CHAPTER

14

Cortical Architecture, Midline Guidance, and Tractography of 3D White Matter Tracts

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14.1 INTRODUCTION

The circuitry of the brain underlies its ability to function. These circuits are formed and refined during development under the influence of a combination of genetic/molecular and activity-dependent mechanisms. Connections between neurons form integrated circuits that receive, process, produce, and store information. The output component of a neuron is its axon, which connects to the dendrites of the neurons that receive its information. The circuitry of the cerebral cortex encompasses both short-range and long-range axonal connections between neurons. Short-range connections form local circuits whereas long-range connections form between different brain regions in the same or opposing cerebral hemisphere. Long-range axonal connections cluster into large white matter tracts containing both myelinated and unmyelinated axons. The correct targeting of axons during brain development is therefore of fundamental importance in establishing the functional circuits of the brain.

The anatomical connections of the nervous system, including the cerebral cortex, have been studied using histological techniques encompassing immunohistochemical staining and tract tracing. More recently, these studies have been complemented by magnetic resonance imaging (MRI) techniques that utilize diffusion MRI (dMRI) as the basis for tractography. Combining these techniques has revolutionized our understanding of the anatomical connectivity of the human brain in both normal and pathological states. It has also enabled more rapid crossover of studies of brain connectivity in human pathologies that can be investigated in animal models. Finally, dMRI can be performed in living human subjects, providing an opportunity to conduct a longitudinal examination of brain connectivity. These studies are providing unprecedented knowledge about brain development, normal brain function, and brain degeneration.

In this chapter, we focus on the development of cerebral connectivity and provide a review of how cortical axonal connections are formed and how they can be studied using the complementary technologies of MRI and histology.

14.2 DEVELOPMENT OF CIRCUITS IN THE BRAIN

14.2.1 Formation of the Neocortex

The neocortex is a highly organized structure present in all mammals that processes sensory, motor, language, emotional, and associative information. It is the largest component of the cerebral cortex and comprises six layers of neurons that are grouped according to their primary input or output circuitry. Two of the most fundamental

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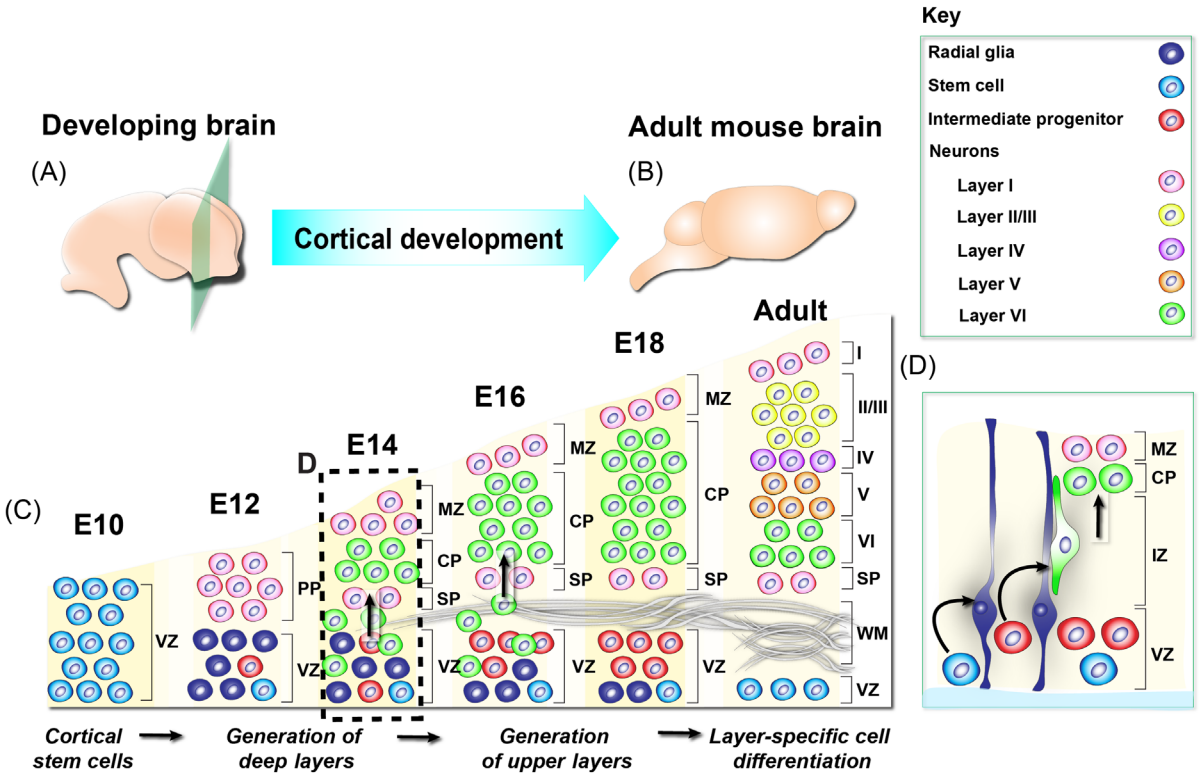

FIGURE 14.1 **Prenatal development of the mammalian neocortex.** Within the developing mouse embryo (A), the neocortex develops from a thin collection of cortical stem cells around embryonic day (E)10, to form a complex structure composed of six layers of mature neurons in the adult (B). Cortical stem cells give rise to radial glia (dark blue cells in C and D), the progenitor cells of all major cell types in the mature brain that provide the scaffold for immature neurons to migrate away from the ventricular zone (VZ) and into the cortical plate (CP). Radial glia generate intermediate progenitor cells (red cells in C and D), which divide and generate the majority of neurons in the brain. The first neurons (pink cells in C) to be born in the cortex form the preplate (PP) around E12, but are split into the marginal zone (MZ) and the subplate (SP) by later born neurons (green cells in C) migrating radially into the CP from E14. Neurons are generated in an inside out fashion, whereby later born neurons (such as those in layer II/III) must migrate past those born earlier. Neurons differentiate into many mature projection neuron subclasses that grow an axon and form local or distant connections. Neurons leaving the VZ for the CP migrate through the intermediate zone (IZ), a region that will contain the axons of cortical neurons forming the white matter (WM). Production of neurons depletes the progenitor cell pool in the mature cortex and very few stem cells remain in the adult.

questions in neuroscience are: how does the complexity of the mature neocortex take shape during development? and how are connections between often distant cortical areas correctly established and maintained? To begin to answer these questions, we must first consider the earliest events in neurodevelopment: the establishment of the cortical plate and its architecture, and subsequent patterning of a primordial cortex of neurons into structurally distinct areas specialized to deal with specific types of inputs and outputs. Disruption to any of these developmental events can have a profound impact on brain wiring and connectivity.

The neocortex emerges from the rostral-most brain vesicle of the neural tube (the prosencephalon), around embryonic day (E)10 in mice and gestational week (GW)5 in humans (Müller and O'Rahilly, 1988). At this stage of development, the neocortex is a thin sheet of pseudostratified neuroepithelial cells that will shortly transform into radial glia: the major progenitors of neurons and glia (Figure 14.1A–C; Aaku-Saraste et al., 1996; Hartfuss et al., 2001; Heins et al., 2002; Noctor et al., 2002). Radial glia are bipolar cells that reside in the ventricular zone of the developing brain; they have both an apical attachment to the underlying ventricular surface and a basal attachment to the pial surface of the brain. Radial glia can undergo either symmetric divisions to produce more radial glia or asymmetric divisions to give rise to a radial glia and an intermediate progenitor or neuron (Figure 14.1D; Chenn and McConnell, 1995; Noctor et al., 2004). Intermediate progenitors undergo mainly symmetric division to produce two daughter neurons, thereby generating the majority of the pyramidal neurons in the brain (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Wu et al., 2005; Sessa et al., 2008; Kowalczyk et al., 2009). In contrast, it is well established that in rodents interneurons are generated outside the cortex in the ganglionic eminences and preoptic area, and migrate tangentially through the marginal zone and intermediate zone to populate the cortical plate (Anderson

et al., 2001, 2002; Gelman et al., 2009). At the end of neurogenesis, radial glia differentiate into ependymal cells or astrocytes (Voigt, 1989; Spassky et al., 2005). This terminal differentiation event depletes the pool of progenitors in the ventricular zone, and very few stem cells remain in the lateral ventricular zone in the adult (Zhao et al., 2008).

The neocortex is a six-layered structure, with layer I closest to the pial surface and layer VI closest to the ventricle during development, and to the white matter in the adult (Figure 14.1C). The first layer of postmitotic neurons in the cortex is called the preplate. The preplate is derived from the early divisions of neuroepithelial cells and is split into the subplate and marginal zone by migrating neurons born in the ventricular zone that form the cortical plate around E13 in mice (Allendoerfer and Shatz, 1994; Marín-Padilla, 1998). Neurons destined for the cortical plate are generated in an inside out fashion, whereby neurons of the deeper layers (V–VI) are born first (from E12 in mice) and migrate away from the ventricular zone along the processes of radial glial cells to reach the correct layer (Figure 14.1; Angevine and Sidman, 1961; Caviness, 1982). Upper layer neurons (born on or after E15 in mice) must migrate past deeper layer neurons to form layers II and III of the neocortex (Caviness, 1982; Tissir and Goffinet, 2003). Neurons in the cortical plate then differentiate into a variety of projection neuron subtypes, which differ in terms of their molecular profile, morphology, electrophysiological properties, and connections (Migliore and Shepherd, 2005; Molyneaux et al., 2007). Differentiation of neurons into subtypes is driven by transcription factor programs that are initiated within progenitor cells or within the immature neurons, and are fundamental for establishing the blueprint of connectivity within the neocortex (reviewed by Molyneaux et al., 2007; Leone et al., 2008; Fame et al., 2011).

14.2.2 dMRI as a Means of Studying Neocortical Development

One means of studying neocortical development is through MRI. MRI is the measurement of the response of protons (most of which are contained in water molecules) to static and oscillating magnetic fields (Stejskal and Tanner, 1965). dMRI is an extension of the MRI method that models the microstructural properties of the brain based on the directionality of the diffusion of hydrogen atoms, on a macroscale (Taylor and Bushell, 1985; Le Bihan et al., 1986; Turner et al., 1990; Le Bihan, 1995). dMRI as a research imaging modality has traditionally been concerned with modeling white matter tracts in the mature brain. The same measurements of water diffusion, however, can be combined with histological techniques to offer a great deal of detail about how brain structure on a microscale or mesoscale correlates with the dynamic events within the developing neocortex. These findings will be discussed throughout the chapter, but the basic concepts underpinning dMRI must first be briefly introduced.

In the brain, the diffusion of water molecules is restricted to varying degrees in different compartments of tissue. Diffusion is unrestricted and random (or "Brownian") within compartments containing cerebrospinal fluid, as they contain no barriers to diffusion (Figure 14.2A and B). Water molecules in the grey matter reside within or between the cell bodies of neurons and glia, and their diffusion is therefore somewhat restricted by barriers such as cellular membranes, although it is still mostly random (Figure 14.2C). This type of diffusion, that is not selectively restricted in specific directions, is termed isotropic diffusion. In situations where cell membranes restrict the direction in which water molecules can move (as is the case within the white matter), the diffusion of water is anisotropic—i.e., it is highly restricted in some directions, but not in others (Figure 14.2D; Thomsen et al., 1987; Chenevert et al., 1990; Moseley et al., 1990, 1991; Le Bihan et al., 1993; Beaulieu, 2002). This physical restriction of the Brownian motion of water molecules underpins dMRI, as the movement of protons between applications of radiofrequency (RF) gradients results in a change in signal that can be measured within the MRI scanner and reconstructed. The critical insight of the early founders of this dMRI technique was that determining the extent of diffusion along a number of directions of an RF gradient (which can vary widely and can influence accurate resolution of diffusion direction) can be used as a noninvasive and indirect measure of the structure and orientation of white matter tracts in the brain.

In a dMRI, a single voxel can be considered a functional unit of protons, and within this voxel (typically 1–2.5 mm^3), the three-dimensional diffusion of water (Figure 14.2) can be modeled as an ellipsoidal tensor defined by the direction of its principal axes (eigenvectors ε_1, ε_2, and ε_3) and their magnitude (eigenvalues; λ_1, λ_2, and λ_3). Fractional anisotropy (FA) describes the shape of the ellipsoid. The isotropic diffusion that occurs in the grey matter is best modeled by a spherical ellipsoid where eigenvalues are close to equal (e.g., $\lambda_1 \sim \lambda_2$, $\sim \lambda_3$) and FA is close to zero. Anisotropic diffusion is modeled by a narrow ellipsoid where the major diffusion eigenvector is near-parallel to the direction of the white matter, there is a significant difference between eigenvalues (e.g., $\lambda_1 >> \lambda_2, >> \lambda_3$), and FA is high (close to 1). Various other scalar values, such as mean diffusivity (the average of the three eigenvalues), axial diffusivity (the principal eigenvalue λ_1), and radial diffusivity (an average of the second and third eigenvalues $[\lambda_2, + \lambda_3]/2$) among others, can be used to describe the structure of tissue within a voxel; these are outlined in Figure 14.2 for various tensor shapes. Diffusion properties are influenced by numerous tissue properties at the microstructural level, including axonal thickness, degree of myelination, cytoskeletal components, orientation and density, as well as dMRI artifacts such as

temperature and head movement (Beaulieu, 2002). As a result, FA is very sensitive to microstructural changes, but is unable to determine the nature of these changes; indeed, it follows that an infinite number of combinations of eigenvectors can result in the same FA value, with each reflecting a different underlying microstructure. FA is regularly implemented as a surrogate measure of the organization of white matter, but should be interpreted with care, and where possible in conjunction with other measures of the tensor model. These concepts will be revisited throughout the chapter and will be built upon to describe how white matter connections, which arise from a complex sequence of developmental events can be modeled noninvasively to describe how connectivity is established.

Water diffusion in the grey matter of the postnatal human brain can be considered restricted but isotropic (Figure 14.2); however, this is not the case in the prenatal neocortex (Mori et al., 2001; McKinstry et al., 2002; Huang et al., 2006, 2009; Ball et al., 2013; Xu et al., 2014). During the second and third trimesters in humans, the FA of the cortex increases to coincide with the radial migration of immature bipolar neurons along the radial glial scaffold that runs perpendicular to the pial surface (Gupta et al., 2005). As the neocortex matures, neuronal morphologies become far more complex, and thalamocortical and corticocortical afferents begin to innervate the cortical plate, giving rise to complex cytoarchitecture that is no longer radially oriented (Gadisseux et al., 1992; Xu et al., 2014). Therefore, somewhat paradoxically, the normal maturation of the neocortex is associated with a decrease in anisotropy and mean diffusivity secondary to a reduction in the principal eigenvalue (λ_1, the axial component of diffusivity).

These microstructural changes are demonstrated in more detail by the refinement of techniques such as dMR microscopy, which allows for the reconstruction of changes in tissue microstructure on the scale of tens of microns in mouse models (Aggarwal et al., 2010, 2014). Progress in these methods is blurring the distinction between the micro- and mesoscale, and the histological correlates of the radial organization of diffusivity in the neocortex (mostly accounted for by radial glial fibers) are intuitively similar to dMRI-based tractography results of the developing cortical plate (Aggarwal et al., 2014; Xu et al., 2014). Using these techniques, the developing cortical plate can be delineated into three distinct layers based on differences in FA: the ventricular zone containing radially organized progenitors, the intermediate zone comprising tangentially oriented early axonal projections, and the radially oriented developing cortical plate (McKinstry et al., 2002; Huang et al., 2009; Xu et al., 2014). As GABAergic interneurons migrate tangentially into the developing cortex, together with the maturation of radial glia into astrocytes and the arrival of afferent axonal projections, the cortex becomes more complex and the clear relationship between microstructure and mesoscale representations of the diffusion signal breaks down. At this point in development, tractography reveals a reorganization of fibers towards tangential and corticocortical (fibers connecting different cortical regions) orientations (Xu et al., 2014). This change coincides with the specification of projection neurons and the sprouting of long-range projections, which will mature into the long-range white matter tracts that allow for information transfer between distant cortical areas in the postnatal brain.

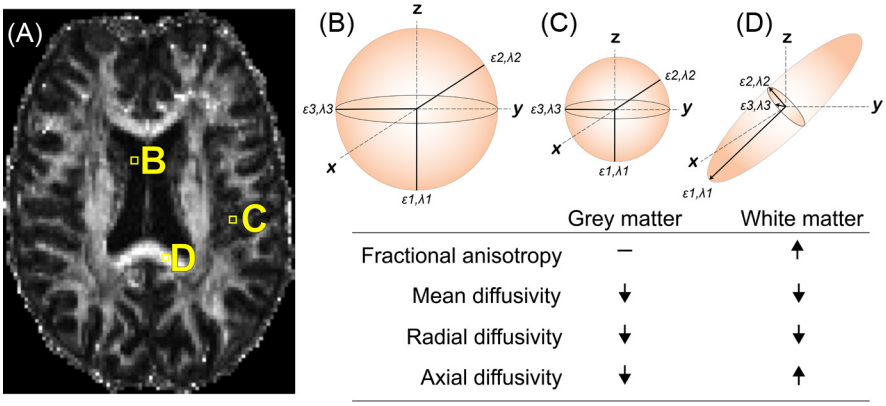

FIGURE 14.2 **Biological basis of the tensor model.** In a dMRI (A, horizontal view of an adult human brain depicted), diffusion within the cerebrospinal fluid (CSF) is unrestricted and isotropic, and is modeled by a spherical tensor (B, depicted right). All eigenvalues (λ1, λ2, and λ3) are approximately equal, such that there is a low FA, high mean diffusivity, and high radial diffusivity. In the grey matter (C), diffusion is nonselectively restricted and can be modeled by a similarly shaped but smaller sphere compared to the CSF, reflecting comparable decreases in λ1, λ2, and λ3. Relative to the CSF, all measures of diffusivity are decreased and the FA remains mostly unchanged. (D) In the white matter, diffusion can be modeled as an ellipsoid with a principal eigenvector (ε1) parallel to the direction of white matter. The FA is increased relative to the CSF; mean diffusivity and radial diffusivity are decreased, reflecting a smaller ε2 and ε3, and axial diffusivity is increased, reflecting a larger λ1 relative to the λ2 and λ3 eigenvalues.

14.2.3 Arealization

A crucial aspect of functional wiring of the brain is that areas that process similar information are interconnected. In part, this is driven by the early patterning of the neocortex into different functional areas. These areas share homology in their development but vary in terms of their final connectivity (inputs and outputs), cytoarchitecture, and repertoire of neural subclasses. The patterning of the cortex into different functional areas is under strict intrinsic control but can later be modified by sensory input (O'Leary and Sahara, 2008; Alfano and Studer, 2013).

The very early secretion of molecules from various signaling centers in the forebrain patterns the neocortex into functionally distinct areas from around E10.5 in mice (Figure 14.3). The best characterized secreted morphogens involved in this initial arealization are members of the fibroblast growth factor (FGF) family of ligands from the rostral patterning center, wingless-related MMTV integrations site (WNT) and bone morphogenic protein (BMP) ligands from the cortical hem dorsally, and sonic hedgehog (SHH) from the prechordal plate ventrally (Figure 14.3A; Munoz-Sanjuan et al., 1999; Grove and Fukuchi-Shimogori, 2003; O'Leary et al., 2007). These ligands diffuse across the cortex, bind to their corresponding receptors on proliferating progenitor cells, and induce regionalized gene programs that specify brain regions by influencing cell proliferation and cell death (Ohkubo et al., 2002; Storm et al., 2006; Fernandes et al., 2007). Each progenitor cell or neuron is subject to a specific combination of identity cues depending on its macroscale location, and it is the interpretation of these cues (on the cellular microscale) that results in the demarcation of cortical areas (Figure 14.3C and D). Four transcription factors are expressed in complementary gradients across the cortex and establish boundaries between functional areas (Figure 14.3B; O'Leary et al., 2007). In addition to their induction by morphogens, reciprocal regulation of these transcription factors also sets up their gradients (Figure 14.3B).

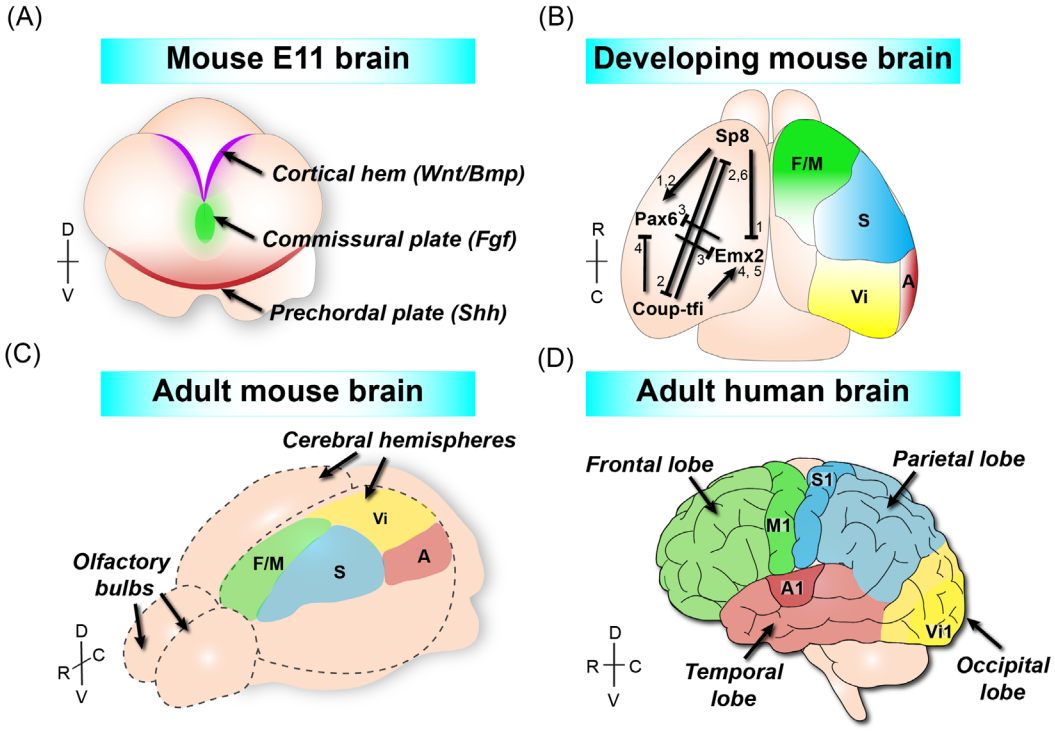

FIGURE 14.3 **Arealization of the developing neocortex.** (A) Early expression of diffusible ligands from three major patterning centers in the forebrain establishes areal identity. *Wnts* and *Bmps* are expressed from the cortical hem, *Fgfs* are expressed from the commissural plate (also known as the rostral patterning center), and *Shh* is expressed from the prechordal plate. Gradients of these morphogens influence the expression of transcription factors that establish boundaries between functionally distinct areas (B). *Sp8* and *Pax6* are expressed in high rostral to low caudal gradients and promote frontal/rostral identity. *Coup-tfi* is expressed in a high caudolateral to low rostromedial gradient and inhibits rostral identity by repressing *Sp8* and *Pax6* expression. *Emx2* is expressed in a high caudal to low rostral gradient and promotes caudal identity. These areal identities have a clear functional distinction in the adult mouse (C) and human (D) brain and form the motor (M, M1), somatosensory (S, S1), auditory (A, A1), and visual (Vi, Vi1) cortices. C=caudal, D=dorsal, F/M=frontal, R=rostral, and V=ventral. Numbers in B refer to the following references: [1]Zembrzycki et al. (2007), [2]Borello et al. (2013), [3]Muzio et al. (2002), [4]Faedo et al. (2008), [5]Hamasaki et al. (2004), [6]Alfano et al. (2014).

Paired box gene 6 (*Pax6*) and Specificity protein 8 (*Sp8*) are expressed in a high rostral to low caudal gradient and are required to establish rostral frontal/motor areas. Mutant mice that are deficient in PAX6 or SP8 display a rostral shift in arealization, such that the frontal/motor and somatosensory areas are diminished, whereas the visual and auditory areas are expanded rostrally (Bishop et al., 2000; Muzio et al., 2002; Sahara et al., 2007; Zembrzycki et al., 2007). *Pax6* conditional knockout mice also show significant reductions in interhemispheric connectivity by dMRI. This is evidenced by a reduction in tractography-generated interhemispheric streamlines associated with (i) decreases in FA and axial diffusivity and (ii) an increase in radial diffusivity, suggesting a decrease in axonal number (Boretius et al., 2009). This change is associated with a reduction in the size of cortical areas that normally give rise to the majority of interhemispheric connections.

Chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1, also known as nuclear receptor subfamily 2, group F, member 1 or NR2F1) is expressed in a high caudolateral to low rostromedial gradient and represses frontal/motor area identity. *Coup-tf1* conditional knockout mice have a remarkably expanded frontal/motor area across most of the cortex, at the expense of somatosensory, visual, and auditory areas (Armentano et al., 2007). Empty spiracles homologue 2 (EMX2) is expressed in a similar high caudomedial to low rostrolateral gradient. In contrast with COUP-TF1, EMX2 is thought to actively promote caudal area identity, as *Emx2* knockout mice have expanded frontal/motor and somatosensory areas and smaller visual and auditory regions (Bishop et al., 2000).

The mechanisms of regional fate specification downstream of these broadly expressed transcription factors are as yet poorly understood; however, three transcription factors have been identified that may play a role: T box brain 1 (*Tbr1*), Basic helix loop helix protein 5 (*Bhlh5*), and *Coup-tf1*. *Tbr1* specifies frontal identity by promoting the expression of the frontal cortex marker gene Autism susceptibility candidate 2 (*Auts2*; Bedogni et al., 2010). *Tbr1* knockout mice show a gene expression pattern consistent with a respecification of frontal areas into more caudal identity. *Bhlh5* specifies the identity of the somatosensory and caudal motor regions (Joshi et al., 2008), and *Bhlh5* null mice have a larger primary visual area and somatosensory barrel field associated with changes in area-specific gene expression, but no change in the graded expression of transcription factors (PAX6, COUP-TF1, EMX2, and SP8 are all normally expressed). Another study found that PAX6 regulates BHLH5 (Holm et al., 2007) suggesting that BHLH5 could be a mediator of arealization downstream of graded transcription factors. The postmitotic expression of *Coup-tf1* has also been shown to be more important for specifying regional identity than *Coup-tf1* expression in progenitor cells. Re-expression of COUP-TF1 in postmitotic neurons of *Coup-tf1* null mice largely rescues the arealization phenotype (Alfano et al., 2014). How these and other genetic pathways downstream of the transcription factors PAX6, COUP-TF1, EMX2, and SP8 result in the acquisition of area-specific characteristics and discrete areal boundaries remains to be elucidated. This is important as subtle changes in arealization, and therefore the connectivity of one or more cortical areas may result from disruption to signaling pathways downstream of early patterning and transcription factor programs.

Arealization is initially set up intrinsically, but can later be modified by sensory input. Sensory input to the cortex comes primarily from thalamocortical neurons. Thalamocortical input can influence the development of a cortical area in terms of its gene expression, cytoarchitecture, and cell morphology (Li et al., 2013). Following sensory deprivation in the visual or auditory system, the corresponding cortical area diminishes in size and is often innervated by sensory afferents from different modalities (Schneider, 1973; Frost, 1982; Sur et al., 1988; Roe et al., 1990; Dehay et al., 1991; Rakic et al., 1991; Roe et al., 1993; Sharma et al., 2000; Hunt et al., 2006). These rewiring events appear to instruct functional changes in areas receiving these ectopic sensory afferents (Frost et al., 2000; Ptito et al., 2001), expanding the processing capacity for that modality, and ultimately resulting in improved performance outcomes (Métin and Frost, 1989; Roe et al., 1990; Sadato et al., 1996; Kujala et al., 1997; Kupers and Ptito, 2014).

Subtler changes in thalamocortical connectivity have been observed in several human disorders. The stabilization of projections between the thalamus and cortex occurs mainly in the third trimester; this critical window of development is therefore disrupted in prematurely born infants. Tractography studies in neonates who have been born prematurely show markedly reduced structural connectivity between the thalamus and neocortex (Ball et al., 2013); changes in this connectivity around birth are associated with poorer cognitive outcome in the ensuing years of development (Ball et al., 2015).

14.2.4 Cortical Connectivity

Excitatory projection neurons in the mature cortex can be subdivided into two major classes based on their axonal projections (Harris and Shepherd, 2015). These are intratelencephalic projection neurons and corticofugal (corticosubcortical) projection neurons. Intratelencephalic projection neurons project to other regions within the cortex

are found in all cortical layers and can be further subdivided into interhemispheric or intrahemispheric projection neurons. Intrahemispheric projection neurons can have single or multiple axonal connections to neurons within different layers of the same cortical column, to other cortical columns within the same functional area (intrinsic or intra-areal), or to other functional areas within the cortex (inter-areal). Axons that form intrahemispheric connections do so by navigating through the grey matter or by contributing to white matter tracts (Watakabe et al., 2014). Interhemispheric connections are formed by long-range projection neurons that form commissures across the interhemispheric midline to innervate both homotopic and heterotopic contralateral cortical areas (Boyd et al., 1971; Yorke and Caviness, 1975; Hedreen and Yin, 1981; Segraves and Rosenquist, 1982; Krubitzer et al., 1998; Catania, 2001; Zhou et al., 2013). The major forebrain commissures connecting the cortical hemispheres in mammals are the corpus callosum, hippocampal commissure, and anterior commissure (depicted for mouse in Figure 14.4A). Callosal neurons are primarily found in layers II–III and V and a proportion are bifurcating, i.e., they project to the contralateral cortex as well as structures in the ipsilateral hemisphere such as the striatum (Wilson, 1987; Koester and O'Leary, 1993; Reiner et al., 2003; Veinante and Deschênes, 2003; Mitchell and Macklis, 2005; Garcez et al., 2007; Hattox and Nelson, 2007; Lickiss et al., 2012; Watakabe et al., 2014). Corticofugal projection neurons are mainly found in the deep layers of the cortex (V–VI); some of the major descending projections are the corticothalamic, corticocollicular, corticopontine, and corticospinal tracts.

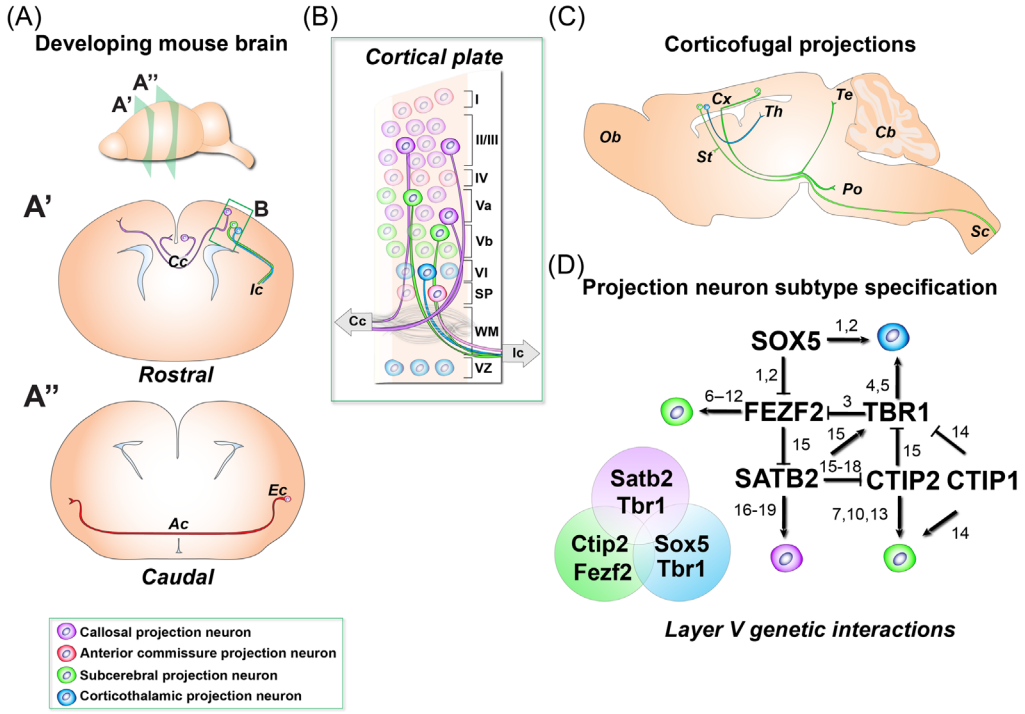

FIGURE 14.4 **Specification of projection neuron subtypes.** (A) Neocortical projection neurons can form connections either within the telencephalon or outside the telencephalon (a corticofugal projection) via the internal capsule (Ic; A') projecting to either the thalamus (corticothalamic projection neurons, blue cells) or subcerebral targets (subcerebral projection neuron, green cells) (B). A subset of intratelencephalic neurons projects across the telencephalic midline via commissures such as the corpus callosum (Cc; A', purple cells) or anterior commissure (Ac; A'', red cells) and connect with either homotopic or heterotopic targets in the contralateral hemisphere. (B) Most callosally projecting and subcerebrally projecting neurons originate from layers II/III and V, whereas corticothalamic projection neurons reside in layer VI (B and C). (D) Projection neuron subtype is specified by a complex network of interactions between transcription factors. *Ctip1*, *Ctip2*, and *Fezf2* are major determinants of subcerebral projection neuron identity, whereas *Satb2* downregulates *Ctip2* expression and is a major determinant of callosal neuron identity. Corticothalamic projection neuron identity is determined by *Sox5* expression secondary to direct repression of *Fezf2*. Cb= cerebellum, Cx= cortex, Ob= olfactory bulb, Po=pons, Sc=spinal cord, St=striatum, Te=tectum, Th=thalamus. Numbers in D refer to the following references: [1]Lai et al. (2008), [2]Shim et al. (2012), [3]Han et al. (2011), [4]O'Leary and Koester (1993), [5]McKenna et al. (2011), [6]Chen et al. (2005), [7]Chen et al. (2008), [8]Molyneaux et al. (2005), [9]Rouaux and Arlotta (2010), [10]Rouaux and Arlotta (2013), [11]De la Rossa et al. (2013), [12]Lodato et al. (2014), [13]Arlotta (2005), [14]Cánovas et al. (2015), [15]Srinivasan et al. (2012), [16]Alcamo et al. (2008), [17]Britanova et al. (2008), [18]Srivatsa et al. (2014), [19]Leone (2014).

14.2.5 The Specification of Neocortical Projection Neurons

The first layers to be generated in the cortex are the subplate and layer VI which contain neurons that form subcortical connections to the thalamus and the spinal cord, and serve as pioneers of the corticothalamic and corticospinal tracts (Figure 14.4B and C; McConnell et al., 1989; Ghosh et al., 1990; De Carlos and O'leary, 1992; Ghosh and Shatz, 1993; McConnell et al., 1994; Clancy et al., 2009; Kanold, 2009; Kanold and Luhmann, 2010; Molnár et al., 2010). The transcription factor Sex determining region Y box 5 (SOX5) is required for the correct generation of these connections. SOX5 is expressed throughout the developing cortical plate at E13.5 but becomes restricted to layers V–VI and the subplate postnatally. It promotes the development of corticothalamic projection neurons by repressing a corticospinal fate through direct repression of *Fezf2* (enhancer of FEZ family zinc finger 2; Lai et al., 2008; Shim et al., 2012). Neurons born at E11.5 that normally generate the subplate and layer VI are disorganized in Sox5 knockout mice, upregulate the transcription factor *Ctip2* (chicken ovalbumin upstream promoter transcription factor-interacting protein 2), and project their axons towards the corticospinal tract (Lai et al., 2008). TBR1 also specifies corticothalamic fate in layer VI projection neurons. Almost all corticothalamic axons highly express TBR1, which is required for timely corticothalamic innervation by birth. TBR1 overexpression in deep layers (at E12.5 and E13.5) is sufficient to redirect corticospinal neurons toward the thalamus (McKenna et al., 2011) because it directly represses the corticospinal-specifying transcription factor FEZF2 (Han et al., 2011). SOX5 and TBR1 are therefore crucial for the development of corticothalamic neurons by suppressing the later born corticospinal fate through repression of *Fezf2* (Figure 14.4D).

Layer Va predominantly contains medially projecting callosal neurons and layer Vb predominantly contains corticofugal neurons, a large majority of which form the corticospinal tract (Figure 14.4C; Harwell et al., 2012). These projection neuron subtypes are generated on the same birth date and are largely intermingled during embryonic development; however, corticofugal projection neurons extend axons 2 days earlier than interhemispheric projection neurons (Richards et al., 1997). Furthermore, subclasses of corticofugal neurons target different subcortical structures (Figure 14.4C). This leads to the question of how such a diverse mix of projection neuron subtypes with the same birth date is specified. FEZF2 is normally expressed in layers V and VI, and specifies corticospinal motor neuron identity by upregulating a suite of genes that promote corticospinal identity and repress callosal and other corticofugal fates (Molyneaux et al., 2005; Rouaux and Arlotta, 2010; la Rossa De et al., 2013; Lodato et al., 2014). Corticocollicular and corticospinal neurons are greatly reduced in *Fezf2* knockout mice (Chen et al., 2005; Molyneaux et al., 2005) and these projection neurons instead project axons through the corpus callosum (Chen et al., 2005, 2008; Molyneaux et al., 2005). This phenotype can be partially rescued by CTIP2 overexpression in cells born at E13.5 (Chen et al., 2008).

Ctip2 knockout mice display a disorganized corticospinal tract that does not project past the pons (Arlotta et al., 2005); therefore CTIP2 is important for the last stages of corticospinal tract guidance. CTIP2 and FEZF2 overexpression in upper layer progenitors at E14.5 induces rewiring of upper layer neurons to a corticospinal projection neuron identity (Chen et al., 2008; Rouaux and Arlotta, 2013). However, FEZF2 does not directly regulate *Ctip2*, and upper layer neurons that are rewired in response to overexpression of FEZF2 cannot project past the pons, as they fail to express CTIP2 (Chen et al., 2008). Similar to CTIP2, CTIP1 directly represses *Tbr1* in layer V and is required for the projection of the corticospinal tract past the pons (Cánovas et al., 2015). *Fezf2*, *Ctip1*, and *Ctip2* are therefore master regulator genes that are required for the correct specification and formation of the corticospinal tract (Figure 14.4D).

Special AT-rich sequence-binding protein 2 (SATB2) is a transcription factor that is highly expressed in layers II–III and V, and is required for the development of callosal neurons from layer V. FEZF2 represses the callosal trajectory of layer V neurons by repressing *Satb2* (Srinivasan et al., 2012). In FEZF2-negative cells, however, SATB2 directly represses *Ctip2* to inhibit the corticofugal fate and initiate a gene expression program that facilitates the acquisition of a callosal fate (Alcamo et al., 2008; Britanova et al., 2008; Srinivasan et al., 2012; Srivatsa et al., 2014). In *Satb2* knockout mice, cells that normally express *Satb2* in layers II–III and V are molecularly respecified to a corticofugal projection neuron type (Alcamo et al., 2008; Britanova et al., 2008). Importantly, cells in both deep and upper layers that normally express SATB2 upregulate CTIP2. However, only deeper layer neurons are able to contribute axons to the corticofugal tract suggesting that upper layer neurons cannot be completely respecified (Leone et al., 2014). Interestingly, early expression of SATB2 is required for the correct formation of the corticospinal tract. When *Satb2* is conditionally knocked out in the cortex (E15.5 or earlier), the corticospinal tract fails to extend past the cerebral peduncle or enter the spinal cord by P15. This phenotype is alleviated when *Satb2* is conditionally knocked out after E16.5 (Leone et al., 2014).

Although these transcription factors regulate many genes that are likely to be involved in the molecular identity of the different projection neuron subtypes, two axon guidance receptors Unc5 homolog c (UNC5C) and Deleted in

colorectal cancer (DCC), and their ligand Netrin1 (NTN1), have been shown to facilitate the downstream guidance of corticofugal versus callosal axons in layer V. DCC and UNC5C mediate an attractive and repulsive response to NTN1, respectively (Keino-Masu et al., 1996; Serafini et al., 1996; Hong et al., 1999; Finger et al., 2002; Srivatsa et al., 2014). Srivatsa et al. (2014) found that SATB2 positively regulates *Unc5c* and inhibits *Dcc*, whereas CTIP2 inhibits *Unc5c*. They showed that overexpressing SATB2, or UNC5C or knocking down DCC in deep layers in the *Satb2* knockout mice led to the redirection of these axons across the corpus callosum, thereby partially rescuing the phenotype. This suggests that *Unc5c* and *Dcc* are axon guidance genes downstream of SATB2 that mediate the callosal versus corticofugal fate of layer V projection neurons. As NTN1 is expressed in the basal ganglia, surrounding the internal capsule (Métin et al., 1997), it is likely that *Unc5c* expression downstream of SATB2 and repression of *Dcc* by SATB2 results in the repulsion of SATB2-expressing callosal axons away from the source of NTN1 where corticofugal neurons must navigate. In the absence of SATB2 and the presence of CTIP2, however, *Unc5c* is repressed and DCC is able to mediate a positive response to NTN1, directing axons toward subcortical targets.

The specification of callosal neurons in upper layers is less well studied. As described above, upper layer neurons in *Satb2* knockout mice, despite molecularly expressing Ctip2, are unable to project corticofugally or callosally (Leone et al., 2014). Instead, their axons project aberrantly to intrahemispheric targets. It should be noted that cells that do not normally express SATB2 continue to cross the midline and form a small corpus callosum in *Satb2* knockout mice. In addition, not all retrogradely labeled callosal neurons express SATB2 (Alcamo et al., 2008; Lickiss et al., 2012), suggesting that a subset of callosal neurons do not require SATB2 to form callosal connections. Furthermore, upper layer neurons continue to project their axons across the corpus callosum when *Satb2* is knocked down only in upper layer progenitors (Zhang et al., 2012), suggesting that *Satb2* may play different roles in callosal axon specification depending on the layer of origin. These cells have defects in axonal growth, somal spacing, and dendritic arborization. Interestingly, the repression of *Ctip2* by SATB2 depends on the coexpression of the proto-oncogene *Ski* (Baranek et al., 2012). SATB2 binds the DNA sequence upstream of *Ctip2*, assembling the Nucleosome remodeling deacetylase (NURD) complex for deacytelation (inactivation) of CTIP2 (Britanova et al., 2008). SKI, however, is required for the recruitment of Histone deacetylase 1 (HDAC1) to this protein complex, and is therefore indispensible for SATB2 repression of *Ctip2* (Baranek et al., 2012). SKI and SATB2 are highly coexpressed in the upper layers, where they repress the expression of *Ctip2* (Baranek et al., 2012). Evidence of this is that *Ski* and *Satb2* knockout mice both show expanded expression of *Ctip2* into the upper layers of the cortex. Interestingly, *Ski* and *Ctip2* are coexpressed in layer V, where *Satb2* and *Ski* are almost never coexpressed (Baranek et al., 2012). Whether SATB2 directly represses *Ctip2* in layer V (without SKI) remains to be investigated; however, layer V neurons regain the ability to cross the corpus callosum in *Satb2/Ctip2* double knockout mice, suggesting that CTIP2 is at least partially responsible for the loss of callosal axons in *Satb2* knockout mice (Srivatsa et al., 2014).

These papers represent a decade of progress toward understanding the transcriptional regulation of projection neuron fate specification. Although several studies have identified the suite of genes downstream of these transcription factors, how these gene programs regulate projection neurons to acquire mature characteristics and make functional connections in the brain remains to be elucidated. Axonal guidance genes, which encode families of ligands and receptors, likely act downstream of these transcription factors to regulate the development of connections within the complex cellular and molecular environment of the brain.

14.2.6 The Corpus Callosum as a Model System for Studying Developing White Matter Tracts

The corpus callosum is the largest white matter tract in eutherian mammals, and is particularly prominent in the human brain, carrying approximately 190 million axons from one cortical hemisphere across the midline of the forebrain and into the opposite cortical hemisphere (Tomasch, 1954; Gazzaniga, 2005). Congenital absence (or agenesis) of the corpus callosum (AgCC) is a relatively common human brain malformation (1:4000 live births; Hetts et al., 2006) that is associated with a large number of neurodevelopmental disorders (Edwards et al., 2014) and results in a wide spectrum of neurological deficits (Paul et al., 2007).

The corpus callosum in AgCC patients can be completely absent (complete AgCC), partially absent (partial AgCC), or reduced in thickness (hypoplasia of the CC). The formation of Probst bundles is a common feature of AgCC. These axons do not cross the midline but instead run anteroposteriorly along the midline (Probst, 1901). All of these phenotypes have been recapitulated in mouse models of AgCC (Ren et al., 2007), which have begun to reveal some of the cellular processes of corpus callosum development that may lead to their emergence. Normal corpus callosum formation depends on a multitude of developmental events, including the correct generation of midline glial cells, and the specification of projection neurons to a callosal identity, as well as the guidance of axons

out of the cortical plate toward the midline of the brain, then across the midline to their targets in the contralateral hemisphere. Corpus callosum development and AgCC provide an excellent system for translational studies that aim to investigate the genetics and anatomy of white matter disorders in humans, using animal models that recapitulate the phenotype for experimental manipulation.

14.2.6.1 Intermediate Zone Guidance

Once callosal neurons have been specified in the cortex, they project axons into the white matter underlying the neocortex and turn medially toward the midline. At this stage, distinct axon guidance receptors downstream of transcription factors are able to guide the axon to turn medially toward the midline and across the corpus callosum, or inferiorly to enter the internal capsule. Callosal axons respond to repulsive cues from the lateral cortex (Zhao et al., 2011), one of which is probably NTN1 in mice. As described in Section 14.2.4, UNC5C expression on callosal axons downstream of SATB2 facilitates the chemorepulsive response to NTN1 expressed in ventral–lateral regions of the mouse forebrain, repelling them from making a lateral turn. T-cadherin (also known as cadherin 13) also regulates this decision. T-cadherin is normally expressed in subcortically projecting neurons of the deeper layers. Suppressing its activity causes subcortical projection neurons to incorrectly project medially toward the midline, and overexpressing T-cadherin in upper layer callosal neurons causes them to project laterally (Hayano et al., 2014). Neurogenin2 (NGN2) plays a complementary role, promoting the medial turning of callosal axons. *Ngn2* knockout mice show partial AgCC without Probst bundles. Interestingly knockdown of *Ngn2* in upper layer neurons results in lateral turning of affected axons but no change in their transcriptional profiles (they continue to express upper layer markers rather than deep layer markers such as *Ctip2*), suggesting that NGN2 is downstream of fate specification (Hand and Polleux, 2011). Another mouse model with a similar phenotype (AgCC without Probst bundles) lacks both the Neuronal differentiation (*Neurod*) *2* and *6* genes. Axons in double knockout mice are able to turn medially out of the cortex toward the midline but fail to grow toward it, instead defasciculating and stalling in the subventricular zone of the cingulate cortex (Bormuth et al., 2013). These examples demonstrate that guidance decisions must be made well before callosal axons reach the midline.

14.2.6.2 Guidepost Cells

Guidepost cells are populations of neurons or glia that express guidance cues at different stages of white matter tract formation. They enable step-by-step navigation of the brain environment, facilitating the guidance of axons across long distances. The corpus callosum crosses the midline rostrally at the boundary between the cingulate cortex and septum (cortico-septal boundary). This region of the midline comprises a heterogeneous mix of both neuronal cells and glia that act as guidepost cells to attract callosal axons toward the midline, channel them through the midline, and repel them into the contralateral hemisphere (Figure 14.5A and B). The dorsal boundary of the corpus callosum is the indusium griseum, which comprises both neurons and glia (the indusium griseum glia; Shu and Richards, 2001). During development, radial glia reside ventral and lateral to the corpus callosum comprising the glial wedge (Shu and Richards, 2001; Shu et al., 2003a). The subcallosal sling is a predominantly neuronal population that forms a characteristic U-shape underneath the corpus callosum (Silver et al., 1982; Shu et al., 2003b). In addition, a population of GABAergic neurons populates the middle and lateral regions of the corpus callosum during midline crossing (Niquille et al., 2009, 2013). All of these guidepost cell populations have been shown to have a functional influence on corpus callosum formation by secreting guidance factors (Table 14.1) or making cellular contacts with the callosal axons.

For the corpus callosum to form correctly, distinct midline glial populations must be generated in the correct proportions and position in order for the midline crossing of callosal axons to occur (Figure 14.5B; Gobius and Richards, 2011). The glial wedge and indusium griseum glia must first correctly differentiate from radial glia within the ventricular zone of the medial cortex and then mature morphologically. These glial populations are some of the first glia to differentiate in the forebrain, as the majority of astrocytes are generated postnatally in the cerebral cortex. Several morphogens, transcription factors, and enzymes are required to produce the glial populations of the cerebral midline, and these are briefly reviewed below.

Nuclear factor one (NFI) transcription factors A and B are important for the transition of the radial glia progenitors into the mature astroglial cells that make up the glial wedge and indusium griseum glia. NFIA and NFIB are expressed in the indusium griseum glia, glial wedge, and subcallosal sling as well as in cortical neurons (Shu et al., 2003a; Piper et al., 2009a). *Nfia* and *Nfib* knockout mice have delayed maturation of these cell populations, which remain radially oriented and lack expression of mature glial markers during embryonic development. Consequently, these knockout mice fail to form a corpus callosum by birth (Neves Das et al., 1999; Shu et al., 2003a; Steele-Perkins et al., 2005; Piper et al., 2009a).

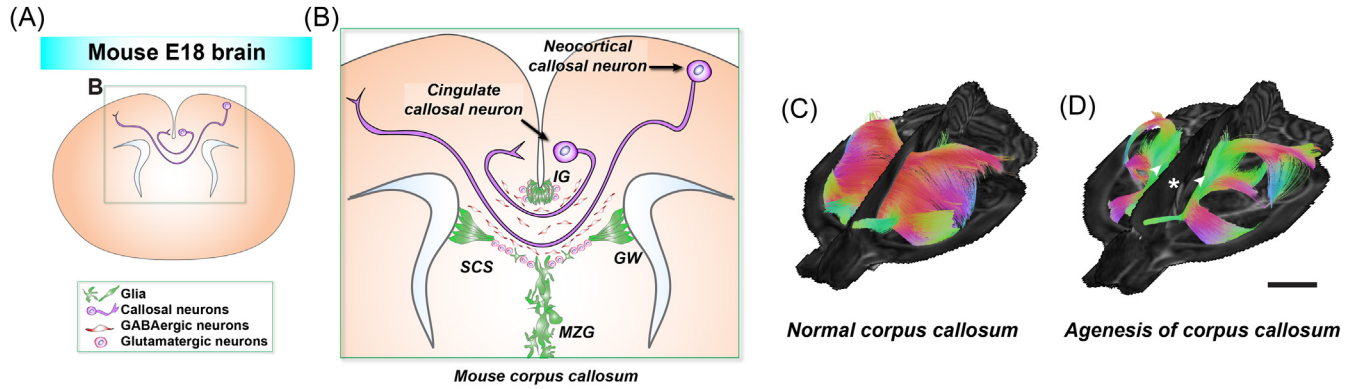

FIGURE 14.5 **Guidepost cells regulate midline axon guidance of the corpus callosum.** (A) The axons that form the corpus callosum originate from neurons within the cingulate cortex and the neocortex. (B) Callosal axon guidance across the midline is facilitated by multiple glial and neuronal populations. The indusium griseum (IG) is positioned dorsal to the corpus callosum and comprises glia and neurons. The glial wedge (GW) consists of glia that arise from the medial ventricular zones and extend processes toward the midline, providing the ventral boundary to the corpus callosum. The subcallosal sling (SCS) is a predominantly neuronal population containing some glia that forms a characteristic U-shape ventral to the developing corpus callosum. The midline is also populated with a population of GABAergic neurons during midline crossing. Gradients of diffusible axon guidance molecules and cell contact guidance cues that are expressed by these cellular populations must first attract/repel callosal axons toward the midline, and then into the contralateral cortex after midline crossing. Midline zipper glia (MZG) are positioned ventral to the corpus callosum and are involved in midline development prior to corpus callosum formation. (C and D). The corpus callosum can be visualized as a large bundle of mediolaterally oriented (colored red) tractography-generated streamlines crossing the telencephalic midline (C). In a mouse with agenesis of the corpus callosum (asterisks in D), Probst bundles are visualized as streamlines running anteroposteriorly (colored green) on either side of the telencephalic midline (arrowheads in D). Scale bar= 2 mm in panels C and D.

Glial wedge cells normally retain a medially oriented polarity and cluster their processes to form a wedge shape. The indusium griseum glia, however, must translocate their cell body to the pial surface and retract their processes from the ventricular zone. This translocation event is regulated by FGF signaling. Glial-specific knockout of the Fgf receptor 1 (*Fgfr1*) results in complete and partial AgCC that is associated with the absence of the indusium griseum glia due to incomplete radial glial translocation to the pial surface. Cells destined to become indusium griseum glia remain attached to the ventricular surface and their soma lies close to the ventricle in *Fgfr1* knockout mice and following cell-specific knockdown of *Fgfr1* (Smith et al., 2006). The heparin sulfotransferases HS6ST1 and HS2ST interact with FGF signaling to inhibit this process, probably normally acting to prevent excessive somal translocation and loss of the glial wedge. In *Hs6st1* and *Hs2st1* knockout mice, AgCC (complete and partial) is associated with an increase in the number of indusium griseum glia at the midline at the expense of the glial wedge (Conway et al., 2011; Clegg et al., 2014). The phosphorylated form of the FGF intracellular signaling effector, extracellular signal-related kinase (ERK) 1/2 is significantly increased at the midline in *Hs6st1* and *Hs2st1* knockout mice, particularly within the nuclei of cells within the medial ventricular zone (Clegg et al., 2014). This callosal phenotype can be rescued in a subset of *Hs6st1* knockout mice that are also heterozygous for *Fgf8* or have been administered an inhibitor targeting the ERK-activating kinase MEK (Clegg et al., 2014), suggesting that downstream activation of FGF signaling is at least partially responsible for the phenotype. An inhibitor of FGF signaling, Sprouty (SPRY) 1/2 also keeps ERK signaling in check. *Spry1/2* double knockout mice have AgCC with Probst bundles, associated with increased phosphorylated ERK1/2, and midline glia that span the entire length of the cingulate cortex from the ventricular zone to the pial surface, thereby ectopically transecting the path of callosal axons (Magnani et al., 2012).

Altered FGF signaling (increased levels FGF8 and phosphorylated ERK1/2) downstream of GLI family zinc finger 3 (GLI3) also results in glial fibrillary acidic protein (GFAP)-positive glia spanning the entire cingulate cortex, and absence of the indusium griseum glia. *Gli3* mutant mice also show expanded expression of the repulsive axon guidance ligand Slit homolog (*Slit*) 2 into the dorsomedial cortex and ectopically in the septum (Magnani et al., 2012; Amaniti et al., 2013, 2015). Similarly, conditional loss of the tumor suppressor Neurofibromatosis 2 (*Nf2*) in the cortex results in a similar glial phenotype, with upregulation of SLIT2 and an increase in glia that extend their processes to the pia and cross the callosal axon path (Lavado et al., 2014). Deleting one copy of *Slit2* restores callosal formation in *Nf2* mouse mutants, suggesting that ectopic SLIT2 expression is preventing callosal crossing in these mice. These findings highlight the importance of correct development of guidepost neurons and disruption to genes involved in glial development associated with AgCC in humans (Edwards et al., 2014).

TABLE 14.1 Expression of Axon Guidance Molecules at the Telencephalic Midline

Callosal axons	IG	GW	MZG	Septum	Subcallosal sling	Guidepost neurons	
						GABAergic interneurons	Glutamatergic neurons
DCC[b]	DRAXIN[i]	DRAXIN[i]	EPHA4[c]	EPHB2[c]	DRAXIN[i]	EPHB2[l]	SEMA3C[l]
EPHB1[c]	EPHA4[c]	EPHA4[c]	EPHB2[c]	EFNB1[c]	EPHA4[c]	EPHB3[a,l]	
EPHB2[c]	EPHB2[c]	EPHB2[c]	EFNB1[c]	EFNB3[c]	SEMA3A[l]	EPHA4[a,l]	
EPHB3[c]	EPHB2[c]	EFNB1[c]	EFNB3[c]	NTN1[b,j]	SEMA3C[d]	EFNA1[l]	
EPHB4[c]	EFNB1[c]	EFNB2[c]		SLIT1[e]	ROBO1[k]	EFNA4[l]	
EFNB1[c]	EFNB3[c]	EFNB3[c]		SLIT3[e]		EFNB1[l]	
EFNB2[c]	NTN1[b,j]	ROBO1[e]		ROBO3[b]		EFNB2[l]	
NRP1[d]	ROBO1[e]	SLIT1[e]				NRP1a[l]	
ROBO1[a,e–g]	ROBO2[e]	SLIT2[e]				NRP2[l]	
ROBO2[a,f–g]	SEMA3C[d]	SLIT3[e]				SEMA3A[l]	
RYK[h]	SLIT1[e]	WNT5A[h]					
	SLIT2[e]						
	SLIT3[e]						
	WNT5A[h]						

GW, glial wedge; IG, indusium griseum glia; MZG, midline zipper glia.
[a]*Very low levels of expression.*
[b]*Fothergill et al. (2014).*
[c]*Mendes et al. (2006).*
[d]*Piper et al. (2009).*
[e]*Unni et al. (2012).*
[f]*Shu and Richards (2001).*
[g]*Andrews et al. (2006).*
[h]*Keeble et al. (2006).*
[i]*Islam et al. (2009).*
[j]*Serafini et al. (1996).*
[k]*Shu et al. (2003b).*
[l]*Niquelle et al. (2011).*

14.2.6.3 Attraction to the Midline

Guidepost cells at the telencephalic midline secrete a wide variety of soluble axon guidance proteins, which signal to surface receptors on callosal axons to mediate corpus callosum formation (Table 14.1). These cues direct the attraction of callosal axons toward and across the midline and their repulsion into the contralateral hemisphere. Many axon guidance genes have been implicated in the development of the corpus callosum, as mice lacking the expression of these genes display AgCC (Table 14.2).

Class 3 semaphorins (SEMA3) and their receptor Neuropilin 1 (NRP1), which is expressed on callosal axons of the cingulate cortex (Piper et al., 2009b), have been shown to regulate the pathfinding of these axons, which are the first population of callosal axons to cross the midline. *Nrp1*$^{Sema-}$ mice (which express NRP1 that lacks the semaphorin-binding domain) and *Sema3c* knockout mice show partial and complete AgCC associated with aberrant guidance of cingulate pioneering axons (Table 14.2; Gu et al., 2003; Niquille et al., 2009; Piper et al., 2009b). SEMA3C is normally expressed in the indusium griseum and subcallosal sling, and can bind and attract callosal axons *in vivo* (Niquille et al., 2009). SEMA3C binding to NRP1 elicits both positive outgrowth and guidance in cingulate cortical explants (Piper et al., 2009b). *Sema3a* is also expressed in the subcallosal sling and in GABAergic interneurons, and has been shown to repel both cingulate cortical and neocortical explants *in vitro.* SEMA3C is therefore likely to attract NRP1-expressing cingulate callosal axons toward the midline, whereas SEMA3A expression from the subcallosal sling is likely involved in preventing the overgrowth of these cingulate axons into the septum, and channeling them across the midline.

The secreted protein, NTN1, is an important attractive guidance cue for commissural axons of the spinal cord (Kennedy et al., 1994; Serafini et al 1996). In contrast, in the cerebral cortex, NTN1 differentially acts as an attractant

TABLE 14.2 Malformation of the Corpus Callosum in Mice Lacking Axon Guidance Genes

Mouse mutant	AgCC	pAgCC	Refs.
Dcc	✓		Fazeli et al. (1997), Ren et al. (2007)
Dcckanga	✓		Fothergill et al. (2014)
Draxin	✓	✓	Islam et al. (2009), Ahmed et al. (2011)
Epha5		✓	Mendes et al. (2006)
Ephb1	✓	✓	Mendes et al. (2006)
Ephb2	✓	✓	Mendes et al. (2006)
EfnB3	✓	✓	Mendes et al. (2006)
Ephb1/b2	✓	✓	Mendes et al. (2006)
Ephb1/b3	✓	✓	Mendes et al. (2006)
Ephb1/Ephb4	✓	✓	Mendes et al. (2006)
Ephb2/b3	✓	✓	Mendes et al. (2006)
Efnb3/Ephb1	✓	✓	Mendes et al. (2006)
Efnb3/Ephb2	✓	✓	Mendes et al. (2006)
Efnb3/Epha4	✓[a]	✓[a]	Mendes et al. (2006)
Fzd3	✓		Wang et al. (2002, 2006)
Ntn1	✓		Fothergill et al. (2014), Serafini et al. (1996)
Nrp1(Sema)	✓	✓	Gu et al. (2003), Piper et al. (2009)
Robo1		✓	Andrews et al. (2006)
Robo1/Robo2		✓	López-Bendito et al. (2007)
Ryk		✓	Keeble et al. (2006)
Slit2		✓	Bagri et al. (2002), Shu et al. (2003c), Unni et al. (2012)
Slit3		✓	Unni et al. (2012)
Slit1/Slit3		✓	Unni et al. (2012)
Slit1/Slit2	✓		Unni et al. (2012)
Sema3c	✓	✓	Niquille et al. (2009)
Efnb1	✓		Bush and Soriano (2009)
Efnb1ΔV[a]	✓		Bush and Soriano (2009)

AgCC, Complete agenesis of the corpus callosum; pAgCC, partial agenesis of the corpus callosum.
[a]*Mice express EFNB1 that lacks a valine required for PDZ reverse signaling.*

depending on the neuronal population (Fothergill et al., 2014). For example, NTN1 attracts cingulate callosal axons to the midline. It does not generally act as an attractant for neocortical callosal axons, but rather modulates the repellent actions of another ligand, SLIT2 (see later).

14.2.6.4 Midline Crossing

Eph receptors (EPH) and their ephrin ligands (EFN) are axon guidance genes that influence growth and guidance through cell–cell contact, as both the receptor and the ligands are anchored to the membrane. Eph receptor tyrosine kinases are divided into two subclasses, A and B, which differ in their binding affinities for ephrins. Ephrins are also divided into an A subclass with a glycosyl-phosphatidylinositol (GPI) anchor to the plasma membrane and a B subclass that possesses transmembrane domains to embed the ligand within the membrane (Egea and Klein, 2007). Ephs and ephrins are widely expressed at the telencephalic midline on both callosal axons and glial guidepost cells (Table 14.1; Mendes et al., 2006). Generally, EFNA class ligands can bind EPHA receptors and EFNB class ligands can bind EPHB receptors, with the exception that EPHA4 binds both EFNA and EFNB ligands (Pasquale, 2005).

Eph-ephrin binding can elicit both forward signaling within the EPH-expressing cell but also reverse signaling within the ephrin-expressing cell, and Ephs and ephrins can also interact on the membrane when expressed in the same cell (Egea and Klein, 2007). For these reasons, the functional role of Eph/ephrin signaling in corpus callosum development remains elusive. *Ephb1*, *Ephb2*, *Epha5*, and *Efnb3* appear to be most important for corpus callosum formation as knockout mice for these genes show partial or complete AgCC with Probst bundles; however, EPHB3 appears to also be involved as *Ephb2/b3* double knockout mice show increased penetrance compared to *Ephb2* single knockout mice (Table 14.2; Hu et al., 2003; Mendes et al., 2006). *In vitro* studies suggest substrate-embedded EFNB1, EFNB2, and EFNA5 stimulate neurite outgrowth of cortical axons (Hu et al., 2003; Mendes et al., 2006), suggesting that ephrins may act as growth-promoting cues during midline crossing.

All three *Slit* family members are expressed in the telencephalic midline and their receptors, roundabout homolog (ROBO)1 and 2 but not 3, are expressed on callosal axons (Table 14.1). Analyses of single and double mutant knockout mice have demonstrated that corpus callosum formation absolutely requires SLIT2 expression, but SLIT1, SLIT3, ROBO1, and ROBO2 are also involved (Table 14.2; Bagri et al., 2002; Shu et al., 2003c; Unni et al., 2012). *Slit2*, *Slit3*, *Robo1*, *Slit1/3*, and *Robo1/2* knockout mice have partial AgCC with Probst bundles, and *Slit1/2* double knockout mice show complete AgCC with Probst bundles (Table 14.2; Unni et al., 2012). DMRI and carbocyanine dye tracing experiments have revealed that *Robo1*, *Robo1/2*, and *Slit2* knockout mice display ipsilateral misguidance of callosal axons ventrally into the septum (López-Bendito et al., 2007; Unni et al., 2012). SLIT2, expressed by the indusium griseum glia and the glial wedge cells, acts to repel callosal axons across the midline and prevent them from entering the septum. This is based on the observations that (i) SLIT2 repels both cingulate and neocortical explants *in vitro* (Shu and Richards, 2001; Unni et al., 2012; Fothergill, Donahoo et al., 2014), (ii) the glial wedge repels callosal axons *in vitro*, an effect which is abolished when ROBO1 and ROBO2 blocking peptides are added to the culture (Shu et al., 2003c), (iii) repression of SLIT2 signaling in the glial wedge on one side of the brain causes axons to be misguided and project aberrantly into the septum, and (iv) overexpression of SLIT2 at the cortico-septal boundary on one side of the brain causes axon misguidance and the formation of Probst bundles on the side ipsilateral to the manipulation (Lavado et al., 2014).

A recent study suggests that axon guidance families interact to generate the appropriate axonal response to different cues. DCC is a transmembrane receptor that is expressed on cingulate callosal axons, while its ligand, NTN1, is expressed in the ventral septum and in the indusium griseum. *Dcc* knockout mice and *Ntn1*-deficient (hypomorphic mutation) mice have fully penetrant complete AgCC with Probst bundles (Table 14.2; Serafini et al., 1996; Fazeli et al., 1997; Ren et al., 2007; Fothergill et al., 2014). The interaction of ROBO1 and DCC receptors in response to simultaneous NTN1 and SLIT2 cues is important for the silencing of SLIT2-mediated repulsion of callosal axons before they reach the midline. In the spinal cord, DCC and ROBO1 proteins interact through their CC1 and P3 intracellular domains, respectively (Stein and Tessier-Lavigne, 2001). In E17 neocortical explants (precrossing), Fothergill et al. (2014) demonstrated that SLIT2 was unable to repel neocortical axons in the presence of NTN1, an effect that was dependent on both ROBO1 and DCC. They showed that the level of DCC protein decreases *in vivo* from E17 until birth, at which point SLIT2 is able to repel neocortical axons. This study was the first to report how callosal axons might respond to multiple guidance cues while navigating the complex midline environment.

14.2.6.5 Exit from the Midline

After callosal axons cross the midline, they must stop being attracted to midline guidepost cells and navigate away from the midline into the contralateral hemisphere. As described above, SLIT2 plays an important role in the initial repulsion of axons from the midline environment (Shu et al., 2003c). The axon guidance receptor Receptor-like tyrosine kinase (RYK) has also been implicated in the regulation of guidance away from the midline. *Ryk* knockout mice have a unique callosal phenotype. Carbocyanine dye tracing in these mice reveals callosal axons crossing the midline and forming Probst bundles in the contralateral hemisphere (Keeble et al., 2006). E18 (postcrossing) but not E16 or E17 neocortical explants are repelled by WNT5A in a RYK- and calcium/calmodulin-dependent protein kinase (CAMK2A)-dependent fashion (Keeble et al., 2006; Hutchins et al., 2011). Furthermore, *Ryk* and *Camk2a* knockdown results in reduced outgrowth and postcrossing misguidance of callosal axons *in vivo* (Hutchins et al., 2011). WNT/RYK signaling to downstream CAMK2A modulates calcium activity within the axon, initiating repulsive growth and guidance away from the midline in mice.

14.2.6.6 Contralateral Targeting

The mechanisms by which callosal axons innervate appropriate heterotopic and homotopic regions in the contralateral cortex, after midline crossing, are less well studied but these processes may be disrupted in a number of neurological and mental disorders (Fenlon and Richards, 2015). However, two general rules governing this guidance

have been identified. The first is that axon position within the callosal tract during midline crossing is important for targeting in the contralateral hemisphere, and the second is that activity matching between cortical hemispheres regulates the targeting of a subset of callosal projections.

Callosal axons from different cortical areas are segregated within the corpus callosum during midline crossing. For example, cingulate axons cross dorsal to neocortical callosal axons, and axons from the medial neocortex (primary motor cortex) cross the midline dorsal to axons from lateral regions of the neocortex (primary somatosensory cortex; Piper et al., 2009b; Zhou et al., 2013). SEMA3A/NRP1 signaling regulates this topography. SEMA3A is expressed within the cortical plate in a high caudal and lateral to low rostral and medial gradient, and NRP1 is highly expressed on callosal axons derived from caudal and medial regions. Cell-autonomous disruption of *Nrp1* expression within the medial primary motor cortex causes mixing of callosal fibers derived from the primary somatosensory and primary motor cortex within the callosal tract at the midline and aberrant targeting of the primary motor cortex to the contralateral somatosensory cortex. Interestingly, *Sema3a* knockout mice display normal midline crossing; however, primary motor and primary somatosensory callosal axons mix at the midline and target the incorrect cortical region in the opposite hemisphere rather than remaining segregated and innervating their homotopic regions (Zhou et al., 2013).

Mouse studies of contralateral callosal targeting have identified that callosal axons derived from the primary somatosensory, motor, and visual areas sparsely innervate homotopic regions and densely innervate the border region between primary and secondary areas. This dense targeting to the border region is highly sensitive to manipulations in activity in both the somatosensory and visual systems (Olavarria and Van Sluyters, 1995; Mizuno et al., 2007; Wang et al., 2007; Ageta-Ishihara et al., 2009; Huang et al., 2013; Suárez et al., 2014). Furthermore, a balance of both intrinsic (endogenous cortical activity) and extrinsic (from the sensory periphery) activity between ipsilateral and contralateral areas appears to be particularly important for targeting (Mizuno et al., 2010; Huang et al., 2013; Suárez et al., 2014). Unilaterally disrupting sensory activity from the periphery or endogenous cortical activity in one hemisphere also disrupts the major projection from the primary somatosensory cortex which targets the border region between the primary and secondary somatosensory regions in the contralateral hemisphere (Mizuno et al., 2007; Wang et al., 2007; Suárez et al., 2014). Furthermore, bilaterally disrupting sensory input or endogenous cortical activity rescues the targeting to the border region (Suárez et al., 2014). The activity-dependent molecular mechanisms that regulate this axon targeting remain to be elucidated.

14.3 dMRI-BASED IMAGING OF BRAIN CONNECTIVITY

14.3.1 Modeling the Development of Normal and Abnormal White Matter Tracts

In addition to investigating the cellular and molecular mechanisms that regulate the development of white matter tracts in the brain, sophisticated neuroimaging techniques allow the investigation of anatomical connectivity in living and postmortem human and animal brains. This provides a means for translational and comparative studies of brain development and plasticity. dMRI generates detailed information regarding axonal white matter tracts based on the diffusion of water molecules within the brain (Mori and Zhang, 2006) as described in Section 14.2.2 in the context of the developing cortical plate. However, the dynamic structural changes of the developing brain are not limited to early cortical development. During preterm and neonatal life, the white matter undergoes significant changes in microstructure and organization. At this time, most long-range white matter tracts are still being established and stabilized, and the mean diffusivity of the white matter decreases while the FA tends to increase (Hüppi et al., 1998; Neil et al., 1998; Mukherjee et al., 2002; Huang et al., 2006). These changes are correlated with the progressive myelination of maturing white matter tracts, as well as earlier changes in the cytoskeletal complexity of axons, changes in axon diameter and packing, population of these tracts with oligodendrocytes, and concomitant decreases in the water content of the tissue (Hüppi et al., 1998; Neil et al., 1998; Huang et al., 2006; Hüppi and Dubois, 2006; Huang et al., 2009). The exact contribution of each of these processes to the changes in the dMRI signal is not entirely clear; however, by further investigating changes in specific components of the dMRI tensor, a clearer picture can be drawn about how different changes in microstructure are reflected in the tensor model. For example, the diffusion parallel to a white matter tract (the principal eigenvalue of a tensor) is unlikely to be affected by an increase in myelination; it does, however, change in response to differences in water content, and barriers to diffusion such as the cytoskeleton (Mukherjee et al., 2002). Decreases in the minor eigenvalues reflect a decrease in the diffusion of water perpendicular to the white matter tract, and so would be expected to correlate with processes such as myelination that occur in early neonatal life (Mukherjee et al., 2002). A significant amount of diffusion is

also likely to occur extracellularly within these tissues. In this case, changes in the two minor eigenvalues may also reflect decreased diffusion between axons as the tissue water content decreases and barriers to extracellular diffusion (such as more axons) increase (Norris, 2001).

dMRI can involve modeling of diffusion in over 100 directions. Under these circumstances, the simple tensor model is inadequate and it becomes more worthwhile to model connectivity between specific brain regions by means of tractography. An initial approach to white matter tractography was to generate streamlines starting from a specified location (the "seed") by interpreting the major eigenvalue of each voxel as the approximate direction of the white matter and propagating a continuous line from voxel to voxel by linking the major eigenvectors into a coherent virtual tract (Conturo et al., 1999; Mori et al., 1999; Mori and van Zijl, 2002). However, the value of this approach is limited because it fails to resolve distinct populations of axons contributing to crossing white matter fibers and different directions of anisotropies within the same voxel (Alexander et al., 2001; Frank, 2001; Alexander et al., 2002; Tuch et al., 2002). This is not ideal, as the complexity of the mammalian brain necessitates that essentially every axonal projection must encounter other axonal projections with different orientations. To overcome this limitation, various algorithms have been proposed to model complex white matter architectures, including diffusion-spectrum imaging (Wedeen et al., 2005), high angular resolution diffusion imaging, q-ball imaging, and constrained spherical deconvolution (Tournier et al., 2004, 2007; Jbabdi and Johansen-Berg, 2011). These more advanced interpretations of the MR signal are based on the assumption that each fiber direction independently contributes to the final tensor model, and they are being further developed to resolve multiple fiber populations, including situations in which crossing fibers meet when axons of different orientations enter the same tract. Using these approaches, it is possible to virtually dissect white matter tracts within the brain, identify novel white matter structures that result from neurodevelopmental disorders, and determine changes in scalar values (such as FA) along white matter tracts.

14.3.2 Corpus Callosum Dysgenesis and Visualizing Axonal Plasticity During Brain Development

Corpus callosum dysgenesis in human or animal brains can be identified by a reduction or complete absence of midline crossing fibers as evident in structural T1- or T2-weighted images or, alternatively, can be identified from modeling with whole-brain tractography or seeded from regions of interest at the midline or in the cortex (Figures 14.5C,D and 14.6). As noted above, AgCC in both human and mouse brains is commonly associated with the formation of Probst bundles, which occur when callosal axons fail to cross the midline and instead form longitudinal intrahemispheric bundles situated in both hemispheres. These bundles run anterior to posterior and can be easily visualized by dMRI and tractography (Figures 14.5C,D and 14.6). As described in Section, mouse models of AgCC demonstrate that the formation of Probst bundles is associated with failure of callosal axons to cross the midline, rather than a defect in axonal generation, specification or navigation toward the midline. The development of Probst bundles has not been extensively investigated but they are electrically active in rodents (Lefkowitz et al., 1991), supporting the possibility that functional connections may exist within the bundle.

In mice, corpus callosum dysgenesis is associated with loss of axon guidance genes (Table 14.2), loss of genes that regulate midline formation as well as guidepost cell differentiation and migration (described in Section). Using *Ntn1* and *Dcc* knockout mice as a model, the anatomy of the Probst bundle has been described using dMRI with tractography combined with carbocyanine dye tracing (Ren et al., 2007). It was demonstrated that axons within the dorsal Probst bundle are more organized, running in a rostral to caudal direction along the midline. In contrast, ventral parts of the Probst bundle appeared more disorganized and had random trajectories. In partial AgCC mouse models, MRI combined with carbocyanine dye tracing can determine which populations of callosal axons contribute to the formation of a Probst bundle. In *Robo1* and *Slit2* knockout mice, tractography modeling of interhemispheric projections has revealed that interhemispheric projections from the visual cortex appear to be reduced, whereas axons from the motor and parietal cortices aberrantly enter both the ipsilateral and contralateral septum (Unni et al., 2012).

The use of dMRI has recently provided a platform for the discovery of the remarkable plasticity in the development of axonal tracts in the brain of human subjects with corpus callosum dysgenesis. dMRI coupled with tractography has identified a series of novel interhemispheric connections, originating from cortical areas, that display an impressive degree of developmental plasticity. Of particular note, ectopic tracts projecting through the anterior and posterior commissures have been identified in individuals with AgCC that do not exist in normal individuals (Tovar-Moll et al., 2014). These novel connections appear to consistently connect homotopic regions of the parietal cortex, and therefore could partially compensate for the lack of interhemispheric connectivity normally mediated by the corpus callosum. In individuals with partial AgCC, where a small remnant of callosal axons still cross the midline

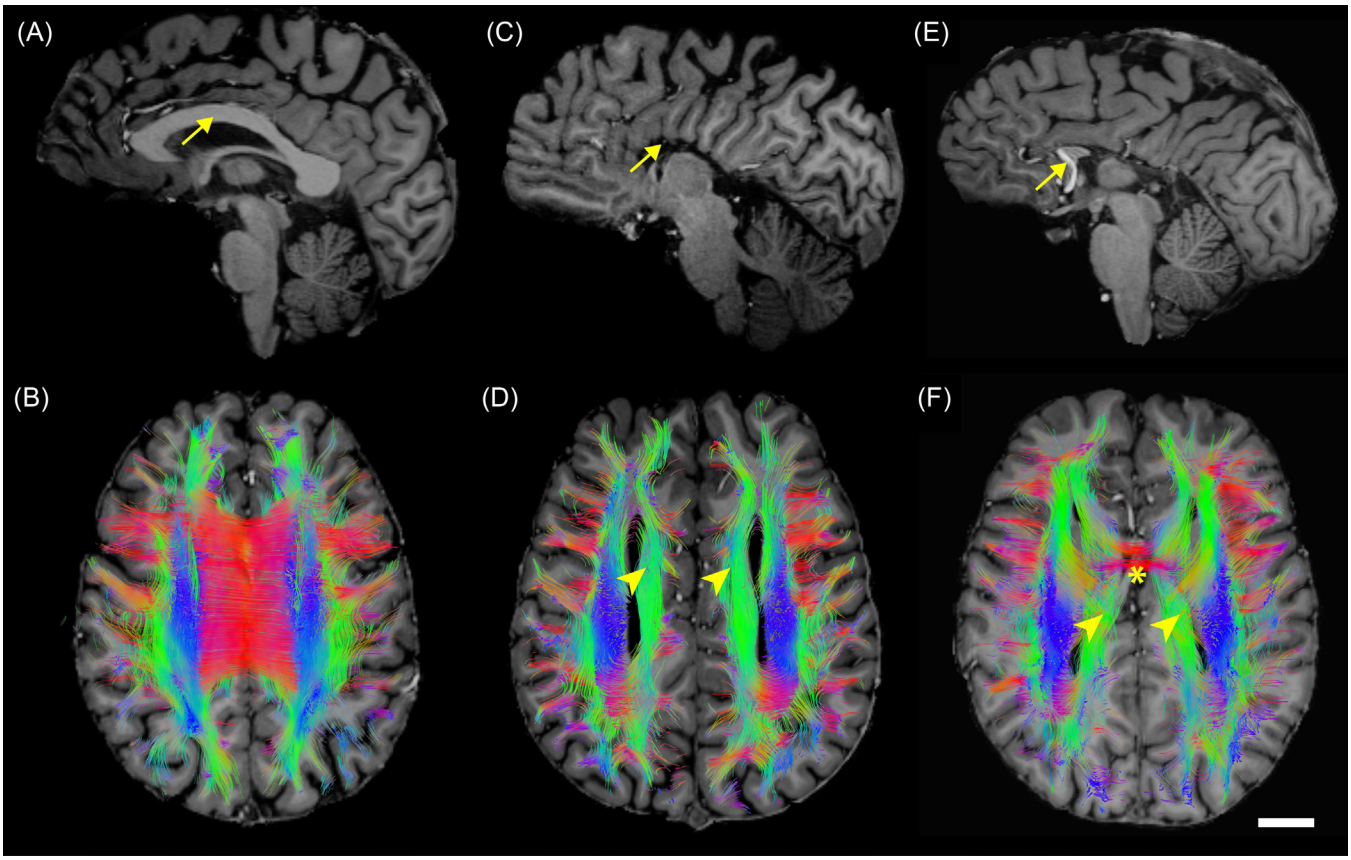

FIGURE 14.6 Anatomical features of agenesis of corpus callosum in humans revealed by T1-weighted MRI and-based tractography. dMRI The normal corpus callosum is a prominent white matter structure that can be easily visualized in a sagittal view of a T1-weighted MR image (A, yellow arrow). dMRI-based tractography reconstructs the corpus callosum as a large band of fibers running mediolaterally (red) connecting the hemispheres (B, horizontal view). In individuals with agenesis of the corpus callosum, the corpus callosum can be completely absent (C, yellow arrow), or a partial remnant may remain (partial agenesis of the corpus callosum, yellow arrow in E). In both cases, callosal axons that would normally cross the midline can be identified by tractography as large, longitudinal Probst bundles (arrowheads in D and F). In partial agenesis, a small remnant of callosal connections can be identified by tractography (asterisk in F). Scale bar= 2 cm.

(Figure 14.6E and F), an additional asymmetric ectopic connection has been identified by tractography connecting the frontal cortex with the contralateral parieto-occipital region (Tovar-Moll et al., 2007; Wahl et al., 2009; Bénézit et al., 2014; Tovar-Moll et al., 2014). This ectopic connection, which has been termed the "sigmoid bundle," represents a dramatic change in brain connectivity compared to the mostly homotopic organization of callosal fibers seen in the normal corpus callosum (Hofer and Frahm, 2006). These ectopic cortical projections appear to be functionally connected as the "strength" of structural connectivity (determined by quantifying the number of streamlines between the two cortical areas) positively correlates with the degree of functional connectivity (correlation in blood-oxygen-level dependent (BOLD) signal) between the two parietal areas (Tovar-Moll et al., 2014).

14.3.3 Validation of dMRI Methods to Model Brain Connectivity

dMRI and tractography allow brain connectivity to be modeled noninvasively in three-dimensional space, and are therefore important tools in human and animal studies. However, dMRI-based tractography is an indirect method based on a measure of water diffusion that can be influenced by multiple microstructural properties leading to several important limitations (Jbabdi and Johansen-Berg, 2011). These include:

1. The direction of a connection cannot be inferred from dMRI and tractography. This is a fundamental limitation of dMRI as the diffusion of water molecules is symmetrical along axial and radial axes.

2. Inability to establish precise cortical origins and terminations (in terms of both identifying cortical layers and determining where a connection first enters the cortex). This is primarily due to high isotropic diffusion within the grey matter that does not allow for accurate fiber tracking along directions of principal diffusion.
3. Ambiguity in determining multiple intravoxel axon trajectories from diffusion measurements. This is particularly important as even advanced algorithms for modeling white matter structure within a voxel can lead to ambiguous interpretations of axonal structure and direction. Consequently, tractography can be highly susceptible to false positives (reconstructing tracts that do not exist biologically) and false negatives (failing to reconstruct tracts that do exist).

It is therefore important to validate tractography findings with some form of "ground truth" of connectivity. Traditionally, this has been achieved *in vivo* with carbocyanine dye tract tracing. This approach has been particularly useful in modeling ectopic white matter tracts in animal models of altered brain connectivity, such as the structure of Probst bundles in AgCC mice (Ren et al., 2007). A major limitation of traditional tract tracing techniques, however, is that only a small population of axons can be studied, corresponding to where the tracer is injected (usually a single injection site in the brain). When multiple injection sites are used, it is difficult to reconstruct each labeled tract in three-dimensional space. One of the most comprehensive projects for establishing a gold standard for mammalian brain connectivity has been the Allen Mouse Brain Connectivity Atlas (Oh et al., 2014; http://connectivity.brain-map.org/). It comprises a mesoscale connectome of the mouse brain based on the digitization and quantification of large numbers of viral tracings of axons. This can overcome the traditional limitations of viewing the three-dimensional structure of labeled axons, but has also presented new challenges in that these data still contain false positive signals and cannot easily distinguish the connection strength or termination sites of axons (Oh et al., 2014). Given the difficulties in tract tracing, it is useful to employ complementary MRI or other imaging techniques, in addition to tracing, to correlate with structural changes determined by tractography. The most important tools for this are functional MRI, which measures changes in the BOLD signal as an indirect measure of brain activity, and magnetoencephalography, which detects changes in magnetic fields that occur secondarily to the electrical activity that occurs normally in the brain.

14.3.4 Modeling Whole-Brain Connectivity

A significant advantage of using dMRI to investigate structural connectivity in the brain is that it can be used to study whole-brain connectivity in three dimensions in a way that is not possible using histological tract tracing. Given the complexity of how the brain is structurally wired, it is not always helpful to consider individual white matter tracts in isolation. Instead, dMRI can reveal complex structural networks between distant brain areas. This idea has been extended by considering the brain as a simple graph, with each anatomical area represented as a node, and each connection as an edge between nodes (Zalesky et al., 2010; Bullmore and Bassett, 2011). Describing connectivity in the human brain using a graph theory approach allows connectional data derived from either dMRI or functional MRI to be described as a macroscale connectome (Sporns, 2011).

14.4 CONCLUSIONS

The anatomical and functional connectivity of the brain underpins its capacity for information processing and storage. As the largest fiber tract in the human brain, the corpus callosum provides an excellent model for understanding the mechanisms that regulate the development of brain wiring. Developmental dysgenesis of the corpus callosum is a common neurodevelopmental defect, the etiology of which can be better understood by applying our knowledge of the variety of developmental events required for the correct formation of this axon tract. A large number of genes have been implicated in mouse callosal development (Donahoo and Richards, 2009) and these are being applied to understanding human callosal disorders (Edwards et al., 2014b).

In addition to genetics, neuroimaging is a critical platform for translational discoveries based on human and mouse studies by providing a wealth of spatial and temporal information about anatomical and functional connectivity. It can be applied longitudinally in living subjects to examine the development and plasticity of axonal wiring in normal and pathological brains, as well as monitoring the effect of treatment. Furthermore, studies that relate cognitive abilities with brain wiring can test hypotheses about the degree to which connectivity underpins brain function. Currently, limitations include an inability to unambiguously resolve crossing fibers and microstructural details and a limited ability to validate tractography findings. In brain development, new advances

are required to improve human fetal imaging *in utero* to provide faster scanning with higher resolution and minimal movement artifacts in a safe manner. Additional methods for neuroimaging anatomical and gene expression changes in smaller groups of neurons, or even individual neurons forming circuits, are urgently required as these will allow real-time changes in brain connectivity and plasticity, and the mechanisms regulating these changes, to be discovered. It is an extremely exciting time for this field of science, as the fruits of decades of basic science research can now be applied to identifying the causes of human disorders of brain wiring and finding treatments that will restore function.

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