schizophrenic patients (Medoff et al., 2001; Schobel et al., 2009). These abnormalities can be normalized by administration of α5GABA A receptor positive allosteric modulator, SH-053-2′F-R-CH3 (Gill et al., 2011), suggesting the involvement of GABA in embryonic MAM treatment-induced deficits. The MAM model provides a direct evidence that subtle embryonic disruptions of neuronal development result in behavioral alterations disorders, although the etiology is absolutely different from that of schizophrenia in humans.

Neuregulin-ERBB Signaling

Neuregulins are a large family of epidermal growth factor (EGF)-like proteins and play divergent roles both in neuronal development and in the neuronal activity homeostasis in the mature central nervous system. Several genetic linkage studies have shown Neuregulin1 (NRG1) as a strong candidate gene for schizophrenia (Badner and Gershon, 2002; Stefansson et al., 2002; Lewis et al., 2003). Some GWASs also support the hypothesis (Li et al., 2006; Munafo et al., 2006; Shi et al., 2009; Agim et al., 2013), although it has not been confirmed by a recent mega-analysis in which international consortia combined the resources to maximize the sample size and identified more than 100 candidate genes for schizophrenia with high levels of statistical significance (Schizophrenia Working Group of the Psychiztric Genomics Consortium, 2014). Most of schizophrenia-associated single nucleotide polymorphisms (SNPs) in NRG1 are localized in the 5′ and 3′ region of the gene. Some of them are associated with the expression level of NRG1 (Law et al., 2006; Weickert et al., 2012). A receptor of NRG1, ERBB4 is also associated with schizophrenia (Benzel et al., 2007; Law et al., 2007; Shi et al., 2009; Agim et al., 2013). It has been reported that a rare chromosome micro-deletion in a schizophrenic patient disrupts this gene, resulting in a truncated protein similar to dominant-negative ERBB4 (Walsh et al., 2008).

Nrg1 generates six types and at least 30 isoforms owing to multiple promoters and alternative splicing (Mei and Nave, 2014). Most pro-Nrg1 isoforms are transmembrane proteins and N-terminal domains containing EGF-like domain are released out of the cell after undergoing proteolytic processing except for TypeIII Nrg1 (cysteine-rich-domain containing Nrg1 (CRD-Nrg1)). This released mature Nrg1 activates ErbB receptor tyrosine kinase such as ErbB2/ErbB3 heterodimer and ErbB4 homodimers. Nrg1 regulates migration of excitatory glutamatergic neurons and γ-aminobutyric acid (GABA)-producing interneurons in the embryonic cortex. Nrg1 promotes the maintenance of radial glial cells in the cortex and induces elongation of radial fiber, which are essential for the radial migration of cortical excitatory neurons and cerebellar granule cells (Anton et al., 1997; Rio et al., 1997). NRG1 is also critical for interneuronal tangential migration (Flames et al., 2004; Li et al., 2012). ErbB4 is expressed in interneuronal progenitors migrating from the MGE to the cortex (Yau et al., 2003; Flames et al., 2004). Type III Nrg1 is expressed in lateral ganglionic eminence, and Type I and II Nrg1 (immunoglobulin (Ig)-domain containing Nrg1 (Ig-Nrg1)) are expressed in the embryonic cortex (Flames et al., 2004). Diffusible Type I and II Nrg1 in the cortex are thought to attract ErbB4-expressing interneurons along a permissive corridor of Type III Nrg1 (Flames et al., 2004), although this model is challenged. In another model, Nrg1, and Nrg3 have been proposed to be repellants for migrating interneurons (Li et al., 2012). Loss of ErbB4 cuases embryonic lethality due to failed development of myocardial trabeculae, which made it difficult to characterize the functions of ErbB4 signaling in interneuronal migration (Gassmann et al., 1995; Kramer et al., 1996). However, heart-rescued ErbB4 knockout mice with cardiac-specific ErbB4 transgene displayed decreased number of GABAergic interneurons in the postnatal cortex (Flames et al., 2004; Fisahn et al., 2009; Li et al., 2012), which clearly showed the essential roles of Nrg1/ErbB4 signaling in interneuronal migration.

Cell-specific gene modification techniques are now starting to elucidate a link between Nrg1/ErbB4 signaling and pathophysiology of schizophrenia. The gain and loss of function of Nrg1/ErbB4 signaling were examined because postmortem studies of schizophrenia reported both increased and decreased NRG1/ERBB4 signaling in schizophrenic patients (Silberberg et al., 2006; Law et al., 2007; Weickert et al., 2012; Joshi et al., 2014). Transgenic mice overexpressing Type I Nrg1 showed deficits in PPI and contextual fear conditioning, and hyperlocomotion (Deakin et al., 2009, 2012; Yin et al., 2013; Luo et al., 2014). If the overexpression of Nrg1 was switched off in adult mice, its effects were reversible (Yin et al., 2013; Luo et al., 2014). The influence of Nrg1 overexpression on neuronal development remains to be elucidated. Nrg1 or ErbB4 heterozygous mice and conditional knockout mice also displayed various behavioral abnormalities: locomotor hyperactivity in open field (OF), impairment in Prepulse inhibition (PPI), and fear conditioning (Stefansson et al., 2002; Golub et al., 2004; Boucher et al., 2007; O'Tuathaigh et al., 2007, 2010; Chen et al., 2008, 2010; Duffy et al., 2008; Ehrlichman et al., 2009; Shamir et al., 2012; Del Pino et al., 2013; Pei et al., 2014)(**Table 1**). Nrg1 heterozygous mice and heart-rescued ErbB4 knockout (KO) mice showed decreased number of cortical PV interneurons (Fisahn et al., 2009; Neddens and Buonanno, 2010; Shamir et al., 2012; Pei et al., 2014) (**Table 1**). However, PV interneuron-specific deletion of ErbB4 did not affect the number of cortical interneurons, which might be due to the slow turnover of ErbB4 (Fazzari et al., 2010; Shamir et al., 2012) (**Table 1**). A comparative behavioral analysis of ErbB4 KO and PV interneuron-specific ErbB4 KO mice demonstrated that PV interneuron-specific deletion is sufficient for hyperactivity and deficits in PPI. The only difference is that ErbB4 KO mice but not PV interneuron-specific ErbB4 KO mice exhibit reduced anxiety-like behaviors and deficits in cued and contextual fear conditioning (Shamir et al., 2012), which might be caused by developmental disorders in ErbB4-deficient interneurons.

Disrupted-in Schizophrenia 1

The disrupted-in schizophrenia 1 (DISC1) gene was discovered at the breakpoint of inherited balanced chromosomal translocation in a Scottish family suffering from major depression, schizophrenia, and bipolar disorder (St Clair et al., 1990; Millar et al., 2000). Following linkage analysis and association studies demonstrated that DISC1 is significantly associated with schizophrenia, bipolar disorder and major depression (Ekelund et al., 2001, 2004; Macgregor et al., 2004; Hamshere et al., 2005; Hashimoto et al., 2006; Liu et al., 2006; Thomson et al., 2014), although it has not been confirmed by a recent mega-analysis of GWASs (Schizophrenia Working Group of the Psychiztric Genomics Consortium, 2014).

DISC1 is a scaffolding protein interacting with multiple proteins: nuclear distribution gene E homolog-like 1 (NDEL1), lissencephaly-1 (LIS1), phosphodiesterase 4B (PDE4B), glycogen

TABLE 1 | Summary of mutant Nrg1/ErbB4 mouse models.

Gene	Locomotion	PPI	Learning and memory	Gross anatomy	Interneuron	References
$Nrg1^{+/-}$ (TM)	↑	→	ND	ND	ND	Boucher et al., 2007
	↑	↓	ND	ND	ND	Stefansson et al., 2002
	↑	ND	Y maze	Small ventricle	ND	O'Tuathaigh et al., 2007, 2010
$Nrg1^{+/-}$ (TM)	→	→	contextual FC ↓ cued FC ↓	ND	PV/Gad67 in HC ↓ (western blotting)	Pei et al., 2014
$Nrg1^{+/-}$ (TypeIII)	→	↓	T-Maze↓	Enlarged lateral ventricle	ND	Chen et al., 2008
$Nrg1^{+/-}$	↑	→	ND	ND	ND	Duffy et al., 2008
(EGF)	→	→	Contextual FC ↓	ND	ND	Ehrlichman et al., 2009
$ErbB4^{+/-}$	↑	→	ND	ND	ND	Stefansson et al., 2002
$ErbB4^{f/-}$ (Nestin-Cre)	↓	ND	Morri Water Maze ↑ (hetero)	ND	ND	Golub et al., 2004
$ErbB4^{-/-}$ (heart-rescued)	↑	↓	Contextual FC ↓ Cued FC ↓	ND	Number of PV neurons ↓	Shamir et al., 2012
$ErbB4^{f/f}$ (PV-Cre)	↑	↓	Contextual FC → Cued FC →	ND	Number of PV neurons→	Shamir et al., 2012
$ErbB4^{f/f}$ (PV-Cre)	ND	ND	Contextual FC ↓	ND	ND	Chen et al., 2010
$ErbB4^{f/f}$ (Lhx6-Cre)	↑	↓	Y-maze ↓	ND	Number of PV neurons→	Del Pino et al., 2013

OF, open field, PPI:prepulse inhibition; FC, fear conditioning; TM, transmembrane region; PV, parvalbumin; HC, hippocampus; ND, not determined.

synthase kinase 3β (GSK3β) and DIX domain-containing 1 (DIXDC1) (Ozeki et al., 2003; Millar et al., 2005; Taya et al., 2007; Mao et al., 2009). DISC1/DIXDC1 functions as a switch between neuronal proliferation and migration. DISC1/DIXDC1 binds to GSK3β and inhibits its activity leading to proliferation of neural progenitors through the inhibition of β-catenin degradation (Mao et al., 2009). CDK5 phosphorylation of DISC1 at S710 and DIXDC1 facilitates neuronal migration by dissociating DISC1 from GSK3β and promoting its binding with NDEL1 (Singh et al., 2010; Ishizuka et al., 2011). DISC1 variants associating with human brain structures and psychiatric phenotypes have been reported to impair this switching mechanism (Singh et al., 2011). Furthermore, knockdown of DISC1 also impairs interneuronal tangential migrations (Steinecke et al., 2012, 2014).

Two hypotheses are proposed on the pathophysiology of the disruption of the DISC1 gene: that the Scottish mutation decreases DISC1 expression and leads to haploinsufficiency; or that the Scottish mutation results in production of carboxy-terminal-truncated DISC1 (amino acids 1-598). This C-terminal truncated DISC1 functions as a dominant negative protein, and impairs microtubule dynamics by blocking interaction between DISC1 and dynein complex (Kamiya et al., 2005). Dynein complex contains LIS1 and NDEL1, and regulates coupling of the nucleus and centrosome, which is indispensable for radial migration of cortical excitatory neurons (Sasaki et al., 2000). Knockdown of DISC1 inhibits cortical neuronal cell migration (Kamiya et al., 2005; Kubo et al., 2010).

Acute knockdown of DISC1 using RNAi leads to drastic neuronal migration deficits. However, DISC1 KO mice ($Disc1^{\Delta 2\text{-}3/\Delta 2\text{-}3}$) astonishingly showed almost normal cytoarchitectures of the cerebral cortex and the HP (Kuroda et al., 2011), whereas the number of PV-positive interneurons reduced in female $Disc1^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice (Nakai et al., 2014). The phenotypes in the proliferation of neuronal progenitors have not been examined in DISC1 KO mice, which will provide important insight into the pathogenesis of DISC1 deficiency. Furthermore, $Disc1^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice did not show schizophrenia-like phenotype but exhibited lower anxiety and higher impulsivity (Kuroda et al., 2011) (**Table 2**). Only female $Disc1^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice exhibited enhanced responsiveness to methamphetamine and deficits in PPI (Kuroda et al., 2011). These milder phenotypes of $Disc1^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice might be explained by a compensation mechanism after chronic loss of DISC1. N-nitroso-N-ethylurea (ENU) mutagenesis was utilized to generate missense mutations of *Disc1*. L100P mutant ($Disc1^{L100P/L100P}$) mice showed reduced brain volume, reduced number of cortical neurons, altered distribution of cortical neurons, interneuonal migration deficits, and schizophrenia-like behavioral abnormalities, although the behavioral phenotypes were not confirmed by another group due to the difference in the genetic background (Clapcote et al., 2007; Lee et al., 2011, 2013; Shoji et al., 2012). 129S6/SvEv 25-bp deletion variant results in the production of a truncated isoform of DISC1 (amino acids 1-542) (Koike et al., 2006). C57BL/6J mice carrying the Disc1 gene from the 129S6/SvEv strain ($Disc1^{\Delta 25\,bp/\Delta 25\,bp}$mice) exhibited enlarged ventricle and

working memory deficits (Koike et al., 2006; Juan et al., 2014). Neuron-specific overexpression of the truncated DISC1 also resulted in drastic phenotypes: enlarged lateral ventricle, reduced number of PV-positive interneurons and schizophrenia-like behavioral abnormalities (Hikida et al., 2007; Pletnikov et al., 2008; Shen et al., 2008; Ayhan et al., 2011) (**Table 2**). Furthermore, a technique of inducible transgene expression enabled a specific expression of truncated DISC1 during only prenatal period, only postnatal period or both periods (Ayhan et al., 2011). Prenatal expression only led to decreased brain volume and decreased number of PV-positive interneurons. Enlarged lateral ventricle seems to be affected by postnatal expression of truncated DISC1. In contrast, enhanced responsiveness to psychostimulant required prenatal and postnatal continuous expression (Ayhan et al., 2011), which suggests that both neurodevelopmental abnormality and neuronal functional impairment caused by truncated DISC1 might be essential for pathogenesis of schizophrenia.

22q11 Deletion Syndrome

22q11.2 deletion syndrome (22q11DS) is the most frequent known genetic cause of schizophrenia (Pulver et al., 1994). 22q11DS accounts for about 1% of schizophrenia cases (Karayiorgou et al., 1995; Manolio et al., 2009). Prior brain imaging studies of human 22q11 DS have indicated reduced cerebral volume, ventricular enlargement and reduced hippocampal volume (Eliez et al., 2000, 2001; Chow et al., 2002; Simon et al., 2005). All of these brain anomalies have also been reported in schizophrenia (Shenton et al., 2001). All of the genes except for one gene in human 22q11.2 locus exist on mouse chromosome 16 (Puech et al., 1997). This has facilitated the generation of mouse models of 22q11 DS, which carry a hemizygous deletion of 22q11-related region of mouse chromosome16 (Lindsay et al., 1999; Paylor and Lindsay, 2006; Stark et al., 2008). These animal models show schizophrenia-related behavioral abnormalities such as working memory deficits, sensory information-processing deficits, and enhanced responsiveness to psychostimulants (Paylor et al., 2001; Stark et al., 2008; Earls et al., 2011; Kimoto et al., 2012), which are recognized as major deficits of schizophrenia (Elvevag and Goldberg, 2000; Green et al., 2000; Swerdlow et al., 2001). Animal models of 22q11DS showed reduced density of layer II–IV projection neurons in a medial PFC (Meechan et al., 2009), reduced volume of a perinatal HP dentate gyrus (Toritsuka et al., 2013), delayed migration of hippocampal dentate neuronal progenitors and cortical interneurons, and altered distribution of PV-positive interneurons (Meechan et al., 2009, 2012; Toritsuka et al., 2013), although it remains to be elucidated these deficits in neurogenesis lead to excitatory/inhibitory imbalance or not. Perinatal hippocampal DG and interneuronal migration abnormalities are caused by Cxcl12/Cxcr4 signaling deficits (Toritsuka et al., 2013). Cxcl12/Cxcr4 signaling might play pivotal roles in the pathogenesis of schizophrenia. Previous studies also suggest a possible involvement of Cxcl12/Cxcr4 signaling in the neurodevelopmental disorders of GD17 MAM-treated animal model of schizophrenia (Paredes et al., 2006). The expression of CXCL12 is decreased in olfactory neurons from sporadic cases with schizophrenia compared with normal controls (Toritsuka et al., 2013).

Among of genes deleted in 22q11DS, *Dgcr8* is a promising candidate gene for schizophrenia-related phenotypes. *Dgcr8* forms the microprocessor complex of microRNA (miRNA) with Drosha, which is essential for miRNA production. Overexpression of *Dgcr8* rescued interneuronal migration deficits of 22q11DS model mice, and the migration of hippocampal DG and interneuronal progenitors were also affected in*Dgcr8*$^{+/-}$ mice (Toritsuka et al., 2013). These observations demonstrated the important roles of *Dgcr8* in the pathogenesis of 22q11DS. miRNA–mediated regulation network fine tunes the balance of signaling and confers robustness to the system (Herranz and Cohen, 2010). miRNA-mediated regulation can buffer increases or reductions in gene dosage (Staton et al., 2011). Haplodeletion of *Dgcr8* causes 20–70% reduction of a specific subsets of mature miRNAs both in *Dgcr8* heterozygous and 22q11DS model mice (Stark et al., 2008). *Dgcr8* heterozygousity might uncover the effects of 22q11 microdeletion through the disruption of miRNA-mediated buffering effects. In mice, heterozygous deletion of *Dgcr8* alone showed working memory

TABLE 2 | Summary of mutant Disc1 mouse models.

Gene	Locomotion	PPI	Learning and memory	Gross anatomy	Interneuron	References
CaMK-DN-DISC1 tg	↑	↓	Y-maze →	Enlarged lateral ventricle	Number of PV neurons ↓	Hikida et al., 2007
BAC DN-DISC1 tg	→	ND	ND	Enlarged lateral ventricle	Number of PV neurons ↓	Shen et al., 2008
Inducible-CaMK DN-DISC1 tg	↑	→	ND	Enlarged lateral ventricle	Number of PV neurons ↓	Pletnikov et al., 2008; Ayhan et al., 2011
Disc1 $^{\Delta 2-3/\Delta 2-3}$	→	↓ (Female)	Y-maze →	Normal	Number of PV neurons ↓ (female)	Kuroda et al., 2011; Nakai et al., 2014
Disc1$^{L100P/L100P}$	↑	↓	T-maze ↓	Brain volume↓	Deficits in the distribution of PV neurons	Clapcote et al., 2007; Lee et al., 2011, 2013; Shoji et al., 2012
Disc1 $^{\Delta 25\,bp/\Delta 25\,bp}$ (C57Bl6)	→	→	T-maze ↓	Enlarged lateral ventricle	ND	Koike et al., 2006; Juan et al., 2014

deficits, sensory information-processing deficits and some of neurodevelopmental abnormalities such as reduced cortical neuronal densities (Stark et al., 2008; Fenelon et al., 2011). The behavioral abnormalities and neurodevelopmental disorders of *Dgcr8*$^{+/-}$ mice are similar but some of them are milder than those of 22q11DS model mice,(Stark et al., 2008; Meechan et al., 2009; Fenelon et al., 2011), which might suggest that additional haplodeletion of other genes in 22q11-related regions might be required for the complete reconstitution of phenotypes of 22q11DS model mice. It remains to be elucidated whether behavioral abnormalities of 22q11DS model mice are directly caused by neuronal migration deficit and Cxcr4 signaling defects.

Concluding Remarks

Elucidating the relationship between neurodevelopmental abnormalities and the pathogenesis of schizophrenia would be exceptionally difficult. In order to dissect the complex causal relations, more sophisticated genetic manipulation would be required. Combination of various techniques such as conditional knockout, inducible transgene expression and virus-mediated gene delivery will enable cell type-specific and developmental stage-specific knockout or rescue experiments. In the future comprehensive profile of neurodevelopmental deficits-behavioral abnormalities will provide significant insights into mental disease pathogenesis of all these neurodevelopmental genes.

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Genetic manipulation of reptilian embryos: toward an understanding of cortical development and evolution

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The mammalian neocortex is a remarkable structure that is characterized by tangential surface expansion and six-layered lamination. However, how the mammalian neocortex emerged during evolution remains elusive. Because all modern reptiles have a homolog of the neocortex at the dorsal pallium, developmental analyses of the reptilian cortex are valuable to explore the origin of the neocortex. However, reptilian cortical development and the underlying molecular mechanisms remain unclear, mainly due to technical difficulties with sample collection and embryonic manipulation. Here, we introduce a method of embryonic manipulations for the Madagascar ground gecko and Chinese softshell turtle. We established *in ovo* electroporation and an *ex ovo* culture system to address neural stem cell dynamics, neuronal differentiation and migration. Applications of these techniques illuminate the developmental mechanisms underlying reptilian corticogenesis, which provides significant insight into the evolutionary steps of different types of cortex and the origin of the mammalian neocortex.

Keywords: amniotes, reptiles, cortex, *in ovo* electroporation, *ex vivo* culture, evolution

INTRODUCTION

The mammalian cerebral cortex is a remarkable brain structure that is responsible for intricate social behaviors and intelligence. The cerebral cortex is characterized by tangential expansion of its surface area, which is particularly enhanced in the primate and human neocortex, and a six-layered laminar structure composed of multiple types of excitatory and inhibitory neurons (Nieuwenhuys, 1994; Kriegstein et al., 2006; Defelipe, 2011; Lui et al., 2011). The basic frameworks of these unique characteristics are accomplished by the dramatic increase in the number of neural stem/progenitor cells and massive irruption of distinct types of neurons, followed by the coordinated migration of differentiated neurons during embryogenesis. Recent advances of developmental neurobiology have illuminated the molecular mechanisms that govern these complicated cellular events during corticogenesis (Campbell, 2005; Flames and Marin, 2005; Dehay and Kennedy, 2007; Molyneaux et al., 2007; Kumamoto and Hanashima, 2014).

On the contrary, the origin and evolutionary process of the mammalian cortex remain elusive. Phylogenic and paleontological evidence indicated that the forerunners of the mammalian lineage diverged from the common ancestors of amniotes at approximately 300 million years ago (Carroll, 1988; Ruta et al., 2003, 2013). Other lineages of amniotes have also diverged into several unique animal groups that include the descent of extant reptiles (Ruta et al., 2003). In recent years, numerous fossil records have been identified from Paleozoic and Mesozoic sediments, which provided significant information on the process of amniote diversification. Three-dimensional tomographic analyses of fossil endocasts suggested that the size of the mammalian cerebral cortex has increased rapidly in accordance with the dependence of olfactory and somatosensory information (Quiroga, 1979; Rowe et al., 2011); however, histological architectures of the ancestral cerebral cortex remains unknown, preventing us from tracing how the cerebral cortex has specifically evolved in the mammalian lineage.

Ontologically, the cerebral cortex is derived from the dorsal pallium (DP), which develops in the dorsal part of the telencephalon in all vertebrate species (Northcutt, 1981; Puelles et al., 2000; Cheung et al., 2007; Aboitiz, 2011). Despite of developmental homology to the cerebral cortex, the DP in non-mammalian amniotes forms in distinct manners: a three-layer lamination is constructed in the reptilian DP, whereas nuclear slabs are formed in the avian DP (Medina and Reiner, 2000; Heyers et al., 2003; Jarvis et al., 2005; Striedter, 2005). Phylogenetically, aves are included in reptiles (Nomura et al., 2013b; Xu et al., 2014), but here we will use the term reptiles to specifically mean "non-avian reptiles" that include lizards, geckoes, turtles and crocodiles. Because reptiles occupy a unique evolutionary position within amniotes, developmental analyses of the reptilian cortex illuminate commonalities and divergence of developmental programs, thus providing significant insights into the origin of the mammalian cerebral cortex. Previous studies identified unique features of reptilian corticogenesis, such as an outside-in pattern of neuronal migration (Goffinet et al., 1986, 1999; Tissir et al., 2003; Aboitiz and Zamorano, 2013), a difference of layer-specific cell types produced in the reptilian dorsal pallium (Reiner, 1991, 1993), a difference regarding the existence of intermediate progenitors (Charvet et al., 2009; Medina and

Abellan, 2009), and lower rates of neurogenesis compared to mouse and other mammalian species (Nomura et al., 2013a). However, modern experimental techniques have not been applied to the analyses of reptilian corticogenesis, largely because of several technical difficulties in collection and manipulation of embryos. First, most reptilian species exhibit seasonal reproduction; thus, a large number of embryos at the desired stages are not constantly available. For example, common lizards/geckoes such as *Lacerta trilineata, Anolis carolinensis*, or *Eublepharis macularius* are frequently used as a model animal in comparative developmental biology (Goffinet et al., 1986; McLean and Vickaryous, 2011; Eckalbar et al., 2012; Sanger et al., 2012). The females of these species produce a limited number of eggs after bleeding. Second, unlike chicken, most reptilian species lay soft-shell eggs, which hampers *in ovo* manipulation of embryos. Although a few pioneering works have reported *in ovo* gene delivery or *ex ovo* culture with snake, lizard and turtle embryos (Nagashima et al., 2007; Matsubara et al., 2014; Tschopp et al., 2014), detailed protocols on embryonic manipulation for reptiles have not been provided.

Here, we describe a method of embryonic manipulation techniques for two reptilian species: the Madagascar ground gecko (*Paroedura pictus*) and the Chinese softshell turtle (*Pelodiscus sinensis*). Surgical techniques on developing reptilian embryos enable us to utilize various experimental approaches. We established the introduction of exogenous genes into the reptilian cortex by *in ovo* electroporation. Furthermore, we developed an *ex ovo* culture system for gecko and turtle embryos, which remarkably increased accessibility to the embryos and improved the efficiency of gene introduction. Successful manipulation techniques of non-mammalian embryos are valuable for studies of the evolutionary developmental biology of the cerebral cortex.

ANIMALS AND DETAILED PROTOCOLS OF NEW TECHNIQUE

MADAGASCAR GROUND GECKO

The Madagascar ground gecko (*P. pictus*) is a ground crawling gecko that commonly lives on Madagascar Island. Gecko, lizard, snakes and Tuatara (Sphenodon) are included in the group of lepidosaurs (**Figure 1A**). The adult size of *P. pictus* is approximately 15–20 cm, and the gecko is easy to handle and breed in captivity (**Figure 1B**). After mating of a pair of male and female geckoes, a female produces 1 or 2 clutches every 10–20 days and continues to lay eggs for several months (Nomura et al., 2013a,b). Embryonic staging of the gecko has been established by Noro et al., who determined that the gecko embryogenesis proceeds much slower than chicken embryogenesis (**Figure 1D**) (Noro et al., 2009; Wise et al., 2009). The gecko does not exhibit temperature-dependent sex determination. The embryo hatches approximately 60 days after oviposition and begins to catch small insects within a few days after the first molting. To feed the geckoes, various sizes of crickets were purchased from a local breeder (Tsukiyono farm, Gunma, Japan) and dusted with mineral supplements (calcium and vitamin D) to prevent rickets. To collect embryos, 4 pairs of wild-type geckoes (total 8 animals) were first obtained from a local store (Kansai Reptile Pro, Osaka, Japan) and maintained in our laboratory (28°C, 12 h of light and dark cycles, 50–60% humidity). More than 100 eggs were obtained from 4 females bred for 6 months.

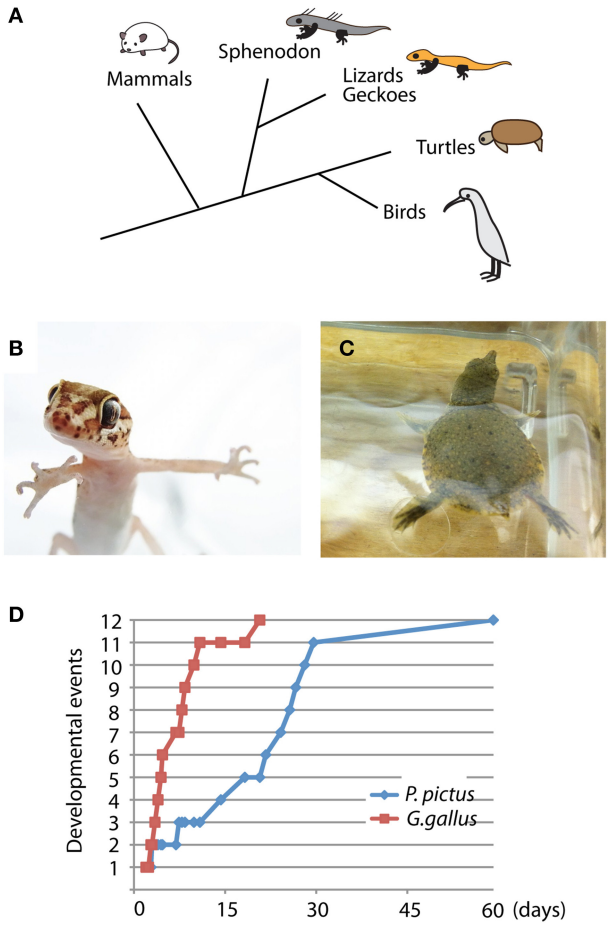

FIGURE 1 | Unique characteristics of Madagascar ground gecko and Chinese softshell turtle. (A) Phylogenic position of the gecko and turtle among amniotes. Lepidosaurs include sphenodon, snake, lizard and gecko, whereas archosaurs include turtle, crocodile and bird. **(B,C)** Young individuals of Madagascar ground gecko (*Paroedura pictus*) **(B)** and Chinese softshell turtle (*Pelodiscus sinensis*) **(C)**. **(D)** Developmental rates of *Paroedura pictus* and *Gallus gallus* (chick). Equivalent developmental stages are based on limb bud and cranial morphology (Wise et al., 2009). Representative developmental events include 1: hindlimb bud develops, 2: hindlimb bud becomes larger than forelimb bud, 4: autopodium develops discrete paddle shape, 5: zeugopodium and stylopodium become distinct, 6: digits develop, 9: phalanges develop, 10: claws develop, 11: scale formation and pigmentation, and 12: hatching. Detailed staging criteria are described in Wise et al. (2009).

CHINESE SOFTSHELL TURTLE

Turtle embryos have been used for anatomical and developmental studies since the nineteenth century (Tokita and Kuratani, 2001). The Chinese softshell turtle (*P. sinensis*) is a freshwater-living turtle that is widely distributed in eastern and southeastern Asia (**Figure 1C**). The adult size of the turtle reaches over 30 cm in carapace length, and sexual maturity takes approximately 5–6 years. Because the turtle exhibits seasonal reproduction, we could obtained fertilized eggs from a local breeder (Daiwa-Yoshoku, Saga, Japan) in the summer from the beginning of June to the end of August. Sex determination is not dependent on temperature.

The developmental stages of the turtle have been established by a previous report (Tokita and Kuratani, 2001). Embryogenesis takes approximately 60 days, and newborn turtles begin foraging after consuming the remaining abdominal yolk. All experimental procedures for reptilian captivity and embryonic manipulation were approved by the experimental animal committee of Kyoto Prefectural University of Medicine (M23-272), and were performed in accordance with the relevant guidelines of the committee.

MANIPULATION AND ELECTROPORATION OF REPTILIAN EMBRYOS

Embryonic manipulation and electroporation are based on the procedures for gene transduction into developing avian embryos with slight modification (**Figure 2**) (Nomura et al., 2008; Nakamura, 2009). However, because the reptilian eggs are much smaller than chicken eggs, *in ovo* manipulation of reptilian embryos requires specific experimental techniques and training of surgical skills under the dissecting microscope (**Figure 2A**).

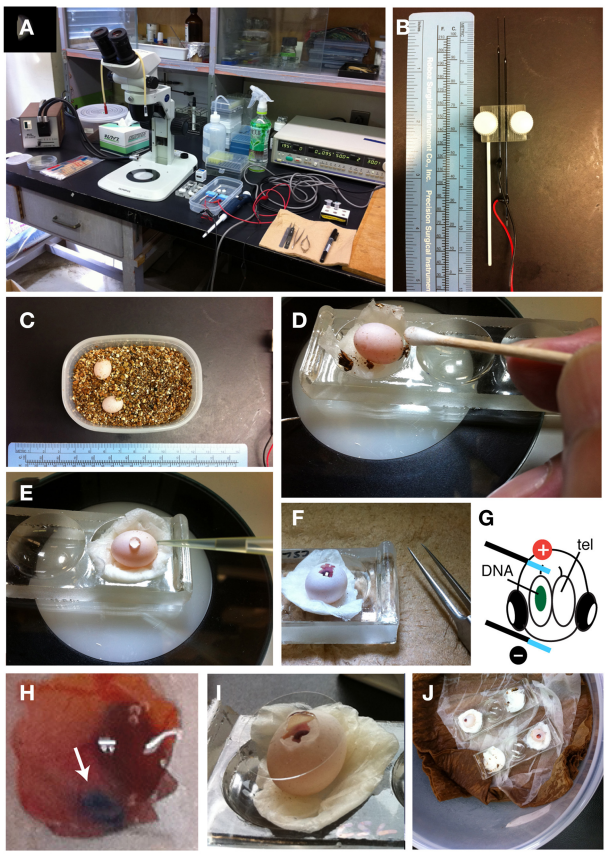

FIGURE 2 | *In ovo* electroporation of gecko embryos. (A) Experimental equipment. **(B)** Needle-type electrodes (CUY200S). **(C)** Two *P. pictus* eggs incubated in a small tapper with vermiculite. **(D)** Sterilization of the egg with 70% ethanol and a cotton stick. **(E)** HBSS was dropped through the hole of the shell. **(F)** The window was opened with fine forceps. **(G)** An illustration showing the position of the electrodes on the embryo. **(H)** High magnification of an electroporated embryo. Green-colored DNA solution was injected in the left lateral ventricle. **(I)** The window was sealed with a cover glass. **(J)** Incubation of operated embryos in the container.

In ovo electroporation of gecko embryos

After oviposition, the laid eggs should be isolated from the mother animal to avoid accidental crushing of the eggs. In our laboratory, the eggs are immediately transferred to a plastic container filled with dried vermiculite (**Figure 2C**). To maintain embryonic respiration, small holes are made through the lid. The egg are approximately 10 mm in diameter and 12–13 mm in length (**Figure 2C**) (Noro et al., 2009). Fertilized eggs are incubated at 28°C in 50–60% humidity until manipulation. *In ovo* electroporation can be performed during 10–15 d.p.o. (days of post-oviposition); after these stages, the eyes and jaws increase rapidly in size, which make it difficult to access and electroporate to brains.

To begin *in ovo* electroporation, the egg is placed on a depression slide (Matsunami, Osaka, Japan) with moistened papers (**Figure 2D**, Prowipe, Elleair, Japan). The egg is sterilized with 70% ethanol and the surface of the shell was wiped with a cotton swab (**Figure 2D**, AspureAP-7, ASONE, Japan). Pieces of vermiculites attached to the eggs are removed at this step. The position of the embryo within the egg is confirmed by illuminating the egg with a fiber optic light (SL FI-150T, Sugihara Lab Inc., Japan). To open the shell, scratch the surface of the shell with fine forceps (VIGOR TW-705#5, B. Jadbow Inc, Switzerland) under a dissecting microscope (SZ61, OLYMPUS, Japan). Because the shell of gecko eggs is extremely fragile, care should be taken to open the shell with a forceps to avoid crushing the egg. After making a small hole in the shell, 50–100 μL of saline (HBSS: Hanks' buffered saline with the addition of 1% penicillin and streptomycin and 0.1% gentamycin) is added through the hole, and the further open by carefully removing the shell (**Figure 2E**). The vitelline and amniotic membranes were cut with microsurgical scissors (**Figure 2F**, RS-5620, ROBOZ, Germany). Next, 50–100 μL of HBSS is further added to the egg to maintain the space for embryonic manipulation.

To prepare the DNA solution for electroporation, purified plasmid DNA vectors are dissolved in sterilized phosphate buffered saline (PBS) with a non-toxic dye (0.01% fast green). Typically, we prepare 2.5–5 μg/μL of plasmid solution for the electroporation. Holding the head of the embryo with a fine forceps, the DNA solution is injected into the left or right side of the lateral ventricle with a fine glass capillary (MODEL G-1, NARISHIGE, Japan) that is connected to a mouth-pipette (Suction tube, Drummond, USA) or mini-injector (BJ100, BEX, Japan). Subsequently, needle-type electrodes (CUY200S, NEPAGENE, Japan) is inserted into the extra-embryonic space. Because DNA is negatively charged, a positive electrode was positioned at the target region (c.f., dorsal cortex), and a negative electrode was placed at the opposite side of the head (lower jaw; **Figure 2G**). The distance between the electrodes and embryos needs to be maintained (approximately 0.5–1 mm) to minimize the risk of tissue damage and hemorrhage by the direct application of electricity. Square waves of electric pulses (32 V, 50 ms, 950 ms interval, 2 or 4 pulses) are passed with an electric stimulator (SEN-3401, Nihon Kohden, Japan) or pulse generator (CUY21EDIT II, BEX, Japan). We compared survival rates of embryos at 48 h after electroporation and found that applying 4 pulses remarkably decreased the viability of gecko embryos (**Table 1**). To prevent microbe

contamination, 50–100 μL of HBSS with antibiotics was applied into the extra-embryonic space. After the electroporation, the shell window was sealed with a micro cover glass (**Figure 2I**, 18 mm, #1, MATSUNAMI, Japan) attached with the tissue glue (1xHistoacryl L, B.Braun, Germany). The operated eggs were kept in a sterilized moist chamber (a plastic container with respiratory holes within the lid) and incubated at 30°C for 24 h to 1 month (**Figure 2J**).

In ovo electroporation of turtle embryos

In ovo manipulation of turtle embryos is similar to the method for gecko embryos with slight modifications. Because the early stages of the turtle embryos are tightly attached to the inside of the shell, frequent rotation of the egg will disrupt normal development of the turtle embryos. Thus, care should be taken to maintain the orientation of the egg after oviposition (**Figure 3A**). Fertilized turtle eggs are incubated in a highly moistened container at 28°C. We usually performed *in ovo* electroporation at stage 10–15 (10–15 days after fertilization).

Table 1 | Efficiency of the *in ovo* electroporation of the gecko embryos.

Stage (d.p.o.)	Number of embryos	Number of pulses	Number of survived embryos (2 days)	*Electroporation efficiency (%)
12	2	2	0	N.D.
13	10	2	9	100
13	2	4	0	N.D.
14	18 (2)**	2 (2)**	11 (2)**	100
14	2	4	0	N.D.
16	4	2	2	100

*Electroporation efficiency was determined by dividing the number of GFP-positive embryos by the number of collected embryos.

**Pilot experiments for the comparison of survival rates.

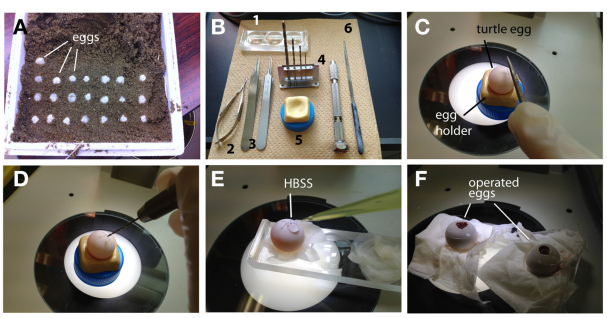

FIGURE 3 | *In ovo*electroporation of turtle embryos. (A) Turtle eggs in the delivery packet. Before transferring the eggs, the top of the shell was marked to maintain an upside-down orientation. **(B)** Tools for surgical manipulation. 1: A depression slide, 2: micro scissors, 3: forceps, 4: mini drill (pinvise), 5: hand-made egg stand, and 6: a metal file. **(C)** A small scar was made on the shell with a metal file. **(D)** A pin vise was used to drill the surface of the egg. **(E)** HBSS was dropped through the small window. **(F)** The window was sealed with a cover glass after electroporation.

The position of an embryo within the egg can be monitored by illuminating the egg with a fiber light. To open the turtle eggs, a small hole is made in the shell by drilling the top of the shell with a micro drill (0.5–0.8 mm in diameter, using a pin vise, TAMIYA, Japan) under the dissecting microscope (**Figures 3B–D**). After opening a small hole on the shell, 50–100 μl of HBSS with antibiotics was added through the hole, and the window was further widened by carefully removing the shell (**Figure 3E**). The chorion and amniotic membranes were cut with microsurgical scissors. After injecting a DNA solution (2.5–5 μl of DNA and 0.1% fast green in PBS) into the lateral ventricle, electroporation is performed with a needle-type electrode (CUY200S), and square pulses (32 V, 50 ms, 950 ms interval, 2 pulses) are applied to the target region of the embryos using an electric stimulator or pulse generator. After electroporation, the shell window was sealed with tissue glue and a micro cover glass as in the case of the gecko eggs (**Figure 3F**). The operated embryos are maintained in a moistened chamber and incubated at 30°C (**Table 2**).

Ex ovo culture of reptilian embryos

Exposing the embryos from the shell to the medium dramatically facilitates accessibility to the embryos and increases the efficiency of electroporation (Buchtova et al., 2008; Tschopp et al., 2014). To allow embryonic development in the medium after electroporation, we established an *ex ovo* culture system for the middle stages of reptilian embryos (**Figure 4** and **Table 3**). To begin *ex ovo* culture, fertilized gecko and turtle eggs are transferred to a glass evaporating dish filled with HBSS, and the shell was cracked within the medium with forceps to carefully expose the embryo from the extra-embryonic membrane (**Figure 4A**). The part of shell on the side of the yolk was kept to preserve the yolk sac (**Figure 4B**). Injection and electroporation can be performed within the evaporating glass (**Figure 4B**). After electroporation, the embryo was carefully transferred to a sterilized glass-made bottle (Ikemoto Rika, Tokyo, Japan) filled with 2 mL of HBSS with antibiotics (1% penicillin and streptomycin, 0.1% gentamycin) and cultured using the whole embryo culture system (Ikemoto Rika, Tokyo, Japan) in which oxygen is constantly supplied (95% oxygen, 5% carbon dioxide, 50 mL/min) to the embryos (**Figures 4C–E**). The culture temperature was maintained at 30°C to match ideal temperature for reptilian embryogenesis. Because the embryos are damaged by bottle rotation, the culture bottles were maintained in a static position during culture. The

Table 2 | Efficiency of the *in ovo* electroporation of turtle embryos.

Stage (TK)	Number of embryos	Number of pulses	Number of survived embryos (1 day)	Number of survived (2 day)	*Electroporation efficiency (%)
13	19	2	14	9	100
15	6	3	3	3	33.3
16	5	2	3	3	0

*Electroporation efficiency was determined by dividing the number of GFP-positive embryos by the number of collected embryos.

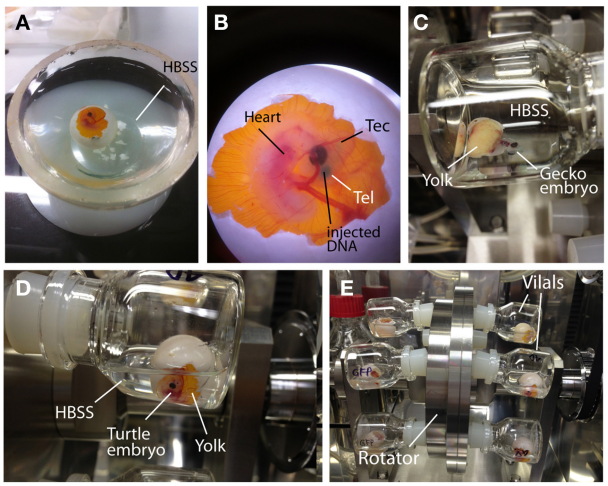

FIGURE 4 | *Ex ovo* culture system for reptilian embryos. (A) Turtle embryos were opened in HBSS. **(B)** A turtle embryo in which a DNA solution (green color) was injected into the lateral ventricle. **(C–E)** Incubation of gecko **(C)** and turtle **(D,E)** embryos in the whole embryo culture system. Electroporated embryos were cultured in glass vials filled with HBSS. Embryo containing vials were inserted into the rotator to supply oxygen continuously. To avoid crushing the embryos, the rotating wheel was not used during the culture.

Table 3 | Efficiency of the *ex ovo* culture of gecko and turtle embryos.

Stage	Number of embryos	Number of survived embryos (24 h)	Number of survived embryos (48 h)	*Electroporation efficiency (%)
Gecko d.p.o.8/9	3	3	2	100
Gecko d.p.o.15	1	1	1	100
Turtle TK16	6	6	2	100

**Electroporation efficiency was determined by dividing the number of GFP-positive embryos by the number of collected embryos.*

culture medium (HBSS) was replaced 24 h after starting the culture. The embryos can be maintained for approximately 2 days in this culture system because embryonic circulation was gradually decreases after 3 days of culture.

EXPRESSION VECTORS

Expression vectors designed for mammalian cells can be used for genetic manipulation in reptilian embryos. In general, the CAG promoter (cytomegalovirus enhancer with chicken ß-actin promoter) provides higher expression of transgenes in amniotic brains, particularly in the neural stem/progenitor cells (Niwa et al., 1991). We used several expression vectors, including pCAX-AFP (a variant form of GFP, Takahashi and Osumi, 2002) and pCAGGS-RFP (Nomura et al., 2008), which express fluorescent reporter proteins under the control of the CAG promoter. Expression vectors with Cre/loxP technology are useful for the restricted expression of transgenes in spatiotemporally controlled manners. The electroporation of Cre-recombinase expression vectors at a lower concentration (1 ng/μL) decreases the recombination frequency, which allows clonal labeling of neural stem/progenitor cells (Kato et al., 2010; Gotoh et al., 2012; Nomura et al., 2013a).

IMMUNOHISTOCHEMSTRY

To perform immunohistochemical analysis, embryos are fixed with standard fixative (4% paraformaldehyde in PBS) for overnight at 4°C and immersed in 20% sucrose for cryoprotection. The samples were embedded in OCT compound (Tissue-tek, SAKURA, Japan), and 14 μm of cryosections are made with a cryostat (LEICA CM1850, Germany). Several commercial antibodies are potentially applicable for immunohistochemistry in gecko and turtle embryos (**Table 4**) (Moreno et al., 2010, 2012), although not all the antibodies provide a single band with naïve brain extracts (**Figure 6** and our unpublished data).

REPRESENTATIVE RESULTS

The expression of exogenous genes can be monitored soon after electroporation. We collected gecko and turtle embryos at several time points after the electroporation, and examined the expression of fluorescent reporters under a fluorescent microscope. At 2–4 days after electroporation, intense GFP expression was detected in the dorsal part of the gecko and turtle telencephalon (**Figures 5A–C**). Even at 1 month after electroporation, reporter fluorescence was still maintained in the brain (**Figures 5G–I**).

At 2 days after electroporation, GFP expression was exclusively detected in mitotic neural stem/progenitor cells that were localized at the ventricular zone of the developing gecko cortex. These neural stem/progenitor cells have a radial fiber similar to the mammalian radial glial cells, but the fibers extend in a curved manner at the neuronal layer as in the case of avian cortical radial fibers (Nomura et al., 2008, 2014). At 4 days after electroporation, GFP-positive cells migrated from the ventricular zone and positioned at the marginal zone (**Figures 5C,E,F**). GFP-positive migrating neurons in the developing gecko cortex exhibited multipolar morphology, similar to intermediate progenitor cells (IPCs) in the subventricular zone (SVZ) of the mammalian neocortex (Miyata et al., 2004; Noctor et al., 2004; Englund et al., 2005). However, unlike mammalian IPCs, we could not detect mitotic activity in the GFP-labeled multipolar cells in the gecko cortex (**Figure 5C**). This result is consistent with our observation that Tbr2-positive cells in the developing gecko cortex are post-mitotic neurons (**Figure 6**) (Nomura et al., 2013a). At 1 month after electroporation, GFP-expressing cells were still detected in the medial and dorsal cortex of gecko embryos (**Figure 5G**). Notably, these GFP-positive cells exhibited reptilian-type pyramidal neurons and extended axonal fibers toward the contra-lateral side of the cortex, which constitutes the pallial commissure in reptiles (**Figures 5H,I**).

Recent studies have shown that the transition from the multipolar to bipolar shape in the migrating neurons is critical for the development of mammalian neocortex (Noctor et al., 2004; Hand et al., 2005; Heng et al., 2008; Ohtaka-Maruyama et al., 2013; Kawauchi, 2014; La Fata et al., 2014). In contrast to the mammalian neocortex, migrating neurons in the developing gecko and turtle cortex still maintained multipolar morphology at 7 days

Table 4 | The list of antibodies for immunohistochemistry of reptilian brains.

Antigen	Provider	Catalog no.	Dilution	Technical note
Sox2	Abcam	ab97959	1:500	
Ctip2	Abcam	ab18465	1:500	
Satb2	Abcam	ab51502	1:500	
Foxp2	Abcam	ab16046	1:500	
Tbr2	Abcam	ab23345	1:500	TSA amplification
Tbr1	Millipore	AB2261	1:500	
ßIII-tubulin	Millipore	MAB1637	1:200	
Phospho- histon H3	Millipore	06-570	1:500	
Phospho- histon H3	Millipore	05-806	1:500	
NeuN	Millipore	MAB377	1:500	
DCX	Santa Cruz Biotech.	sc-8066	1:500	
Pax6	MBL	PD022	1:500	
Pax6*	DSHB	PAX6	1:500	Antigen retrieval with 2N HCl, 37°C, 15 min and TSA amplification
Rbpj-k	Cosmo Bio	SIM-2ZRBP2	1:500	Antigen retrieval with 2N HCl, 37°C, 15 min and TSA amplification

**Specificity was examined with western blot in previous reports (Moreno et al., 2010, 2012).*

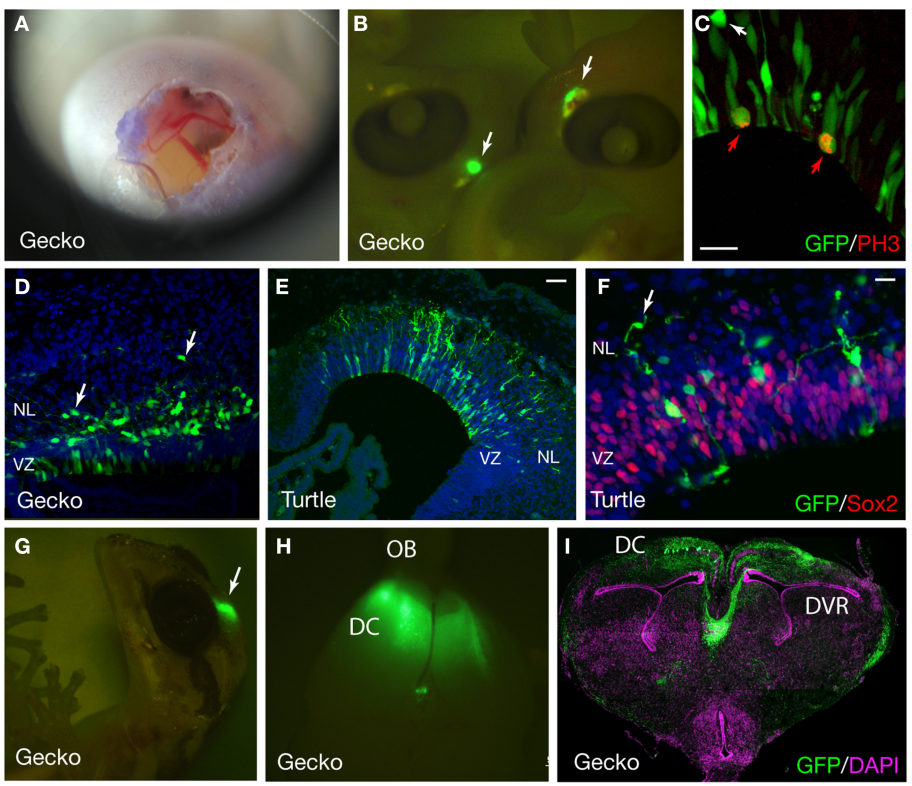

FIGURE 5 | GFP expression in the developing gecko and turtle cortex. (A) Developing gecko embryo after electroporation. The image was captured using an iPhone4S camera through a magnifier. **(B)** Gecko embryos at 4 days after electroporation. GFP was expressed at the dorsal part of the telencephalon (arrows). **(C)** GFP expression in the cortical neural stem/progenitor cells. Mitotic GFP-positive cells were labeled with an anti-phospho histoneH3 (PH3) antibody (red arrows). A GFP-positive cell at the outside of the ventricular zone was not mitotic (white arrow). **(D)** The distribution of GFP-positive cells in the gecko cortex at 7 days after electroporation. Arrows indicate migrating neurons **(E,F)** GFP expression in the developing turtle cortex at 4 days after electroporation. Arrows indicate migrating neurons. **(G–I)** GFP expression in the gecko cortex at 1 month after electroporation. VZ, ventricular zone; NL, neuronal layer; OB, olfactory bulb; DC, dorsal cortex; DVR, dorsal ventricular ridge. Scale bars: 25 μm **(C,F)**, 50 μm **(E)**.

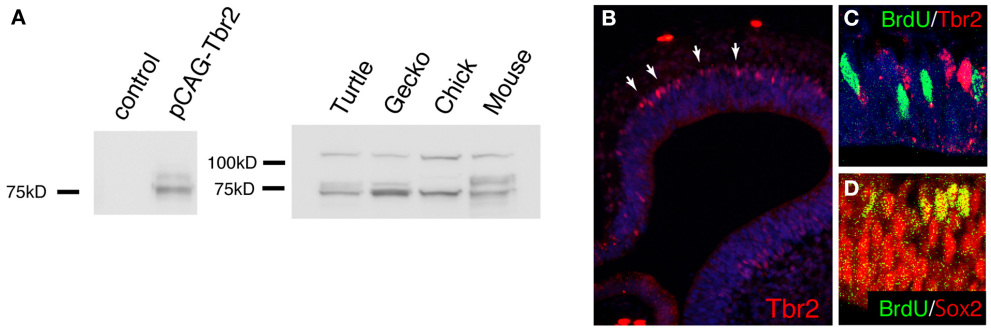

FIGURE 6 | The expression of Tbr2 in the developing gecko cortex. (A) Western blot with anti-mouse Tbr2 antibody. The left panel shows lysate from HEK293 cells transfected with the expression vector for mouse Tbr2. The control lane was whole cell lysate without transfection. A major band was detected at the predicted molecular weight (72 kD) for mouse Tbr2. A slightly lager band was possibly due to post-translational modification. The right panel shows western blot of embryonic turtle (st17), gecko (d.p.o.18), chick (E7), and mouse (E14) brain lysate. Together with the bands of predicted molecular weight (72 kD), additional larger bands were detected in all examined species. **(B–D)** Immunohistochemistry of the developing gecko cortex (d.p.o. 18) with anti-Tbr2 antibody. Tbr2-positive cells were detected at the basal side of the ventricular zone (white arrows). **(C)** Tbr2-positive cells did not overlap with BrdU-incorporated cells. **(D)** All BrdU-incorporated cells were Sox2-positive. Detailed immunohistochemistry and BrdU incorporation protocols were described previously (Nomura et al., 2013a).

after electroporation (**Figures 5D**, **7A–L**). To quantify the orientation of leading process in migrating neurons, the angle of the longest process in each neuron relative to the ventricular surface was quantified in mouse, gecko, turtle and chicken cortex/dorsal pallium. Comparison of leading process orientation demonstrated that all migrating neurons in the mammalian cortical plate are vertically aligned: thus, all neuronal processes are directed to the pial surface. In contrast, migrating neurons in the reptilian and avian marginal zone are not tightly aligned and extend leading process in various directions (**Figures 7M–P**). Thus, the strict alignment of bipolar migrating neurons in the cortical plate is a unique characteristic in the developing mammalian cortex. However, we also confirmed that the expression of mammalian cortical plate markers, such as Tbr1, CTIP2, and SATB2, is also detected in the developing gecko cortex (**Figures 7Q–T**) (Nomura et al., 2013a), suggesting that some of the molecular characteristics of the cortical plate neurons are conserved between the mammalian and reptilian cortex.

DISCUSSION

Comparative analyses of extant amniote brains are powerful approaches to understand the evolutionary processes of the mammalian neocortex and homologous structures in non-mammalian lineages (Molnar et al., 2006; Aboitiz, 2011; Medina et al., 2013). Previous histological studies revealed that the stellate morphology of migrating neurons in the developing reptilian cortex resemble migrating neurons in the early stages of mammalian neocortex (Goffinet, 1983). Based on the ontogenic analyses, Marin-Padilla hypothesized that mammalian neocortex has dual origins: the superficial and deepest neurons (layer I and IV) retain ancestral phenotypes that are reminiscent of the amphibian or reptilian cortex, whereas the later-born cortical plate neurons (layer II-V) are recently acquired during mammalian evolution (Marin-Padilla, 1971, 1978). Our *in vivo* cell tracing analyses indicated that (1) multipolar neurons in the reptilian cortex do not exhibit mitotic activity and (2) multipolar-to-bipolar transition of migratory modes is not detected during the reptilian corticogenesis. These data support the idea that both amplification of IPCs (Martinez-Cerdeno et al., 2006; Cheung et al., 2007; Charvet et al., 2009; Puzzolo and Mallamaci, 2010) and unipolar cortical plate neurons with a "locomotive mode" are derived developmental processes in the mammalian neocortex (Aboitiz et al., 2001), through which the expansion of neuron numbers and multiple laminar structures evolved. However, the morphological similarities of migrating neurons are not always associated with common cellular dynamics and gene expression patterns. Thus, the reptilian neurons are not simply equivalent to the early stages of mammalian cortical neurons or ancestral neuronal subtypes.

In addition to cell tracing of migrating neurons, we applied several developmental techniques to analyze reptilian corticogenesis, such as (1) lineage tracing of neural stem/progenitor cells, (2) quantification of reporter activities for signaling molecules, and (3) gain- and loss-of-function analyses of specific genes in the developing reptilian cortex (Nomura et al., 2013a). These experimental approaches unveiled further unique characteristics of reptilian neural stem/progenitor cells. For example, the rates of proliferation and differentiation of reptilian cortical progenitors are very slow and contribute to the production of a lower number of cortical neurons. Some of these characteristics depend on Notch signaling, and experimental manipulation of a Notch downstream effector dramatically increased neuronal production in geckoes. We hypothesized that after the diversification of mammalian and non-mammalian amniote lineages, some critical changes in neural stem cell regulation might have occurred in the ancestral mammals and thus provided the expansion of cortical areas and massive generation of excitatory neurons (Nomura et al., 2013a,b, 2014).

Recently, whole genome sequences of Chinese softshell turtle and sew turtle have been performed, which have confirmed that turtles must be positioned phylogenetically in archosaur groups in amniotes (Wang et al., 2013). The data also demonstrated that conserved and derived genetic programs in turtle

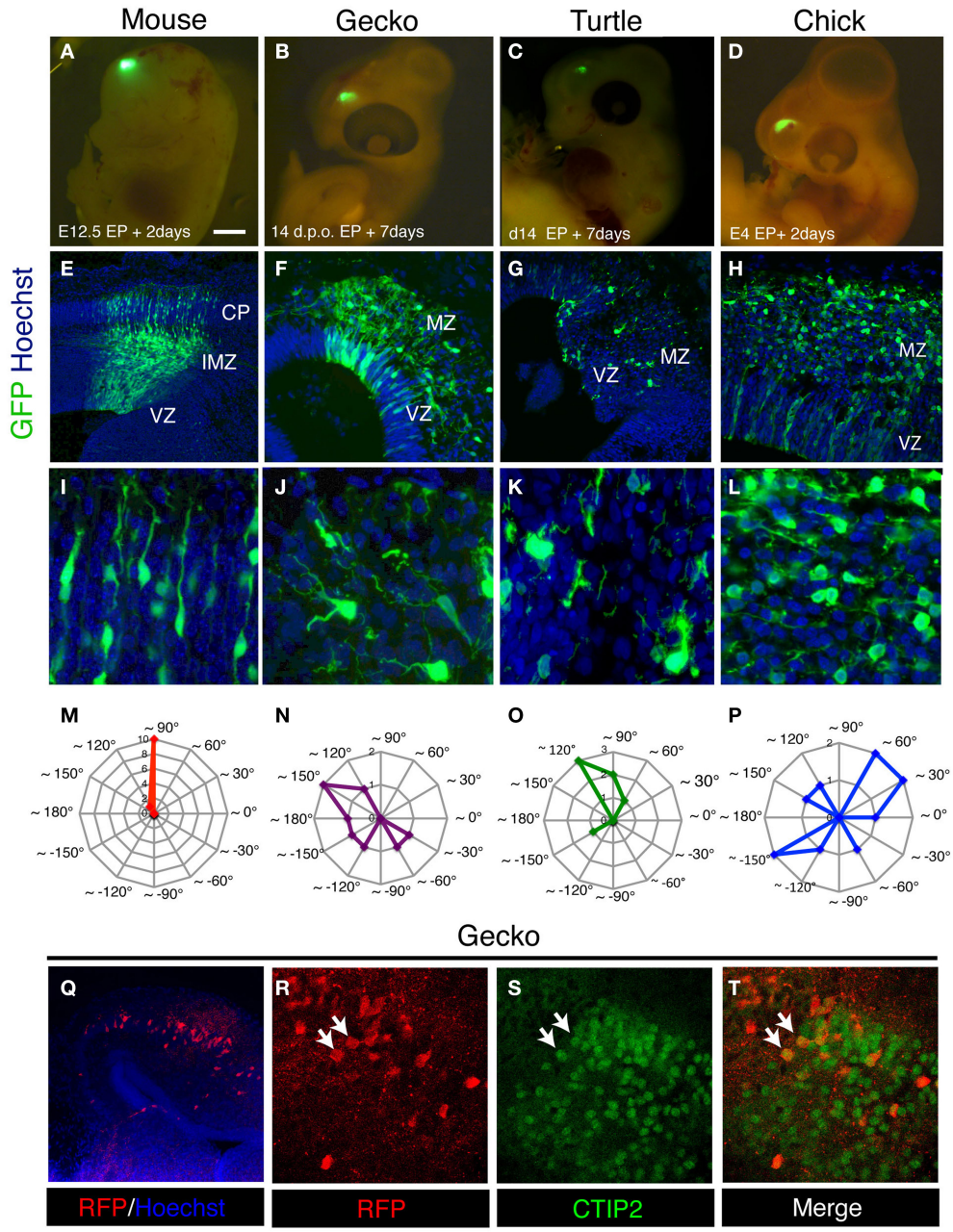

FIGURE 7 | Characteristics of migrating neurons in the developing amniote pallia. (A–D) Electroporation of GFP-expression vector into the developing mouse **(A)**, gecko **(B)**, turtle **(C)**, and chick **(D)** pallia. **(E–L)** Distribution and morphology of GFP-positive migrating neurons in the mouse neocortex **(E,I)** and the gecko **(F,J)**, turtle **(G,K)** and chick **(H,L)** pallia. **(M–P)** Contour graphs of the longest process orientation of mouse (**M**; the data were taken from the cortical plate), gecko **(N)**, turtle **(O)**, and chick **(P)**. The angles of the processes were calculated against the ventricular plane. Each contour line represents the number of cells. **(Q–T)** The expression of CTIP2 in RFP-positive pallial neurons in the developing gecko cortex (white arrows in **R–T**).

embryogenesis contributed to the evolution of the turtle-specific body plan (Wang et al., 2013). Although genome analyses of Madagascar ground gecko have not been accomplished, draft genomes of green anole lizard (*Anolis carolinensis*), a related species to the gecko, have been published (Alfoldi et al., 2011). Genomic information of the Anolis lizard revealed unique characteristics in its genomic composition, such as homogenization of the GC content and higher number of mobile elements than other amniotes (Alfoldi et al., 2011). Additional studies of the comparative genomics of reptiles will clarify how genetic and epigenetic changes contributed to brain evolution in distinct lineages of amniotes. Genomic sequences of the Chinese softshell turtle and Anolis lizard are available at the website of the Ensemble Genome Browser (http://asia.ensembl.org/index.html).

Currently, we have only successfully performed *in ovo* electroporation during a narrow window of time (d.p.o. 10–16 for gecko embryos and stages 10–15 for turtle embryos). Because reptilian eyes and jaws rapidly increase in size during embryogenesis, positioning the electrodes to target dorsal cortex is technically difficult at later embryonic stages. Application of an *ex ovo* culture system for gecko and turtle embryos is also limited for 3–4 days, most likely due to the lack of some essential nutrients and/or sufficient oxygen supply. Further improvements of gene delivery tools and/or culture conditions are required to manipulate embryos at any developmental stage.

Electroporation with a transposon-mediated genomic integration system provides permanent lineage tracing in mammalian and non-mammalian vertebrates (Garcia-Moreno et al., 2014; Loulier et al., 2014). Furthermore, recent advances in genome editing tools, such as TALEN (transcription activator-like effector nuclease) and CRISPR/Cas (clustered regulatory interspaced short palindromic repeats/CRISPR-associated proteins), extend the possibility of genetic manipulation in a variety of organisms (Aida et al., 2014; Kaneko et al., 2014; Pal et al., 2014). The *in vivo* delivery of CRISPR/Cas vectors induces direct somatic recombination in target tissues, which enables the site-specific mutation of endogenous genes (Xue et al., 2014; Yin et al., 2014). The application of these new research strategies to the study of comparative brain development provides a new avenue for the understanding of the origin and evolution of amniote brains, particularly the mammalian cerebral cortex.

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