Title
The role of gap junctions in the development of the neocortex

Author
Elias, Laura Allyn Barker

Publication Date
2008-06-05

Peer reviewed|Thesis/dissertation

The role of gap junctions in the development of the neocortex

by

Laura Allyn Barker Elias

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

[redacted]

For my mom and dad, Wendy Elizabeth and Jack George Barker
I hope I can one day give my own children the love and support you have given me, as it is a gift of immense proportions.

For my sister, Carryn Adrianna Barker
You have taught me the meaning of life and the joy in every moment.

For my husband, Guillermo Munoz Elias
The unwritten story between these pages is our story - how our lives became forever intertwined here in San Francisco.

In memory of my Grandfather, William Kleinberg, and the deep appreciation for scientific discovery he instilled in my family.

Acknowledgements

I would like to thank Arnold Kriegstein for his continual support and scientific guidance as well as the many opportunities he has given me both in the laboratory and beyond; I have learned much from his mentorship. I am very grateful to the members of the Kriegstein Laboratory who have been my colleagues and friends during this process. Tristan Sands, David Castaneda, and Ashkan Javaherian, Stephen Noctor, and Veronica Martinez-Cerdeno played a primary role in teaching me all of the techniques that I have used in my research. Doris Wang contributed to this project during her summer rotation as a collaborator and has continued to contribute her ideas through discussions. The whole laboratory including the newer members, Corey Harwell, Jan Lui, David Hansen, and Phillip Parker have given me valuable feedback on the direction of my project. William Walantus and Jeanelle Agudelo make the laboratory run smoothly and provide expert technical assistance for which I am very grateful.

Importantly, I would also like to thank my husband, Guillermo Elias, for his personal and scientific support. The countless scientific discussions we have had during graduate school have played a major role in guiding my scientific progress.

In addition, I would also like to thank my thesis committee members, Arturo Alvarez-Buylla, John Rubenstein, and Samuel Pleasure for their ideas and suggestions during my thesis work as well as Susan McConnell for being the outside member of my defense committee. Furthermore, the Neuroscience Program faculty and students have greatly expanded my knowledge and appreciation of neuroscience and provided me with an intellectually simulating environment in which to pursue my PhD. I would like to specifically thank the laboratories in which I rotated during my first year (Lily Jan, Robert

Edwards, and Roger Nicoll). I also want to thank Hee Chung and Magda Santos, my postdoctoral rotation advisors, for their time and patience in teaching me the basics of molecular biology and neuroscience.

This work was supported by grants from the National Institutes of Health (to Arnold Kriegstein), the Sandler Family and Genentech Graduate Fellowship (to Laura Elias), the California Institute for Regenerative Medicine Graduate Fellowship (to Laura Elias), and the J.G. Bowes Research Fund.

Contributions

The work in this thesis was conducted in the laboratory of Arnold R. Kriegstein at the University of California San Francisco. Laura A.B. Elias conceived of and carried out all experiments except as noted below. Doris D. Wang developed methods for and carried out the cell transplant and cell autonomy BrdU experiments as well as the whole-cell patch clamp recordings in C6 cells. Arnold R. Kriegstein, as the principle investigator, provided conceptual and technical guidance for all aspects of the project.

William Walantus provided expert technical assistance with the *in utero* surgeries, and Jeanelle Agudelo provided expert assistance with perfusions and cryostat sectioning. Tara Calcagni provided expert technical assistance with the FACS sorting. Dale Laird (University of Western Ontario) provided the connexin plasmids, Albert Lai (UCLA) provided the C6 cell lines, Ke Hu (Indiana University) provided the actin–cherry plasmid, and Richard Vallee (Columbia Univeristy) provided the centrin II–dsRed plasmid. Artwork on page ii and in Chapter 5, Figure 1b was provided by Kenneth X. Probst.

This work was reprinted from:

Elias LAB & Kriegstein AR. (2008) Gap junctions: multifaceted regulators of cerebral cortical development. *Trends in Neurosciences*. 31(5):243-250

Elias LAB, Wang DD & Kriegstein AR (2007) Gap junction adhesion is necessary for radial migration in the neocortex. *Nature.* 448(7156): 901-907

Abstract

Radial glia, the neuronal stem cells of the embryonic cerebral cortex, reside deep within the developing brain and extend radial fibres to the pial surface, along which embryonic neurons migrate to reach the cortical plate. Here we show that the gap junction subunits connexin 26 (Cx26) and connexin 43 (Cx43) are expressed at the contact points between radial fibres and migrating neurons, and acute downregulation of Cx26 or Cx43 impairs the migration of neurons to the cortical plate. Unexpectedly, gap junctions do not mediate neuronal migration by acting in the classical manner to provide an aqueous channel for cell-cell communication. Instead, gap junctions provide dynamic adhesive contacts that interact with the internal cytoskeleton to enable leading process stabilization along radial fibres as well as the subsequent translocation of the nucleus. These results indicate that gap junction adhesions are necessary for glial-guided neuronal migration, raising the possibility that the adhesive properties of gap junctions may have an important role in other physiological processes and diseases associated with gap junction function.

Table of Contents

List of Figures

Chapter 1:

General Introduction

Cortical development

Development of a mature brain from the pseudo-stratified neuroepithelial cells that constitute the neural plate and subsequent neural tube is dependent on the proper regulation of proliferation, differentiation, and migration of a myriad of neural stem and progenitor cells such that the brain has the correct number and types of cells as well as the correct morphology. During the initial stages of central nervous system development, regional patterning and specification delineate the anterior position of the future telencephalon or forebrain as well as the dorsal-ventral positioning of the forebrain structures including the neocortex and basal ganglia (Figure 1).

The telencephalon is induced in part by a patterning center at the anterior neural ridge expressing fibroblast growth factor 8 (Fgf8), which positively regulates a critical telencephalic winged-helix transcription factor, forkhead box G1 (Foxg1 or BF1) (Dou et al., 1999; Shimamura and Rubenstein, 1997; Tao and Lai, 1992; Xuan et al., 1995). The anterior-posterior and dorsal-ventral specification of the telencephalon is delineated by a network of interactions between patterning centers including Fgf 8 at the anterior neural ridge, bone morphogenic protein 4 (BMP4) and Wnt3a in the roof plate/cortical hem, and sonic hedgehog (Shh) in the ventral telencephalon (Chiang et al., 1996; Crossley et al., 2001; Furuta et al., 1997; Hebert et al., 2002; Lee et al., 2000; Ohkubo et al., 2002; Shimamura and Rubenstein, 1997). In this manner, the dorsal region of the telencephalon is induced to form the necortex. These dorsally fated cells can be distinguished from their ventral counterparts by the expression of transcription factors including Emx1 and 2, Pax6, Neurogenin 1 (Ngn1) and Ngn2, and chick ovalbumin upstream transcription factor 1 (COUP-TF1) (Bishop et al., 2000; Bishop et al., 2002; Fode et al., 2000; Liu et al.,

2000); the more ventrally situated cells expressing Dlx1 and 2, Gsh1 and 2, and Mash1 will form the basal ganglia (Anderson et al., 1997b; Casarosa et al., 1999; Fode et al., 2000; Hsieh-Li et al., 1995; Porteus et al., 1994; Toresson et al., 2000; Yun et al., 2001). The dorsal medial cells of the cortical hem which become the hippocampus and choroid plexus can be distinguished from the neocortex by their lack of expression of LIM homeobox gene 2 (Lhx2), which represses midline fate (Bulchand et al., 2001; Monuki et al., 2001). Following this initial regional fate specification, neural stem cells lining the ventricular surface begin to proliferate and divide asymmetrically, producing neurons that migrate from the germinal zones to their final position in the brain parenchyma.

Radial glial cells, descendents of the neuroepithelial cells that constitute the neural tube, are a specialized subclass of astroglial cells with both immunocytochemical and structural characteristics of glia (Kriegstein and Gotz, 2003; Rakic, 2003). Morphologically, radial glial cell are characterized by a long radial process that terminates in the basement membrane of the cortical pial surface while their cell bodies reside in the ventricular zone (VZ) near the surface of the lateral ventricle and undergo interkinetic nuclear migration during cell cycle progression (Misson et al., 1988; Rakic, 2003; Stagaard and Mollgard, 1989). During neocortical brain development, radial glia serve two purposes: first, as stem cells of the developing cortex, dividing asymmetrically and giving rise to neurons or intermediate progenitor cells (Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2002), and second, as guides along which the newborn neurons migrate to reach the correct lamina of the cortical plate, where they will become excitatory pyramidal cells in the adult cortex (Rakic, 1971, 1972, 1988) (Figure 1).

The first-born neurons of the neocortex form the preplate which is then split into the subplate and the marginal zone, the latter contains reelin expressing Cajal-Retzius cells which play an important role in orchestrating the pattern of neuronal migration to the cortical plate (Caviness, 1982; D'Arcangelo et al., 1995; Marin-Padilla, 1971; Ogawa et al., 1995). Subsequently, neurons populate the cortical lamina in an inside-out fashion such that later born neurons migrate to increasingly superficial layers (Angevine and Sidman, 1961). The acquisition of laminar fate is dependent on a combination of cell extrinsic and intrinsic factors. Later born neurons are restricted to upper-layer fates, however, early born neurons can acquire upper-layer fates in a cell cycle dependent manner such that their fate is determined by the environment in which they commence their final mitotic division (Frantz and McConnell, 1996; McConnell and Kaznowski, 1991). Neocortical interneurons also reside in increasingly superficial layers in accordance with their birth order, however they originate in the ganglionic eminances of the ventral telelencephalon and migrate tangentially to the neocortex (Anderson et al., 1997a; Fairen et al., 1986; Miller, 1985) (Figure 1).

The adult cerebral cortex is an intricate laminar structure composed of six distinct layers of neurons that together are highly adapted to process complex information. The mechanisms that control the morphological development of the cerebral cortex are of particular interest, as evolutionary advancements in cognition might be closely linked with changes in developmental patterns (Kriegstein et al., 2006). Contact, signaling, and communication between cells in the developing neuroepithelium regulates cerebral cortical development. Interestingly, gap junction proteins are highly expressed during

embryonic development in distinct spatial–temporal patterns (Lo, 1996) and may play an important role in neocortical brain development.

Gap junctions

Gap junctions are large diameter channels that form an aqueous pore between the cytoplasm of two adjacent cells (Figure 2). Gap junctions are made up of two hemichannels on opposing membranes that join through hydrophobic interactions (Harris, 2001). A hemichannel or connexon is composed of six connexin (Cx) subunits, each having four transmembrane domains (M1-M4) and two extracelluar loops. The inner pore of the gap junction is approximately 1.5nm in diameter and allows the passage of electrical current along with inorganic ions and small molecules. Hemichannels can also exist in an unopposed form thus creating a pore to the extracellular space. Functional transfer of cAMP, ATP, inositol 1,4,5-triphosphate, Ca^{2+}, sugars, nucleotides, and amino acids have been highlighted in the literature (Dunlap et al., 1987; Johnson and Sheridan, 1971; Pitts and Simms, 1977; Rieske et al., 1975; Saez et al., 1989; Subak-Sharpe et al., 1969; Tsien and Weingart, 1974). Gap junctional conductances and the affinity for specific molecules vary between the different Cx subunits (Harris, 2007). In addition, gap junctions can be gated by a number of physiological variables. For example, gap junctions close in response to increases in the concentration of H^+ or divalent ions including Ca^{2+} as well as signaling cascades that include phosphorlyation of the C-terminal tail and second messengers including cyclic monophosphates (Harris, 2001).

Functionally, during development gap junctions have been broadly implicated in the control of embryonic patterning and morphogenesis by mediating information flow

between coupled populations of cells through the creation of morphogenic gradients and synchronization of electrical and/or metabolic activities (Levin, 2002, 2007).

Various diseases have been mapped to human Cx genes. For example, X-linked Charcot-Marie-Tooth syndrome is a peripheral neuropathy caused by Cx32 mutations (Bergoffen et al., 1993), nonsyndromic sensineural deafness is caused by Cx26 mutations (Kelsell et al., 1997), and oculodentodigital dysplasia is a pleiotropic disorder with neurological manifestations caused by Cx43 mutations (Loddenkemper et al., 2002; Paznekas et al., 2003).

Gap junctions in the developing brain

Gap junctions were first described in the mature brain in the late 1970s. Soon after the identification and cloning of the rat gap junction subunits Cx32 and Cx26 from the liver (Nicholson et al., 1987; Paul, 1986; Zhang and Nicholson, 1989) and the cloning of Cx43 from the heart (Beyer et al., 1987), it was shown that Cx26 and Cx43 are highly expressed in the developing embryonic cortex whereas Cx32 is upregulated after birth; this was the first evidence that there is a complex cell-specific expression pattern of Cx proteins in the developing and mature brain (Dermietzel et al., 1989). Today we know that there are at least 20 genes encoding Cxs in rodents and humans, and that at least 5 of them are highly expressed in the rodent embryonic cerebral cortex including Cx26, Cx36, Cx37, Cx43 and Cx45, each with a distinct spatial and temporal pattern of expression (Cina et al., 2007; Nadarajah et al., 1997). Cx26, Cx37 and Cx45 are largely evenly distributed from the VZ to the cortical plate, whereas Cx36 and Cx43 are highly expressed in the VZ and less so in the cortical plate (Cina et al., 2007).

The formation of functional gap junctions in the developing cortex was originally demonstrated by transfer of low but not high molecular weight dyes between clusters of neuroepithelial cells in the VZ; the lack of transfer seen with high molecular weight dyes excludes the possibility of cell coupling through cytoplasmic bridges (Bittman et al., 1997; Lo Turco and Kriegstein, 1991). In addition, electrophysiological recordings demonstrated that neuroepithelial cells have a low input resistance as a result of the expanded intracellular volume of coupled cells, a volume that can be effectively reduced by closing the gap junctions (Lo Turco and Kriegstein, 1991). Electron microscopy has confirmed the presence of gap junction plaques between cells lining the ventricular surface (Nadarajah et al., 1997). Coupling has also been observed between neural progenitor cells derived from the embryonic ganglionic eminences using dye transfer techniques *in vitro* (Duval et al., 2002).

The identity of coupled cells in the cortical neuroepithelium remains somewhat elusive in the literature. Coupled clusters of cells were initially hypothesized to contain neuroblasts (Lo Turco and Kriegstein, 1991) and then later neural progenitors as well as at least some radial glial cells (Bittman et al., 1997; Bittman and LoTurco, 1999). However, radial glial cells have more recently been identified as the neural progenitors of the embryonic cortex dividing asymmetrically to produce neurons or intermediate progenitor cells (Malatesta et al., 2003; Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2002). We suggest that coupled clusters of cells are composed largely of radial glial cells. Initial experiments did not detect all the radial fibers because they were limited by the sensitivity of microscopic techniques that were only able to detect the fiber on the brightest cell filled directly by the patch pipette. More advanced confocal microscopy

allows visualization of radial glial fibers associated with secondarily filled cells in coupled clusters in the VZ (S.C. Noctor and A.R.K., unpublished).

Gap junctions regulate radial glial proliferation

Radial glial gap junction coupling is regulated progressively during cortical development and within each cell-cycle division and might play an important role in controlling the pattern of neurogenesis (Bittman et al., 1997; Bittman and LoTurco, 1999; Lo Turco and Kriegstein, 1991; Weissman et al., 2004) (Figure 3). The number of cells in a cluster of coupled cells decreases from embryonic day 15 in the rat, suggesting that coupling is more prevalent during mid-neurogenesis than in late neurogenesis (Lo Turco and Kriegstein, 1991). However, this decrease in overall cell coupling is accompanied by an increase in gap junction hemichannel-mediated Ca^{2+} waves. It has been shown that gap junction hemichannels mediate Ca^{2+} waves in the developing VZ through the release of ATP that binds to purinergic P2Y1 receptors on neighboring radial glia, thereby activating an IP3-mediated release of Ca^{2+} from internal stores (Cotrina et al., 1998; Weissman et al., 2004). It was origionaly thought that such hemichanels would remain closed due to high extracellular Ca^{2+} levels, but hemichannel mediated release has been described in a number of brain regions (Bennett et al., 2003; Pearson et al., 2005b; Weissman et al., 2004; Ye et al., 2003). Interestingly, Ca^{2+} wave frequency, size and distance increase in late neurogenesis (Weissman et al., 2004). The observation that similar levels and types of Cxs are expressed in mid- and late neurogenesis (Bittman and LoTurco, 1999; Cina et al., 2007) suggests that Cx proteins are regulated at a molecular level such that they underlie the formation of gap junction-coupled clusters of cells

during mid-neurogenesis and the hemichannel-mediated spread of Ca^{2+} waves during late neurogenesis.

How do gap junction-mediated cell coupling and Ca^{2+} waves differ in their functional consequences and their impact on neurogenesis? In rat organotypic slices at E16, a time point when both cell coupling and Ca^{2+} waves are present, the pharmacological block of gap junction channels or P2Y1 receptors, which blocks Ca^{2+} wave propagation, both result in a decrease in the entry of cells into S phase of the cell cycle (Bittman et al., 1997; Weissman et al., 2004). Cultured cortical explants or dissociated cells from E12–13 mice also show cell-cycle inhibition in the presence of pharmacological gap junction blockers (Cheng et al., 2004; Goto et al., 2002), as do neurospheres derived from ganglionic eminence progenitors (Duval et al., 2002). However, these studies do not explore whether functional coupling or hemichannel-mediated Ca^{2+} waves are involved. In the development of the chick retina, increases in intracellular Ca^{2+} are correlated with the initiation of interkinetic nuclear migration (IKNM), the stereotyped migration of the nucleus during the cell cycle (Pearson et al., 2005b). Retinal Ca^{2+} waves, mediated by gap junction coupling and ATP release through hemichannels, regulate cell proliferation and IKNM (Pearson et al., 2005a; Pearson et al., 2005b). However, it is not clear whether the effects on IKNM are independent from those on the cell cycle. In the cortex, IKNM does not proceed in the presence of cell-cycle inhibitors, suggesting a dependence on cell-cycle progression (Ueno et al., 2006). Furthermore, as IKNM occurs throughout neurogenesis, and Ca^{2+} waves predominate in late neurogenesis, another signaling mechanism would be expected during early development. Overall, it remains to be seen what functional role the switch from gap

junction radial glial coupling to hemichannel-mediated Ca^{2+} wave propagation plays in the progression from mid- to late neurogenesis.

Not only is gap junction and hemichannel formation regulated during the course of neurogenesis, but it is also regulated with each passage through the cell cycle. M phase cells, identified by their morphology and position near the ventricle, are excluded from cell clusters, suggesting that cells uncouple when they pass through M phase (Bittman et al., 1997). Cells appear to recouple in S phase during early neurogenesis or in late S phase or early G2 during late neurogenesis and remain coupled until the subsequent mitosis (Bittman et al., 1997). The delay in recoupling during late neurogenesis is correlated with and might be related to the increase in Ca^{2+} wave initiation as open hemichannels, which initiate the Ca^{2+} waves, are restricted to S phase cells (Weissman et al., 2004). Thus, there is a dynamic regulation of coupling, uncoupling and hemichannel opening throughout each cell cycle.

Does regulation of Cx expression underlie the changes in coupling and hemichannel formation during the cell cycle? During both mid- and late neurogenesis, Cx26 is expressed in more dividing cells during M/G1 than during S/G2; conversely, Cx43 is expressed in more dividing cells during S/G2 than in M/G1 (Bittman and LoTurco, 1999). Thus, the uncoupling of cells in M phase cannot be simply correlated with lower overall Cx expression levels. Alternatively, it is possible that the changes in the types of Cxs expressed affect the level of coupling. Interestingly, Cx43 expression is reduced during M phase in other cell types, which might result from $p34^{cdc2}$ protein kinase phosphorylation that triggers degradation (Laird, 2005). The first posttranslational modifications of Cx26 have recently been reported but the functions of these

modifications are unknown (Locke et al., 2006). Furthermore, it seems likely that Cx43 plays a more important role than Cx26 in the mediation of Ca^{2+} waves. Cx43 is particularly permeable to ATP (Goldberg et al., 2002), and is expressed at high levels during S phase in late neurogenesis (Bittman and LoTurco, 1999), a time point when ATP is released through hemichannels to initiate Ca^{2+} waves (Weissman et al., 2004), and hemichannels composed of Cx43 mediate Ca^{2+} waves by releasing ATP in the chick retinal pigment epithelium (Pearson et al., 2005a). Nonetheless, the fluctuation in Cx levels does not appear extremely large, and more than 50% of cells express Cx26 or Cx43 during all phases of the cell cycle. It is therefore likely that the ability of Cxs to form functional gap junctions or hemichannels is also tightly regulated by signaling interactions during the cell cycle. Other Cx proteins expressed in the developing cortex including Cx45, Cx36 and Cx37 may also be regulated with and contribute to cell-cycle progression. Additionally, during their migration to the cortex, intermediate progenitor cells in the sub-ventricular zone (SVZ) can undergo an expansive, symmetric division (Noctor et al., 2001). It is unknown whether intermediate progenitors express Cxs and, if so, whether their divisions are also regulated by gap junctions.

Gap junctions do not act in isolation to control progenitor proliferation in the developing cortex. Soluble growth factors such as bFGF are thought to be critical in the regulation of cell division and might, in some cases, do so by controlling gap junction expression and coupling. An interesting story has emerged linking the actions of basic fibroblast growth factor (bFGF, otherwise known as FGF 2) to Cx43 (Cheng et al., 2004; Nadarajah et al., 1998). bFGF plays a very important role as a positive regulator of cerebral cortical size and progenitor proliferation during development (Ghosh and

Greenberg, 1995; Murphy et al., 1990; Vaccarino et al., 1995; Vaccarino et al., 1999). bFGF and its receptors are expressed in the proliferative VZ during development, suggesting that it might act as a paracrine signal between progenitor cells (Vaccarino et al., 1999). Microinjection of bFGF into the *in vivo* developing cortex increases neuronal number and cerebral cortical size, whereas bFGF knockout mice have decreased neuronal numbers and decreased cortical size (Vaccarino et al., 1999). In fact, bFGF along with EGF is used to maintain neural progenitor cells grown as neurospheres *in vitro*, but the effect of bFGF is only evident when cells are in contact with each other, suggesting that cell–cell contact and signaling are necessary for its actions (Ghosh and Greenberg, 1995). In cortical progenitors, *in vitro* treatment with bFGF increases the expression of Cx43 but not Cx26 mRNA and protein in a concentration-dependent manner and increases dye coupling between cells as well as proliferation (Cheng et al., 2004; Nadarajah et al., 1998). bFGF binds to receptor tyrosine kinases (RTKs) and signals through p42/p44 mitogen-activated protein (MAP) kinases (otherwise known as extracellular signal regulated kinases; ERK1/2) to increase the expression levels of Cx43 (Cheng et al., 2004; Nadarajah et al., 1998). Furthermore, blocking gap junctions pharmacologically inhibits the proliferative effect of bFGF. Cx43 expression increases proliferation in cultures not treated with bFGF but does not increase proliferation in cultures already treated with bFGF. Together, this suggests that the upregulation of Cx43 through MAP kinase signaling is necessary and sufficient for the proliferative effects of bFGF (Cheng et al., 2004; Nadarajah et al., 1998).

Thus, gap junctions are regulated with the cell cycle and contribute to cell cycle progression by mediating intercellular coupling and Ca^{2+} wave initiation. While this

aspect of gap junction function in the developing cortex has been most widely studied, gap junctions may have roles in other developmental processes including neuronal differentiation and neuronal migration.

Gap junctions regulate neuronal differentiation

The expression of Cxs is dynamic during the time period of neuronal differentiation. During early postnatal ages in the developing cortex, the overall expression of Cx43 increases, that of Cx26 initially increases and then decreases by the third postnatal week and that of Cx32 increases (Dermietzel et al., 1989; Nadarajah et al., 1997). These fluctuations are accompanied by changes in the cell types that express each Cx. Cx43 and Cx26 are expressed in neurons and radial glia during development (Nadarajah et al., 1997), but their expression is largely restricted to astrocyes in the adult, whereas Cx26 and Cx45 are the most abundant Cxs in neurons and Cx32 is found in oligodendrocytes. Are the changes in Cx expression levels important for neuronal maturation?

Cx43 is highly expressed in newborn neurons derived from cultured immortalized hippocampal progenitor cells; however, upon differentiation, Cx43 levels are reduced whereas Cx40 and then Cx33 increase (Rozental et al., 1998). The decrease in Cx43 might be specific to neuronal and not glial differentiation, as RT4 rat peripheral neurotumor cells decrease levels of Cx43 when differentiated into neurons but not when differentiated into glia (Donahue et al., 1998). In cultured human NT2/D1 and rat P19 cells, both of which are pluripotent carcinoma cell lines in which cells are coupled and express Cx43, differentiation along the neural lineage with retinoic acid reduces levels of

Cx43 expression (Bani-Yaghoub et al., 1997; Belliveau et al., 1997). Interestingly, treatment of NT2/D1 or P19 cells with pharmacological gap junction blockers, but not inactive analogs, profoundly reduces the number of mature neurons generated, as assayed by microtubule-associated protein 2 (MAP2) expression, and increases the proportion of cells expressing immature markers such as Nestin and Vimentin (Bani-Yaghoub et al., 1999a; Bani-Yaghoub et al., 1999b). Together, this work suggests that functional gap junction channels mediate signaling that promotes neural differentiation.

Recent studies suggest that Cx32, Cx43 and Cx31 might play a role in neurite outgrowth (Belliveau et al., 2006; Unsworth et al., 2007). Neural crest-derived PC12 cells do not express appreciable levels of endogenous Cxs, but when Cx32 or Cx43 are overexpressed during NGF-induced neural differentiation, neurite length is increased about twofold (Belliveau et al., 2006). Interestingly, this increase in neurite growth is a result of hemichannel-mediated ATP release and its binding to purinergic receptors, as the effect can be mimicked by ATP treatment and blocked with purinergic antagonists or with gap junction channel blockers (Belliveau et al., 2006). Furthermore, NGF treatment stimulates the opening of hemichannels (Belliveau et al., 2006). Contrary to this finding, expression of Cx31 in SH-SY5Y human epithelial-derived neuroblastoma cells increases neurite length independent of Cx trafficking to the cell membrane (Unsworth et al., 2007). Thus, signaling or transcriptional changes that result from Cx31 expression produce increased neurite outgrowth (Unsworth et al., 2007). Further studies of neuronal differentiation *in vivo*, rather than in cell culture, will be critical to understanding the role of Cxs in neuronal differentiation. Gap junctions also contribute to the generation of

cortical circuits in neonatal animals by mediating oscillatory patterns of electrical activity (Khazipov and Luhmann, 2006).

Do gap junctions regulate glial-guided neuronal migration?

Following asymmetric radial glial divisions, newborn neurons migrate to the cortical plate along radial glial fibers (for further discussion of glial-guided migration see: Chapter 2, Introduction). It has been suggested that gap junctions might mediate communication between the radial fiber and the migrating neuron therefore playing an important role in glial-guided neuronal migration (Nadarajah et al., 1997). Interestingly, Cx43 is expressed in radial glial fibers as well as closely associated migrating neurons (Nadarajah et al., 1997). Using electron microscopy, gap junctions have been localized between nestin-positive radial glia and nestin-negative cells (Nadarajah et al., 1997), further suggesting the possibility that gap junctions could serve as the basis for communication between the radial fiber and migrating neuron. However, the possibility that gap junctions are involved in glial-guided neuronal migration in the neocortex has not been previously explored in depth.

Figure 1. Development of the rat neocortex

Coronal section through the forebrain of the developing rat brain at embryonic day 11 (E11), E16 and E22 (figure adapted from: Altman and Bayer, 1995). The dorsal regions of the neural tube at E11 go on to form the neocortex (blue arrow) and hippocampus (orange arrow) while the ventral regions form the basal ganglia (green arrow). Boxed inset (figure adapted from: (Noctor et al., 2004). In the neocortex radial glial cells (green) divide asymmetrically in the VZ (*) to produce neurons or intermediate progenitor cells (blue) which have the capacity to undergo a symmetric neurogenic division in the SVZ (**) (Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2002). The newborn excitatory neurons then migrate radially into the cortical plate along radial glial fibers (arrow) (Rakic, 1971, 1972, 1988). Inhibitory neurons (yellow) are born in the ganglionic eminences of the ventral telencephalon and migrate tangentially into the neocortex (Anderson et al., 1997a).

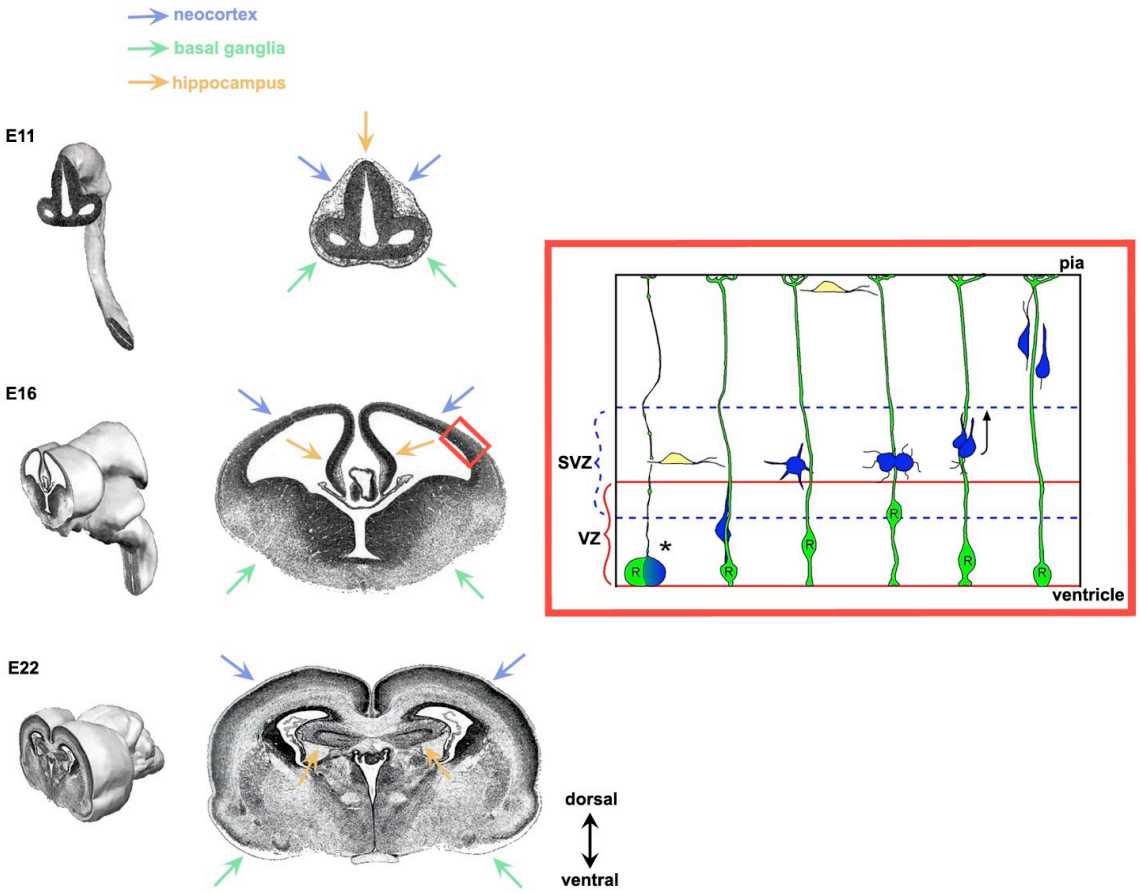

neocortex
basal ganglia
hippocampus
E11
E16
E22
pia
SVZ
VZ
R
*
ventricle
dorsal
ventral

Figure 2. Gap junction structure

Gap junctions are large diameter channels that allow for the exchange of ions or small molecules between adjacent cells. Cxs, four transmembrane domain proteins, are the basic subunits of gap junctions. Cxs form hexamers termed connexons (or hemichannels) that can open to mediate exchange with the extracellular environment. Connexons on opposing membranes join through hydrophobic interactions to form a gap junction (Harris, 2001).

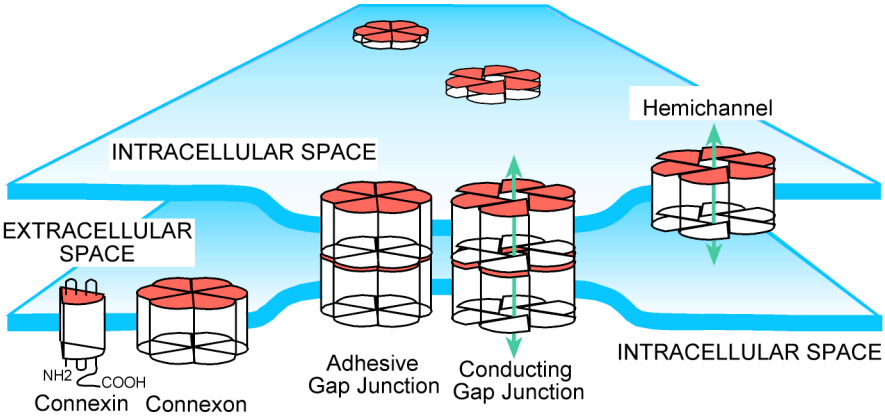
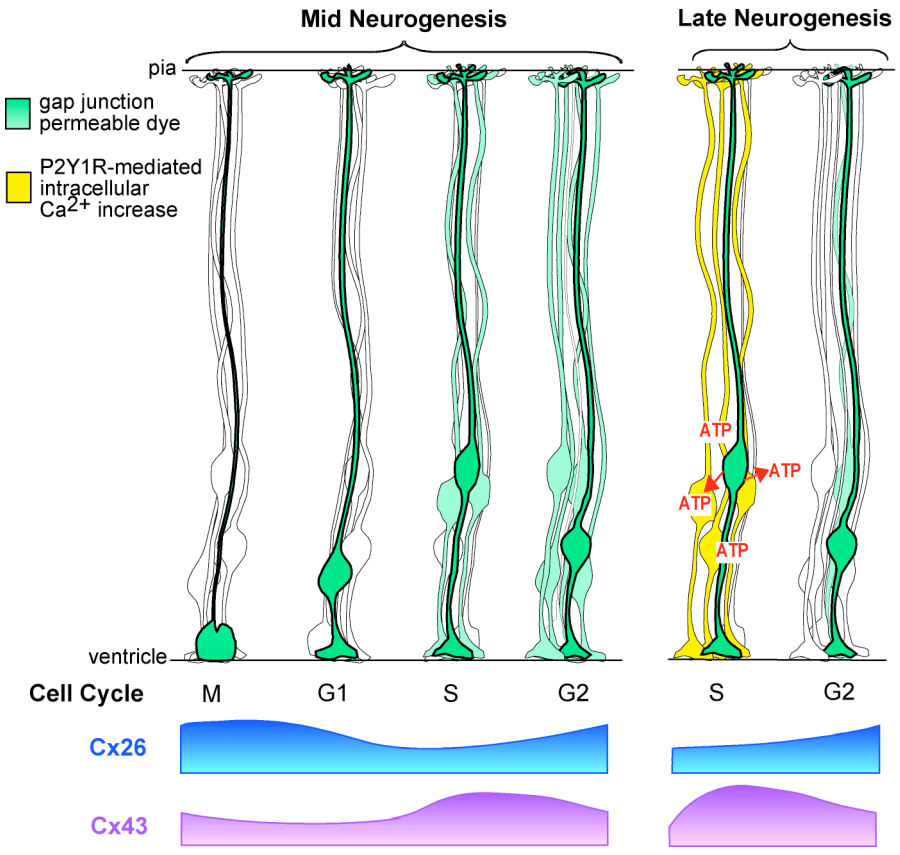

Hemichannel
INTRACELLULAR SPACE
EXTRACELLULAR SPACE
NH2
COOH
Connexin
Connexon
Adhesive Gap Junction
Conducting Gap Junction
INTRACELLULAR SPACE

Figure 3. The role of gap junction coupling and hemichannels in radial glial cell proliferation.

Radial glial coupling and hemichannel activity are regulated during the course of neurogenesis and within each cell cycle. Radial glial cell gap junction coupling is greatest during mid-neurogenesis and decreases in late neurogenesis (Lo Turco and Kriegstein, 1991). During the cell cycle, cells uncouple from clusters during M phase and recouple during S phase in mid-neurogenesis or late S or G2 phase in late neurogenesis (Bittman et al., 1997). During late neurogenesis, hemichannels on S phase radial glia initiate Ca^{2+} waves by releasing ATP which binds to P2Y1 receptors on adjacent cells inducing an IP3-mediated release of Ca^{2+} from intracellular stores (Weissman et al., 2004). Additionally, during each cell cycle, the levels of Cx26 and Cx43 fluctuate such that Cx26 and Cx43 levels are at their peak during M phase and S phase, respectively (Bittman and LoTurco, 1999). Pharmacologically blocking coupling or Ca^{2+} waves inhibits entry into S phase of the cell cycle (Lo Turco and Kriegstein, 1991; Weissman et al., 2004).


Mid Neurogenesis
Late Neurogenesis
pia
gap junction permeable dye
P2Y1R-mediated intracellular Ca2+ increase
ventricle
ATP
ATP
ATP
ATP
Cell Cycle
M
G1
S
G2
S
G2
Cx26
Cx43

Chapter 2:

Methods

Immunohistochemistry and imaging

Immunohistochemistry was performed on embryonic brains subsequent to transcardial purfusion with PBS followed by 4% paraformaldehyde (PFA) (diluted from 16% PFA EM grade, Electron Microscopy Sciences 15710) in Phosphte Buffered Saline (PBS). Brains were post fixed over night in 4% PFA in PBS, dehydrated in 20% and then 30% sucrose in PBS, frozen in optimal cutting temperature embedding medium (Tissue-Tec, 4583), and cryostat sectioned at a thickness of 14μm. For Cx staining the above protocol was modified in the following ways. Following purfusion of 4% PFA in PBS, a purfusion of PBS alone was performed and the brains were not post-fixed to ensure light fixation. Brains were dehydrated as described and cryostat sectioned at 10μm. Cx stains were performed less than a week after sectioning or sectioned brains were stored at -80^{O}C. For endogenous Cx knockdown assays, dissociated cortical cells were plated on Poly-L-Lysine coated coverslips, allowed to adhere for 2hrs, and fixed in 4% PFA in PBS for 10 minutes and then washed with PBS.

Citrate antigen retrieval was preformed for Cx and Ki67 staining prior to blocking. Slices were washed with PBS, incubated in Citrate Buffer (1.2mM Citric Acid, 8.2mM Sodium Citrate, pH 6.0 with HCl) for 5 minutes at room temperature (RT), brought to a pre-boil in the microwave and incubated for 30 minutes, transferred to RT Citrate Buffer, and washed with PBS. Blocking Solution contained 2% gelatin, 10% serum, and 1% Triton-X-100 in PBS. Sections/cells were incubated overnight in primary antibodies in a 1:5 dilution of Blocking Solution: rabbit anti-Cx43 (1:50, Zymed), mouse anti-Cx43 (Zymed), rabbit anti-Cx26 (1:25, Zymed SI-2800), chicken anti-Vimentin (1:1000, Chemicon), mouse anti-β-III tubulin (TUJ1) (1:200, Covance), chicken anti-GFP (1:500,

Aves Labs), rabbit anti-Ki67 (1:1000, Novocastra), mouse anti-Bromodeoxyuridine Alexa Flour® 647 (1:50, Molecular Probes), mouse anti-Nestin (1:100, Chemicon), rabbit anti ZO-1 (1:100, Zymed), mouse anti N-cadherin (1:100, Zymed), and mouse anti β1-integrin (1:100, BD Transduction Laboratories). TUNEL staining was performed using TMR red Cell Death Detection Kit (Roche). Secondary antibodies included Alexa 488/568 conjugates (1:1000, Molecular Probes) or Cy3/Cy5 conjugates (1:100, Jackson). Standard images were acquired on an Olympus Fluoview 300 laser-scanning confocal microscope. 'Standard' images of Cx staining represent collapsed ≤3μm stacks collected at 1μm steps, and other images represent single optical sections. 'High resolution' images were collected on an Olympus Fluoview 1000 at 0.5μm steps. Images were analyzed using Photoshop 7.0 or Photoshop CS2.

Short hairpin RNA (shRNA) design and plasmid constructs

shRNA oligonucleotides were inserted into the dual promoter construct pLentiLox3.7 using the restriction enzymes Hpa1 and Xho1 for expression under the U6 promoter while EGFP was expressed under the CMV promoter (Lois et al., 2002). The following loop sequence was used: TTGATATCCG. The following target sequences were used for Cx26: TCTGGAATTTGCATCCTGCTA (alternative target: GCAGCGTCTGGTGAAGTGTAA) and Cx43: GCAATTACAACAAGCAAGCTA (alternative target: GGCTTGCTGAGAACCTACATCATCA). The control shRNA oliognucleotiedes with three point mutations (underlined) were as follows for Cx26: TCTTGAATATGCATCGTGCTA and Cx43: GTAATTGCAACAAGAAAGCTA. The

following target sequences were used for the P2Y1 receptor: P2Y1-shRNA(2) GGAGTGAGGCCAATTTACA, P2Y1-shRNA(3) GAGTACCTGCGAAGTTATT.

Rat Cx26 and Cx43 clones in the pEYFP-N1 vector (Clonetech) were a gift of D. Laird. Cx26 is cloned into the XhoI and EcoRI sites, and Cx43 is cloned into the KpnI and BamHI sites. QuickChange® site-directed mutagenesis (Stratagene) was used to introduce the following point mutations as underlined: Cx26CM, CTCTGTGTCCGGAATCTGCATACTGCTCAACATCACAGAGCTGTG; Cx43CM, CCTCGTGCCGGAATTATAACAAACAAGCCAGCGAGCAAAACTGGG; Cx43C61S, CTCAACAACCTGGCTCCGAAAACGTCTGCTATGAC; Cx26T135A, TCCCTGTGGTGGGCCTACACCACCAGC; Cx43T54A, GGCTTGCTGAGAGCCTACATCATCAGCATCC; Cx26 1-216, ATCACAGAGCTGTGCTATCTG TTCATTA**GAATTC**GCTCAGGGAAGTCC; Cx43 1-238, TTCAAAGGCGTTAA**GGATCC**CGTGAAGGGAAG (introduced restriction site in bold).

The P2Y1 receptor was cloned with the following primers: AGCTCGAGTGCCTGAGTTGGAAAGAAG (sense), TCGAATTCGGGCGTAGTCGGGCACGTCGTAGGGGTACAAACTTGTGTCTCCG TT (anti-sense), and inserted the pEYFPN1 vector (Clonetech) using the Xho1 and EcoR1 restriction sites . The anti-sense primer introduced an HA tag and removed the stop codon.

Cell transfections and western blots

Western blots were performed on Cos-7 cells. Cos-7 cells were maintained in DMEH21 with 10% Fetal Bovine serum, 1% Sodium Pyruvate, 1% glutamine, and 1% Penicillin/Streptomycin and split 1:10 with 0.25% Trypsin-EDTA. Cos-7 cells were transfected with FuGENE6 transfection reagent (Roche) with 1μg of DNA, 200μl Opti MEM, and 5μl FuGENE6 per 35mm dish. Samples were collected 3 days post transfection in RIPA buffer (150mM NaCl, 0.1%SDS, 1%TritonX100, 50mM TrisHCl pH6.8, 1mM EDTA, 0.5% Deoxycholate, 10% Glycerol) plus protease inhibitors, normalized for protein concentration, denatured at 37^{o}C for 30min in 4X loading buffer (6mM Tris pH6.8, 5% β-Mercaptoethanol, 2% SDS, .05% Bromophenol Blue), and run using the Criterion Precast Gel System on a 12.5% Tris-HCl gel (Bio-Rad) at 200V. Protein was transferred onto a PVDF membrane on a Criterion Blotter (Bio-Rad) at 100V for 30 minutes in Transfer Buffer (0.22% CAPS, 20% Methanol, 0.06% 10N NaOH).

Membranes were blocked with 5% milk in TBST (0.05% Tween20, 0.15M NaCl, 10mM Tris pH8.0). Membranes were probed with rabbit anti-Cx43 (1:1000, Zymed) or rabbit anti-Cx26 (1:2000, Zymed 71-0500) overnight followed by anti-rabbit peroxidase conjugate (1:2000, Sigma) or mouse anti-HA peroxidase for P2Y1-HA detection (1:2000, Sigma H 6533) for 1 hour. All blots were stripped and re-probed with mouse anti-γ-tubulin (1:500, Sigma) overnight and anti-mouse peroxidase conjugate (1:400, Sigma) for 1 hour. Antibodies were diluted in 1% milk in TBST. Antibodies were detected using ECL Detection Reagents (GE Healthcare, RPN2109). For quantification all western blots were normalized to the levels of the loading control γ-tubulin and band intensity was measured in Photoshop.

C6 cells were maintained in 81% F-12 Ham's (Invitrogen, 11765), 15% Horse Serum, 2.5% Fetal Bovine Serum, 1% Penicillin/Streptomycin, 10mM L-glutamine and split 1:5 with 0.5% Trypsin-EDTA. C6 cells were transfected with FuGENE6 transfection reagent (Roche) with 0.3μg of DNA, 20μl Opti MEM, and 2μl FuGENE6 per 16mm dish.

In-utero injection and electroporation and cell transplants

Animals were maintained according to protocols approved by the Institutional Animal Care and Use Committee at UCSF. Plasmids were introduced into the developing cortex *in vivo* by intraventricular injection and electroporation (Saito and Nakatsuji, 2001) (Figure 1). DNA was prepared in an endotoxin free manner (Qiagen Endo-free Mega Prep), and mixed with Fast Green dye (Sigma) prior to surgery to allow tracking of the injection. DNA was loaded into a glass micropipet with plunger (Drummond PCR micropipets 1-10μl) that had been bevelled (World Precision Instruments, 1300M) using a diamond abrasive plate (Sutter, 104D). Intraventricular injections were carried out in E16 timed pregnant Sprague Dawley rats as previously described (Noctor et al., 2001). Dams were anesthetized with xylazine/ketamine, and the uterine horns were exposed in a sterile hood environment and hydrated with saline. 1μl of DNA was injected per brain into the left lateral ventricle at the following concentrations: shRNA constructs, 1.7μg/μl; rescue constructs, at a molar ratio of 0.67:1 (Cx26 rescue construct: Cx26-shRNA) or 0.45:1 (Cx43 rescue construct: Cx43-shRNA); Cx26T135A and Cx43T154A 1.7μg/μl; Tomato 1μg/μl; Actin-cherry 1.7μg/μl; Centrin II-dsRed 2.0μg/μl; HcRed 2.5μg/μl. Electroporations were performed using an Electro Square Porator ECM830 (Genetronics)

(5 pulses, 50V, 100ms, 1s interval). The electroporation tweezertrodes (BTX Genetronics, 7mm diameter) were orientated such that the DNA was driven into the cells lining the dorsal lateral wall of the ventricle. Following electroporation, the uterine horns were placed back inside the dam, and antibiotic/antimitotic was administered to reduce the chance of infection as well as buphenorphine for pain management. A full video protocol of the in-utero electroporation process is available (Walantus et al., 2007).

For analysis of endogenous knockdown, electroporations were performed at E16 and cortices were dissociated at E18, allowed to adhere in culture for 2 hrs, fixed, and stained. For cell transplants, electroporations were performed at E16, GFP^+ cells were dissected from electroporated corticies at E17 in ACSF (125mM NaCl, 2.5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 1.25mM NaH_2PO_4, 25mM $NaHCO_3$ and 20mM glucose) bubbled with 5% CO_2 and 95% O_2, the cells were dissociated and resuspended in culture media containing 25% Hanks BBS, 66% Basal Medium Eagle (Gibco), 5% Fetal bovine serum, 1X Pen/Strep, 0.66% glucose, 1% N2-supplement (Invitirogen). 50,000 cells per brain were intraventricularlly injected into WT E17 embryos, and embryos were fixed at E21

Quantification of cortical cell distribution

The shRNA cell distribution phenotype was quantified by taking a total of 20 evenly spaced rostral to caudal sections per brain from 3 brains per condition. A threshold was applied in Photoshop and the fraction of cells in each of 5 rows of equal area (A-E) stacked parallel to the VZ surface was quantified in each slice and then averaged across slices. The Cx43-shRNA rescue experiments were quantified in a similar

manner by counting the fraction of cells in the CP (layers C,D and E) in 10 rostral to caudal sections per brain for 4-6 brains per condition. For the Cx26-shRNA rescue experiments, 5 medial to caudal sections per brain for 4-6 brains per condition were quantified. For translplant experiments, confocal stacks of all sections with cells that integrated into the cortex were collected and the number of cells in each area (A-E) was counted. All quantifications were performed in a blind manner.

Connexin adhesion assay

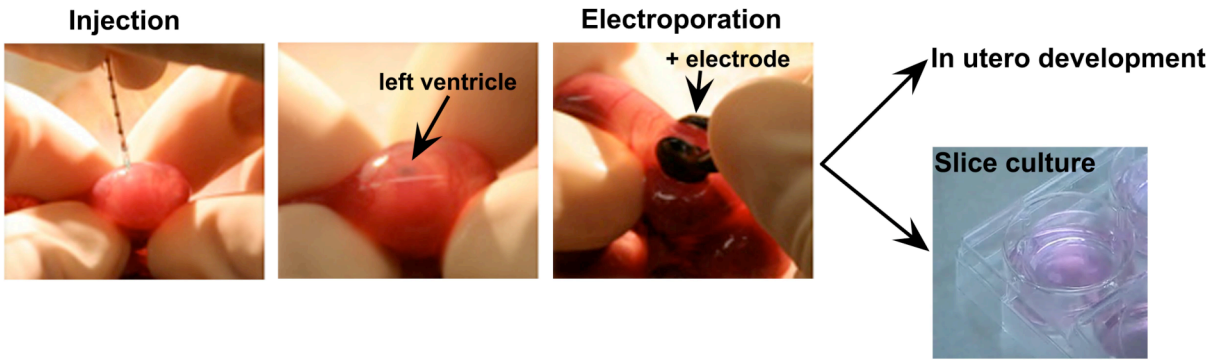
Electroporations were performed at E16 and cells were dissected at E19 in ACSF bubbled with 5% O_2 95% CO_2. Cells were transferred to PBS 2% Fetal Bovine Serum immediately prior to sort. Cells were sorted for GFP on a FACSAria (Becton Dickinson) using a 100μm nosel, 488 laser, the 530/30 filter and collected in culture media (25% Hanks BBS, 66% Basal Medium Eagle (Gibco), 5% Fetal bovine serum, 1X Pen/Strep, 0.66% glucose, 1% N2-supplement (Invitirogen)). Collected cells were labelled with Vybrant DiD cell labelling solution (Molecular Probes) in serum free culture media for 20 min and washed according to instructed protocol. 10,000 cells were plated per well of 100% confluent of C6 cells grown on 24 well collagen biocoat® plates (Becton Dickinson). C6 (containing the TVA receptor) and C6+Cx43 (containing the TVA receptor and Cx43-EYFP) cells were a gift of Albert Lai (Lai et al., 2006). Cells were incubated for 30 min shaking at 250 rpm, and then washed 4X with PBS and fixed. Each experiment included three or more wells of each condition. 5 pictures of each well were taken (middle, right, left, top, bottom), and quantifications were carried out in a blind manner.

Slice culture and time-lapse imaging

Electroporations were performed at E16. At E18 brains were dissected in ice cold ACSF bubled with 5% O_2 95% CO_2, and embedded in 4% low melt agarose (Fisher, BP165-25) in ACSF pre-warmed to 50^0C and cooled on ice. 300μm thick vibratome (Leica) sections were prepared in ice cold ACSF bubbled with 5%CO_2 95%O_2. Slices were transferred to room temperature ACSF bubbled with 5% O_2 95% CO_2, and then plated on Millicell-CM inserts (Millipore, Bedford, MA) in culture medium (25% Hanks BBS, 66% Basal Medium Eagle (Gibco), 5% Fetal bovine serum, 1X Pen/Strep, 0.66% glucose, 1% N2-supplement (Invitirogen)) and allowed to equilibrate overnight before imaging. Confocal images, were collected on the Olympus Fluoview 300 at 2-5um steps (to minimize bleaching) every hour for 8-10hrs. Stacks containing the cell of interest were collapsed and analyzed in Photoshop. A full video protocol of the slice culture technique is available (Elias and Kriegstein, 2007).

Figure 1. *In-utero* intraventricular injections and electroporations

In-utero intraventricular injections and electroporations were used to introduce exogenous plasmid constructs into the developing *in vivo* cortex. Plasmid DNA was loaded into glass micropipets and mixed with fast green dye for visualization. The uterine horn of the dam was exposed at embryonic day 16, and the DNA was injected into the left lateral ventricle of each embryo. Upon injection, the filling of the lateral ventricle with the fast green dye/DNA mix can be observed. Subsequently charge is passed across the developing brain using tweezertrodes such that the DNA is driven into the cells lining the dorsal lateral wall of the left ventricle. The embryos are then placed back inside the dam to allow for *in-vivo* development, or alternatively, brain slices are made and maintained in culture for time-lapse imaging.


Injection
Electroporation
left ventricle
+ electrode
In utero development
Slice culture

Chapter 3:

Gap junctions are necessary for glial-guided radial migration in the developing neocortex

Introduction: glial-guided neuronal migration

Excitatory cortical neurons are generated in the proliferative regions adjacent to the dorsal wall of the lateral ventricles in the telencephalon. However, the new-born neurons must migrate radially away from the ventricle to assume their final position in the cortical plate. Early on in cortical development this distance is quite small, but as the cortex expands, neurons must travel a formidable distance to reach their increasingly superficial destination. The observation that clonally generated neurons are spatially restricted to columns in the cortex (the radial unit hypothesis), suggested that instructional cues guide the migration of neurons based on their position at birth (Rakic, 1972, 1978, 1988). In fact, radial glial fibers, which extend from the ventricle and send an endfoot to the basement membrane along the pial surface, serve as cellular highways to guide neuronal migration. Newborn neurons migrate in very close association with a radial glial fiber and interstitial junctions containing filamentous material have been found between migrating neurons and radial fibres (Cameron and Rakic, 1994; Edmondson and Hatten, 1987; Gadisseux et al., 1990; Gregory et al., 1988; Rakic, 1972, 1978, 1988, 2003). Time-lapse imaging of clonally related cells has confirmed that cortical neurons often migrate along their parental radial glial fibre (Noctor et al., 2001). Glial-guided neuronal migration is the most widely described form of migration for excitatory cortical neurons. Alternative modes of migration such as somal translocation, in which the neuron inherits the radial fiber and maintains an attachment to the basement membrane during migration, have also been postulated and may play a role during early corticogenesis (Miyata et al., 2001; Nadarajah et al., 2001).

Glial-guided migration involves the saltatory movement of the neuroblast along the radial glial guide, beginning with the forward movement of the neuronal leading process and followed by the displacement of the nucleus and cell soma (Edmondson and Hatten, 1987; Komuro and Rakic, 1995; Noctor et al., 2001; Rakic, 1978; Schaar and McConnell, 2005; Tsai et al., 2007). Neurons extend a long leading process from the cell soma that moves forward continuously (Schaar and McConnell, 2005; Tsai et al., 2007). A swelling or dilation forms in the leading process proximal to the cell body and the centrosome, always maintaining a position in front of the nucleus (Tanaka et al., 2004), moves forward in a continuous manner therefore entering the swelling while the nucleus pauses (Tsai et al., 2007). A microtubule network with the plus ends extending in a radial array from the centrosome forming a cage like structure around the nucleus is responsible for orchestrating the saltatory movement of the nucleus into the swelling (Gregory et al., 1988; Schaar and McConnell, 2005; Solecki et al., 2004; Tanaka et al., 2004; Tsai et al., 2007).

Studies of human disorders as well as mouse models have elucidated a number of molecules important for glial-guided migration. Interestingly the majority of human cortical malformations have been linked to defects in microtubule and actin dynamics. Classical lissencephaly caused by LIS1 and doublecortin (DCX) are both involved in microtubule dynamics (Bielas, 2004; des Portes et al., 1998; Gleeson et al., 1998; Reiner et al., 1993). Filamin I (FLN1), the cause of human periventricular heterotopia, is involved in actin dynamics (Fox et al., 1998; Sheen et al., 2002). It is thought that, microtubule dynamics play a role in the extension of the leading process, the localization of the centrosome, and the translocation of the nucleus, while actin plays a role in the

formation of fillopodia at the leading processes and more recently has been described to play a role in the translocation of the nucleus (Schaar and McConnell, 2005; Tsai et al., 2007).

Surprisingly few molecules have been identified to mediate the critical interaction between the radial glial fiber and the migrating neuron. The soluble form of Neuregulin, Glial Growth Factor, is released by migrating neurons and binds to ErbB2/4 receptors on radial glia, and is important for radial glial maitnence as well as neuronal migration (Anton et al., 1997; Rio et al., 1997). In addition Neuregulin-1 signaling to ErbB2 receptors on radial glial cells is important for maintaining radial glial identity by repressing astrocytic differentiation (Ghashghaei et al., 2007; Schmid et al., 2003).

With regards to molecules that provide adhesion between the migrating neuron and radial fibre, Astrotactin has been described as an adhesion molecule in the cerebellar cortex (Adams et al., 2002; Edmondson et al., 1988), but has not yet been clearly implicated in neocortical development. Integrins, extracellular matrix receptors composed of an α and β subunit, have been implicated in neural-glial recognition and adhesion (Anton et al., 1999; Schmid et al., 2004). Alternatively, it has been proposed that integrins may only be important for anchoring the radial glial endfoot at the basement membrane and maintaining basement membrane integrity, thereby secondarily affecting neuronal migration (Belvindrah et al., 2007). α3 integrins are expressed in migrating neurons and αV integrins are expressed in radial glia (Anton et al., 1999; McCarty et al., 2005). Cell culture studies using function blocking antbodies for α3, αV, and β1 integrins suggest that $\alpha3\beta1$ integrin expressed in migrating neurons is involved in glial recognition, and αV integrin expressed in radial glia is involved in neuronal adhesion

(Anton et al., 1999). However, discrepancies remain as to whether the α3 integrin knockout mice (Kreidberg et al., 1996) have cortical lamination defects (Anton et al., 1999; Belvindrah et al., 2007; Schmid et al., 2004). Furthermore, although Nestin-cre mediated deletion of β1 integrin in radial glia results in severe cortical lamination defects (Graus-Porta et al., 2001), deletion mediated by Nex-Cre in intermediate progenitors/differentiating neurons does not lead to lamination abnormalities (Belvindrah et al., 2007), suggesting the loss of β1 integrin may primerely effect the anchoring of radial glial fibers at the basement membrane. Other integreins and integrin signaling molecules have also been implicated in the maintnance of the radial glial-basement membrane interaction and result in neuronal lamination defects (Beggs et al., 2003; Georges-Labouesse et al., 1998; Haubst et al., 2006; Niewmierzycka et al., 2005).

Another widely studied model of disrupted migration, the mouse mutant reeler, has inverted cortical layering (D'Arcangelo et al., 1995; Ogawa et al., 1995). Reelin, produced by Cajal Retzius cells in layer I of the cortical plate, has been proposed to serve as a stop signal for migrating neurons in a signaling pathway that includes very low density lipoprotein receptor (VLDLR), apolipoprotein E (ApoER2), and disabled-1 (Dab1), which all result in similar cortical lamination defects (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Howell et al., 1997; Sheldon et al., 1997; Trommsdorff et al., 1999; Ware et al., 1997). It has also been suggested that reelin disrupts neruoblast-glial interactions by binding with β1 integrin and initiating a signaling cascade through Dab1 that leads to the separation of the neuroblast and the radial fiber (Dulabon et al., 2000; Sanada et al., 2004; Schmid et al., 2005). Secreted protein acidic and rich in cysteine like 1 (SPARC-like 1) may also play a role in cortical lamination by reducing the adhesivitiy

of neurons once the reach the cortical plate (Gongidi et al., 2004). Further work will be needed to clarify the role of integrins, reelin, and other signaling molecules in mediating the adhesion between the migrating neuron and radial fibre during glial-guided migration. In addition, the possibility that gap junctions play a role in glial-guided migration has been suggested as Cx43 is expressed in both migrating neurons and radial fibers (Nadarajah et al., 1997).

Gap junctions are expressed at contact points between migrating neurons and radial fibres

The gap junction subunits expressed in the developing cortex include Cx26, Cx36, Cx43, and Cx45 (Figure 1) (Cina et al., 2007; Dermietzel et al., 1989; Nadarajah et al., 1997). Recent reports suggest that Cx37 is also expressed in the embryonic cortex (Cina et al., 2007). I chose to focus my studies on Cx26, a β1 Cx family member, and Cx43, an α1 Cx family member. Cx26 and Cx43 differ in their expression distribution in the embryonic cortex. Whereas Cx26 is evenly distributed from the VZ through the intermediate zone (IZ) to the cortical plate (CP), Cx43 is highly expressed at the ventricular surface and its levels are reduced in the CP (Fig. 2a,b,e,f). The expression of both Cxs outside of the VZ proliferative region suggests that gap junctions may regulate functions in addition to radial glial proliferation.

To address the possibility that gap junctions may be involved in neuronal migration, we determined the pattern of Cx26 and Cx43 expression with respect to β-*III* tubulin^{+} migrating neurons and Vimentin^{+} radial glial fibres. Both gap junction subunits are expressed in migrating neurons and along radial fibres (Figure 2,3). In fact, Cx26 and

Cx43 are highly localized in neurons to the regions of contact with radial fibres, consistent with the idea that gap junctions may have a role in mediating communication between migrating neurons and their radial guides (Figure 2 c,d,g,h). Using confocal microscopy, one can visualize gap junction proteins at the points of contact between migrating neurons and radial fibres (Figure 3). Interestingly, Cx26 and Cx43 junctions may be functionally distinct as Cx26 and Cx43 puncta do not co-localize with each other in the embryonic cortex (Figure 4); they belong to separate families that do not make heterotypic junctions or heteromeric hemichannels (Falk, 2000).

Cx26 and Cx43 are necessary for neuronal migration

To investigate the role of gap junctions in cortical development, I developed acute loss-of-function manipulations for each Cx utilizing RNA interference. Short hairpin RNA (shRNA) constructs that produced significant knockdown of rat Cx26 or Cx43 by western blot in Cos-7 cells were selected (Cx26-shRNA or Cx43-shRNA respectively) (Figure 5a,b). It was confirmed that the same hairpin sequences with three point mutations (Ctrl-shRNA) failed to produce significant knockdown and that the shRNA was specific to the Cx of interest and did not affect the levels of the other Cx (Figure 5a,b). Furthermore, Cx26-shRNA and Cx43-shRNA were able to knockdown endogenous Cx protein in the developing rat cortex (Figure 5c,d).

We next examined the functional effects of Cx26 or Cx43 down-regulation in the intact developing neocortex. Ctrl-shRNA, Cx26-shRNA, or Cx43-shRNA plasmids were introduced to the embryonic cortex by *in-utero* intraventricular injection and electroporation at embryonic day 16 (E16). Ctrl-shRNA expressing cells can be observed

entering the cortical plate starting at E18, and by E21 the majority of cells have migrated to the cortical plate (Figure 6a). Expression of Cx26-shRNA or Cx43-shRNA resulted in a striking cellular redistribution pattern compared to Ctrl-shRNA at both E18 and E21 (Figure 6a). At E18 almost no Cx26-shRNA or Cx43-shRNA expressing cells are observed in the cortical plate, and at E21 more cells are localized to the intermediate zone and fewer in the cortical plate. We quantified this effect by dividing the cortex into 5 equal areas and determining the fraction of the total number of shRNA-expressing (GFP^+) cells in each area (Figure 6b). At E18 there was a significant change in the fraction of cells in all areas except in the VZ; most clearly there was a loss of cells in the lower (l) and upper (u) cortical plate (Figure 6b). The effect was more dramatic at E21. The percent of cells in each area, except the VZ/SVZ, was significantly different. Most strikingly, there was an accumulation of cells in the intermediate zone with far fewer cells in the cortical plate (Figure 6b).

The intermediate phenotype of the Cx26-shRNA, apparent in the representative image and quantification at E21 (Figure 6), is due to a rostral caudal gradient such that Cx26-shRNA exerts a stronger effect on neuronal migration in caudal regions while it has very little effect in rostral regions (Figure 7a). This phenotypic gradient can be correlated with an expression gradient as Cx26 is expressed at relatively low levels in the rostral cortex compared to the caudal cortex (Figure 7b). In contrast, Cx43-shRNA does not show a rostral-caudal phenotypic gradient, and Cx43 is expressed evenly in the rostral-caudal axis (Figure 7).

In order to confirm the specificity of the shRNAs and eliminate the possibility of off-target effects, rescue experiments were performed to restore the appropriate levels of Cx expression. First, in order to rescue the migration phenotype, conservative mutations (CM) were introduced into the shRNA-targeted regions of Cx26-EYFP or Cx43-EYFP (Cx26CM or Cx43CM respectively), which prevented shRNA-induced knockdown in Cos-7 cells (Figure 8a,c). These constructs, or EYFP alone, were co-electroporated with the shRNA constructs at a set molar ratio. The co-expression of Cx43CM or Cx26CM with its respective shRNA construct significantly rescued the fraction of cells that migrate to the cortical plate, demonstrating the target-specificity of the shRNA effect (Figure 8b,d). Furthermore, target-specificity was also confirmed by using alternative shRNA targets for Cx26 and Cx43, which also reduce the migration of cells to the cortical plate (Figure 9).

Because radial glia express Cxs, it was possible that the defect in neuronal migration was due to a general disruption in the radial glial scaffolding. However, immunohistochemical analysis of the radial glial scaffolding as well as the migration of wild type (WT) neurons in electroporated regions appeared unpreturbed (Figure 10). To further address the possibility that changes in the radial glial scaffolding were responsible for the migration defect, transplant assays were performed. Brains were electroporated at E16 and shRNA-expressing (GFP^+) cells were microdissected and intraventricularly injected into WT brains at E17, which were fixed at E21 (Figure 11a). In contrast to donor Ctrl-shRNA expressing cells, which were able to engraft and migrate toward the upper layers of the cortex, the Cx26-shRNA and Cx43-shRNA expressing donor cells were able to engraft into the host brain but had a significantly reduced migration capacity

(Figure 11b). These data strongly suggest that Cx expression in neurons is required for migration.

We next examined secondary defects potentially able to affect cell distribution independent of migration. Neurons expressing Cx-shRNA were able to exit the cell cycle and begin to differentiate normally (Figure 12a,b), suggesting that their altered distribution is not secondary to an inability to exit the cell cycle and begin to differentiate. Furthermore, TUNEL staining showed no change in apoptosis (Figure 12c). Additionally, the expression pattern of the adherens junction component N-cadherin, the tight junction component Zona Occludens-1, and β1-integrin appeared unchanged, suggesting that Cx knockdown does not overtly disrupt expression of other cell-cell adhesion proteins (Figure 13,14). Together these data suggest that the defect in cell migration is not secondary to a defect in cell cycle exit, differentiation, cell death, or down-regulation of other adhesion proteins. These results together suggest that Cx26 and Cx43 are necessary for glial-guided neuronal migration in the developing cerebral cortex.

Figure 1. Cx expression in the developing forebrain

Cx26, Cx36, Cx43 and Cx45 are expressed in the developing forebrain at embryonic day 16 (E16) as shown in one hemisphere. Both dorsal structures such as the cerebral cortex and ventral structures such as the ganglionic eminences express Cxs.

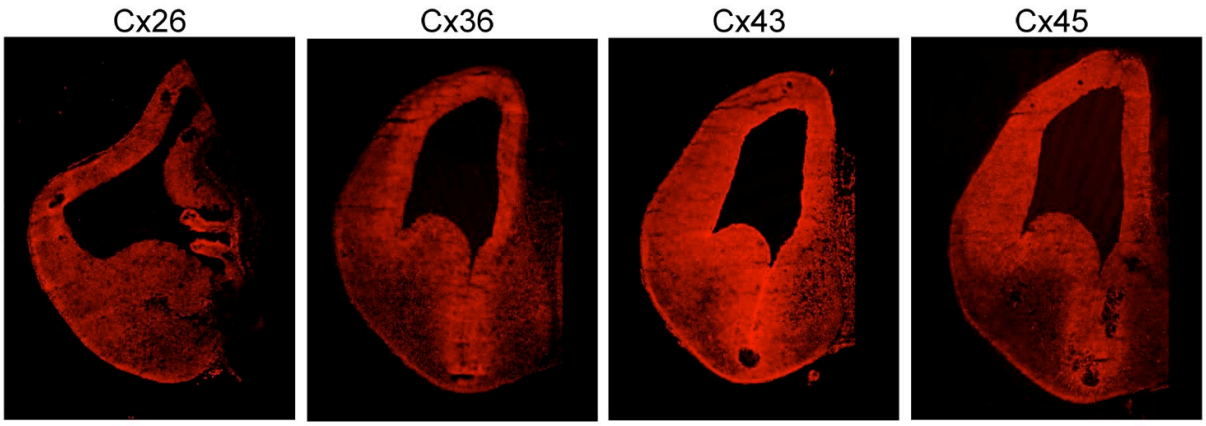

Cx26
Cx36
Cx43
Cx45

Figure 2. Pattern of Cx26 and Cx43 expression in the embryonic cortex

a,**e**, Immunohistochemistry of cortical sections at E16 showing overlay of Cx26 (a) or Cx43 (e) (red), Vimentin (green) to label radial fibres, and β-III tubulin (blue) to label immature neurons. **b,f,** Red channel in (a,e) showing expression pattern of Cx26 (b) or Cx43 (f). Cx26 is evenly distributed from the VZ to the cortical plate while Cx43 is highly expressed at the surface of the ventricle and less so in the cortical plate. (IZ = intermediate zone; CP = cortical plate) **c,g**, Migrating neuron from boxed area in panel (a,e). Clockwise: overlay, Vimentin (green), Cx26 (c) or Cx43 (g) (red), and β-III tubulin (blue). Cx26 and Cx43 are expressed in neurons and radial glia (white arrows). **d,h**, Overlay of Cx26 (d) or Cx43 (h) and β-III tubulin in migrating neuron (from c,g) showing that the localization of Cx puncta in neurons is enriched in regions of overlap with radial fibres. Scale bars, 5μm.

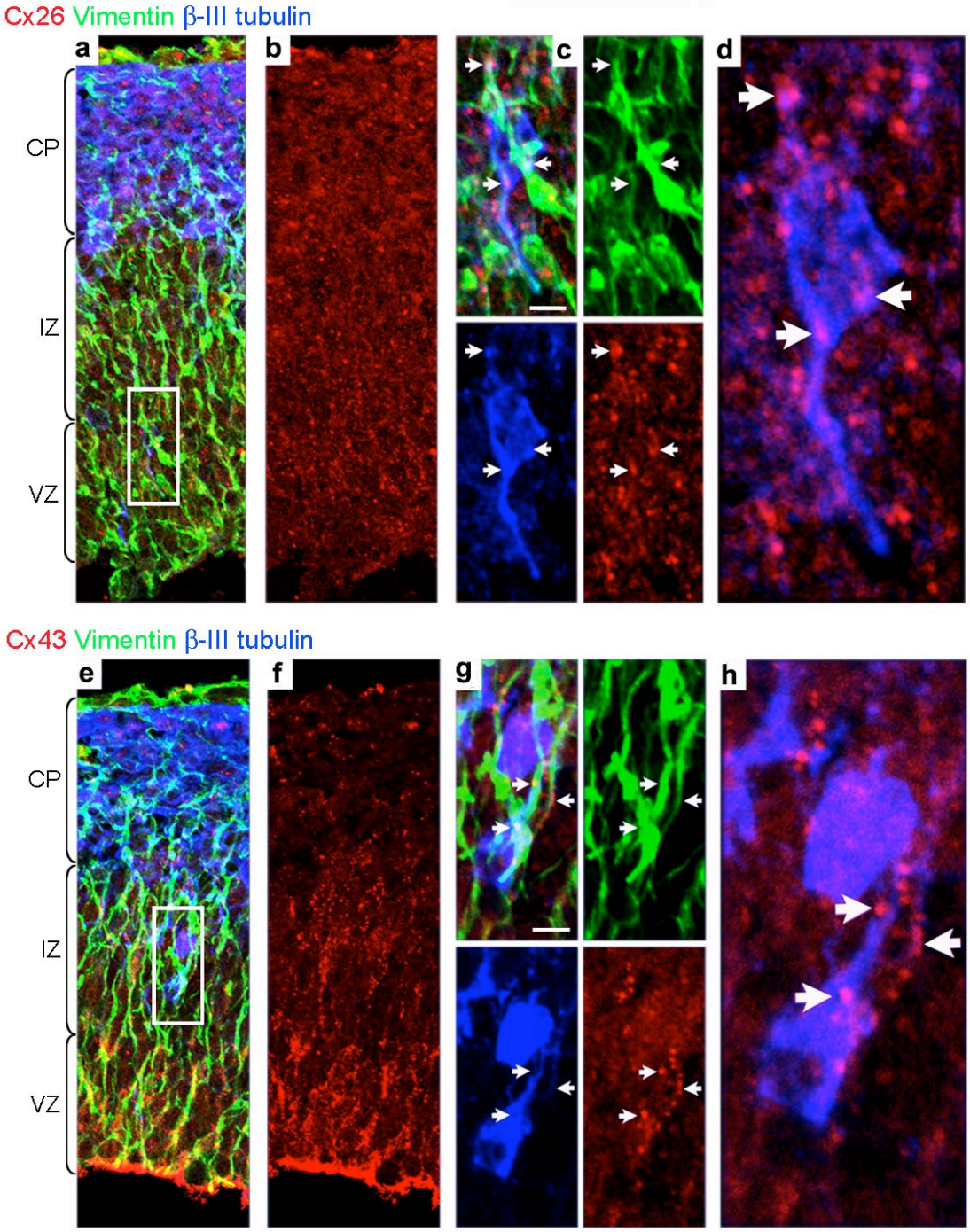

Cx26 Vimentin β-III tubulin
a
b
c
d
CP
IZ
VZ
Cx43 Vimentin β-III tubulin
e
f
g
h
CP
IZ
VZ

Figure 3. Cx26 and Cx43 puncta localize to points of contact between radial fibres and migrating neurons

The relationship between Cx26 (a,b) or Cx43 (c,d) puncta, Vimentin^{+} fibres (green), and β-III tubulin^{+} neurons (blue). **a,** Cx26 puncta at the leading portion of the neuronal cell body (outlined). Cross sections through the X and Y axis, bottom and right panels respectively, show individual Cx puncta (white arrows) at the interface between the Vimentin^{+} radial fibre and β-III tubulin^{+} migrating neuron. **b,** 3-dimentional rotation of region at crosshair in 'a' highlighting the relationship between the Cx26 punta, the migrating neuron, and the radial fibre (puncta of interest are circled at 0^{O}). **c,** Cx43 puncta at the leading process of a migrating neuron (outlined). Cross sections through the X and Y axis, bottom and right panels respectively, show individual Cx puncta (white arrows) at the interface between the Vimentin^{+} radial fibre and β-III tubulin^{+} migrating neuron. **d,** 3-dimentional rotation of region at crosshair in 'c' highlighting the relationship between the Cx43 punta, the migrating neuron, and the radial fibre (puncta of interest are circled at 0^{O}). Images taken at 'high resolution' (see methods). Scale bars, 5μm.

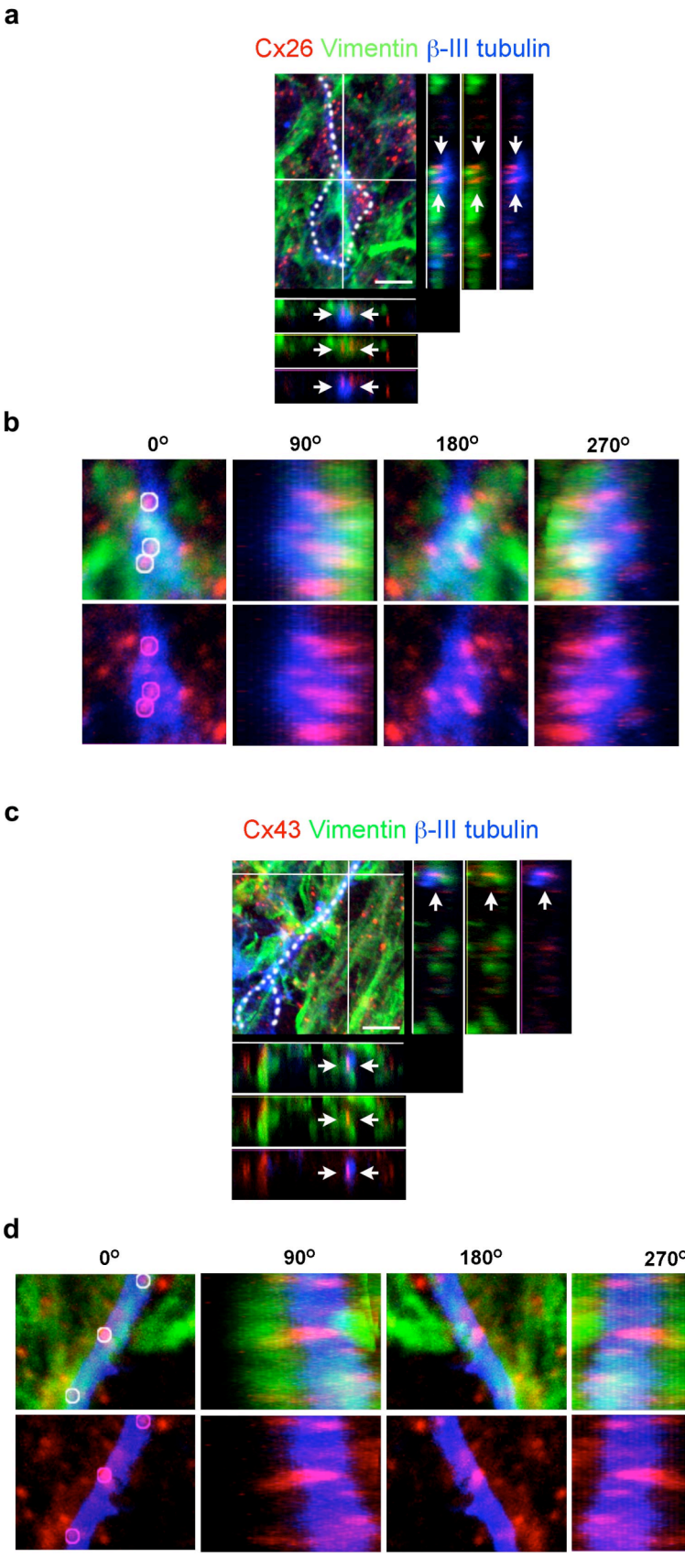

a
Cx26 Vimentin β-III tubulin
b
0°
90°
180°
270°
c
Cx43 Vimentin β-III tubulin
d
0°
90°
180°
270°

Figure 4. Cx26 and Cx43 puncta do not co-localize

a, Immunohistochemistry at E16 for Vimentin (blue) to label radial fibers, Cx26 (green), and Cx43 (red). **b,c**, Close-up of boxed region in panel (a) highlighting the largely non-overlapping expression patterns of Cx26 (green) and Cx43 (red). Images taken at 'high resolution'. Scale bar,10μm.

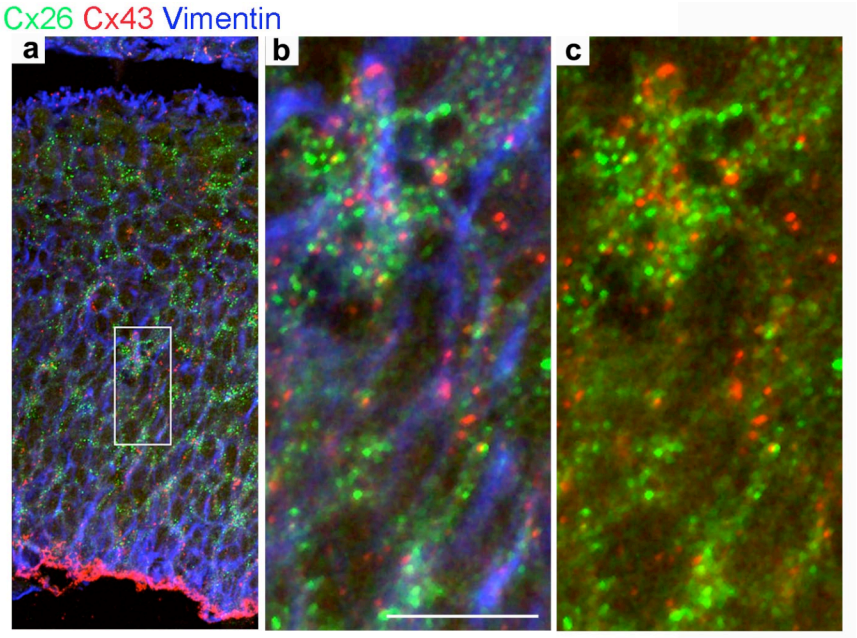

Cx26 Cx43 Vimentin
a
b
c

Figure 5. Short hairpin RNA (shRNA) reduces the expression of Cx26 or Cx43

a,**b,** Knockdown of Cx26 (a) and Cx43 (b) in Cos-7 cells. Candidate shRNAs for Cx26 and Cx43 were inserted into the pLLox3.7 vector under control of the U6 promoter with EGFP under control of the CMV promoter (Lois et al., 2002). Knockdown was tested by co-expression in Cos-7 cells with rat Cx26 or Cx43, and Cx levels were normalized to γ-tubulin for quantification. Cx26-shRNA (a) or Cx43-shRNA (b) significantly reduced Cx26 or Cx43 expression respectively. Ctrl-shRNA (Cx26-shRNA or Cx43-shRNA with three point mutations) did not effect expression, and shRNAs did not cross react with the non-targeted Cx (* $p<0.01$, t-test, Cx26+: pLLox3.7 n=8, Cx26-shRNA n=8, Ctrl 26-shRNA n=7, Cx43-shRNA n=3; Cx43+: pLLox3.7 n=7, Cx43-shRNA n=6, Ctrl 43-shRNA n=5, Cx26-shRNA n=4). Values represent mean±s.e. **c,d**, Endogenous knockdown of Cx26 (c) and Cx43 (d) in the developing cortex. Constructs were intraventricularly injected and electroporated at E16 into the developing neocortex, and cells were dissected, dissociated, and fixed two days post electroporation. Cx26-shRNA (c) or Cx43-shRNA (d) significantly reduced the fraction of GFP^+ cells expressing Cx26 or Cx43 respectively above a set threshold as assayed by immunohistochemistry (*** $p<0.0001$, Fisher's Exact Test, Cx26: Ctrl-shRNA n=241, Cx26-shRNA n=233; Cx43: Ctrl-shRNA n=187, Cx43-shRNA n=267).

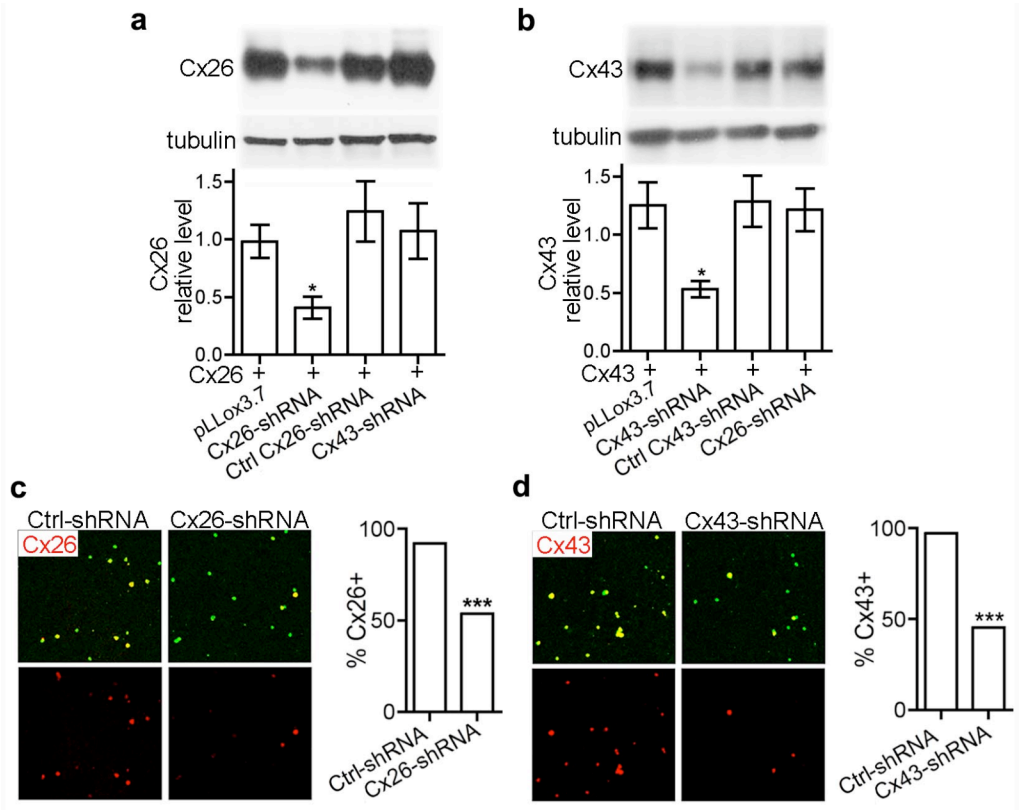

a
Cx26
tubulin
Cx26 relative level
1.5
1.0
0.5
0.0
*
Cx26 + + + +
pLLox3.7
Cx26-shRNA
Ctrl Cx26-shRNA
Cx43-shRNA
b
Cx43
tubulin
Cx43 relative level
1.5
1.0
0.5
0.0
*
Cx43 + + + +
pLLox3.7
Cx43-shRNA
Ctrl Cx43-shRNA
Cx26-shRNA
c
Ctrl-shRNA
Cx26-shRNA
Cx26
100
50
0
% Cx26+
***
Ctrl-shRNA
Cx26-shRNA
d
Ctrl-shRNA
Cx43-shRNA
Cx43
100
50
0
% Cx43+
***
Ctrl-shRNA
Cx43-shRNA

Figure 6. Cx26-shRNA and Cx43-shRNA expression impairs neuronal migration

a, Coronal sections from brains electroporated at E16 with shRNA constructs. Cx26-shRNA or Cx43-shRNA expression reduces the fraction of cells in the CP (white arrowheads) at E18 and E21. **b**, Quantification of shRNA knockdown-mediated cell distribution effect. (Left) Representative optical section with grid overlay of five equal areas (A-E) and quantification threshold. (Right) There is a significant change in the distribution of cells in all areas except A at E18 and E21 (1-way ANOVA, E18: A $p=0.0745$, B $p=0.0010$, C $p=0.0106$, D $p\leq0.0001$, E $p<0.0001$; E21: A $p=0.3625$, B $p\leq0.0001$, C $p=0.0003$, D $p\leq0.0001$, E $p\leq0.0001$; n=3 brains/condition) (l = lower; m = middle; u = upper; IZ = intermediate zone; CP = cortical plate). Values represent mean±s.e.

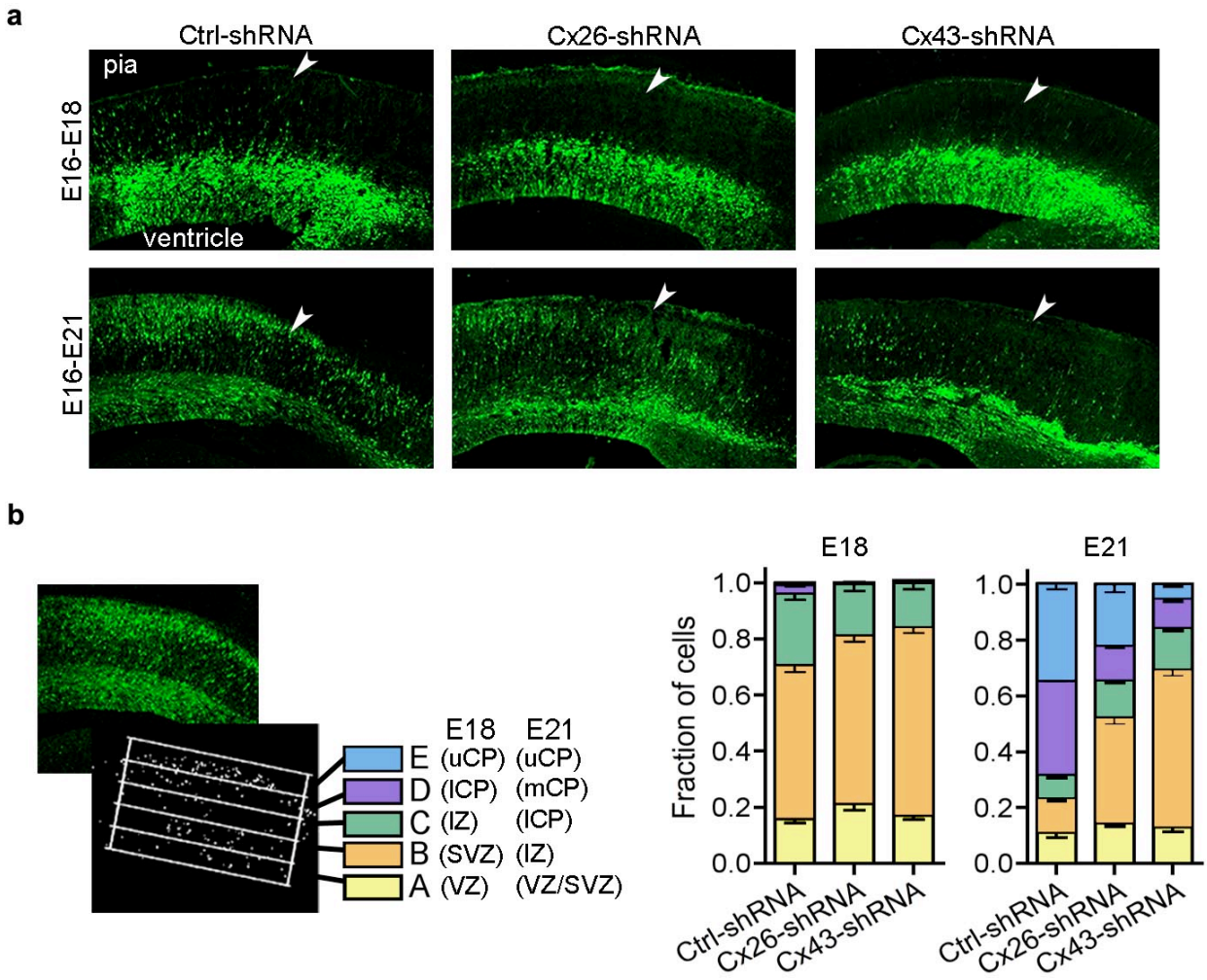

a
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
pia
ventricle
E16-E18
E16-E21
b
E18 E21
E (uCP) (uCP)
D (ICP) (mCP)
C (IZ) (ICP)
B (SVZ) (IZ)
A (VZ) (VZ/SVZ)
E18
E21
Fraction of cells
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA

Figure 7. Rostral-caudal phenotypic and expression gradient of Cx26 but not Cx43

a, Distribution of electroporated cells at E21 in the intermediate zone (area B, top) and the upper cortical plate (area E, bottom) in rostral, medial, and caudal coronal sections. The distribution of cells expressing Cx26-shRNA is more impaired in caudal sections. **b**, In sagittal sections at E16 the rostral (r) and medial levels of Cx26 staining are significantly lower than caudal (c) levels; there is no change in the level of Cx43 expression (* $p < 0.01$, t-test, Cx26 n=4, Cx43 n=3). Values represent mean±s.e.

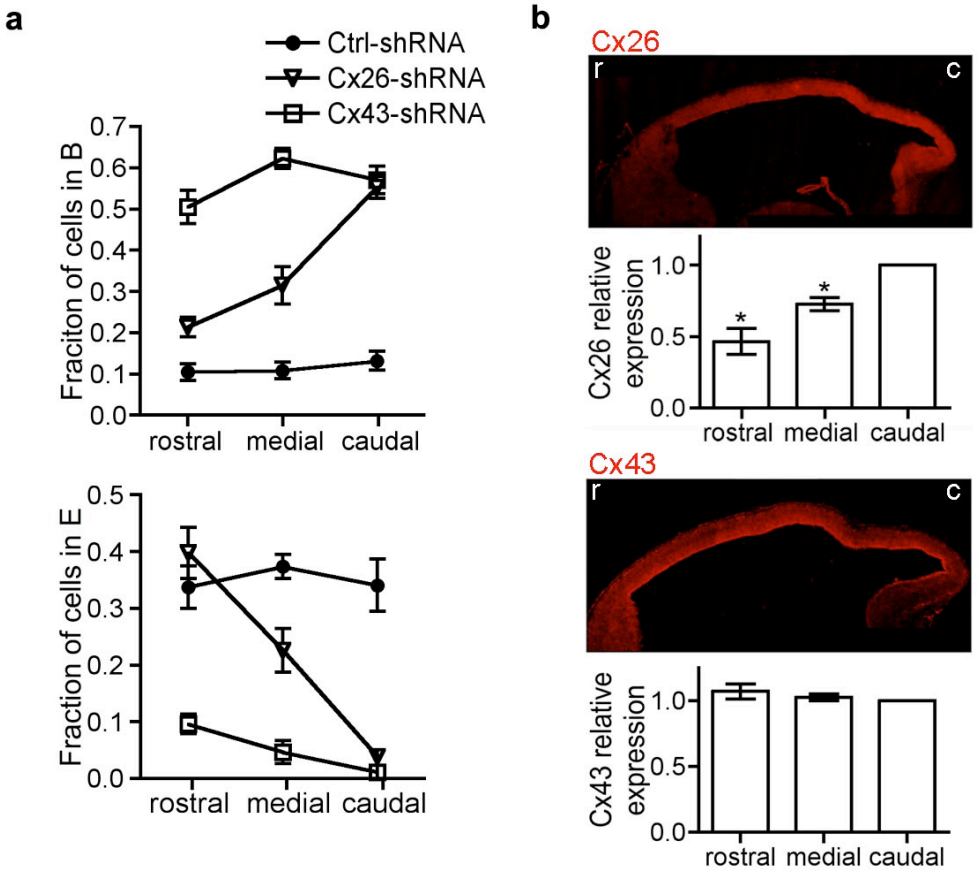

a
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
Fraciton of cells in B
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
rostral medial caudal
Fraction of cells in E
0.5
0.4
0.3
0.2
0.1
0.0
rostral medial caudal
b
Cx26
r
c
Cx26 relative expression
1.0
0.5
0.0
*
*
rostral medial caudal
Cx43
r
c
Cx43 relative expression
1.0
0.5
0.0
rostral medial caudal

Figure 8. Rescue of Cx-shRNA induced migration defect by co-expression of Cxs with conservative mutations that prevent knockdown

a, Four conservative mutations were introduced in region of Cx43 targeted by Cx43-shRNA (Cx43CM). Cx43-shRNA does not significantly reduce the expression levels of Cx43CM in Cos-7 cells as measured by western blot (pLLox3.7 vs. Cx43-shRNA, t-test p=0.80, pLLox3.7 n=4, Cx43-shRNA n=4). **b,** Co-electroporation of EYFP or Cx43CM with the Cx43-shRNA construct at fixed molar ratio of 0.45:1. Co-expression of Cx43CM significantly increases the fraction of cells that migrate to the cortical plate by E21 (*** $p<0.0001$, t-test, EYFP n=6 brains, Cx43CM n=4). **c,** Four conservative mutations were introduced in region of Cx26 targeted by Cx26-shRNA (Cx26CM). Cx26-shRNA does not significantly reduce the expression levels of Cx26CM in Cos-7 cells as measured by western blot (pLLox3.7 vs. Cx26-shRNA, t-test p=0.74, pLLox3.7 n=3, Cx26-shRNA n=4). **d)** Co-electroporation of EYFP or Cx26CM with the Cx26-shRNA construct at fixed molar ratio of 0.67:1. Co-expression of Cx26CM significantly increases the fraction of cells that migrate to the cortical plate by E21 in medial and caudal sections (*** $p\leq0.0001$, t-test, EYFP n=4 brains, Cx26CM n=5). Values represent mean±s.e.

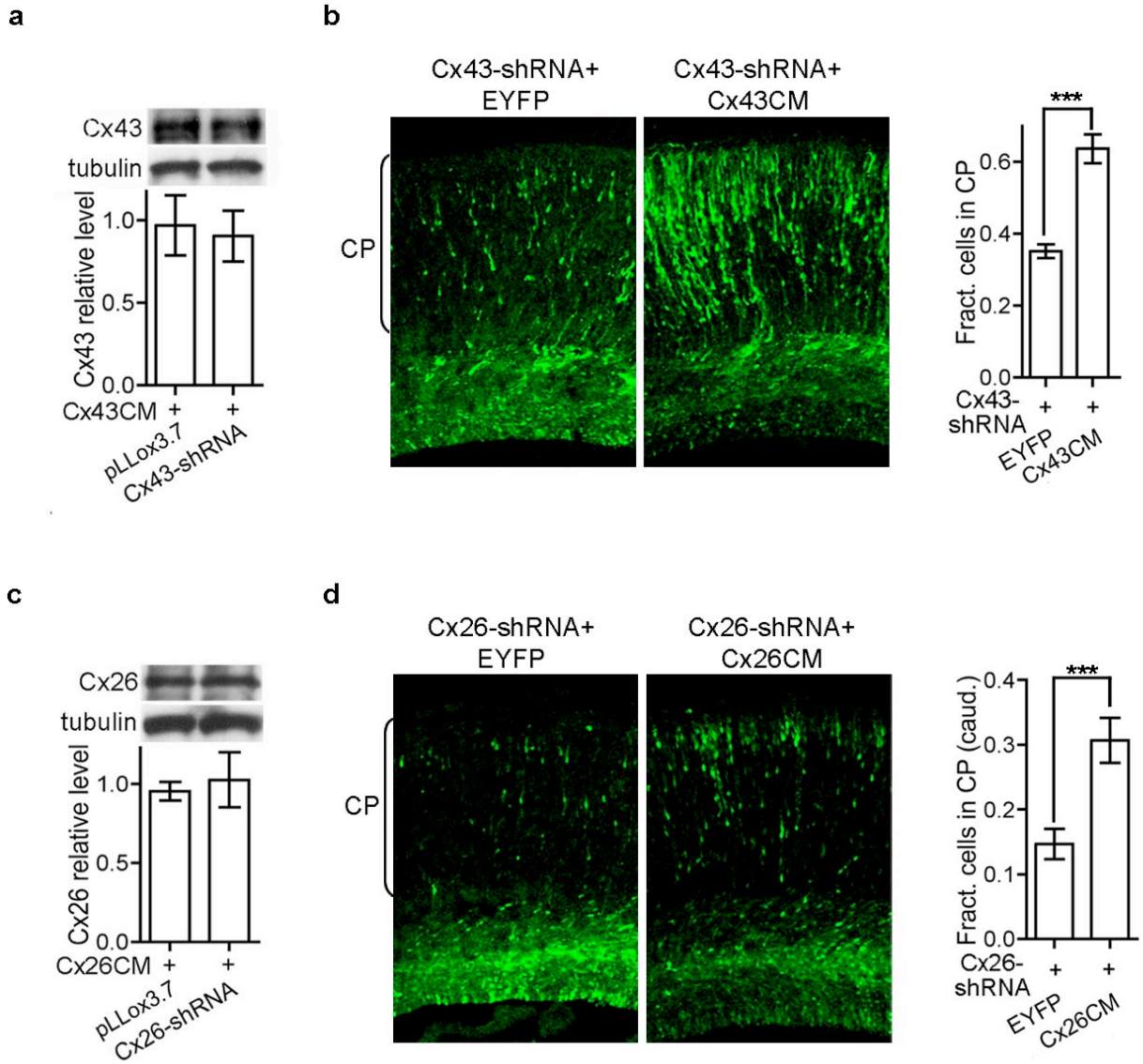

a
Cx43
tubulin
Cx43 relative level
1.0
0.5
0.0
Cx43CM + +
pLLox3.7
Cx43-shRNA
b
Cx43-shRNA+
EYFP
Cx43-shRNA+
Cx43CM
CP
***
Fract. cells in CP
0.6
0.4
0.2
0.0
Cx43-
shRNA + +
EYFP
Cx43CM
c
Cx26
tubulin
Cx26 relative level
1.0
0.5
0.0
Cx26CM + +
pLLox3.7
Cx26-shRNA
d
Cx26-shRNA+
EYFP
Cx26-shRNA+
Cx26CM
CP
***
Fract. cells in CP (caud.)
0.4
0.3
0.2
0.1
0.0
Cx26-
shRNA + +
EYFP
Cx26CM

Figure 9. Cx26-shRNA and Cx43-shRNA alternative targets (AT) phenocopy the migration defect

a, Empty vector (pLLox3.7) or Cx26AT-shRNA was co-expressed with Cx26 in Cos-7 cells. Cx26AT-shRNA significantly reduced Cx26 expression by western blot (* $p \leq 0.01$, t-test, n=8). **b,** Representative section from a brain electroporated at E16 with Cx26AT-shRNA and fixed at E21. The majority of the cells fail to migrate to the cortical plate. **c,** Empty vector (pLLox3.7) or Cx43AT-shRNA was co-expressed with Cx43 in Cos-7 cells. Cx43AT-shRNA significantly reduced Cx43 expression by western blot (** $p < 0.001$, t-test, n=3). **d,** Representative section from a brain electroporated at E16 with Cx43AT-shRNA and fixed at E21. The majority of the cells fail to migrate to the cortical plate. Cells appear to remain closer to the ventricular surface, which may be due to the increased knockdown observed with this construct compared with the original Cx43-shRNA construct. Values represent mean±s.e.

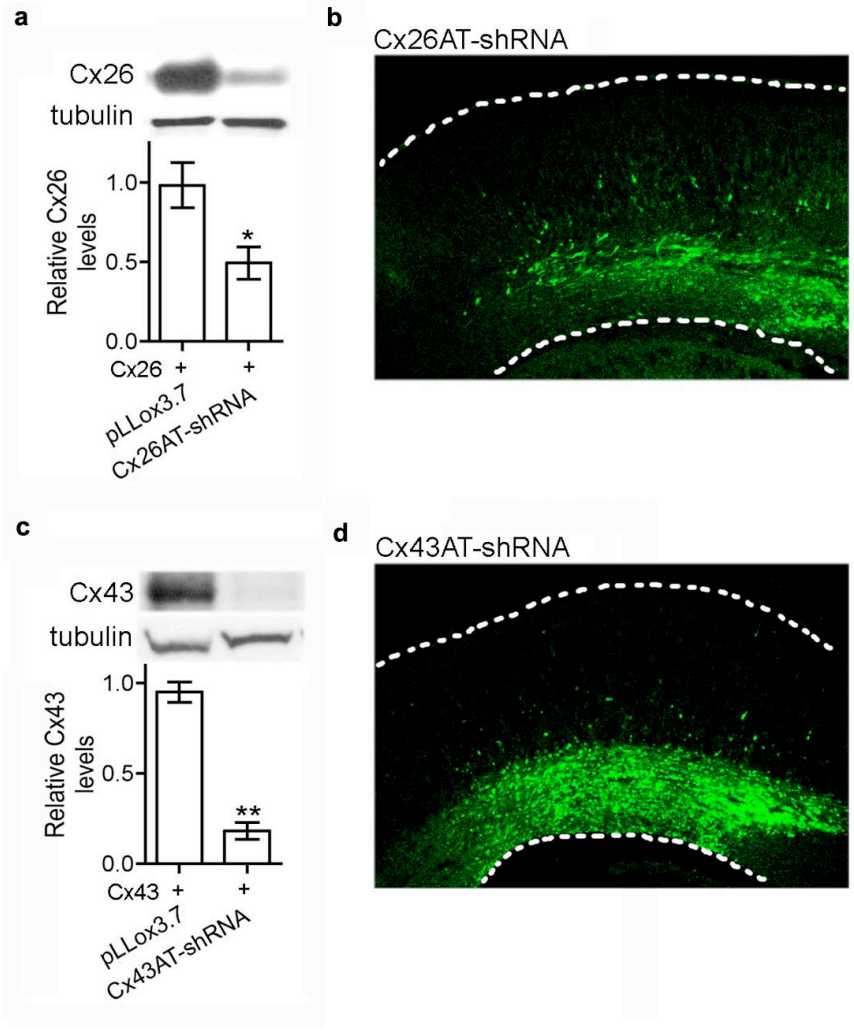

a
Cx26
tubulin
Relative Cx26 levels
1.0
0.5
0.0
*
Cx26 + +
pLLox3.7
Cx26AT-shRNA
b
Cx26AT-shRNA
c
Cx43
tubulin
Relative Cx43 levels
1.0
0.5
0.0
**
Cx43 + +
pLLox3.7
Cx43AT-shRNA
d
Cx43AT-shRNA

Figure 10. Electroporation of Cx26-shRNA or Cx43-shRNA does not disrupt the radial glial scaffold

a, Immunohistochemistry for the radial glial marker Nestin (red) was performed in brains electroporated at E16 and fixed at E21. No change in the appearance of the radial glial scaffolding was observed. **b**, In order to determine if wild type neurons could migrate on an shRNA background, wild type neurons born in the same time period as the electroporated neurons were labeled with 3 pulses of BrdU (20mg/pulse) spaced 24hrs apart, starting at the time of electroporation. The population of GFP^-, $BrdU^+$ neurons did not have a significant change in distribution, suggesting that the wild type neurons are, for the most part, unaffected by Cx knockdown in neighbouring cells ($p \geq 0.05$, 2-way ANOVA). Such neurons are likely able to migrate on untransfected radial glial fibres. Values represent mean±s.e.

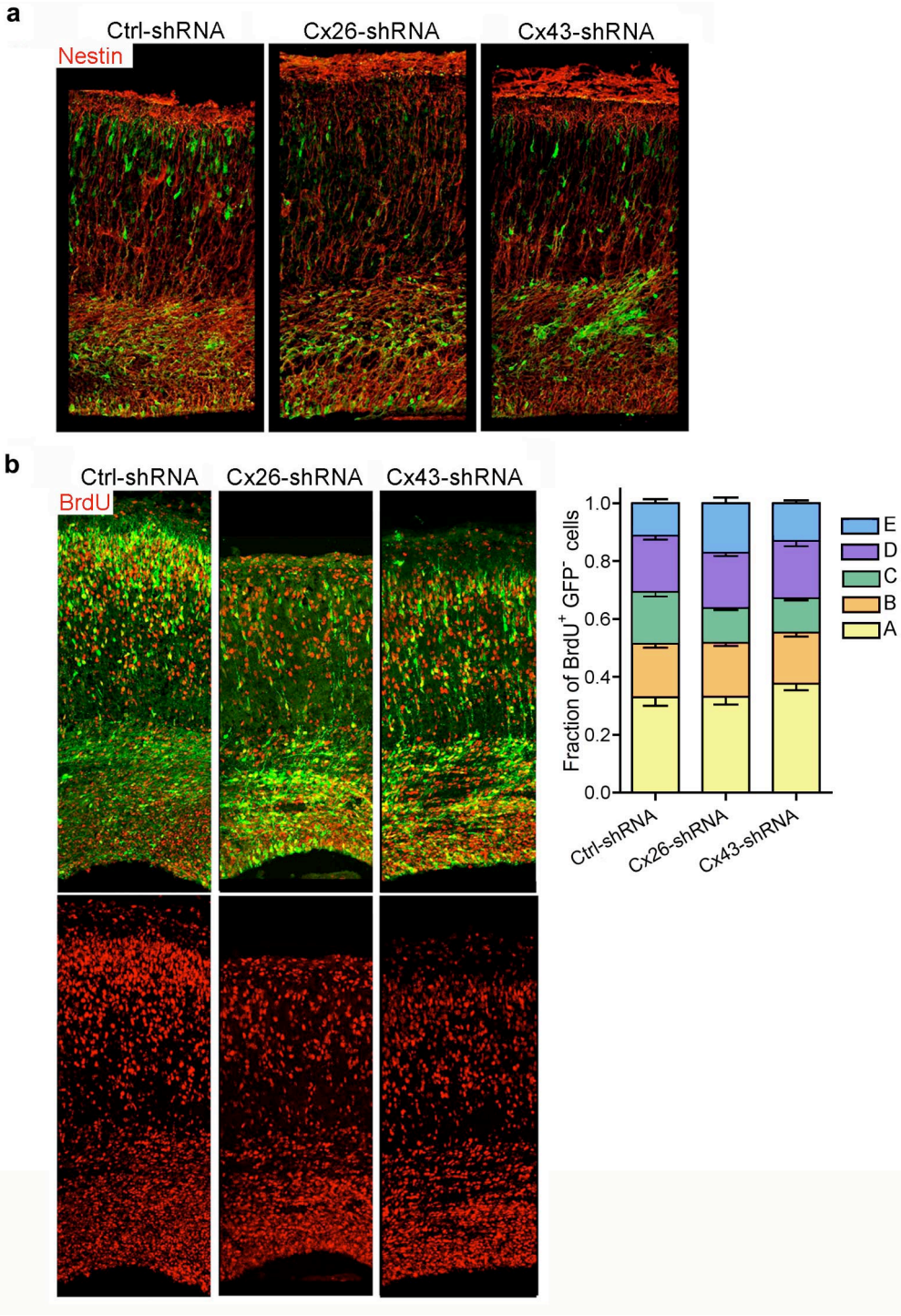

a
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
Nestin
b
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
BrdU
Fraction of BrdU+ GFP- cells
1.0
0.8
0.6
0.4
0.2
0.0
E
D
C
B
A
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA

Figure 11. Cx expression in neurons is necessary for migration

a, Transplant assays were performed to determine if shRNA expressing neurons were able to migrate on wild type radial glia. Brains were electroporated at E16, GFP^+ cells were microdissected at E17 and 50,000 cells per brain were injected into WT E17 embryos and fixed at E21. **b,** Control-shRNA cells transplanted into a WT host brain are able to integrate and migrate while transplanted Cx26-shRNA or Cx43-shRNA cells have reduced migration capacity (Chi-square: Ctrl. vs. shRNA, A $p<0.0001$, B $p<0.0001$, C $p>0.05$, D $p<0.0001$, E $p<0.000$, Ctrl-shRNA n= 225 cells, Cx26-shRNA n=421, Cx43-shRNA n= 698).

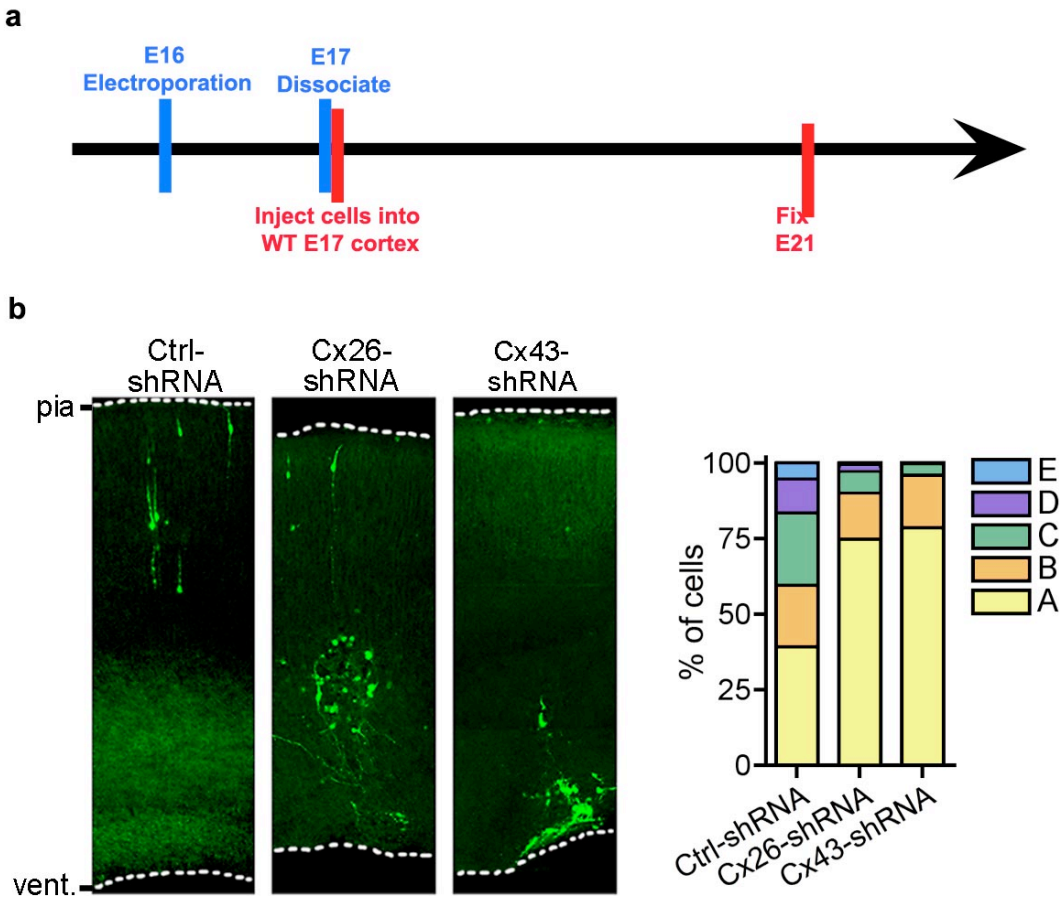

a
E16
Electroporation
E17
Dissociate
Inject cells into
WT E17 cortex
Fix
E21
b
Ctrl-
shRNA
Cx26-
shRNA
Cx43-
shRNA
pia
vent.
100
75
50
25
0
% of cells
E
D
C
B
A
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA

Figure 12. Electroporation of Cx26-shRNA or Cx43-shRNA does not produce a change in cell cycle exit, the proportion of differentiating cells, or cell death

a, Electroporation was performed at E16, a BrdU pulse was administered 24 hrs later, and brains were fixed 48 hrs post-electroporation. Immunohistochemistry for BrdU, Ki67, and GFP was performed. The percent of GFP^+, $BrdU^+$ cells (in the cell cycle when BrdU was administered) that were also $Ki67^-$ (no longer in the cell cycle) was calculated in rostral, medial, and caudal sections from at least three brains per condition. There was no significant difference in the percent of cells exiting the cell cycle (Chi-square: Ctrl-shRNA vs. Cx26-shRNA p= 0.08, Ctrl-shRNA vs. Cx43-shRNA p=0.53, Ctrl-shRNA n=1802, Cx26-shRNA n=1805, Cx43-shRNA n=1849). **b,** Electroporation at E16 of Cx26-shRNA or Cx43-shRNA does not produce a change in the percentage of $Ki67^+$ cells in the cell cycle or β-III $tubulin^+$ differentiating neurons at E21 as quantified in rostral, medial, and caudal sections from at least 3 brains per condition (Chi-square: Ctrl-shRNA vs. Cx26-shRNA p=0.21, Ctrl-shRNA vs. Cx43-shRNA p=0.81, Ctrl-shRNA n=1013, Cx26-shRNA n=839, Cx43-shRNA n=823). **c,** Electroporation was performed at E16 and brains were fixed at E18. TUNEL staining was performed on rostral, medial, and caudal sections from at least three brains per condition. No difference in the percent of $TUNEL^+$ cells was observed (Chi-square: Ctrl-shRNA vs. Cx26-shRNA p=0.76, Ctrl-shRNA vs. Cx43-shRNA p=0.55, Ctrl-shRNA n=2226, Cx26-shRNA n=2492, Cx43-shRNA n=2237). TUNEL staining was also performed at E21, however due to the extremely low number of $TUNEL^+$ cells in all conditions at this age the proportions were not quantified; no difference was qualitatively observed.

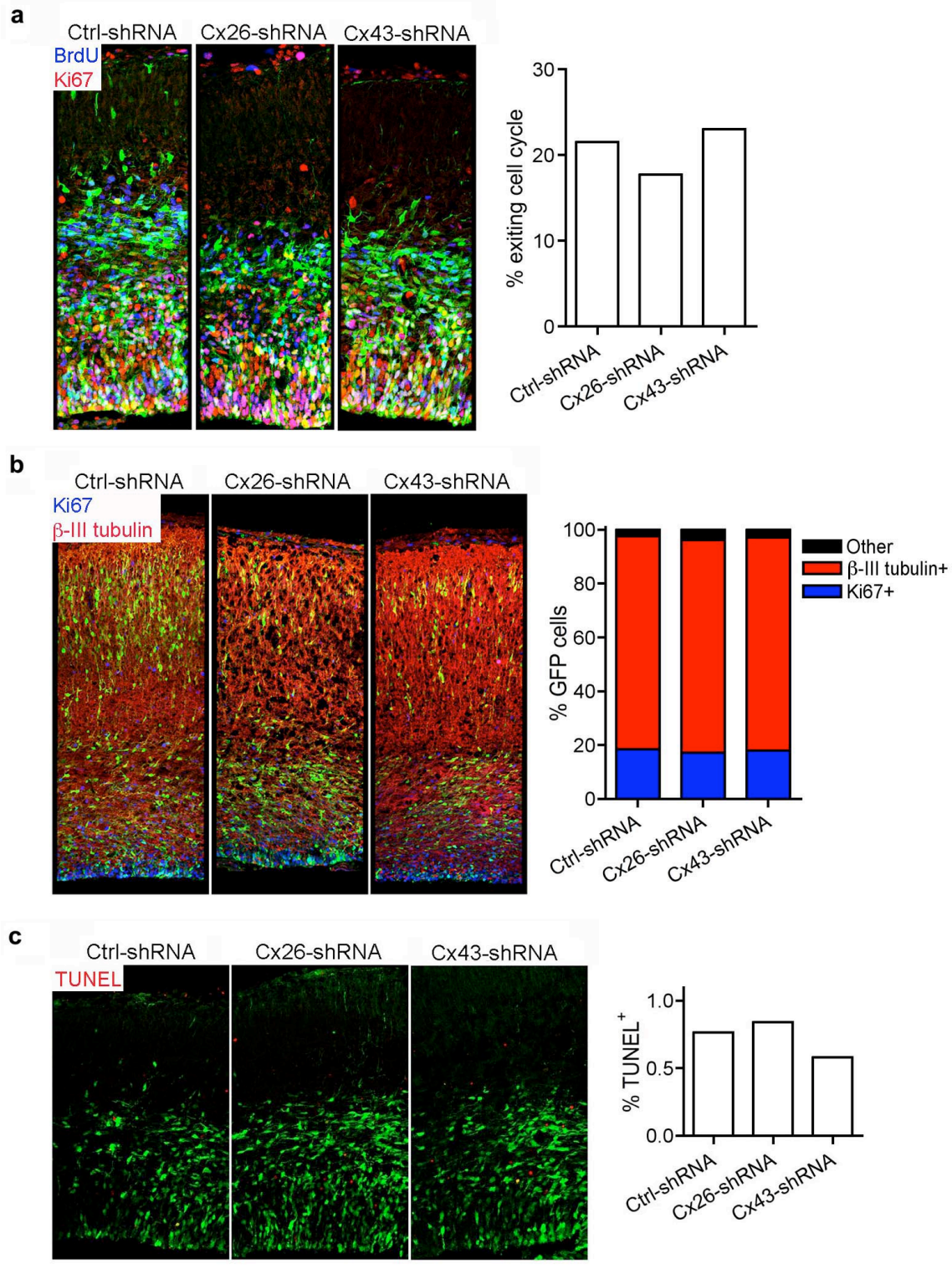

a
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
BrdU
Ki67
% exiting cell cycle
30
20
10
0
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
b
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
Ki67
β-III tubulin
% GFP cells
100
80
60
40
20
0
Other
β-III tubulin+
Ki67+
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
c
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
TUNEL
% TUNEL+
1.0
0.5
0.0
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA

Figure 13. Expression of N-Cadherin (N-Cad) and Zona Occludens-1 (ZO-1) in Cx-shRNA expressing cells is normal

a, Immunohistochemistry of coronal cortical sections two days after electroporation for the adherens junction component N-Cad (red) and the tight junction component ZO-1 (blue). **b**, Close-ups of the expression pattern of N-Cad (red) and ZO-1 (blue) in electroporated radial glial cells. **c,** Close-ups of the expression pattern of N-Cad (red) and ZO-1 (blue) in electroporated migrating neurons. No clear differences were observed in expression patterns. Images taken at 'high resolution'. Scale bar,10μm.

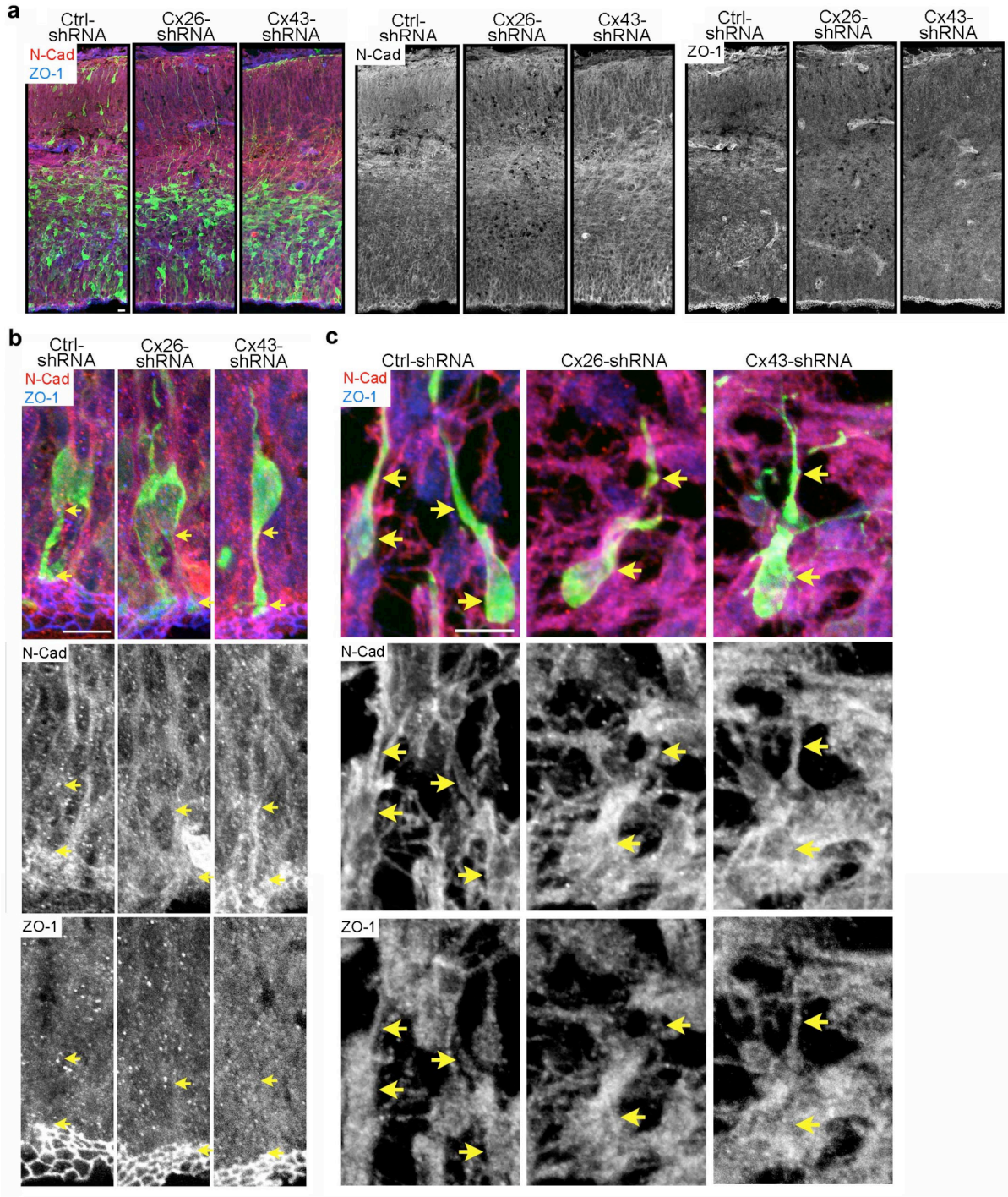
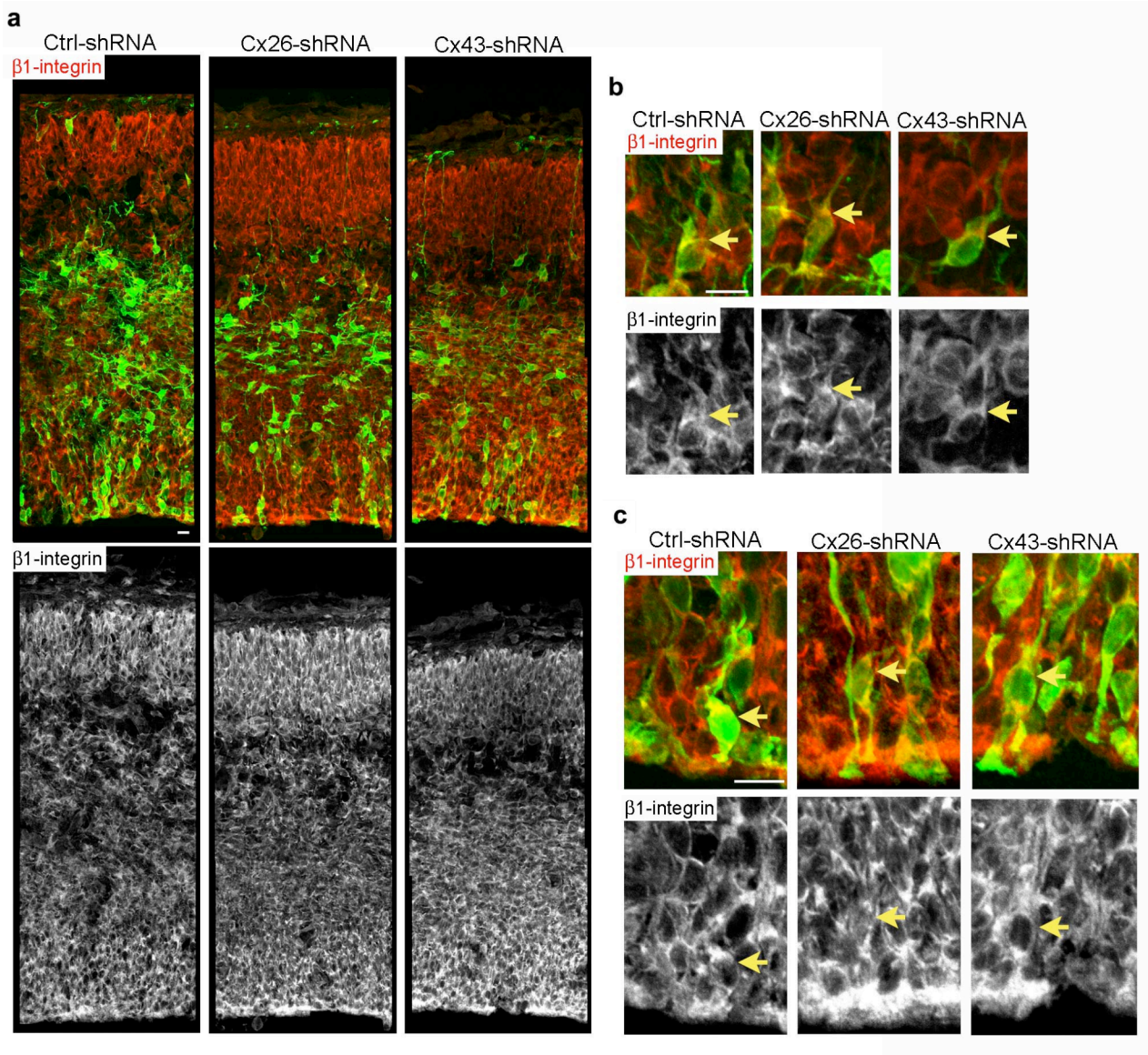

a
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
N-Cad
ZO-1
b
c
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA

Figure 14. Expression of β1-integrin in Cx-shRNA expressing cells is normal

a, Immunohistochemistry of cortical coronal sections two days after electroporation for the adhesion molecule β1-integrin (red). **b**, Close-ups of the expression pattern of β1-integrin (red) in electroporated migrating neurons. **c**, Close-ups of the expression pattern of β1-integrin (red) in electroporated radial glial cells. No clear differences were observed in expression patterns. Images taken at 'high resolution'. Scale bar, 10μm.


a
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
β1-integrin
β1-integrin
b
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
β1-integrin
β1-integrin
c
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
β1-integrin
β1-integrin

Chapter 4:

The adhesive properties of gap junctions mediate neuronal migration

Introduction: gap junctions and migration

Gap junctions have been previously described to play a role in the migration of other cell types including neural crest cells and gliomas. Neural crest cells migrate in sheets away from the dorsal neuroepithelium to produce a variety of cell types including the peripheral nervous system and cardiac tissues. In fact, genetic deletion of Cx43 causes perinatal lethality due to conotruncal heart malformations and pulmonary outflow obstruction (Reaume et al., 1995). Interestingly, transgenic overexpression of Cx43 in neural crest cells also leads to similar heart malformations (Ewart et al., 1997; Huang et al., 1998). Cx43 is expressed in migrating neural crest cells, and genetic deletion of Cx43 inhibits neural crest migration while transgenic overexpression of Cx43 enhances migration (Lo et al., 1997; Lo et al., 1999). Interestingly, studies that dissociate Cx43 expression and dye coupling have shown that Cx43 expression levels, not dye coupling levels, predict migration behavior (Xu et al., 2006; Xu et al., 2001). Wnt-1 deficient neural crest cells, which express Cx43 but are not dye coupled, do not have any change in cell motility; the authors hypothesize that interactions between Cx43 and p120 catenin may modulate cell motility (Xu et al., 2001). Further studies suggest that Cx43 may interact with vinculin and the actin cytoskeleton as well as modulating cellular responses to integrin and semaphorin 3A signaling (Xu et al., 2006).

The spread of astrocytic tumors, gliomas, into the brain parenchyma is also enhanced by Cx43 expression (Lin et al., 2002; Oliveira et al., 2005). Comparisons between a rat glioma line that does not express gap junctions and the same line engineered to express Cx43 demonstrates that Cx43 expression is necessary and sufficient for the invasion of the brain parenchyma rather than restricted migration along

the vasculature (Lin et al., 2002). Mechanistically, this study demonstrated that the adhesive properties of Cx43 are necessary for migration although the authors also concluded that the formation of functional channels with astrocytes were also necessary (Lin et al., 2002). However, the hypothesis that functional channels with astrocytes are necessary for migration is based on the use of a Cx40 chimera that has a closed channel, but that also does not make gap junction contacts with Cx43 (Haubrich et al., 1996), the Cx primarily expressed in astrocytes, and thus this conclusion should be revisited. Further studies will be necessary to clarify the mechanism by which gap junctions modulate cell motility and migration in both neural crest cell migration as well as glioma cell migration.

What is the mechanism by which gap junctions mediate neuronal radial migration? We sought to differentiate among three plausible functions. Gap junctions can electrically and chemically couple cells to allow the exchange of current or small molecules, mediate extracellular release of substrates such as ATP through hemichannels, provide an adhesive contact between two cells, or/and mediate signaling (Figure 1).

Adhesions, not channels, mediate migration

In order to elucidate the mechanism by which gap junctions mediate the radial migration of neurons in the cortex, selective rescue experiments were performed in which Cx-shRNA was co-electroporated with Cx constructs having various functional properties. We previously established that the Cx43-shRNA and Cx26-shRNA induced migration defect can be rescued by co-expression of Cx43CM or Cx26CM respectively (Chapter 3, Figure 8; Figure 4).

To determine whether functional channels are necessary to rescue the migration defect, a conserved tyrosine in the third transmembrane domain of Cx43CM and Cx26CM was mutated. These dominant negative Cx mutants are able to make adhesions but have a closed channel and thus are not able to mediate exchange between cells or with the extracellular environment (Cx43CMT154A, Cx26CMT135A) (Beahm et al., 2006). Consistent with their previous characterization, Cx43CMT154A and Cx26CMT135A are able to form adhesive plaques at the junctions between cell membranes in Cos-7 cells but do not have open channels as measured by the ability of C6 glioma cells to uptake extracellular propidium iodide through hemichannels as well as by physiologically measuring the input resistance of C6 cells expressing Cx43CMT154A (Figure 2). Cx43CMT154A and Cx26CMT135A localize similarly to Cx43CM and Cx26CM when electroporated into the cortex (Figure 3). Remarkably, these mutants, that can make adhesions but not channels, rescued the migration of cells to the CP (Figure 4). The ability of the closed channel mutants to rescue the migration defect strongly suggests that the channel (either hemichannel or gap junction channel) does not contribute to the role of gap junctions in neuronal migration.

It has been shown that Ca^{2+} is important for neuronal migration (Komuro and Rakic, 1996) and that gap junctions mediate Ca^{2+} waves in the developing cortex by releasing ATP through hemichannels that activates purinergic P2Y1 receptors on radial glia (Weissman et al., 2004). Thus, to further test the possibility that hemichannel-mediated Ca^{2+} waves play a role in neuronal migration, Ca^{2+} waves were inhibited by pharmacologically antagonizing the P2Y1 receptor with suramin or by knocking down the P2Y1 receptor with multiple shRNA constructs. No migration defect was observed,

suggesting that hemichannel-mediated Ca^{2+} waves are not involved in cortical neuronal migration (Figure 5).

To determine whether the adhesive properties of gap junctions are necessary for migration, one of the conserved extracellular cystines was mutated (Cx43CMC61S) to produce a Cx43 channel that does not make adhesions and thus cannot form gap junctions (Lin et al., 2002). As previously reported Cx43CMC61S does not make adhesive plaques at the boundaries between Cos-7 cells (Figure 2). However Cx43C16S maintains a functional channel as assayed by the ability to uptake extracellular propidium iodide through hemichannles in C6 glioma cells as well as input resistance recordings (Figure 2). Cx43CMC16S differs from Cx43CM in its expression pattern when electroporated into the cortex as it is localized diffusely rather than in punctate junctions (Figure 3). Interestingly, Cx43CMC61S failed to rescue the Cx43-shRNA induced migration defect, suggesting that the adhesive properties of gap junctions are necessary for radial migration (Figure 4).

In addition, we wanted to determine whether signalling via carboxyl-terminal (c-terminal) interactions with cytosolic proteins are important. Cx26 has a short uncharacterized intracellular c-terminus, while Cx43 has a longer c-terminus that interacts with a variety of proteins including Zona Occludens-1, V-Src, and tubulin (Giepmans and Moolenaar, 1998; Giepmans et al., 2001; Lin et al., 2001). Both c-terminal truncations of the closed channel mutants (Cx43CMT154A 1-238 and Cx26CMT135A 1-216) were able to rescue the migration defect (Figure 4). These results suggest that signalling mediated by the cytoplasmic c-termini do not play a significant role during migration. However, the possibility remains that intermolecular interactions

with other parts of the Cx protein may be important. In summary, we found that adhesion, but not the channel or the c-terminus, is necessary for the role of gap junctions during neuronal migration.

Gap junctions provide adhesion

In order to demonstrate that gap junctions can mediate adhesion in cortical cells, we built upon previous work which showed that overexpression of Cx43, but not Cx43C61S, increases adhesion in C6 glioma cells (Lin et al., 2002), a cell type without endogenous gap junction expression (Naus et al., 1991). We used C6 cells with or without stable transfection of Cx43-EYFP (Lai et al., 2006) to provide a glial substrate upon which we could place cortical cells and test their ability to adhere. Cortical cells were electroporated with Ctrl-shRNA, dissected and FACS sorted for GFP, labelled with DiD, and plated on C6 cells or C6 cells expressing Cx43-EYFP (C6+Cx43). We found a 2-fold increase in the number of DiD^+ cells adhering to the C6+Cx43 substrate compared to the C6 substrate, suggesting that cortical cells can use endogenous Cx43 as a means of cell-cell adhesion (Figure 6a).

To test whether Cx43-shRNA reduces the ability of cells to adhere to the C6+Cx43 cells, Ctrl-shRNA or Cx43-shRNA FACS sorted cells were plated on C6 cells or C6+Cx43 cells. There was a significant reduction in the ratio of the number of Cx43-shRNA to Ctrl-shRNA cells on the C6+Cx43 substrate compared to the C6 substrate (Figure 6b). Thus, we show that cortical cells can use endogenous Cx43 to adhere to a glial substrate and that knocking down Cx43 reduces their ability to adhere to a Cx43 expressing substrate but not a control substrate.

Classical adhesion molecules typically provide a link with internal cytoskeletal components. Previous work in neural crest cells demonstrates that Cx43 co-localizes and co-immunoprecipitates with numerous actin binding proteins, and that filamentous actin is disorganized in neural crest cells from the Cx43 knockout mouse (Xu et al., 2006). To explore Cx-actin interactions, an Actin-cherry clone was co-electroporated with GFP. Electroporated Actin appears diffusely distributed in radial glia and new-born neurons and becomes punctate in multipolar SVZ cells and migrating neurons (Figure 7a). Co-electroporation of Cx26T135A or Cx43T154A (to avoid toxicity associated with over-expression of WT Cx, Figure 3b) with Actin-cherry revealed that both Cx26 and Cx43 puncta frequently co-localize with the Actin puncta, with the Cx puncta slightly offset (Figure 7b). Interestingly, cells expressing Cx26-shRNA or Cx43-shRNA, have significantly fewer Actin puncta than Cont-shRNA expressing cells (Figure 7c). This suggests that both Cx26 and Cx43 can interact with the actin cytoskeleton.

Connexin dynamics during migration

To investigate the dynamic behaviour of migrating neurons, we performed time-lapse live imaging. We observed that control migrating neurons typically start with a bifurcated leading process, one of which prevails, and the neuron's nucleus then translocates forward into a dilation that forms in the leading process (Edmondson and Hatten, 1987; Komuro and Rakic, 1995; Noctor et al., 2001; Rakic, 1978; Schaar and McConnell, 2005; Tsai et al., 2007) (Figure 8a). Interestingly, migrating neurons with multiple leading processes can show differential expression of Cx43 or Cx26 in these processes (Figure 9a). Hence, we hypothesized that gap junction adhesions play a role in stabilizing the leading process along a radial glial fiber. Indeed, Cx43-shRNA and Cx26-

shRNA expressing neurons are unable to stabilize their processes and continue to extend multiple branches (Figure 8b). This phenotype is reminiscent of neural crest cells from Cx43KO mice that show increased protrusive activity but decreased directional migration (Xu et al., 2006).

In order to analyze the dynamics of Cx puncta during neuronal migration, the Cx43T154A-EYFP or the Cx26T135A-EYFP plasmid was co-electroporated with Tomato to visualize the cells, and followed by time-lapse microscopy. The closed pore Cx mutants were expressed in radial glia and migrating neurons in a pattern that resembled WT expression, but avoided the toxicity associated with the overexpression of WT Cx (Figure 3). Furthermore, because these mutants were sufficient to rescue migration, we can assume that they behave similarly to WT Cxs with respect to neuronal migration.

The localization of Cx43T154A puncta in the branches of bifurcated leading processes was predictive of the dominant branch that would be maintained over time, and the absence of puncta was predictive of the transient branch (Figure 9b,c). In the rare case in which a radial fiber was visible, stabilization of the branch along the fiber was observed (Figure 9b, Cell 2). When Cx26T135A was observed in the branches of bifurcated neurons, it also localized to the dominant branch (Figure 9d); however, Cx26T135A was not frequently present in the branches. Cx26T135A was often localized to puncta in the cell body. Interestingly, Cx26T135A puntca in the cell body move into the swelling prior to the movement of the nucleus in approximately 81% of translocation events (n=56) (Figure 10a,b). Cx43T154A puncta found in the cell body

followed a similar pattern in approximately 57% of translocation events (n=24) (Figure 10d). This stereotyped pattern of Cx puncta movement, which mimics the pattern of centrosome movement, suggests that gap junctions may provide the hypothesized adhesion contact associated with the centrosome in the dilation of translocating neurons (Schaar and McConnell, 2005; Tsai et al., 2007). This hypothesis is supported by the observation that Cx26T135A and Cx43T154A puncta co-localize with Centrin II, where the Cx puncta are slightly offset towards the cell membrane (Figure 10c,e). Other patterns of Cx dynamics in the trailing process and between radial fibers and neurons were also observed (Figure 11). Overall, the dynamics of Cx puncta localization and of shRNA electroporated cells suggest that both Cx43 and Cx26 play important roles in leading process stabilization and nuclear translocation, with Cx26 playing a more prominent role at the soma and Cx43 in the branches. Such a functional separation is consistent with the observation that Cx26 and Cx43 do not make heteromeric gap junctions, and localize to separate domains in gap junction plaques (Falk, 2000) (Chapter 3, Figure 4).

Figure 1. Gap junction functions: coupling, hemichannels, adhesion and signalling

Gap junctions have a variety of functions: (1) Cell coupling: the classical action of gap junctions is to allow electrical current, small molecules, metabolites or ions to travel between cells, including but not limited to cAMP, ATP, IP3, glucose, glutamate, Ca^{2+} and K^{+}. (2) Hemichannels: in addition to forming the opposing subunits for gap junctions, hemichannels can also exist in an unopposed form on the cell membrane, thus mediating exchange with the extracellular environment. It was originally thought that hemichannels would remain in a closed state because of high levels of extracellular Ca^{2+}; however, recent evidence suggests that hemichannels mediate functional release of small molecules such as ATP under physiological conditions (Goodenough and Paul, 2003). (3) Adhesion: the formation of gap junctions between adjacent cells can provide an adhesive force between cells and interact with the internal cytoskeleton (Lin et al., 2002). (4) Signaling: gap junctions, especially the C terminus of the Cx subunit, have been implicated in junction-independent cell-signaling functions (Giepmans, 2004).

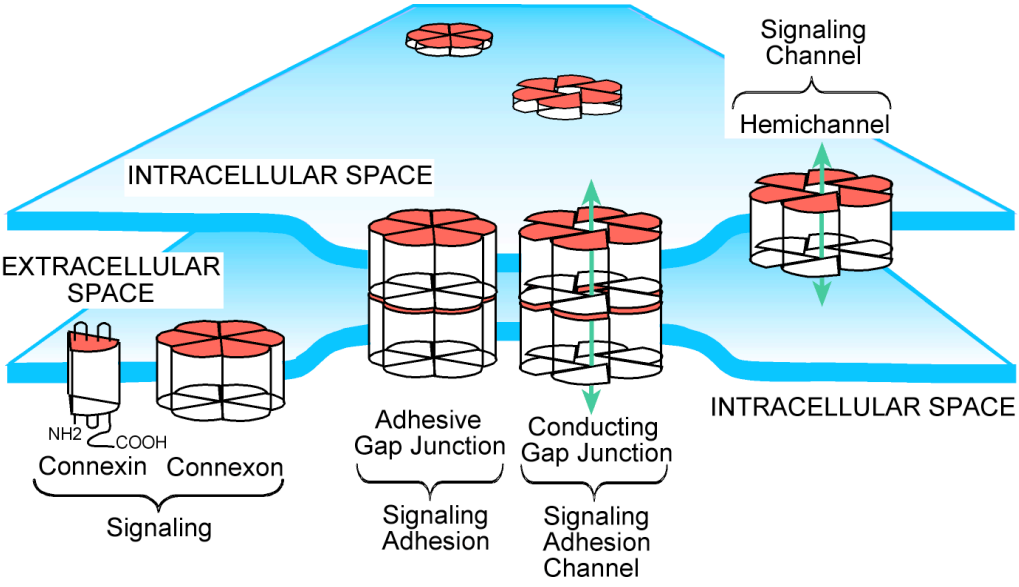

Signaling
Channel
Hemichannel
INTRACELLULAR SPACE
EXTRACELLULAR
SPACE
INTRACELLULAR SPACE
NH2
COOH
Connexin
Connexon
Signaling
Adhesive
Gap Junction
Signaling
Adhesion
Conducting
Gap Junction
Signaling
Adhesion
Channel

Figure 2. Point mutations and truncations in the Cx26-EYFP and Cx43-EYFP clones.

a, Summary of point mutations and their functional consequences. The introduction of conservative mutations (CM) in the region targeted by the shRNA does not change the amino acid sequence and thus has no functional consequences. The C61S mutation in Cx43 impairs the ability to make adhesions (Lin et al., 2002) but allows the formation of hemichannels (e). The dominant negative T154A mutation in Cx43 and T135A mutation in Cx26 allow the formation of functional adhesions but have a closed channel (Beahm et al., 2006). The c-terminus, which has been shown to mediate intracellular signaling, especially in Cx43, was removed to prevent intracellular signaling (Cx43CMT154A 1-238, Cx26CMT135A 1-216). **b**, Expression of Cx26 variants in Cos-7 cells. All constructs are able to form gap junction plaques (red arrows). **c**, Expression of Cx43 variants in Cos-7 cells. All variants, except the C61S point mutant, are able to form gap junction plaques (red arrows). **d, e,** C6 glioma cells were used to test the ability of the Cx mutants to form hemichannels, as C6 cells do not express endogenous Cx proteins (Naus et al., 1991). 24 hrs after transfection, cells were loaded with propidium iodide (Lai et al., 2006). Cells were then washed in PBS, fixed, and the number of loaded cells was quantified. Cx26CM but not Cx26CMT135A is able to form hemichannels (d). Cx43CM and Cx43CMC61S are able to form hemichannels while Cx43CMT154A is not (e). The percent of cells loaded with Cx43CMC61S is lower than that of Cx43CM, probably because in the case of Cx43C61S dye cannot pass from cell to cell through gap junctions and thus loads cells sporadically rather than in clumps as seen in the Cx43CM condition (*** $p<0.0001$, Chi-square, EYFP n=1106, Cx26 n=615, Cx26T135A n=1125, Cx43CM

n=1135, Cx43CMC61S n=1285, Cx43CMT154A n=1193). **f**, The input resistance (R_i) of C6 cells 24hrs after transfection was assayed with whole cell voltage clamp recordings holding the cells at -70mV. Cells expressing Cx43CMT154A have a high R_i, similar to cells expressing EYFP, suggesting that Cx43CMT154A does not make open channels. Cx43CM and Cx43CMC61S expressing cells have a low R_i compared to EYFP expressing cells, suggesting that they do make open channels. Values represent means $\pm$ s.e.

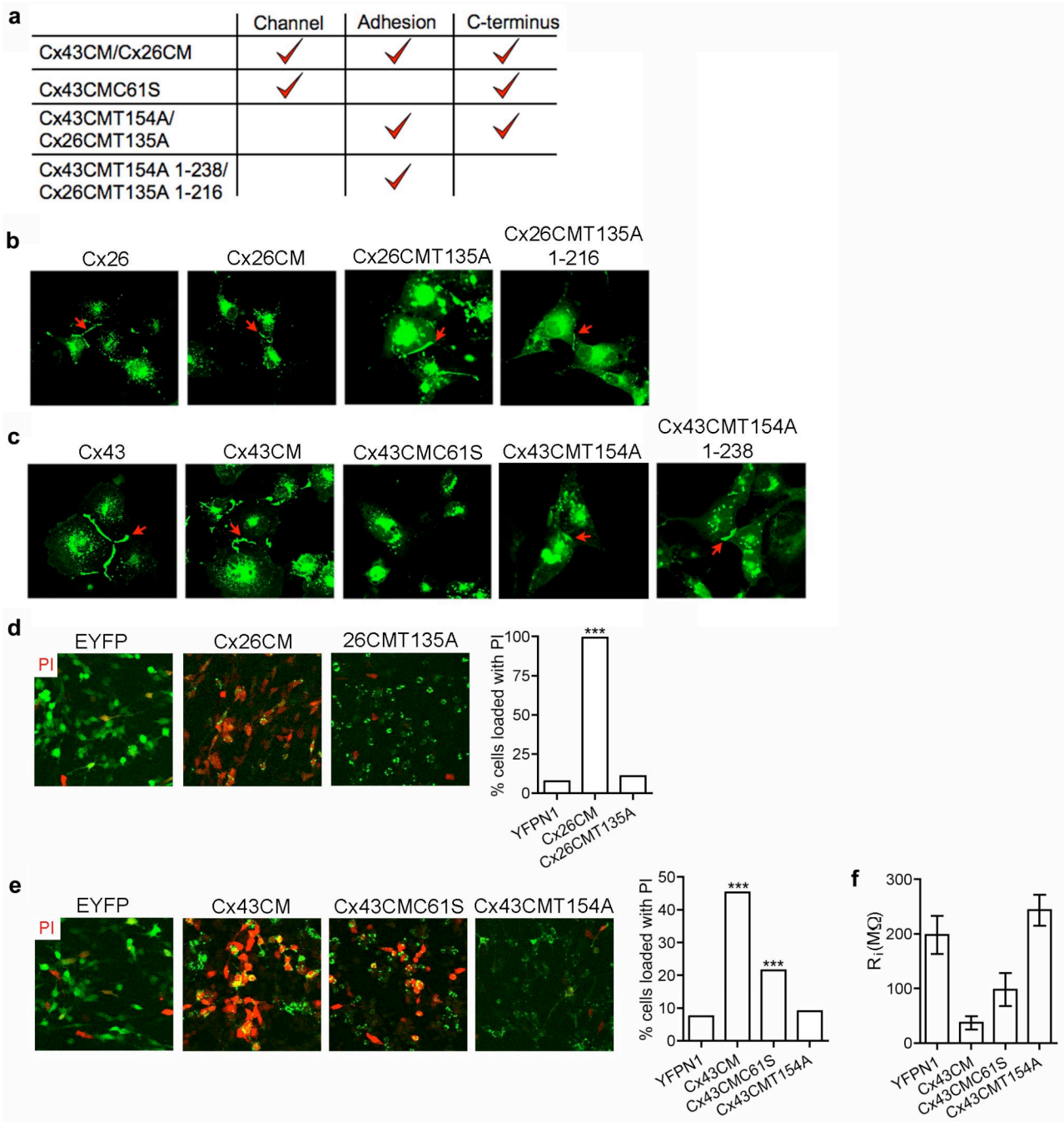

a
Channel
Adhesion
C-terminus
Cx43CM/Cx26CM
Cx43CMC61S
Cx43CMT154A/
Cx26CMT135A
Cx43CMT154A 1-238/
Cx26CMT135A 1-216
b
Cx26
Cx26CM
Cx26CMT135A
Cx26CMT135A
1-216
c
Cx43
Cx43CM
Cx43CMC61S
Cx43CMT154A
Cx43CMT154A
1-238
d
EYFP
PI
Cx26CM
26CMT135A
% cells loaded with PI
***
YFPN1
Cx26CM
Cx26CMT135A
e
EYFP
PI
Cx43CM
Cx43CMC61S
Cx43CMT154A
% cells loaded with PI
***
***
YFPN1
Cx43CM
Cx43CMC61S
Cx43CMT154A
f
Ri (MΩ)
YFPN1
Cx43CM
Cx43CMC61S
Cx43CMT154A

Figure 3. Expression patterns of Cx26-EYFP and Cx43-EYFP clones in the cortex. Cx26-EYFP and Cx43-EYFP clones were co-electroporated with Tomato to visualize the cells. **a**, Expression of Cx clones 24hrs after electroporation at E16. All clones except Cx43CMC61S are expressed in a punctate pattern reflecting their ability to make adhesive junctions. Cx43CMC61S is expressed in a more diffuse pattern (*). **b**, Expression of clones 3 days after electroporation at E16. Only the closed pore Cx mutants and closed pore c-terminal truncation mutants can be visualized 3 days after electroporation, as the other pore forming constructs are lethal presumably due to the formation of excessive hemichannels. Cx43CMT154A puncta were distributed throughout the cortex but concentrated in the VZ, whereas Cx26CMT135A puncta were distributed more evenly, similar to the endogenous expression patterns (data not shown). In neurons, Cx26CMT135A and Cx26CMT135A 1-216 puncta are primarily localized in the leading portion of the cell body although they are also occasionally seen in the leading and trailing processes (also see Figures 9-11). Cx43CMT154A and Cx43CMT154A 1-238 puncta are localized in the cell body and the leading and trailing processes (also see Figures 9-11). Images taken at 'high resolution'. Scale bar, 10μm.

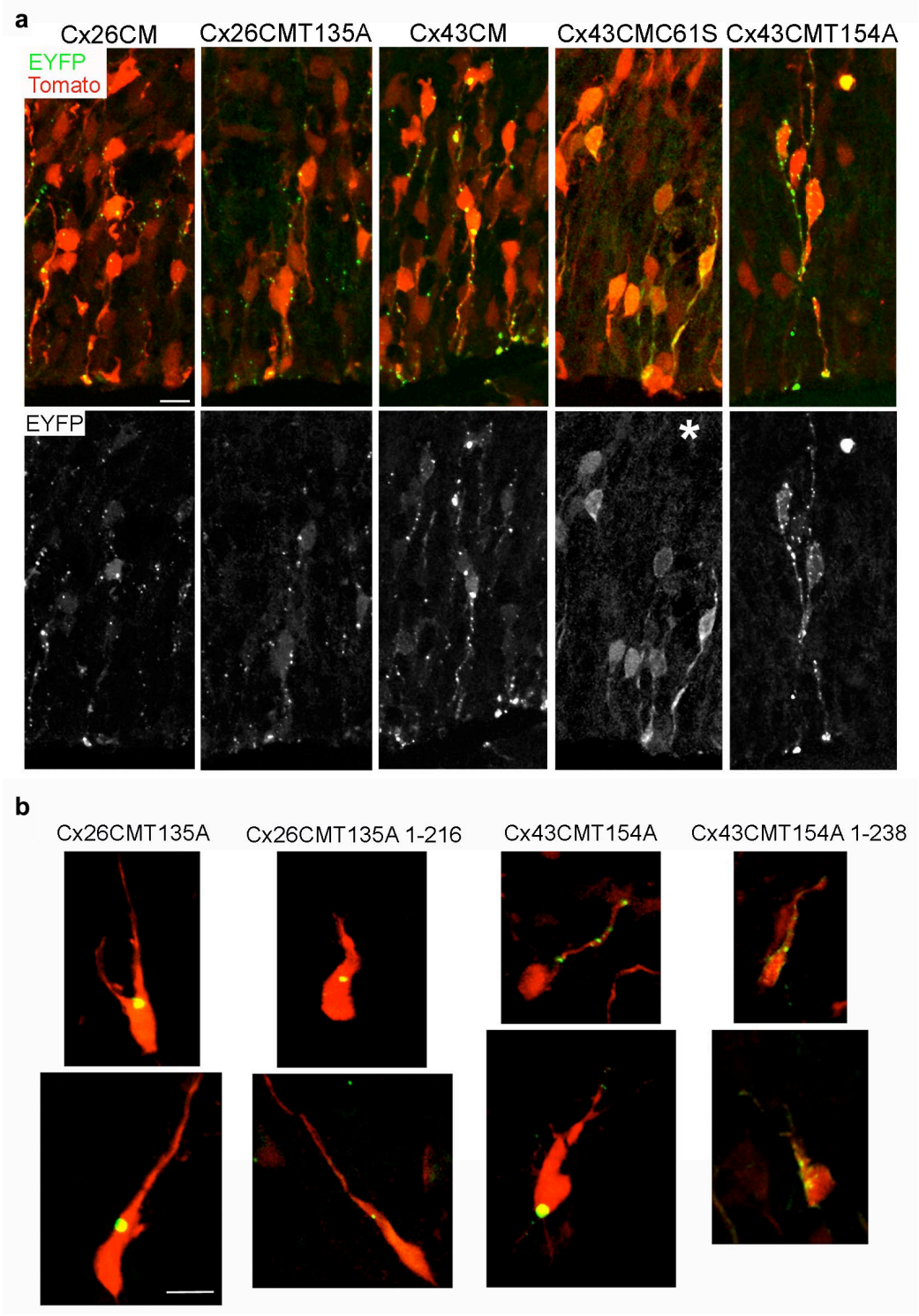
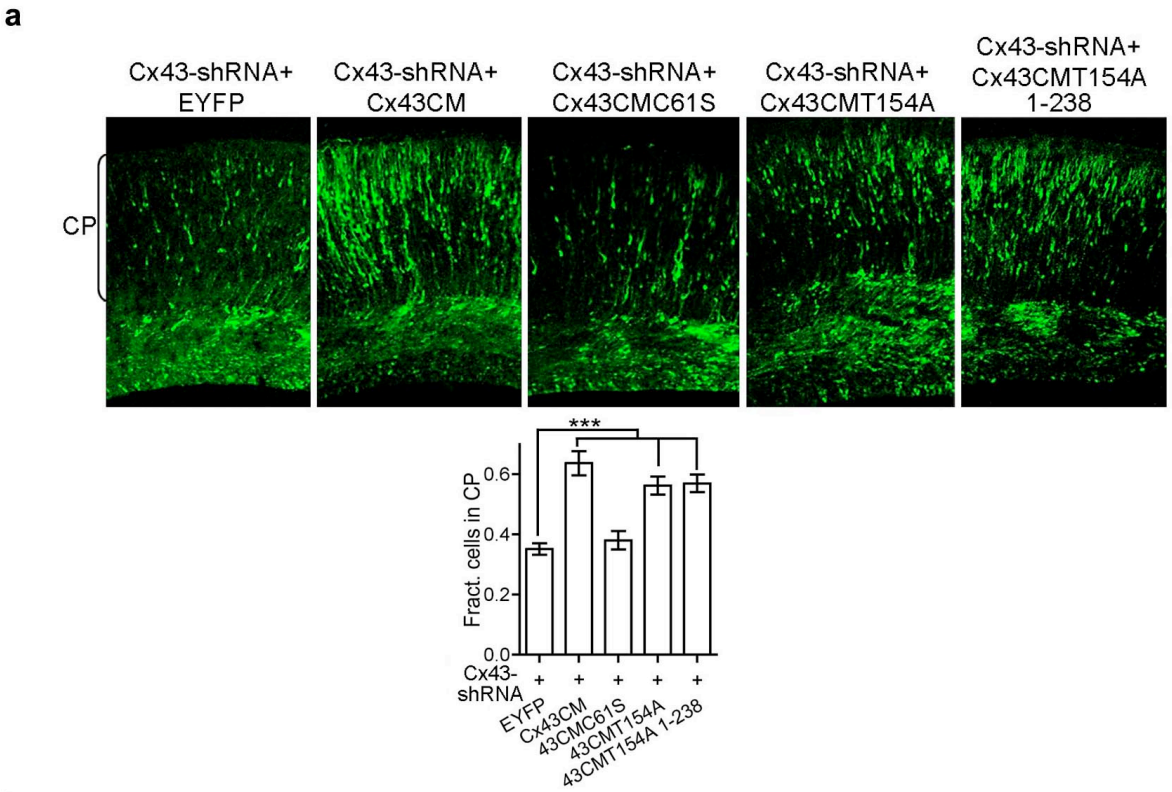

a
Cx26CM
Cx26CMT135A
Cx43CM
Cx43CMC61S
Cx43CMT154A
EYFP
Tomato
EYFP
*
b
Cx26CMT135A
Cx26CMT135A 1-216
Cx43CMT154A
Cx43CMT154A 1-238

Figure 4. Rescue of Cx-shRNA induced migration phenotype by Cx mutants that make adhesions but not channels.

a, Cx43CM (channel, adhesion, c-terminus), Cx43CMT154A (adhesion, c-terminus), or Cx43CMT154A 1-238 (adhesion) significantly increased the fraction of cells in the cortical plate compared to EYFP when co-expressed with Cx43-shRNA (*** $p<0.0001$, t-test, EYFP n=6 brains, Cx43CM n=4, Cx43CMT154A n=5, Cx43CMT154A 1-238 n=6). Cx43CMC61S (hemichannel, c-terminus) did not rescue the migration defect (EYFP vs. Cx43CMC61S, t-test p=0.4727, Cx43C61S n=4 brains). **b,** Cx26CM (channel, adhesion, c-terminus), Cx26CMT135A (adhesion, c-terminus), or Cx26CMT135A 1-216 (adhesion) increased the fraction of cells in the cortical plate compared to EYFP when co-expressed with Cx26-shRNA (*** $p\leq0.0001$, t-test, EYFP n=4 brains, Cx26CM n=5, Cx26CMT135A n=5, Cx26CMT135A 1-216 n=5). (CP = cortical plate). Values represent means±s.e.


a
Cx43-shRNA+ EYFP
Cx43-shRNA+ Cx43CM
Cx43-shRNA+ Cx43CMC61S
Cx43-shRNA+ Cx43CMT154A
Cx43-shRNA+ Cx43CMT154A 1-238
CP
***
Fract. cells in CP
0.0
0.2
0.4
0.6
Cx43-shRNA
+ + + + +
EYFP
Cx43CM
43CMC61S
43CMT154A
43CMT154A 1-238

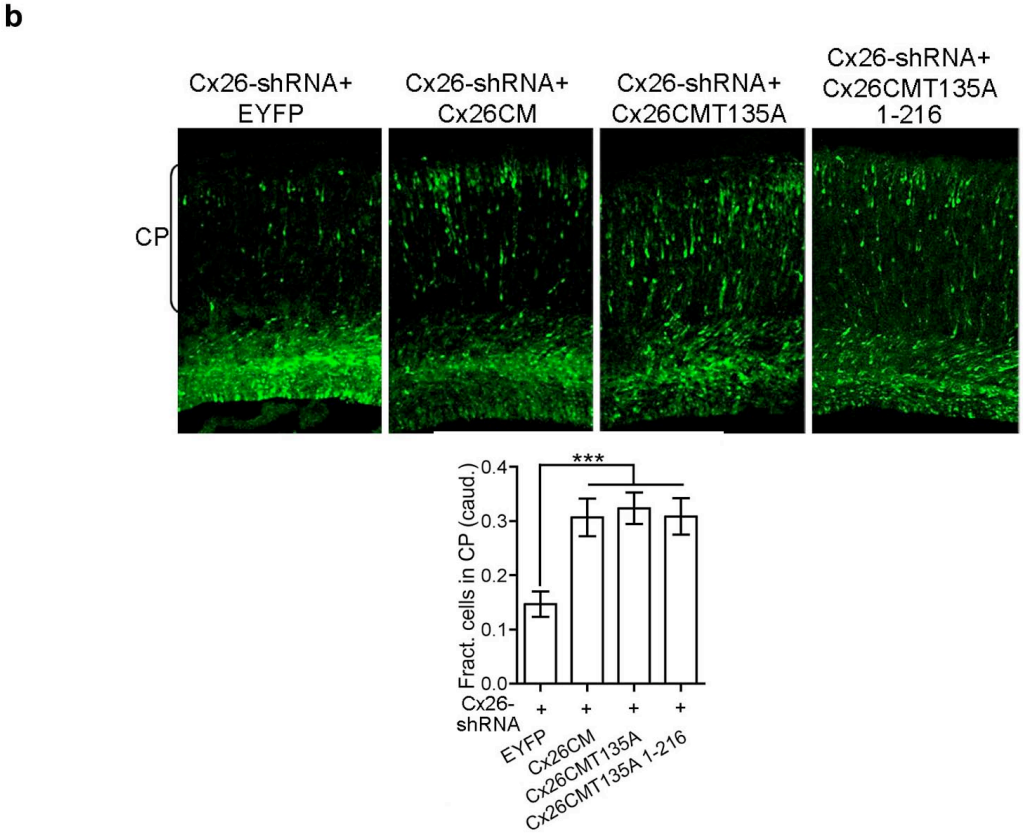

b
Cx26-shRNA+ EYFP
Cx26-shRNA+ Cx26CM
Cx26-shRNA+ Cx26CMT135A
Cx26-shRNA+ Cx26CMT135A 1-216
CP
***
Fract. cells in CP (caud.)
0.0
0.1
0.2
0.3
0.4
Cx26-shRNA
+ + + +
EYFP
Cx26CM
Cx26CMT135A
Cx26CMT135A 1-216

Figure 5. Blocking Ca^{2+} waves in the developing cortex does not inhibit neuronal migration a, Slices were electroporated with EGFP at E16 and organotypic slice cultures were made at E17. Half of the slices were incubated in the presence of suramin (100μm), the P2Y1 receptor antagonist, for 4 days and images were taken at t=0 and t=4 days (media was replaced on day two). **b,** The migration index was quantified by subtracting the fraction of cells in the top half of the cortex at t=0 (cells that had already migrated) from the fraction of cells in the top half of the cortex at t=4 days. No change was observed in the migration index ($p=0.36$, t-test, control n=14, suramin n=13). **c,** Three candidate shRNAs were tested for their ability to knock down the P2Y1 receptor by co-expression with P2Y1-HA in Cos-7 cells. Two constructs produced significant knockdown by western blot for the HA tag (** $p<0.001$, *** $p<0.0001$, t-test, pLLox3.7 vs. shRNA, n=4). **d,** Representative coronal section from a brain electroporated with P2Y1-shRNA at E16 and fixed at E21. **e,** Cell distribution was quantified as previously described (Chapter 3, Figure 6), and no defect in migration was observed when compared to previous controls.

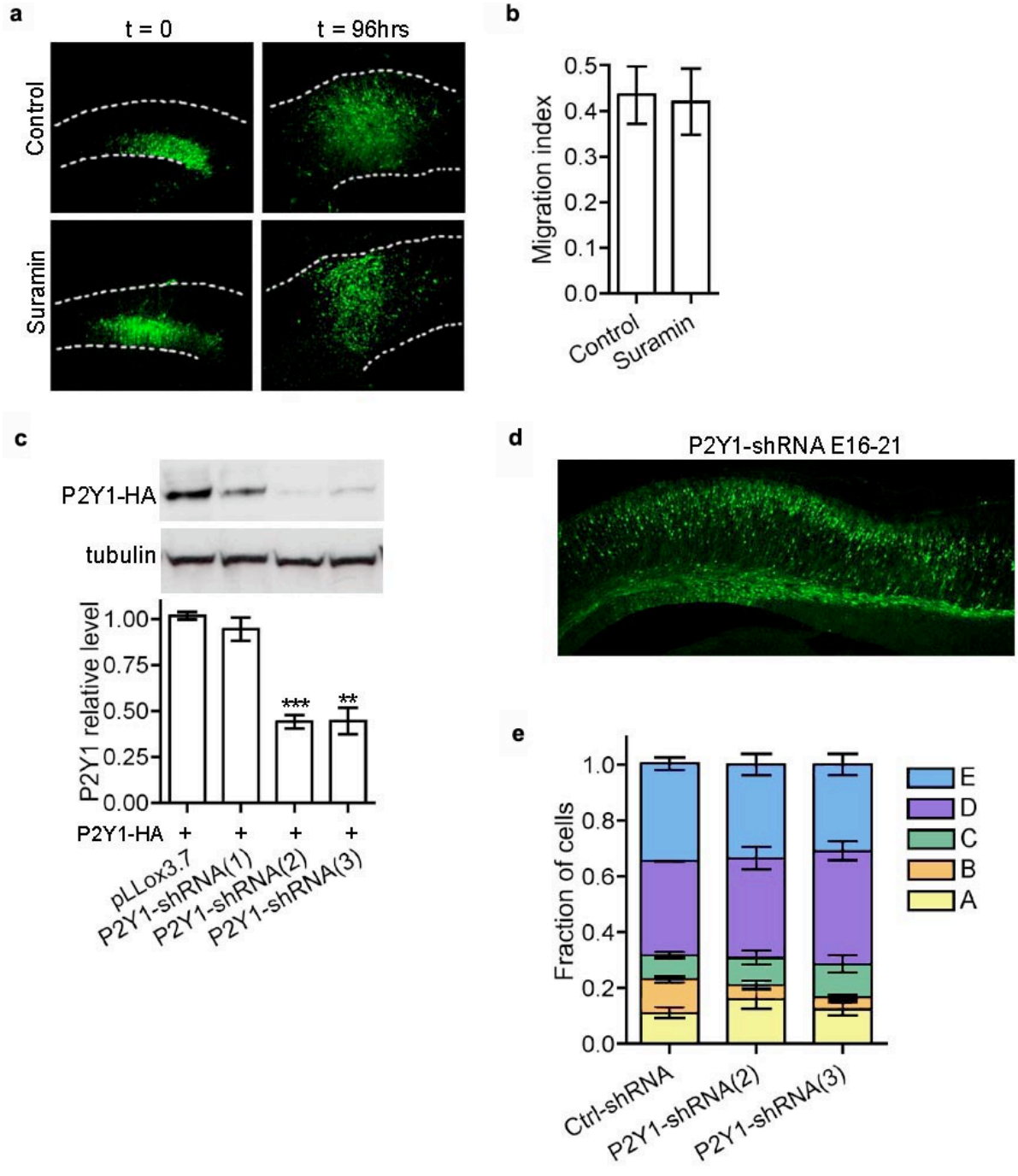

a
t = 0
t = 96hrs
Control
Suramin
b
Migration index
0.5
0.4
0.3
0.2
0.1
0.0
Control
Suramin
c
P2Y1-HA
tubulin
P2Y1 relative level
1.00
0.75
0.50
0.25
0.00
***
**
P2Y1-HA + + + +
pLLox3.7
P2Y1-shRNA(1)
P2Y1-shRNA(2)
P2Y1-shRNA(3)
d
P2Y1-shRNA E16-21
e
Fraction of cells
1.0
0.8
0.6
0.4
0.2
0.0
E
D
C
B
A
Ctrl-shRNA
P2Y1-shRNA(2)
P2Y1-shRNA(3)

Figure 6. Gap junctions promote cortical cell adhesion

a, Control DiD labelled (red), FACS sorted cortical cells were allowed to adhere to a glial substrate of confluent C6 or C6+Cx43 cells. Right panel shows quantification of the number of adherent DiD^+ cells per image frame (** $p \leq 0.001$, t-test, n=3 experiments). Values represent mean±s.e. **b,** DiD labelled, FACS sorted Ctrl-shRNA or Cx43-shRNA expressing cells were plated on a C6 or C6+Cx43 glial substrate. The ratio of Cx43-shRNA to Ctrl-shRNA cells that adhered to the C6+Cx43 substrate was 20% less than the C6 substrate (** $p<0.001$, t-test, C6 n=3 experiments, C6+Cx43 n=4 experiments).

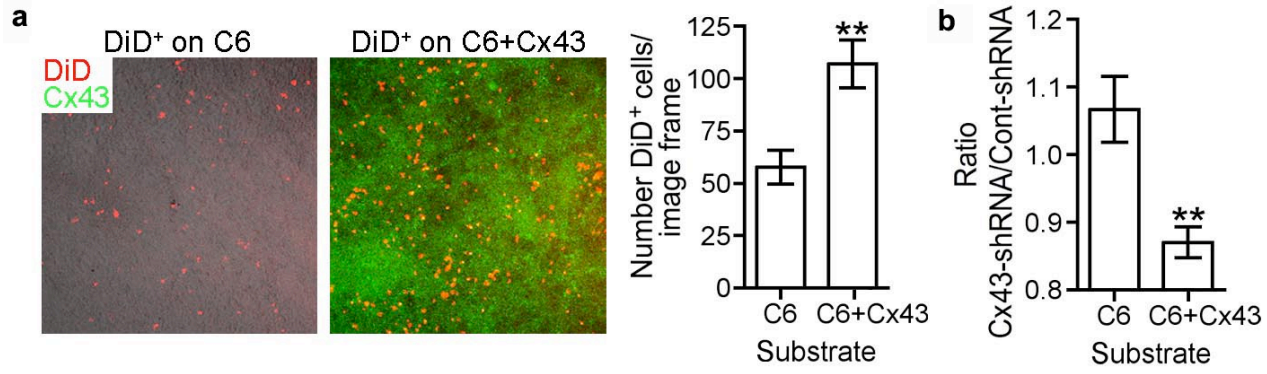

a
DiD+ on C6
DiD+ on C6+Cx43
DiD
Cx43
Number DiD+ cells/ image frame
125
100
75
50
25
0
**
C6
C6+Cx43
Substrate
b
Ratio Cx43-shRNA/Cont-shRNA
1.2
1.1
1.0
0.9
0.8
**
C6
C6+Cx43
Substrate

Figure 7. Cx26 and Cx43 interact with the actin cytoskeleton in the developing cortex

a, Expression of GFP and Actin-cherry three days after co-electroporation at E16. Actin is localized in a diffuse pattern in radial glia (1) and a punctate pattern in multipolar SVZ cells (2) and migrating neurons (3,4). In bipolar migrating neurons, Actin puncta localize to the leading process (4) or, if present, the dilation in the leading process (3). Scale bar, 10μm. **b,** Cx26T135A-EYFP (left) or Cx43T154A-EYFP (right) puncta co-lozalize with Actin-cherry (red) (cell bodies outlined in white), with their center of fluorescence 0.5-1μm apart in the z-plane (arrows). Graph shows a frequency histogram of the distance between the center of the puncta. **c,** Fewer Actin puncta are observed in cells expressing Cx26-shRNA or Cx43-shRNA than Ctrl-shRNA (** $p<0.001$, t-test, Ctrl-shRNA n=37 cells, Cx26-shRNA n=21, Cx43-shRNA n=44). Images taken at 'high resolution'. Values represent mean±s.e. Scale bars, 5μm. Images taken at 'high resolution'.

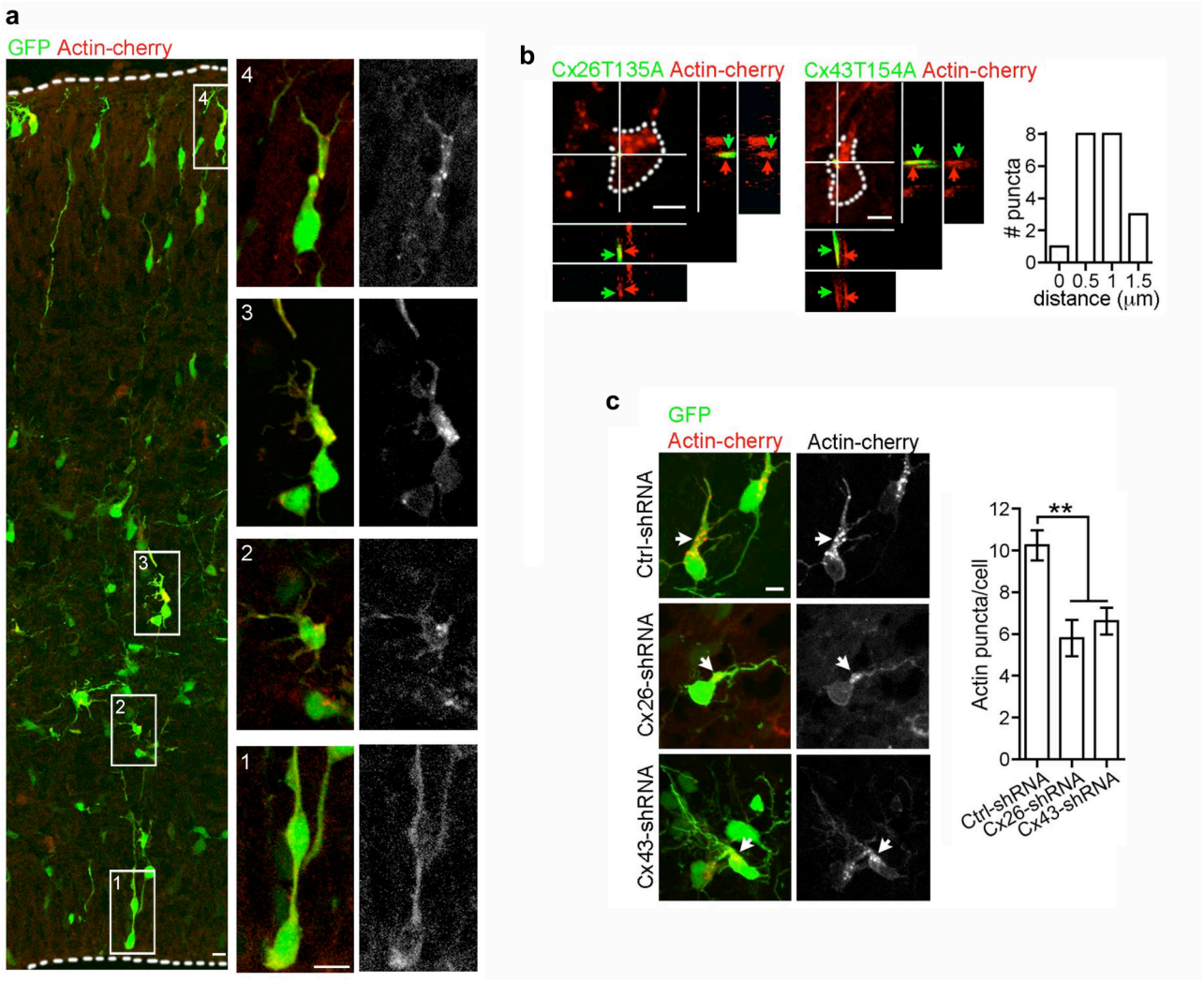

a
GFP Actin-cherry
4
3
2
1
b
Cx26T135A Actin-cherry
Cx43T154A Actin-cherry
puncta
distance (μm)
c
GFP
Actin-cherry
Actin-cherry
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA
Actin puncta/cell
**
Ctrl-shRNA
Cx26-shRNA
Cx43-shRNA

Figure 8. Gap junction adhesions play a role in the stabilization of the migrating neuron's leading process and in the translocation of the nucleus

a, Time-lapse of two control migrating neurons (white arrowheads) demonstrating the sequence through which bifurcated neurons (t=0-1hr) select a dominant leading process (t=1-2hr) and translocate their nucleus into the swelling that forms in the leading process. **b,** Time-lapses of neurons expressing Cx43-shRNA (top) or Cx26-shRNA (bottom). Neurons fail to stabilize a dominant process (note multiple transient processes), and the Cx26-shRNA neuron fails to translocate its nucleus.

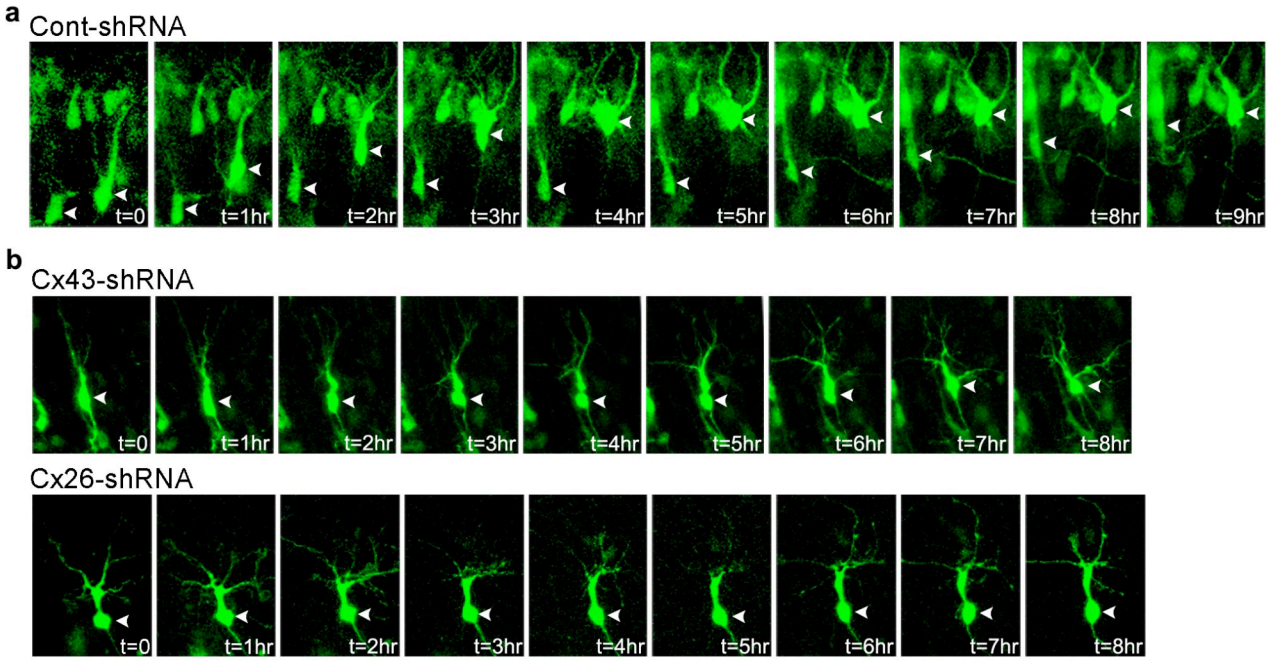

a
Cont-shRNA
t=0
t=1hr
t=2hr
t=3hr
t=4hr
t=5hr
t=6hr
t=7hr
t=8hr
t=9hr
b
Cx43-shRNA
t=0
t=1hr
t=2hr
t=3hr
t=4hr
t=5hr
t=6hr
t=7hr
t=8hr
Cx26-shRNA
t=0
t=1hr
t=2hr
t=3hr
t=4hr
t=5hr
t=6hr
t=7hr
t=8hr

Figure 9. Gap junction adhesions localize to the dominant branch of migrating neurons

a, Immunohistochemistry for Cx43 (left) or Cx26 (right) in a β-III tubulin^{+} bifurcated neuron or neuronal process (relative Cx expression, white arrows>yellow arrow). Scale bars, 5μm. **b**, Time-lapses of Cx43T154A puncta in branches of bifurcated neurons. The dominant process (white arrowheads) contains numerous Cx puncta (circled in white when multiple branches) while transient processes (blue arrowheads) do not. **e**, The number of puncta the dominant and transient process. (*** $p \leq 0.0001$, paired t-test, n=13). Values represent mean±s.e. **d**, Time-lapse of Cx26T135A-EYFP puncta in branches of bifurcated neurons. The dominant process (white arrowheads) contain Cx puncta while transient processes (blue arrowheads) do not. White circles highlight the location of Cx puncta at time points when the neurons have multiple processes.

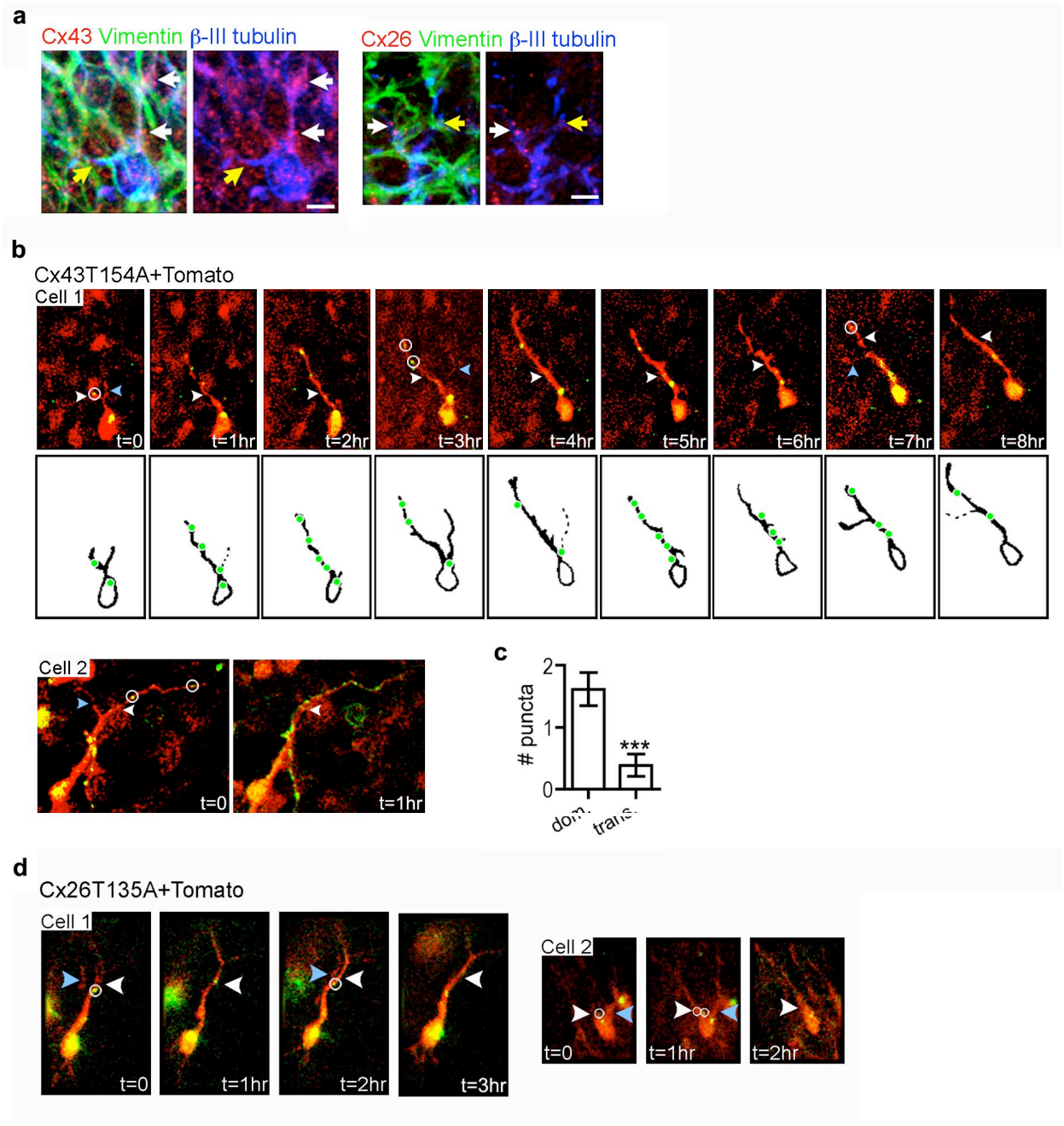

a
Cx43 Vimentin β-III tubulin
Cx26 Vimentin β-III tubulin
b
Cx43T154A+Tomato
Cell 1
t=0
t=1hr
t=2hr
t=3hr
t=4hr
t=5hr
t=6hr
t=7hr
t=8hr
Cell 2
t=0
t=1hr
c
puncta
2
1
0
***
dom.
trans.
d
Cx26T135A+Tomato
Cell 1
t=0
t=1hr
t=2hr
t=3hr
Cell 2
t=0
t=1hr
t=2hr

Figure 10. Gap junction adhesions enter the swelling in the leading process prior to the nuclear translocation and co-localize with the centrosome

a, Time-lapse of Cx26T135A punctum in the cell body of a migrating neuron. The punctum (white arrowhead) moves into the dilation in the leading process prior to the translocation of the cell body (blue arrowhead). **b**, Brains expressing Cx26T135A-EYFP and Tomato were fixed at E19 and stained with the nuclear dye Syto63 demonstrating the localization of the Cx puncta with respect to the nucleus. The puncta can be found adjacent to the nucleus in cells without a dilation (1st panel). In cells with a dilation the Cx puncta can be found adjacent to the nucleus (2nd panel) or in the dilation (3rd and 4th panel). **c,** Cx26T135A and Centrin II-dsRed co-localize (white arrows) in the dilation of a migrating neuron (left), with their center of fluorescence approximately 1μm apart in the z-plane (middle). Frequency histogram of the distance between the center of the puncta (right). Scale bar, 1μm. **d**, Time-lapse of Cx43T154A-EYFP punctum in the cell body of a migrating neuron. The punctum (white arrowhead) moves into the dilation in the leading process prior to the translocation of the soma (blue arrowhead). **e**, Expression of Cx43T154A-EYFP, Centrin II-dsRed, and HcRed at E19. Cx43T154A and Centrin II co-localize where the center of fluorescence of Cx43T154A and Centrin II approximately 1μm apart as shown in the z-plane. Frequency histogram of the distance between the center of Cx43T154A and Centrin II puncta (right).

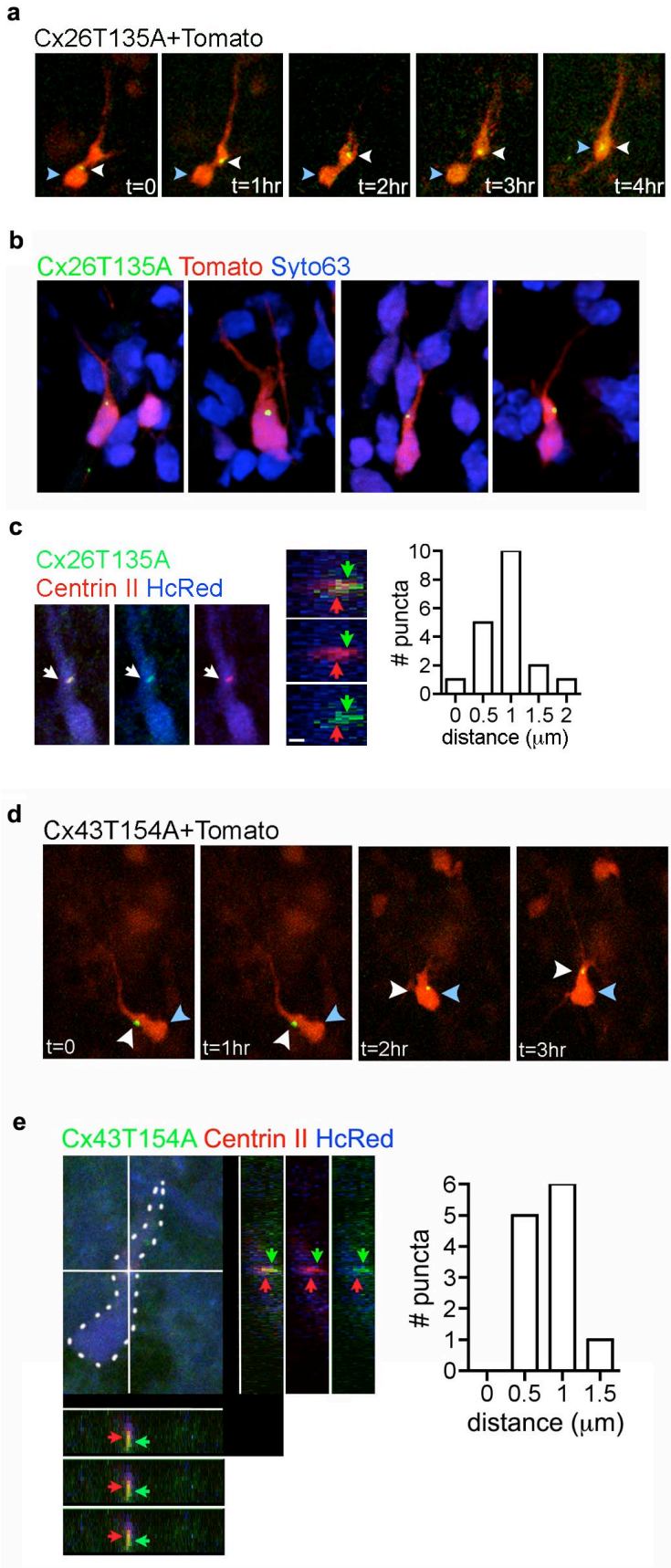
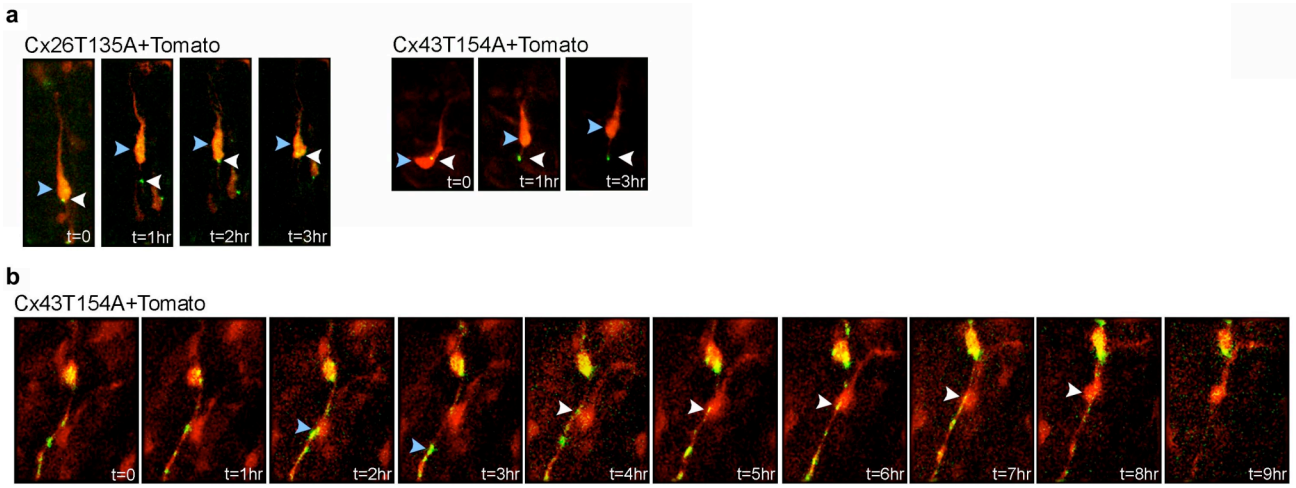

a
Cx26T135A+Tomato
t=0
t=1hr
t=2hr
t=3hr
t=4hr
b
Cx26T135A Tomato Syto63
c
Cx26T135A
Centrin II HcRed
puncta
distance (μm)
d
Cx43T154A+Tomato
t=0
t=1hr
t=2hr
t=3hr
e
Cx43T154A Centrin II HcRed
puncta
distance (μm)

Figure 11. Other observed patterns of Cx puncta dynamics in migrating neurons

a, Time-lapses of Cx26T135A and Cx43T154A puncta in the trailing processes of migrating neurons. The puncta (white arrowheads) begin in the cell body and are left behind in the trialing process when the cell body (blue arrowheads) translocates. The puncta are later re-localized back to the cell body. **b**, A time-lapse was performed in the VZ where radial glial fibers and newborn neurons are strongly labeled with Tomato and Cx43T154A-EYFP. Puncta between neurons and fibers appear able either to move with the cell along the fiber (white arrowheads) or to disassemble (blue arrowheads).


a
Cx26T135A+Tomato
t=0
t=1hr
t=2hr
t=3hr
Cx43T154A+Tomato
t=0
t=1hr
t=3hr
b
Cx43T154A+Tomato
t=0
t=1hr
t=2hr
t=3hr
t=4hr
t=5hr
t=6hr
t=7hr
t=8hr
t=9hr

Chapter 5:

General Conclusions

This study indicates that Cx26 and Cx43 containing gap junctions are localized to the contact points between migrating neurons and radial-glial fibers in the developing neocortex and are necessary for glial-guided radial migration. Functionally, gap junctions can promote adhesion in cortical cells, and their adhesive but not their channel properties mediate migration. These dynamic gap junction adhesions between migrating neurons and radial glial fibers regulate properties of neuronal migration including branch stabilization and nuclear translocation.

Connexins and glial-guided neuronal migration

Observations from Cx43 knockout mice and Cx43 conditional knockout mice also support a functional role for gap junctions in mediating neuronal migration along radial glial fibers (Fushiki et al., 2003; Wiencken-Barger et al., 2007). In the Cx43 genetic knockout mouse, there is a change in the distribution of BrdU-labeled cells in the cortex such that more cells are found in the intermediate zone and fewer are found in the cortical plate (Fushiki et al., 2003).

Mice with a conditional deletion of Cx43 driven by Cre expression under the human GFAP

promoter also have a phenotype suggestive of a failure in neuronal migration (Wiencken-Barger et al., 2007). Interestingly, the mouse background has a substantial effect on the severity of the phenotype, such that those on a C57Bl/6J background do not display a phenotype whereas those on a 129SVEV background have a clear brain phenotype, of which a particularly severe inbred line has been termed Shuffler (Wiencken-Barger et al., 2007). The biological significance of the background effect in these mice is yet to be

fully understood, but might relate to the potential for compensation by other Cx proteins in different mouse lines. Although the smaller cortex, cerebellum and hippocampus in Shuffler mice are most likely a result of proliferative defects, other phenotypes suggest compounding migratory problems. Most strikingly, in the cerebellum, granule cell ectopies and the increased thickness of the external granule layer are suggestive of a failure of granule cells to migrate radially to the internal granule layer. Additionally, the delamination of Purkinje cells in the cerebellum and ectopic clusters of cells in the cortex that fail to reach the cortical plate suggest a general radial migration defect as well. It is not clear from these studies whether migration defects result from a loss of Cx43 in neurons, radial fibers, or both, because disorganization of radial fibers is also observed (Wiencken-Barger et al., 2007). In the work described here, using *in utero* injection and electroporation of shRNA constructs targeting Cx26 and Cx43 in the developing rat cortex, cells expressing Cx-shRNA are unable to migrate to the cortical plate and remain in the intermediate zone (Figure 1). No effects were observed on cell death, early differentiation or ability to exit the cell cycle suggesting a primary defect in neuronal migration. Furthermore, the requirement for Cx expression in migrating neurons was established using transplant assays showing that Cx-shRNA-expressing neurons are unable to migrate on wild-type radial glia.

Functional redundancy and compensation among Cx family members

The migration defect described here with shRNA-mediated acute knockdown is more dramatic than the phenotype described in the Cx43KO mouse (Fushiki et al., 2003). This may be due to developmental compensation in the genetic knockout arising from

redundancy among Cxs. Indeed, we show at least one other Cx, Cx26, has a very similar role in neuronal migration. As only Cx43 has been previously implicated in cell migration, the role of Cx26 is interesting and implies that other Cxs, including β1 family Cxs, may be involved in migration. In addition, our work suggests that different Cxs may have somewhat distinct endogenous functional roles in neural migration, with Cx26 playing a dominant role in nuclear translocation and Cx43 in branch stabilization.

This brings up a common thread in gap junction research, that of compensation. In fact, when the Cx43 genetic knockout was first described, no apparent neurological defects were reported (Reaume et al., 1995). This might be a result of genetic compensation by other Cx family members and is reminiscent of the disconnect in other areas of gap junction research, such as embryonic axis formation, between the observation of strong phenotypes with pharmacological or knockdown studies and mild or absent phenotypes with genetic manipulations (Levin, 2002, 2007). To test this rigorously, approaches to studying other highly redundant protein families could be employed (Elias et al., 2006). Double and triple knockouts of Cx26, Cx43 and possibly Cx45, Cx36 or Cx37 could be created and studied. The embryonic lethality of some Cx proteins could complicate such an approach, but it might be possible to combine traditional and conditional genetic knockouts as well as targeted shRNA knockdown to eliminate multiple Cx family members.

To complicate matters, pannexins, homologs of invertebrate gap junction innexin proteins, might also contribute to gap junction function in the developing brain (Litvin et al., 2006). Pannexins are four-transmembrane domain proteins that can form gap junctions and hemichannels in *Xenopus* oocytes (Bruzzone et al., 2003). Pannexin 1 is

expressed at early embryonic time points in the cortex peaking around mouse embryonic day 18, whereas pannexin 2 expression increases postnatally (Ray et al., 2005; Vogt et al., 2005). As pannexin channels are blocked by pharmacological agents used to block Cx channels such as carbenoxolone, pannexin-mediated gap junction function might have previously been attributed to Cxs (Bruzzone et al., 2005). However, there is at present no evidence for the formation of functional gap junctions by pannexins in the brain (Huang et al., 2007a). Alternatively, pannexins might act as hemichannels mediating ATP release and Ca^{2+} waves as seen in other cell types (Huang et al., 2007b; Locovei et al., 2006). It is thus still unclear what role pannexins might play in cortical development.

Connexins as adhesive molecules

Surprisingly we were able to rescue the Cx-shRNA induced migration defect by co-expressing Cx mutants that are able to form adhesions but not channels, but we were not able to rescue the effect with mutants that are unable to form adhesions (Figure 1). To our knowledge, a functional role for gap junctions based on adhesion, rather than channel activity, has not been described to date. Might gap junction adhesions play a role in other gap junction-mediated cellular processes? It is thought that gap junction channels as well as their interactions with molecules such as p120catenin, integrin, and the actin cytoskeleton are important for neural crest cell migration (Xu et al., 2006; Xu et al., 2001), and that glioblastoma invasion of the brain parenchyma requires functional gap junctions between tumor cells and astrocytes, however this conclusion should be reassessed as the chimera used in this study does also does not make adhesive junctions with Cx43 expressed in astrocytes (Haubrich et al., 1996; Lin et al., 2002). The migration

of lung and skin cancer cells has also been associated with gap junction expression although no mechanism has been proposed (el-Sabban and Pauli, 1994; Ito et al., 2000). In addition, the neurological disorder Charcot-Marie-Tooth syndrome, caused by mutations in Cx32, may not only result from impaired gap junction communication but also from the loss of gap junction adhesions stabilizing Schmidt-Lanterman incisures. Further studies may elucidate an important role for the adhesive properties of gap junctions in the migration of neural crest cells and cancer cells, as well as in other diseases and physiological processes associated with gap junction function.

Future work needs to explore the way in which gap junction adhesions interact with the internal cytoskeleton. We observed that gap junction adhesions in migrating neurons co-localize with actin puncta and the centrosome (Figure 1), and Cx43 has been shown to bind several actin-interacting proteins including vinculin and drebrin (Butkevich et al., 2004; Xu et al., 2006). Furthermore, in avian tenocytes, a mechanosensitive cell type, Cx43 colocalization with actin increases with mechanical strain and can be reduced by inhibition of myosin II, suggesting a dynamic functional interaction between the actin cytoskeleton and Cx43 (Wall et al., 2007). The mechanism by which gap junctions co-localize with the centrosome, a structure that plays a very important role in coordinating migration (Higginbotham and Gleeson, 2007), is yet to be understood. Interestingly, the C terminus of Cx43 directly binds microtubules (Giepmans et al., 2001), but this interaction does not appear to be necessary for migration as removal of the C terminus of Cx43 or of the very short C terminus of Cx26 (for which there is no evidence of microtubule binding) does not disrupt the ability to mediate migration. Furthermore, as Cx43 associates with other adhesion molecules including N-cadherin and

integrin (Meyer et al., 1992; Xu et al., 2006; Xu et al., 2001), it will be of interest to explore their signaling relationships. Thus, further investigation concerning the mode of Cx interaction with the actin cytoskeleton and the centrosome as well as other adhesion molecules such as integrins will provide insights into the mechanistic role of gap junction adhesions during migration. For instance, the low expression of Cx43 in the cortical plate suggests a possible interaction with integrins, which play a role in the cessation of migration (Anton et al., 1999; Dulabon et al., 2000; Schmid et al., 2005), and may aid in the detachment of neurons from radial fibers.

Gap junctions: multifaceted regulators of embryonic cortical development

Gap junctions regulate the proliferation of neural progenitors as well as the migration and differentiation of young neurons in the embryonic neocortex. The functional capabilities of gap junctions extend beyond the classical notion of intercellular coupling and include hemichannel-mediated exchange with the extracellular environment, intracellular signaling, and cell–cell adhesion, as shown in this study. Thus, although gap junctions act in the classical manner coupling neural progenitors (Bittman et al., 1997; Bittman and LoTurco, 1999; Lo Turco and Kriegstein, 1991; Nadarajah et al., 1997), they also act as hemichannels mediating the spread of calcium waves across progenitor cell populations(Weissman et al., 2004), and as adhesive molecules guiding neuronal migration. Gap junctions are thus emerging as multifaceted regulators of cortical development playing diverse roles in intercellular communication.

All of the functional studies we have discussed suggesting that gap junctions regulate radial glial cell-cycle dynamics and neuronal differentiation have used

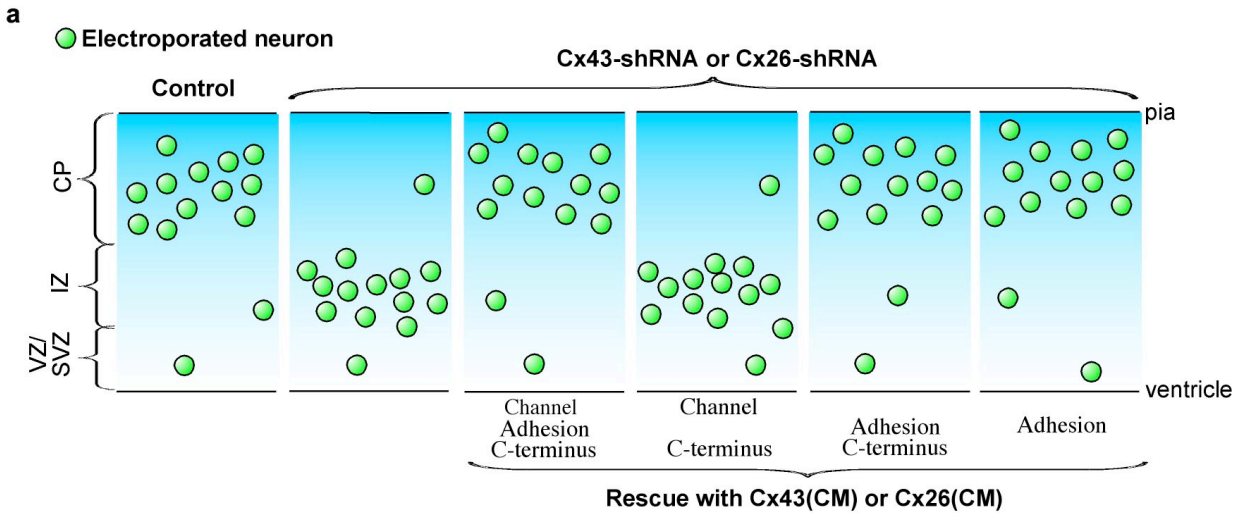
pharmacological manipulations (Chapter 1), whereas the studies that suggest a role for gap junctions in migration have used shRNA knockdown or genetic manipulations (Fushiki et al., 2003; Wiencken-Barger et al., 2007). Pharmacology allows determination of channel function when it is involved in gap junction intercellular exchange or hemichannel opening. As expected if migration is independent of channel function, none of the pharmacological studies note a migration defect. Alternatively, knocking down or genetically eliminating a Cx protein interferes with all aspects of its function including channel, adhesion and signaling capabilities. Although we have discussed migratory defects in relation to the loss of Cx proteins, is there also evidence for proliferation defects when the levels of Cx proteins are manipulated? In the conditional Cx43 knockout Shuffler strain, the smaller hippocampus, cortex and cerebellum are suggestive of a proliferation defect (Wiencken-Barger et al., 2007). Furthermore, shRNA-induced knockdown of Cx26 or Cx43 has effects on radial glial proliferation (L.A.B.E. and A.R.K., unpublished). However, the genetic knockout of Cx43 does not have a phenotype suggestive of proliferative defects which may be due to compensation (Fushiki et al., 2003).

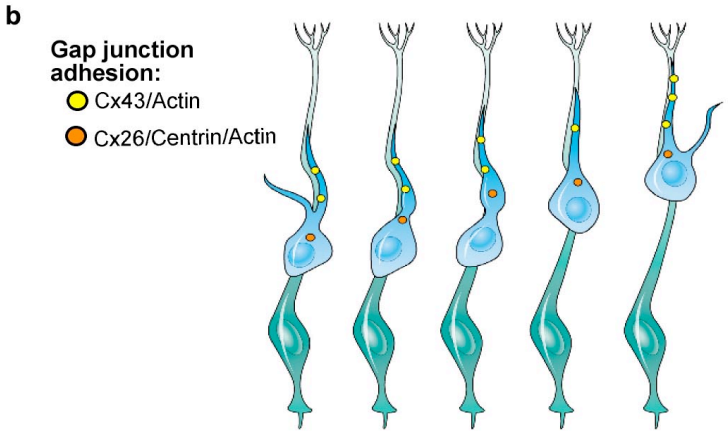
In conclusion, gap junction channels, hemichannels and adhesions act in distinct ways to mediate radial glial division, neuronal migration and neuronal differentiation. The scope of gap junction-mediated signaling continues to expand when considering the largely unknown downstream signaling pathways, which are arguably the most fascinating and least understood aspects of gap junction biology. For example, identifying the functionally relevant molecules exchanged between cells through gap junction channels is, at present, very difficult. Although it is typically thought that small

metabolites and ions are the main signaling molecules, the possibility that short RNAs pass through gap junctions suggests alternative possibilities (Valiunas et al., 2005). In understanding the role of gap junctions in radial glial division, it will be critical to identify the molecules exchanged between coupled cells and how they regulate the cell cycle. Likewise, the current data suggest that hemichannels on radial glial cells mediate ATP release that activates purinergic receptors and initiates Ca^{2+} waves in the cortical VZ (Weissman et al., 2004), but it is not known how purinergic Ca^{2+} waves in turn regulate proliferation. A recent study of *Xenopus* development demonstrates that purinergic signaling induces the transcription factor Pax6, thereby initiating eye development (Masse et al., 2007). Pax6 also plays a critical role in radial glial divisions in the cortex (Gotz and Barde, 2005), and it is possible that Pax6 levels might be affected by purinergic activation. Furthermore, the gap junction adhesions that mediate radial migration as described here are likely involved in a dynamic crosstalk with cytoskeletal elements and other signaling molecules to mediate neuronal migration and it will be interesting to dissect these pathways. Finally, recent reports also suggest that gap junction expression might coordinate the expression of networks of genes, the so-called Cx transcriptome, which might have diverse effects that are largely unexplored (Spray and Iacobas, 2007). Thus, when designing future studies to discern the role of gap junctions in cortical development, not only will it be necessary to consider the possible roles of gap junctions as channels, hemichannels, adhesions or signaling molecules but also how these functions are integrated into the cells' other signaling pathways and communication machinery.

Figure 1. The role of gap junction adhesion in neuronal radial migration

a, In order to study the role of gap junctions in neuronal migration, we delivered shRNAs targeting Cxs (Cx-shRNA) into the VZ of the developing cortex using *in utero* injection and electroporation techniques. Control electroporated neurons are able to migrate to the cortical plate (CP), whereas those expressing Cx26-shRNA or Cx43-shRNA are unable to migrate to the CP and remain in the intermediate zone (IZ). The shRNA-induced migration defect can be rescued by co-expression of Cx43 or Cx26 with conservative mutations (CM) that confer resistance to shRNA knockdown. Interestingly, selective rescue experiments using a variety of Cx mutants that selectively impair the channel, adhesion or the C terminus suggest that adhesion, but not the channel or C terminus, is necessary for migration. **b,** Time-lapse imaging of Cx-shRNA-expressing neurons as well as the dynamics of Cx puncta localization in migrating neurons suggests that gap junction adhesions play two major roles in neuronal migration. (i) Gap junction adhesions, especially Cx43 but also Cx26, localize to the dominant but not the transient branch of bifurcated migrating neurons, thereby stabilizing the dominant leading process (yellow puncta). (ii) Gap junction adhesions, especially Cx26 but also Cx43, localize to the cell body of migrating neurons, specificall co-localizing with the centrosome, and move into the dilatation in the leading process before the translocation of the nucleus, possibly stabilizing the centrosome during nuclear translocation (orange punctum). Actin puncta also co-localize with gap junction adhesions and might provide a link to the internal cytoskeleton.


a
Electroporated neuron
Cx43-shRNA or Cx26-shRNA
Control
pia
CP
IZ
VZ/
SVZ
ventricle
Channel
Adhesion
C-terminus
Channel
C-terminus
Adhesion
C-terminus
Adhesion
Rescue with Cx43(CM) or Cx26(CM)


b
Gap junction
adhesion:
Cx43/Actin
Cx26/Centrin/Actin

Chapter 6:

References

Adams, N.C., Tomoda, T., Cooper, M., Dietz, G., and Hatten, M.E. (2002). Mice that lack astrotactin have slowed neuronal migration. Development *129*, 965-972.

Anderson, S.A., Eisenstat, D.D., Shi, L., and Rubenstein, J.L. (1997a). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. Science *278*, 474-476.

Anderson, S.A., Qiu, M., Bulfone, A., Eisenstat, D.D., Meneses, J., Pedersen, R., and Rubenstein, J.L. (1997b). Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late born striatal neurons. Neuron *19*, 27-37.

Angevine, J.B., Jr., and Sidman, R.L. (1961). Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. Nature *192*, 766-768.

Anton, E.S., Kreidberg, J.A., and Rakic, P. (1999). Distinct functions of alpha3 and alpha(v) integrin receptors in neuronal migration and laminar organization of the cerebral cortex. Neuron *22*, 277-289.

Anton, E.S., Marchionni, M.A., Lee, K.F., and Rakic, P. (1997). Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. Development *124*, 3501-3510.

Bani-Yaghoub, M., Bechberger, J.F., and Naus, C.C. (1997). Reduction of connexin43 expression and dye-coupling during neuronal differentiation of human NTera2/clone D1 cells. J Neurosci Res *49*, 19-31.

Bani-Yaghoub, M., Bechberger, J.F., Underhill, T.M., and Naus, C.C. (1999a). The effects of gap junction blockage on neuronal differentiation of human NTera2/clone D1 cells. Exp Neurol *156*, 16-32.

Bani-Yaghoub, M., Underhill, T.M., and Naus, C.C. (1999b). Gap junction blockage interferes with neuronal and astroglial differentiation of mouse P19 embryonal carcinoma cells. Developmental genetics *24*, 69-81.

Beahm, D.L., Oshima, A., Gaietta, G.M., Hand, G.M., Smock, A.E., Zucker, S.N., Toloue, M.M., Chandrasekhar, A., Nicholson, B.J., and Sosinsky, G.E. (2006). Mutation of a conserved threonine in the third transmembrane helix of alpha- and beta-connexins creates a dominant-negative closed gap junction channel. J Biol Chem *281*, 7994-8009.

Beggs, H.E., Schahin-Reed, D., Zang, K., Goebbels, S., Nave, K.A., Gorski, J., Jones, K.R., Sretavan, D., and Reichardt, L.F. (2003). FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies. Neuron *40*, 501-514.

Belliveau, D.J., Bani-Yaghoub, M., McGirr, B., Naus, C.C., and Rushlow, W.J. (2006). Enhanced neurite outgrowth in PC12 cells mediated by connexin hemichannels and ATP. J Biol Chem *281*, 20920-20931.

Belliveau, D.J., Bechberger, J.F., Rogers, K.A., and Naus, C.C. (1997). Differential expression of gap junctions in neurons and astrocytes derived from P19 embryonal carcinoma cells. Developmental genetics *21*, 187-200.

Belvindrah, R., Graus-Porta, D., Goebbels, S., Nave, K.A., and Muller, U. (2007). Beta1 integrins in radial glia but not in migrating neurons are essential for the formation of cell layers in the cerebral cortex. J Neurosci *27*, 13854-13865.

Bennett, M.V., Contreras, J.E., Bukauskas, F.F., and Saez, J.C. (2003). New roles for astrocytes: gap junction hemichannels have something to communicate. Trends Neurosci *26*, 610-617.

Bergoffen, J., Scherer, S.S., Wang, S., Scott, M.O., Bone, L.J., Paul, D.L., Chen, K., Lensch, M.W., Chance, P.F., and Fischbeck, K.H. (1993). Connexin mutations in X-linked Charcot-Marie-Tooth disease. Science *262*, 2039-2042.

Beyer, E.C., Paul, D.L., and Goodenough, D.A. (1987). Connexin43: a protein from rat heart homologous to a gap junction protein from liver. The Journal of cell biology *105*, 2621-2629.

Bielas, S., Higginbotham, H., Koizumi, H., Tanaka, T., Gleeson, J.G. (2004). Cortical neuronal migration mutants suggest separate but intersecting pathways. Annu. Rev. Cell Dev. Biol. *20*, 593-618.

Bishop, K.M., Goudreau, G., and O'Leary, D.D. (2000). Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science *288*, 344-349.

Bishop, K.M., Rubenstein, J.L., and O'Leary, D.D. (2002). Distinct actions of Emx1, Emx2, and Pax6 in regulating the specification of areas in the developing neocortex. J Neurosci *22*, 7627-7638.

Bittman, K., Owens, D.F., Kriegstein, A.R., and LoTurco, J.J. (1997). Cell coupling and uncoupling in the ventricular zone of developing neocortex. J Neurosci *17*, 7037-7044.

Bittman, K.S., and LoTurco, J.J. (1999). Differential regulation of connexin 26 and 43 in murine neocortical precursors. Cereb Cortex *9*, 188-195.

Bruzzone, R., Barbe, M.T., Jakob, N.J., and Monyer, H. (2005). Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes. J Neurochem *92*, 1033-1043.

Bruzzone, R., Hormuzdi, S.G., Barbe, M.T., Herb, A., and Monyer, H. (2003). Pannexins, a family of gap junction proteins expressed in brain. Proc Natl Acad Sci U S A *100*, 13644-13649.

Bulchand, S., Grove, E.A., Porter, F.D., and Tole, S. (2001). LIM-homeodomain gene Lhx2 regulates the formation of the cortical hem. Mechanisms of development *100*, 165-175.

Butkevich, E., Hulsmann, S., Wenzel, D., Shirao, T., Duden, R., and Majoul, I. (2004). Drebrin is a novel connexin-43 binding partner that links gap junctions to the submembrane cytoskeleton. Curr Biol *14*, 650-658.

Cameron, R.S., and Rakic, P. (1994). Identification of membrane proteins that comprise the plasmalemmal junction between migrating neurons and radial glial cells. J Neurosci *14*, 3139-3155.

Casarosa, S., Fode, C., and Guillemot, F. (1999). Mash1 regulates neurogenesis in the ventral telencephalon. Development *126*, 525-534.

Caviness, V.S., Jr. (1982). Neocortical histogenesis in normal and reeler mice: a developmental study based upon [3H]thymidine autoradiography. Brain Res *256*, 293-302.

Cheng, A., Tang, H., Cai, J., Zhu, M., Zhang, X., Rao, M., and Mattson, M.P. (2004). Gap junctional communication is required to maintain mouse cortical neural progenitor cells in a proliferative state. Dev Biol *272*, 203-216.

Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., and Beachy, P.A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature *383*, 407-413.

Cina, C., Bechberger, J.F., Ozog, M.A., and Naus, C.C. (2007). Expression of connexins in embryonic mouse neocortical development. J Comp Neurol *504*, 298-313.

Cotrina, M.L., Lin, J.H., Alves-Rodrigues, A., Liu, S., Li, J., Azmi-Ghadimi, H., Kang, J., Naus, C.C., and Nedergaard, M. (1998). Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci U S A *95*, 15735-15740.

Crossley, P.H., Martinez, S., Ohkubo, Y., and Rubenstein, J.L. (2001). Coordinate expression of Fgf8, Otx2, Bmp4, and Shh in the rostral prosencephalon during development of the telencephalic and optic vesicles. Neuroscience *108*, 183-206.

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D.S., Sheldon, M., and Curran, T. (1999). Reelin is a ligand for lipoprotein receptors. Neuron *24*, 471-479.

D'Arcangelo, G., Miao, G.G., Chen, S.C., Soares, H.D., Morgan, J.I., and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature *374*, 719-723.

Dermietzel, R., Traub, O., Hwang, T.K., Beyer, E., Bennett, M.V., Spray, D.C., and Willecke, K. (1989). Differential expression of three gap junction proteins in developing and mature brain tissues. Proc Natl Acad Sci U S A *86*, 10148-10152.

des Portes, V., Pinard, J.M., Billuart, P., Vinet, M.C., Koulakoff, A., Carrie, A., Gelot, A., Dupuis, E., Motte, J., Berwald-Netter, Y*., et al.* (1998). A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. Cell *92*, 51-61.

Donahue, L.M., Webster, D.R., Martinez, I., and Spray, D.C. (1998). Decreased gap-junctional communication associated with segregation of the neuronal phenotype in the RT4 cell-line family. Cell Tissue Res *292*, 27-35.

Dou, C.L., Li, S., and Lai, E. (1999). Dual role of brain factor-1 in regulating growth and patterning of the cerebral hemispheres. Cereb Cortex *9*, 543-550.

Dulabon, L., Olson, E.C., Taglienti, M.G., Eisenhuth, S., McGrath, B., Walsh, C.A., Kreidberg, J.A., and Anton, E.S. (2000). Reelin binds alpha3beta1 integrin and inhibits neuronal migration. Neuron *27*, 33-44.

Dunlap, K., Takeda, K., and Brehm, P. (1987). Activation of a calcium-dependent photoprotein by chemical signalling through gap junctions. Nature *325*, 60-62.

Duval, N., Gomes, D., Calaora, V., Calabrese, A., Meda, P., and Bruzzone, R. (2002). Cell coupling and Cx43 expression in embryonic mouse neural progenitor cells. J Cell Sci *115*, 3241-3251.

Edmondson, J.C., and Hatten, M.E. (1987). Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. J Neurosci *7*, 1928-1934.

Edmondson, J.C., Liem, R.K., Kuster, J.E., and Hatten, M.E. (1988). Astrotactin: a novel neuronal cell surface antigen that mediates neuron-astroglial interactions in cerebellar microcultures. The Journal of cell biology *106*, 505-517.

el-Sabban, M.E., and Pauli, B.U. (1994). Adhesion-mediated gap junctional communication between lung-metastatatic cancer cells and endothelium. Invasion Metastasis *14*, 164-176.

Elias, G.M., Funke, L., Stein, V., Grant, S.G., Bredt, D.S., and Nicoll, R.A. (2006). Synapse-specific and developmentally regulated targeting of AMPA receptors by a family of MAGUK scaffolding proteins. Neuron *52*, 307-320.

Elias, L.A.B., and Kriegstein, A.R. (2007). Organotypic slice culture of E18 rat brains. Journal of Visualized Experiments, http://www.jove.com/index/details.stp?ID=235.

Ewart, J.L., Cohen, M.F., Meyer, R.A., Huang, G.Y., Wessels, A., Gourdie, R.G., Chin, A.J., Park, S.M., Lazatin, B.O., Villabon, S., and Lo, C.W. (1997). Heart and neural tube defects in transgenic mice overexpressing the Cx43 gap junction gene. Development *124*, 1281-1292.

Fairen, A., Cobas, A., and Fonseca, M. (1986). Times of generation of glutamic acid decarboxylase immunoreactive neurons in mouse somatosensory cortex. J Comp Neurol *251*, 67-83.

Falk, M.M. (2000). Connexin-specific distribution within gap junctions revealed in living cells. J Cell Sci *113 (Pt 22)*, 4109-4120.

Fode, C., Ma, Q., Casarosa, S., Ang, S.L., Anderson, D.J., and Guillemot, F. (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes & development *14*, 67-80.

Fox, J.W., Lamperti, E.D., Eksioglu, Y.Z., Hong, S.E., Feng, Y., Graham, D.A., Scheffer, I.E., Dobyns, W.B., Hirsch, B.A., Radtke, R.A., *et al.* (1998). Mutations in filamin 1 prevent migration of cerebral cortical neurons in human periventricular heterotopia. Neuron *21*, 1315-1325.

Frantz, G.D., and McConnell, S.K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron *17*, 55-61.

Furuta, Y., Piston, D.W., and Hogan, B.L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development *124*, 2203-2212.

Fushiki, S., Perez Velazquez, J.L., Zhang, L., Bechberger, J.F., Carlen, P.L., and Naus, C.C. (2003). Changes in neuronal migration in neocortex of connexin43 null mutant mice. J Neuropathol Exp Neurol *62*, 304-314.

Gadisseux, J.F., Kadhim, H.J., van den Bosch de Aguilar, P., Caviness, V.S., and Evrard, P. (1990). Neuron migration within the radial glial fiber system of the developing murine cerebrum: an electron microscopic autoradiographic analysis. Brain Res Dev Brain Res *52*, 39-56.

Georges-Labouesse, E., Mark, M., Messaddeq, N., and Gansmuller, A. (1998). Essential role of alpha 6 integrins in cortical and retinal lamination. Curr Biol *8*, 983-986.

Ghashghaei, H.T., Weimer, J.M., Schmid, R.S., Yokota, Y., McCarthy, K.D., Popko, B., and Anton, E.S. (2007). Reinduction of ErbB2 in astrocytes promotes radial glial progenitor identity in adult cerebral cortex. Genes & development *21*, 3258-3271.

Ghosh, A., and Greenberg, M.E. (1995). Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron *15*, 89-103.

Giepmans, B.N. (2004). Gap junctions and connexin-interacting proteins. Cardiovasc Res *62*, 233-245.

Giepmans, B.N., and Moolenaar, W.H. (1998). The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein. Curr Biol *8*, 931-934.

Giepmans, B.N., Verlaan, I., Hengeveld, T., Janssen, H., Calafat, J., Falk, M.M., and Moolenaar, W.H. (2001). Gap junction protein connexin-43 interacts directly with microtubules. Curr Biol *11*, 1364-1368.

Gleeson, J.G., Allen, K.M., Fox, J.W., Lamperti, E.D., Berkovic, S., Scheffer, I., Cooper, E.C., Dobyns, W.B., Minnerath, S.R., Ross, M.E., and Walsh, C.A. (1998). Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. Cell *92*, 63-72.

Goldberg, G.S., Moreno, A.P., and Lampe, P.D. (2002). Gap junctions between cells expressing connexin 43 or 32 show inverse permselectivity to adenosine and ATP. J Biol Chem *277*, 36725-36730.

Gongidi, V., Ring, C., Moody, M., Brekken, R., Sage, E.H., Rakic, P., and Anton, E.S. (2004). SPARC-like 1 regulates the terminal phase of radial glia-guided migration in the cerebral cortex. Neuron *41*, 57-69.

Goodenough, D.A., and Paul, D.L. (2003). Beyond the gap: functions of unpaired connexon channels. Nat Rev Mol Cell Biol *4*, 285-294.

Goto, T., Takahashi, T., Miyama, S., Nowakowski, R.S., Bhide, P.G., and Caviness, V.S., Jr. (2002). Developmental regulation of the effects of fibroblast growth factor-2 and 1-octanol on neuronogenesis: implications for a hypothesis relating to mitogen-antimitogen opposition. J Neurosci Res *69*, 714-722.

Gotz, M., and Barde, Y.A. (2005). Radial glial cells defined and major intermediates between embryonic stem cells and CNS neurons. Neuron *46*, 369-372.

Graus-Porta, D., Blaess, S., Senften, M., Littlewood-Evans, A., Damsky, C., Huang, Z., Orban, P., Klein, R., Schittny, J.C., and Muller, U. (2001). Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. Neuron *31*, 367-379.

Gregory, W.A., Edmondson, J.C., Hatten, M.E., and Mason, C.A. (1988). Cytology and neuron-glial apposition of migrating cerebellar granule cells in vitro. J Neurosci *8*, 1728-1738.

Harris, A.L. (2001). Emerging issues of connexin channels: biophysics fills the gap. Q Rev Biophys *34*, 325-472.

Harris, A.L. (2007). Connexin channel permeability to cytoplasmic molecules. Prog Biophys Mol Biol *94*, 120-143.

Haubrich, S., Schwarz, H.J., Bukauskas, F., Lichtenberg-Frate, H., Traub, O., Weingart, R., and Willecke, K. (1996). Incompatibility of connexin 40 and 43 Hemichannels in gap junctions between mammalian cells is determined by intracellular domains. Molecular biology of the cell *7*, 1995-2006.

Haubst, N., Georges-Labouesse, E., De Arcangelis, A., Mayer, U., and Gotz, M. (2006). Basement membrane attachment is dispensable for radial glial cell fate and for proliferation, but affects positioning of neuronal subtypes. Development *133*, 3245-3254.

Hebert, J.M., Mishina, Y., and McConnell, S.K. (2002). BMP signaling is required locally to pattern the dorsal telencephalic midline. Neuron *35*, 1029-1041.

Hiesberger, T., Trommsdorff, M., Howell, B.W., Goffinet, A., Mumby, M.C., Cooper, J.A., and Herz, J. (1999). Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. Neuron *24*, 481-489.

Higginbotham, H.R., and Gleeson, J.G. (2007). The centrosome in neuronal development. Trends Neurosci *30*, 276-283.

Howell, B.W., Hawkes, R., Soriano, P., and Cooper, J.A. (1997). Neuronal position in the developing brain is regulated by mouse disabled-1. Nature *389*, 733-737.

Hsieh-Li, H.M., Witte, D.P., Szucsik, J.C., Weinstein, M., Li, H., and Potter, S.S. (1995). Gsh-2, a murine homeobox gene expressed in the developing brain. Mechanisms of development *50*, 177-186.

Huang, G.Y., Cooper, E.S., Waldo, K., Kirby, M.L., Gilula, N.B., and Lo, C.W. (1998). Gap junction-mediated cell-cell communication modulates mouse neural crest migration. The Journal of cell biology *143*, 1725-1734.

Huang, Y., Grinspan, J.B., Abrams, C.K., and Scherer, S.S. (2007a). Pannexin1 is expressed by neurons and glia but does not form functional gap junctions. Glia *55*, 46-56.

Huang, Y.J., Maruyama, Y., Dvoryanchikov, G., Pereira, E., Chaudhari, N., and Roper, S.D. (2007b). The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. Proc Natl Acad Sci U S A *104*, 6436-6441.

Ito, A., Katoh, F., Kataoka, T.R., Okada, M., Tsubota, N., Asada, H., Yoshikawa, K., Maeda, S., Kitamura, Y., Yamasaki, H., and Nojima, H. (2000). A role for heterologous gap junctions between melanoma and endothelial cells in metastasis. J Clin Invest *105*, 1189-1197.

Johnson, R.G., and Sheridan, J.D. (1971). Junctions between cancer cells in culture: ultrastructure and permeability. Science *174*, 717-719.

Kelsell, D.P., Dunlop, J., Stevens, H.P., Lench, N.J., Liang, J.N., Parry, G., Mueller, R.F., and Leigh, I.M. (1997). Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. Nature *387*, 80-83.

Khazipov, R., and Luhmann, H.J. (2006). Early patterns of electrical activity in the developing cerebral cortex of humans and rodents. Trends Neurosci *29*, 414-418.

Komuro, H., and Rakic, P. (1995). Dynamics of granule cell migration: a confocal microscopic study in acute cerebellar slice preparations. J Neurosci *15*, 1110-1120.

Komuro, H., and Rakic, P. (1996). Intracellular Ca2+ fluctuations modulate the rate of neuronal migration. Neuron *17*, 275-285.

Kreidberg, J.A., Donovan, M.J., Goldstein, S.L., Rennke, H., Shepherd, K., Jones, R.C., and Jaenisch, R. (1996). Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. Development *122*, 3537-3547.

Kriegstein, A., Noctor, S., and Martinez-Cerdeno, V. (2006). Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. Nat Rev Neurosci *7*, 883-890.

Kriegstein, A.R., and Gotz, M. (2003). Radial glia diversity: a matter of cell fate. Glia *43*, 37-43.

Lai, A., Le, D.N., Paznekas, W.A., Gifford, W.D., Jabs, E.W., and Charles, A.C. (2006). Oculodentodigital dysplasia connexin43 mutations result in non-functional connexin hemichannels and gap junctions in C6 glioma cells. J Cell Sci *119*, 532-541.

Laird, D.W. (2005). Connexin phosphorylation as a regulatory event linked to gap junction internalization and degradation. Biochim Biophys Acta *1711*, 172-182.

Lee, S.M., Tole, S., Grove, E., and McMahon, A.P. (2000). A local Wnt-3a signal is required for development of the mammalian hippocampus. Development *127*, 457-467.

Levin, M. (2002). Isolation and community: a review of the role of gap-junctional communication in embryonic patterning. J Membr Biol *185*, 177-192.

Levin, M. (2007). Gap junctional communication in morphogenesis. Prog Biophys Mol Biol *94*, 186-206.

Lin, J.H., Takano, T., Cotrina, M.L., Arcuino, G., Kang, J., Liu, S., Gao, Q., Jiang, L., Li, F., Lichtenberg-Frate, H.*, et al.* (2002). Connexin 43 enhances the adhesivity and mediates the invasion of malignant glioma cells. J Neurosci *22*, 4302-4311.

Lin, R., Warn-Cramer, B.J., Kurata, W.E., and Lau, A.F. (2001). v-Src phosphorylation of connexin 43 on Tyr247 and Tyr265 disrupts gap junctional communication. The Journal of cell biology *154*, 815-827.

Litvin, O., Tiunova, A., Connell-Alberts, Y., Panchin, Y., and Baranova, A. (2006). What is hidden in the pannexin treasure trove: the sneak peek and the guesswork. J Cell Mol Med *10*, 613-634.

Liu, Q., Dwyer, N.D., and O'Leary, D.D. (2000). Differential expression of COUP-TFI, CHL1, and two novel genes in developing neocortex identified by differential display PCR. J Neurosci *20*, 7682-7690.

Lo, C.W. (1996). The role of gap junction membrane channels in development. J Bioenerg Biomembr *28*, 379-385.

Lo, C.W., Cohen, M.F., Huang, G.Y., Lazatin, B.O., Patel, N., Sullivan, R., Pauken, C., and Park, S.M. (1997). Cx43 gap junction gene expression and gap junctional communication in mouse neural crest cells. Developmental genetics *20*, 119-132.

Lo, C.W., Waldo, K.L., and Kirby, M.L. (1999). Gap junction communication and the modulation of cardiac neural crest cells. Trends Cardiovasc Med *9*, 63-69.

Lo Turco, J.J., and Kriegstein, A.R. (1991). Clusters of coupled neuroblasts in embryonic neocortex. Science *252*, 563-566.

Locke, D., Koreen, I.V., and Harris, A.L. (2006). Isoelectric points and post-translational modifications of connexin26 and connexin32. Faseb J *20*, 1221-1223.

Locovei, S., Bao, L., and Dahl, G. (2006). Pannexin 1 in erythrocytes: function without a gap. Proc Natl Acad Sci U S A *103*, 7655-7659.

Loddenkemper, T., Grote, K., Evers, S., Oelerich, M., and Stogbauer, F. (2002). Neurological manifestations of the oculodentodigital dysplasia syndrome. Journal of neurology *249*, 584-595.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868-872.

Malatesta, P., Hack, M.A., Hartfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F., and Gotz, M. (2003). Neuronal or glial progeny: regional differences in radial glia fate. Neuron *37*, 751-764.

Malatesta, P., Hartfuss, E., and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development *127*, 5253-5263.

Marin-Padilla, M. (1971). Early prenatal ontogenesis of the cerebral cortex (neocortex) of the cat (Felis domestica). A Golgi study. I. The primordial neocortical organization. Zeitschrift fur Anatomie und Entwicklungsgeschichte *134*, 117-145.

Masse, K., Bhamra, S., Eason, R., Dale, N., and Jones, E.A. (2007). Purine-mediated signalling triggers eye development. Nature *449*, 1058-1062.

McCarty, J.H., Lacy-Hulbert, A., Charest, A., Bronson, R.T., Crowley, D., Housman, D., Savill, J., Roes, J., and Hynes, R.O. (2005). Selective ablation of alphav integrins in the central nervous system leads to cerebral hemorrhage, seizures, axonal degeneration and premature death. Development *132*, 165-176.

McConnell, S.K., and Kaznowski, C.E. (1991). Cell cycle dependence of laminar determination in developing neocortex. Science *254*, 282-285.

Meyer, R.A., Laird, D.W., Revel, J.P., and Johnson, R.G. (1992). Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. The Journal of cell biology *119*, 179-189.

Miller, M.W. (1985). Cogeneration of retrogradely labeled corticocortical projection and GABA-immunoreactive local circuit neurons in cerebral cortex. Brain Res *355*, 187-192.

Misson, J.P., Edwards, M.A., Yamamoto, M., and Caviness, V.S., Jr. (1988). Mitotic cycling of radial glial cells of the fetal murine cerebral wall: a combined autoradiographic and immunohistochemical study. Brain Res *466*, 183-190.

Miyata, T., Kawaguchi, A., Okano, H., and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron *31*, 727-741.

Monuki, E.S., Porter, F.D., and Walsh, C.A. (2001). Patterning of the dorsal telencephalon and cerebral cortex by a roof plate-Lhx2 pathway. Neuron *32*, 591-604.

Murphy, M., Drago, J., and Bartlett, P.F. (1990). Fibroblast growth factor stimulates the proliferation and differentiation of neural precursor cells in vitro. J Neurosci Res *25*, 463-475.

Nadarajah, B., Brunstrom, J.E., Grutzendler, J., Wong, R.O., and Pearlman, A.L. (2001). Two modes of radial migration in early development of the cerebral cortex. Nat Neurosci *4*, 143-150.

Nadarajah, B., Jones, A.M., Evans, W.H., and Parnavelas, J.G. (1997). Differential expression of connexins during neocortical development and neuronal circuit formation. J Neurosci *17*, 3096-3111.

Nadarajah, B., Makarenkova, H., Becker, D.L., Evans, W.H., and Parnavelas, J.G. (1998). Basic FGF increases communication between cells of the developing neocortex. J Neurosci *18*, 7881-7890.

Naus, C.C., Bechberger, J.F., Caveney, S., and Wilson, J.X. (1991). Expression of gap junction genes in astrocytes and C6 glioma cells. Neurosci Lett *126*, 33-36.

Nicholson, B., Dermietzel, R., Teplow, D., Traub, O., Willecke, K., and Revel, J.P. (1987). Two homologous protein components of hepatic gap junctions. Nature *329*, 732-734.

Niewmierzycka, A., Mills, J., St-Arnaud, R., Dedhar, S., and Reichardt, L.F. (2005). Integrin-linked kinase deletion from mouse cortex results in cortical lamination defects resembling cobblestone lissencephaly. J Neurosci *25*, 7022-7031.

Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S., and Kriegstein, A.R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. Nature *409*, 714-720.

Noctor, S.C., Flint, A.C., Weissman, T.A., Wong, W.S., Clinton, B.K., and Kriegstein, A.R. (2002). Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. J Neurosci *22*, 3161-3173.

Noctor, S.C., Martinez-Cerdeno, V., Ivic, L., and Kriegstein, A.R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci *7*, 136-144.

Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Seike, M., Ikenaka, K., Yamamoto, H., and Mikoshiba, K. (1995). The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron *14*, 899-912.

Ohkubo, Y., Chiang, C., and Rubenstein, J.L. (2002). Coordinate regulation and synergistic actions of BMP4, SHH and FGF8 in the rostral prosencephalon regulate morphogenesis of the telencephalic and optic vesicles. Neuroscience *111*, 1-17.

Oliveira, R., Christov, C., Guillamo, J.S., Debouard, S., Palfi, S., Venance, L., Tardy, M., and Peschanski, M. (2005). Contribution of gap junctional communication between

tumor cells and astroglia to the invasion of the brain parenchyma by human glioblastomas. BMC Cell Biol *6*, 7.

Paul, D.L. (1986). Molecular cloning of cDNA for rat liver gap junction protein. The Journal of cell biology *103*, 123-134.

Paznekas, W.A., Boyadjiev, S.A., Shapiro, R.E., Daniels, O., Wollnik, B., Keegan, C.E., Innis, J.W., Dinulos, M.B., Christian, C., Hannibal, M.C., and Jabs, E.W. (2003). Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. American journal of human genetics *72*, 408-418.

Pearson, R.A., Dale, N., Llaudet, E., and Mobbs, P. (2005a). ATP released via gap junction hemichannels from the pigment epithelium regulates neural retinal progenitor proliferation. Neuron *46*, 731-744.

Pearson, R.A., Luneborg, N.L., Becker, D.L., and Mobbs, P. (2005b). Gap junctions modulate interkinetic nuclear movement in retinal progenitor cells. J Neurosci *25*, 10803-10814.

Pitts, J.D., and Simms, J.W. (1977). Permeability of junctions between animal cells. Intercellular transfer of nucleotides but not of macromolecules. Experimental cell research *104*, 153-163.

Porteus, M.H., Bulfone, A., Liu, J.K., Puelles, L., Lo, L.C., and Rubenstein, J.L. (1994). DLX-2, MASH-1, and MAP-2 expression and bromodeoxyuridine incorporation define molecularly distinct cell populations in the embryonic mouse forebrain. J Neurosci *14*, 6370-6383.

Rakic, P. (1971). Guidance of neurons migrating to the fetal monkey neocortex. Brain Res *33*, 471-476.

Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. J Comp Neurol *145*, 61-83.

Rakic, P. (1978). Neuronal migration and contact guidance in the primate telencephalon. Postgrad Med J *54 Suppl 1*, 25-40.

Rakic, P. (1988). Specification of cerebral cortical areas. Science *241*, 170-176.

Rakic, P. (2003). Elusive radial glial cells: historical and evolutionary perspective. Glia *43*, 19-32.

Ray, A., Zoidl, G., Weickert, S., Wahle, P., and Dermietzel, R. (2005). Site-specific and developmental expression of pannexin1 in the mouse nervous system. Eur J Neurosci *21*, 3277-3290.

Reaume, A.G., de Sousa, P.A., Kulkarni, S., Langille, B.L., Zhu, D., Davies, T.C., Juneja, S.C., Kidder, G.M., and Rossant, J. (1995). Cardiac malformation in neonatal mice lacking connexin43. Science *267*, 1831-1834.

Reiner, O., Carrozzo, R., Shen, Y., Wehnert, M., Faustinella, F., Dobyns, W.B., Caskey, C.T., and Ledbetter, D.H. (1993). Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit-like repeats. Nature *364*, 717-721.

Rieske, E., Schubert, P., and Kreutzberg, G.W. (1975). Transfer of radioactive material between electrically coupled neurons of the leech central nervous system. Brain Res *84*, 365-382.

Rio, C., Rieff, H.I., Qi, P., Khurana, T.S., and Corfas, G. (1997). Neuregulin and erbB receptors play a critical role in neuronal migration. Neuron *19*, 39-50.

Rozental, R., Morales, M., Mehler, M.F., Urban, M., Kremer, M., Dermietzel, R., Kessler, J.A., and Spray, D.C. (1998). Changes in the properties of gap junctions during neuronal differentiation of hippocampal progenitor cells. J Neurosci *18*, 1753-1762.

Saez, J.C., Connor, J.A., Spray, D.C., and Bennett, M.V. (1989). Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. Proc Natl Acad Sci U S A *86*, 2708-2712.

Saito, T., and Nakatsuji, N. (2001). Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. Dev Biol *240*, 237-246.

Sanada, K., Gupta, A., and Tsai, L.H. (2004). Disabled-1-regulated adhesion of migrating neurons to radial glial fiber contributes to neuronal positioning during early corticogenesis. Neuron *42*, 197-211.

Schaar, B.T., and McConnell, S.K. (2005). Cytoskeletal coordination during neuronal migration. Proc Natl Acad Sci U S A *102*, 13652-13657.

Schmid, R.S., Jo, R., Shelton, S., Kreidberg, J.A., and Anton, E.S. (2005). Reelin, integrin and DAB1 interactions during embryonic cerebral cortical development. Cereb Cortex *15*, 1632-1636.

Schmid, R.S., McGrath, B., Berechid, B.E., Boyles, B., Marchionni, M., Sestan, N., and Anton, E.S. (2003). Neuregulin 1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex. Proc Natl Acad Sci U S A *100*, 4251-4256.

Schmid, R.S., Shelton, S., Stanco, A., Yokota, Y., Kreidberg, J.A., and Anton, E.S. (2004). alpha3beta1 integrin modulates neuronal migration and placement during early stages of cerebral cortical development. Development *131*, 6023-6031.

Sheen, V.L., Feng, Y., Graham, D., Takafuta, T., Shapiro, S.S., and Walsh, C.A. (2002). Filamin A and Filamin B are co-expressed within neurons during periods of neuronal migration and can physically interact. Hum Mol Genet *11*, 2845-2854.

Sheldon, M., Rice, D.S., D'Arcangelo, G., Yoneshima, H., Nakajima, K., Mikoshiba, K., Howell, B.W., Cooper, J.A., Goldowitz, D., and Curran, T. (1997). Scrambler and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice. Nature *389*, 730-733.

Shimamura, K., and Rubenstein, J.L. (1997). Inductive interactions direct early regionalization of the mouse forebrain. Development *124*, 2709-2718.

Solecki, D.J., Model, L., Gaetz, J., Kapoor, T.M., and Hatten, M.E. (2004). Par6alpha signaling controls glial-guided neuronal migration. Nat Neurosci *7*, 1195-1203.

Spray, D.C., and Iacobas, D.A. (2007). Organizational principles of the connexin-related brain transcriptome. J Membr Biol *218*, 39-47.

Stagaard, M., and Mollgard, K. (1989). The developing neuroepithelium in human embryonic and fetal brain studied with vimentin-immunocytochemistry. Anatomy and embryology *180*, 17-28.

Subak-Sharpe, H., Burk, R.R., and Pitts, J.D. (1969). Metabolic co-operation between biochemically marked mammalian cells in tissue culture. J Cell Sci *4*, 353-367.

Tanaka, T., Serneo, F.F., Higgins, C., Gambello, M.J., Wynshaw-Boris, A., and Gleeson, J.G. (2004). Lis1 and doublecortin function with dynein to mediate coupling of the nucleus to the centrosome in neuronal migration. The Journal of cell biology *165*, 709-721.

Tao, W., and Lai, E. (1992). Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. Neuron *8*, 957-966.

Toresson, H., Potter, S.S., and Campbell, K. (2000). Genetic control of dorsal-ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. Development *127*, 4361-4371.

Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R.E., Richardson, J.A., and Herz, J. (1999). Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell *97*, 689-701.

Tsai, J.W., Bremner, K.H., and Vallee, R.B. (2007). Dual subcellular roles for LIS1 and dynein in radial neuronal migration in live brain tissue. Nat Neurosci *In Press*.

Tsien, R.W., and Weingart, R. (1974). Proceedings: Cyclic AMP: cell-to-cell movement and inotropic effect in ventricular muscle, studied by a cut-end method. The Journal of physiology *242*, 95P-96P.

Ueno, M., Katayama, K., Yamauchi, H., Nakayama, H., and Doi, K. (2006). Cell cycle progression is required for nuclear migration of neural progenitor cells. Brain Res *1088*, 57-67.

Unsworth, H.C., Aasen, T., McElwaine, S., and Kelsell, D.P. (2007). Tissue-specific effects of wild-type and mutant connexin 31: a role in neurite outgrowth. Hum Mol Genet *16*, 165-172.

Vaccarino, F.M., Schwartz, M.L., Hartigan, D., and Leckman, J.F. (1995). Basic fibroblast growth factor increases the number of excitatory neurons containing glutamate in the cerebral cortex. Cereb Cortex *5*, 64-78.

Vaccarino, F.M., Schwartz, M.L., Raballo, R., Nilsen, J., Rhee, J., Zhou, M., Doetschman, T., Coffin, J.D., Wyland, J.J., and Hung, Y.T. (1999). Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. Nat Neurosci *2*, 848.

Valiunas, V., Polosina, Y.Y., Miller, H., Potapova, I.A., Valiuniene, L., Doronin, S., Mathias, R.T., Robinson, R.B., Rosen, M.R., Cohen, I.S., and Brink, P.R. (2005).

Connexin-specific cell-to-cell transfer of short interfering RNA by gap junctions. The Journal of physiology *568*, 459-468.

Vogt, A., Hormuzdi, S.G., and Monyer, H. (2005). Pannexin1 and Pannexin2 expression in the developing and mature rat brain. Brain Res Mol Brain Res *141*, 113-120.

Walantus, W., Elias, L.A.B., and Kriegstein, A.R. (2007). In utero intraventricular injection and electroporation of E16 rat enbryos. Journal of Visualized Experiments, <http://www.jove.com/index/Details.stp?ID=236& VID=222>.

Wall, M.E., Otey, C., Qi, J., and Banes, A.J. (2007). Connexin 43 is localized with actin in tenocytes. Cell Motil Cytoskeleton *64*, 121-130.

Ware, M.L., Fox, J.W., Gonzalez, J.L., Davis, N.M., Lambert de Rouvroit, C., Russo, C.J., Chua, S.C., Jr., Goffinet, A.M., and Walsh, C.A. (1997). Aberrant splicing of a mouse disabled homolog, mdab1, in the scrambler mouse. Neuron *19*, 239-249.

Weissman, T.A., Riquelme, P.A., Ivic, L., Flint, A.C., and Kriegstein, A.R. (2004). Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron *43*, 647-661.

Wiencken-Barger, A.E., Djukic, B., Casper, K.B., and McCarthy, K.D. (2007). A role for Connexin43 during neurodevelopment. Glia *55*, 675-686.

Xu, X., Francis, R., Wei, C.J., Linask, K.L., and Lo, C.W. (2006). Connexin 43-mediated modulation of polarized cell movement and the directional migration of cardiac neural crest cells. Development *133*, 3629-3639.

Xu, X., Li, W.E., Huang, G.Y., Meyer, R., Chen, T., Luo, Y., Thomas, M.P., Radice, G.L., and Lo, C.W. (2001). Modulation of mouse neural crest cell motility by N-cadherin and connexin 43 gap junctions. The Journal of cell biology *154*, 217-230.

Xuan, S., Baptista, C.A., Balas, G., Tao, W., Soares, V.C., and Lai, E. (1995). Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. Neuron *14*, 1141-1152.

Ye, Z.C., Wyeth, M.S., Baltan-Tekkok, S., and Ransom, B.R. (2003). Functional hemichannels in astrocytes: a novel mechanism of glutamate release. J Neurosci *23*, 3588-3596.

Yun, K., Potter, S., and Rubenstein, J.L. (2001). Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon. Development *128*, 193-205.

Zhang, J.T., and Nicholson, B.J. (1989). Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. The Journal of cell biology *109*, 3391-3401.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses and dissertations. Copies of all UCSF theses and dissertations will be routed to the library via the Graduate Division. The library will make all theses and dissertations accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis or dissertation to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

L Ellis 6/3/2008

Author Signature Date