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Understanding the Mechanisms of Callosal Development Through the Use of Transgenic Mouse Models

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The cerebral cortex is the area of the brain where higher-order cognitive processing occurs. The 2 hemispheres of the cerebral cortex communicate through one of the largest fiber tracts in the brain, the corpus callosum. Malformation of the corpus callosum in human beings occurs in 1 in 4000 live births, and those afflicted experience an extensive range of neurologic disorders, from relatively mild to severe cognitive deficits. Understanding the molecular and cellular processes involved in these disorders would therefore assist in the development of prognostic tools and therapies. During the past 3 decades, mouse models have been used extensively to determine which molecules play a role in the complex regulation of corpus callosum development. This review provides an update on these studies, as well as highlights the value of using mouse models with the goal of developing therapies for human acallosal syndromes.
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The corpus callosum is the largest fiber tract in the mammalian brain. It connects neurons in the left and right cerebral hemispheres and is essential for the coordinated transfer of information between them. For the corpus callosum to form, several critical developmental events must occur in sequence. These include the patterning and formation of the midline, (which later acts as a substrate for pioneering callosal axons), the generation of callosal neurons and their axons, and the targeting and growth of these axons (a process regulated by specific midline glial structures). Finally, callosal axons must locate and innervate their targets in the contralateral hemisphere (Fig 1). As soon as they are as formed, developing neurons are nourished by the vasculature of the brain, and thus, like all fiber tracts in the brain, callosal axons are susceptible to vascular insult or cell death mechanisms both during development, and in the adult. Here we discuss the current state of research, using mouse models to investigate these developmental mechanisms, to provide insight into how the corpus callosum forms in human beings, and the mechanisms that could underlie agenesis or dysgenesis of the corpus callosum.

Malformation of the corpus callosum can result in either the complete absence of the corpus callosum (defined as "agenesis of the corpus callosum," ACC) or partial absence or thinning of the corpus callosum (defined here as "dysgenesis").[1] Because of the wide diversity of developmental mechanisms regulating the midline crossing of callosal axons, there is a spectrum of phenotypic variations of agenesis and dysgenesis of the corpus callosum.[2] When callosal fibers fail to cross the midline, they often remain ipsilateral and form longitudinal axon fascicles (known as Probst bundles). Described in 1901,[3] Probst bundles are likely to be a product of callosal axon misguidance caused by disruption of the midline structures and/or their secreted molecules. Probst bundles form longitudinally in both cerebral hemispheres (adjacent to the midline), with recent analyses revealing that the axons project along the rostrocaudal axis of the forebrain (and do not form "whorls," as classically described) in both mice and human beings.[4-8] The morphology of Probst bundles has been demonstrated in both acallosal human cases and mouse models using the three-dimensional technique of diffusion tensor magnetic resonance imaging, which identifies fiber tracts by the direction of isotropic water movement[7,9] (reviewed in this issue, Wahl 2009). These studies demonstrate that because the axons are able to arrive at the

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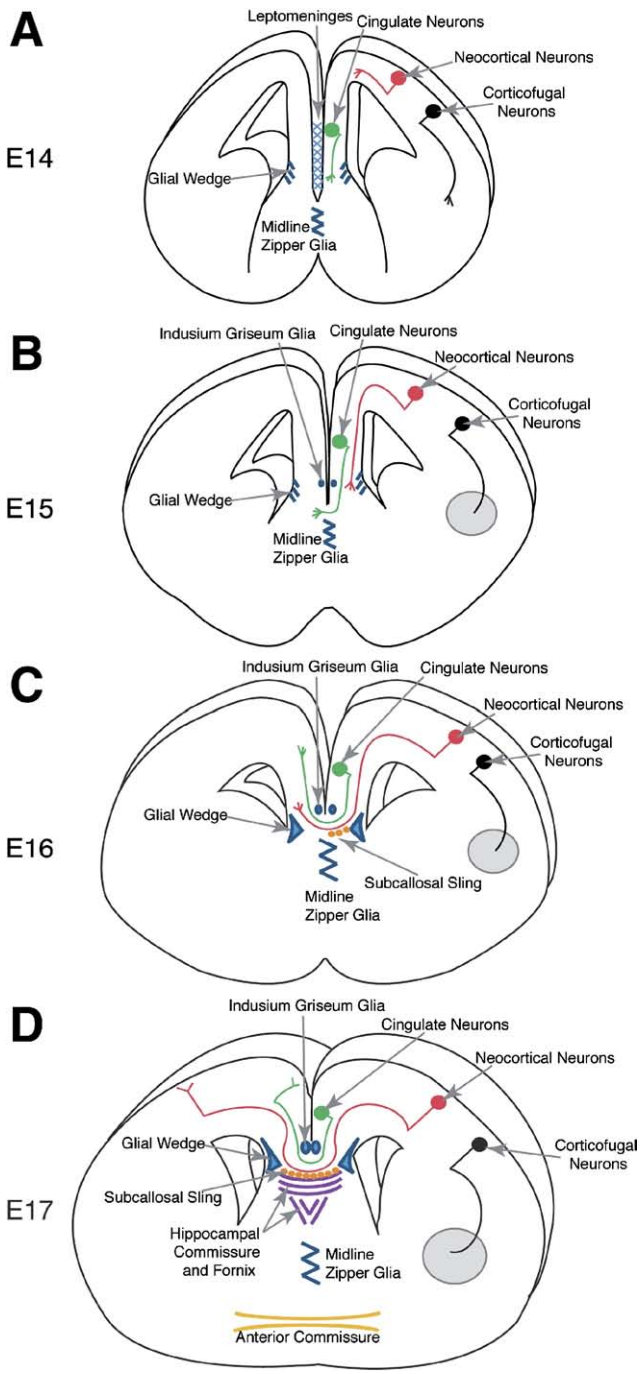

Figure 1 Corpus callosum development in the mouse. (A) Callosal formation firstly requires the establishment of a substrate, which is achieved through the fusion of the telencephalic midline. This involves either the elimination or exclusion of the leptomeninges found between the hemispheres. (B) The pioneers of the corpus callosum originate from the cingulate cortex (medial-most part of the cortex) and reach the midline betweeen embryonic day 14 (E14) and E15. (C) Axons from the neocortex then grow along the pathway defined by the pioneers, expanding the corpus callosum by E17. (C) On arrival of callosal axons at the midline, midline cellular populations (glial wedge, indusium griseum, and the subcallosal sling), and the extracellular cues that they secrete, assist in the turning and channeling of these axons across the midline. (D) Axon guidance of the caudal corpus callosum may also be facilitated by the hippocampal commissure. After the axons have entered the contralateral hemisphere, they traverse dorsolaterally before innervating homotopic areas of the contralateral cortex. (Color version of figure is available online.)

midline, Probst bundles result from failure of the callosal fibers to cross the midline and not defective growth of the axons per se. This phenotype is conserved between ACC patients and most of the > 65 different mouse models of ACC (Table 1). A newer method of acquiring and processing diffusion tensor magnetic resonance imaging information (called high angular resolution diffusion imaging (HARDI) and q-ball imaging) has also revealed fascinating information about the projection of callosal axons in cases of dysgenesis of the corpus callosum (partial ACC) where a callosal remnant remains. Sherr et al[86] (also see Wahl, 2009 this issue) have found that the position of the remnant along the rostrocaudal axis cannot be used to predict the origin of the fibers that cross the midline. Their results demonstrate that different patients, with similar anatomical magnetic resonance imaging scans, can have dramatically different axonal connectivity patterns. These differences in callosal connectivity may underlie the behavioral differences observed in these patients. Although clearly visible by anatomical magnetic resonance imaging, ACC may therefore represent a more generalized brain-wiring defect than that observed by T1-weighted images alone, highlighting the value of HARDI and q-ball imaging in revealing more detailed anatomy.

Mouse models provide a way to experimentally elucidate the molecular mechanisms that cooperate to enable the formation and maintenance of the corpus callosum, particularly as cortical organization is conserved in most mammals[87] and many of the genes known to be involved in callosal formation in the mouse display similar expression profiles within the embryonic human brain.[9] Complications in the study of mouse models arise from the use of the wide variety of mouse strains and also the different techniques used to generate genetic modifications. Such complications are similarly encountered in studies mapping human genetic disorders. However, mice used for research are usually the products of diligent inbreeding, a process that causes the retention of genetic instability within many strains (such as 129SV and BALB/c, Table 1) that can result in sporadic occurrence of callosal agenesis.[77,78] However, this instability can be overcome by backcrossing the genetically manipulated heterozygous mouse onto a more robust strain (such as C57BL/6).[82] Provided back crossing is corporated, mouse models can be used to identify a large cohort of single-gene mutations that cause corpus callosum malformations (Table 1). Many of these genes represent excellent candidates for human screens to identify the genetic basis of acallosal syndromes.

As outlined earlier in the text, understanding the development of the corpus callosum involves an appreciation of the sequence of developmental and molecular events required for callosal axon growth and targeting (Fig 1). In the remainder of this review, we will summarize how various mouse models have elucidated the different mechanisms controlling callosal development, and highlight how these studies have illuminated our understanding of callosal malformations in human beings.

Patterning of the Forebrain and Formation of the Commissural Plate

The cerebral cortex is first identified early in development as a single vesicle known as the prosencephalon. The prosencephalon undergoes a process of rapid proliferation and expansion, and over time, the dorsal wall invaginates to form 2 telencephalic hemispheres that will become the cerebral hemispheres in adults.[88] The induction of tissue identity is first established by the expression of proteins called morphogens. Morphogens are crucial in establishing signaling domains, called patterning centers, early in development (before prosencephalic invagination), which then dictate the specialization of areas within the forebrain through the regulation of transcription factors[89,90] (Fig 2). Forebrain morphogens include members of the bone morphogenic protein (BMP)[91] and Wnt protein[92] families, which are expressed by the cortical hem, fibroblast growth factors (FGF), expressed at the midline and in the cortex,[93] and Sonic hedgehog (Shh),[94] which is expressed ventrally. These signaling domains interact to establish regional identity of the forebrain,[89,95,96] as well as patterning of the forebrain midline, including the formation of the commissural plate.

The commissural plate is defined as the anatomical domain through which all interhemispheric forebrain commissures (the corpus callosum, the hippocampal commissure, and the anterior commissure) cross the midline during development[97] (Fig 3). Although the anatomy of this domain has been characterized during human development, the molecular and cellular composition that regulates the formation of the 3 forebrain commissures is yet to be fully determined. One factor that is known to be a potent regulator of forebrain patterning is Fgf8, which is expressed by the commissural plate[93] and regulates expression of other midline molecules.[98] In mice deficient in Fgf8 (or its receptor FGFR1), the telencephalic midline and all 3 forebrain commissures are disrupted,[74,75,99] which indicates that Fgf8 plays an integral role in this system.

Telencephalic Midline Fusion: Holoprosencephaly and Interhemispheric Cysts

For callosal axons to cross the midline into the contralateral hemisphere, a substrate through which the axons can grow must first be present. Thus, the inversion of the dorsal prosencephalic wall to form 2 telencephalic hemispheres and the subsequent fusion of the telencephalic midline are imperative for the development of later axon tracts that facilitate interhemispheric communication and information processing. Two main pathologies have been described in human fetuses where the midline substrate is either absent or disrupted, being replaced by a single ventricle or a fluid-filled space (or cyst): holoprosencephaly (which is due to a failure of telencephalic hemisphere formation), and interhemispheric cyst formation (a build-up of fluid at the midline which disrupts the morphology of the telencephalic midline structures). The formation of an interhemispheric midline cyst, or the disruption of midline fusion, is highly correlated with malformations of the corpus callosum.[100-103]

Holoprosencephaly occurs when the prosencephalic vesicle fails to invaginate to forms 2 telencephalic hemispheres; instead, a singular hollow vesicle remains and many midline structures are lost. Holoprosencephaly, which occurs in 0.49-1.3 of 10,000 live-births[104-107] with a higher incidence level of 1 in 250 at prenatal stages, encompasses a variety of phenotypes, including alobar, semilobar, lobar, and middle interhemispheric holoprosencephaly.[108-110] Holoprosencephaly impedes callosal formation because of the loss of the midline substrate. In cases, such as lobar, semilobar, and middle interhemispheric holoprosencephaly (all of which are partial holoprosencephalies), a remnant of the corpus callosum remains intact in the areas that are unaffected at the midline.[110-111] Analysis of callosal fiber connectivity using HARDI and q-ball imaging in these patients would provide interesting information about the ability of callosal axons to project to their correct targets in this disorder and may reveal novel insights into the basis of this disorder.

Mouse models have proven very successful in unraveling the molecular mechanisms that give rise to holoprosencephaly (Fig 2), most commonly demonstrating a crucial role for patterning molecules in the development of the midline. Shh was the first molecule identified in human beings as causing holoprosencephaly when mutated.[112] In the mouse forebrain, Shh is normally expressed at the ventral midline.[94] Holoprosencephaly occurs when Shh is deficient in transgenic mice[113,114] and also when it is ectopically expressed in the dorsal midline, thus disrupting cortical hem signaling centers.[115] Holoprosencephaly also occurs when the receptor for Shh (Patched) is deficient.[116] Zic2 is another ventral patterning molecule, a deficiency of which has been found to produce holoprosencephaly in the mouse.[117,118] Both Shh and Zic2 have been identified from genetic screens of human holoprosencephalic patients.[117] The midline fuses in a ventral to dorsal direction[119] and in cases where the signaling centers are deficient, midline formation is impaired, possibly leading to defects in the subsequent fusion of the telencephalic midline.

Interhemispheric cysts produce different phenotypes from those associated with holoprosencephaly. In particular, the telencephalic hemispheres have a definable (though disrupted) midline in the presence of a cyst, whereas, in holoprosencephaly the cortex is continuous, showing no morphologic differences at the midline as compared with the lateral cortex.[120] Therefore, these 2 midline anomalies rise from different mechanisms—holoprosencephaly from a failure of the dorsal prosencephalon to invaginate and form the 2 hemispheres, whereas the cysts cause disturbances to the established telencephalic midline, possibly by an increase of ventricular pressure.[120] An alternate hypothesis is that some interhemispheric midline cysts result from defects in midline fusion. Interhemispheric cysts, including those that associ-

Table 1 Genetically Modified Mice That Display Malformations of the Corpus Callosum

Gene	Callosal Phenotype		Associated Defects			Ref
	ACC	pACC	MG	HC	AC	
Guidance molecules/receptors						
Deleted in colorectal cancer (DCC)	Y (100%)			Y	Y	6,10
Draxin	Y (58%)	Y (42%)	Y	Y (100%)	Y (100%)	11
EphA5		Y (100%)	N	Y (46%)		12,13
EphB1	Y (43%)	Y (87%)				14
EphB2	Y (13%)	Y (61%)				14
EphrinB3	Y (64%)	Y (84%)				14
EphB1/EphB2	Y (60%)	Y (80%)				14
EphB1/EphB3	Y (18%)	Y (90%)				14
EphB1/EphA4	Y (12%)	Y (26%)				14
EphB2/EphB3	Y (67%)	Y (100%)				14
EphrinB3/EphB1	Y (87%)	Y (100%)				14
EphrinB3/EphB2	Y (45%)	Y (100%)				14
EprinB3/EphA4	Y (29%)	Y (64%)				14
Forebrain embryonic zinc fingerlike (Fezl)	Y	Y		N		15
Frizzled3	Y			Y	Y	16,17
Netrin-1	Y (100%)			Y (100%)	Y	18
Neuropilin-1^{sema} (Npn-1^{sema})	Y	Y		Y		19,20
Roundabout homolog 2 (Robo-1)		Y	N	Y	N	21
Robo-1/Robo-2		Y (100%)			Y	22
Receptor-like tyrosine kinase (Ryk)		Y (25%)	N	N	N	23
Slit-2		Y	N	N	N	24,25
Transcription factors						
Chicken ovalbumin upstream promoter transcription factor I (COUP-TFI)	Y (63%)	Y (16%)		Y	Y	26
Empty spiracles homolog 1 (Emx1)	Y	Y		N	Y	27,28
Empty spiracles homolog 2 (Emx2)		Y		Y	Y	28,29
Forkhead box C1 (Foxc1)	Y					30
Homeobox gene expressed in ES cells 1 (Hesx1)	Y (75%)			Y (50%)	Y (75%)	31
Nuclear factor IA (Nfia)	Y (100%)		Y	Y	Y	32,33
Nuclear factor IB (Nfib)	Y	Y	Y			34
Paired box gene 6 (Pax6)		Y		Y		35
Special AT-rich sequence binding domain (Satb2)		Y	N	N	N	36,37
Tailless (Tlx)		Y		Y	Y	38,39
Ventral anterior homeobox 1 (Vax1)	Y			Y	Y	40
Extracellular matrix molecules						
Ankyrin		Y (62%)				41
Laminin γ1		Y				42
L1 cell adhesion molecule (L1 CAM)	Y (17%)	Y (83%)			Y	43
Signaling/cytoplasmic molecules						
β-Amyloid precursor protein (β-APP)	Y	Y		Y	Y	44,45
Arx		Y		Y		46
β-catenin	Y					47
CREB1		Y		N	Y	48
Disrupted in schizophrenia (DISC1)		Y (100%)			N	49
Doublecortin-like kinase (Dclk)	Y			Y	N	50,51
Doublecortin (DCX)/Dclk	Y	Y		Y	Y	50,51
Exostosin-1 (Ext1)	Y			Y	Y	52
Focal adhesion kinase (FAK)		Y				53
FE65/FE65L1	Y (100%)					54
Growth associated protein 43 (GAP 43)	Y (100%)		Y	Y (100%)	Y (100%)	55,56
JNK-interacting protein 3 (Jip3 or Jsap1)	Y (100%)			N	Y	57,58
Microtubule-associated protein 1 B (Map 1B)	Y (80%)	Y (20%)		Y	N	59
Myristoylated alanine-rich protein kinase C substrate (MARCKS)	Y (93%)	Y (7%)		Y	Y	60
macMARCKS (MLP or F52)	Y (100%)			N	N	61
Mena	Y (55%)			Y	N	62

Table 1 (continued)

Gene	Callosal Phenotype		Associated Defects			Ref
	ACC	pACC	MG	HC	AC	
Mena Vasodilator-stimulated phosphoprotein (VASP)	Y (100%)		Y	Y (100%)	Y (100%)	63
N-ethylmaleimide-sensitive factor attachment protein, alpha (α-SNAP)	Y			Y	N	26
Nuk		Y (~20%)			Y (100%)	64,65
P35		Y (100%)		N	Y	66,67
P190A RhoGAP	Y (100%)			Y	Y	68
P190B RhoGAP		Y		Y	Y	69
Protein-tyrosine-phosphatase sigma (PTPσ)		Y (100%)				70
Rac1	Y (100%)			Y	Y	71
Sek4	Y (37.5%)				N	64
Sek4-/-; Nuk-/-	Y (89%)			N	Y (100%)	64
Trio		Y (100%)			Y (100%)	72
Growth factors/receptors						
Fibroblast growth factor 8 (FGF8)	Y					73
Fibroblast growth factor receptor 1 (FGFR1)	Y		Y	Y	Y	74,75
Insulin-like growth factor binding protein 1 (IGFBP-1)		Y (100%)				76
ACC mouse strains						
BALB/cCF	Y (20%)					77
BALB/cWah1	Y (14%)			Y (8%)		78,79
BTBR T/+tf/tf	Y (100%)			Y (83%)		80
Chakragati (Ckr)		Y				81
C129F2	Y (23%)	Y (58%)		Y (7%)		82
ddN	Y (8%)	Y		N	N	83,84
I/LnJ	Y (100%)					85
129/J	Y (17%)	Y (70%)				77
129S1/SvImJ	Y (20%)	Y (10%)		Y (5%)		77
9XCA/Wah	Y (100%)			Y (100%)		79

ate with callosal agenesis, are phenotypically diverse between patients. However, they can be categorized depending on whether they are associated with the ventricular system (communicating cysts) or not (noncommunicating cysts), and whether there are other obvious phenotypic malformations associated with them.[101,103]

Recently, a mouse known as *hydrocephalous with hop gait* (hyh) has been identified as displaying an interhemispheric cyst correlated with callosal agenesis.[121,122] Genetic screens to identify the genes influencing the phenotypes seen in this mouse have revealed a mutation in the protein α-SNAP,[123,124] which plays a role in cellular membrane fusion and intracellular transport.[125,126] In addition to the hyh mouse, the mouse strains BALBc/Wah1 and 129 J, as well as the *L1* and *p190-rhoGAP* mutant mice, display spontaneous ACC, suggested to be secondary to defective interhemispheric fusion.[43,68,78] Thus, there is some evidence that a subset of interhemispheric cysts results from inadequate midline fusion rather than increases in intraventricular pressure.

Specification of Callosal Neurons

Thus far, we have highlighted the importance of the formation of the telencephalic midline substrate in forebrain development. However, of equal importance is the development and maturation of neurons making up the cerebral cortex. The cerebral cortex is a highly organized six-layered structure and the processes that orchestrate its formation include cellular proliferation, migration, and subsequent differentiation. Normal formation of the corpus callosum is dependent on the generation of callosal neurons from progenitor cells (within the ventricular zone), as well as their migration to, and differentiation within, their cortical layer. Malformation of the cortical architecture is often correlated with callosal dysgenesis. The transcription factor Emx2, for example, regulates both the proliferation[127] and migration[128] of glutamatergic neurons and thus, when there is an Emx2 deficiency, the cortex is thinner, with disruptions to its architecture, and the number of neurons that project an axon medially is reduced, producing a thinner corpus callosum.[19,29]

Information regarding the direction of the axonal projection (callosal or subcortical) is genetically specified within the neuron before it reaches the cortical plate.[129] Transcription factors can regulate the development of callosal projections by 3 different mechanisms: (1) the specification of neurons as callosal projection neurons,[36,37,75,130,131] 2) the cell-autonomous modification of gene expression within the callosal neuron to promote or repress the expression of specific molecules

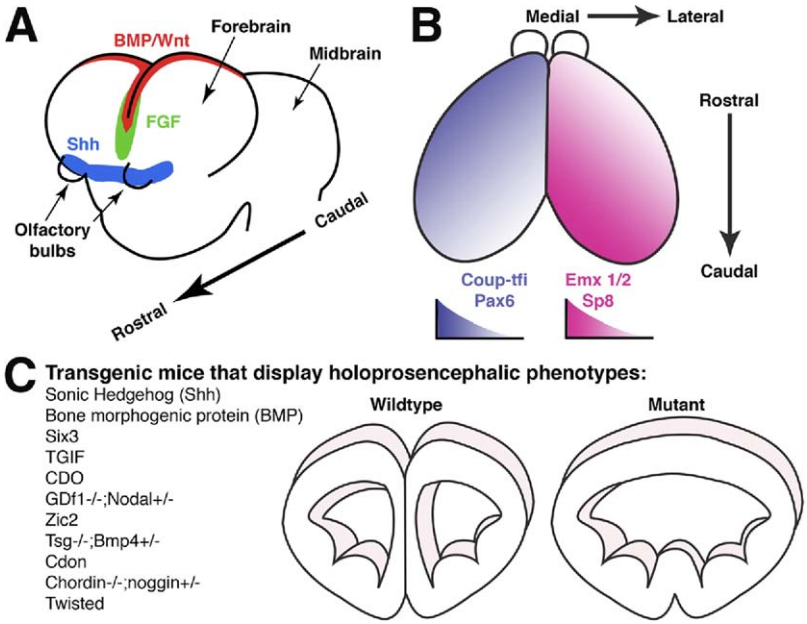

Figure 2 Signaling centers in the early development of the forebrain. Early in development, morphogens are expressed in specific locations and their expression regulates later expression of transcription factors. Expression domains of morphogens determine cell identity and this can be modulated by their graded expression. (A) The three-dimensional nature of morphogens requires their expression in dorso-ventral, mediolateral, and rostrocaudal axes. Wnts and Bmps are expressed high dorso-medially and low laterally. Fgfs are expressed at the rostral telencephalic midline. Shh is expressed ventrally. (B) Later in development, cortical areas (motor, somatosensory and visual) are determined by the overlapping expression patterns of transcription factors. (B) The graded expression of these proteins plays a key role in cortical arealization. (C) Many midline molecules have been found to cause holoprosencephaly when they are mutated in transgenic mice.[115,206-217] Schematic gives an example of a normal coronal brain at E12-13 compared with a holoprosencephalic brain. (Color version of this figure is available online.)

(particularly the expression of receptors and cytoskeletal factors that mediate axon growth through external cues), or (3) by mediating the development and/or the expression of guidance factors in other cellular populations (such as midline glial populations and the subcallosal sling), thereby indirectly regulating callosal axon pathfinding.

Cell-autonomous regulation of callosal formation can occur through the expression of cortex-specific transcription factors, such as Emx1,[27,28] Emx2,[28,29] and Satb2.[36,37,130] Although Emx1 and Emx2 are specifically expressed within the dorsal telencephalon, Satb2 is the first transcription factor with an expression profile that is specific for upper-layer callosal neurons.[36,37,130] Additionally, the removal of this transcription factor causes aberrant axon pathfinding but only to the subpopulation of callosal axons that would normally express it.[36,37] Midline guidance molecules known to contribute to callosal development were analyzed in these mutants and were found to be normal.[36] These studies provide the first evidence for a cell-autonomous role by a transcription factor in callosal development.

Conversely, transcription factors that could regulate callosal development through the mediation of extrinsic cell populations include nuclear factor 1 A (Nfia)[32] and B (Nfib).[34] Nfia and Nfib, although expressed in the cortical plate, are not expressed within callosal neurons.[132] *Nfia* and *Nfib* mutant mice display a number of morphologic malformations in the forebrain, including defects in the formation of midline glial populations as well as defective migration of the subcallosal sling.[32,34] Thus, Nfia and Nfib could be regulating callosal formation through the development of midline structures (discussed later in the text), which in turn mediate axon guidance across the midline.

Mechanisms of Callosal Axon Guidance

After the establishment of a midline substrate and cortical architecture, cortical wiring is achieved by the postmitotic neuron extending a specialized projection (axon) into the cortical substrate to seek out a target and establish a connection. Axonal extension and pathfinding are regulated both by intrinsic and extrinsic guidance mechanisms. The environment that axons traverse contains a variety of molecules, known as guidance factors, that influence the direction of the distal tip of the axon (a specialized structure known as the growth cone) based on the receptors that it expresses[133] (Fig 4). Guidance factors can act in either a short-range manner [where they are expressed on the cell surface such that cell-cell interactions are required for guidance; such factors include the Ephs and Ephrins, the Semaphorins (Semas) and the Neuropilins (Npns)] or they can act over a long range (molecules are secreted into the extracellular environment, such as occurs with the Netrins and the Slits). Guidance

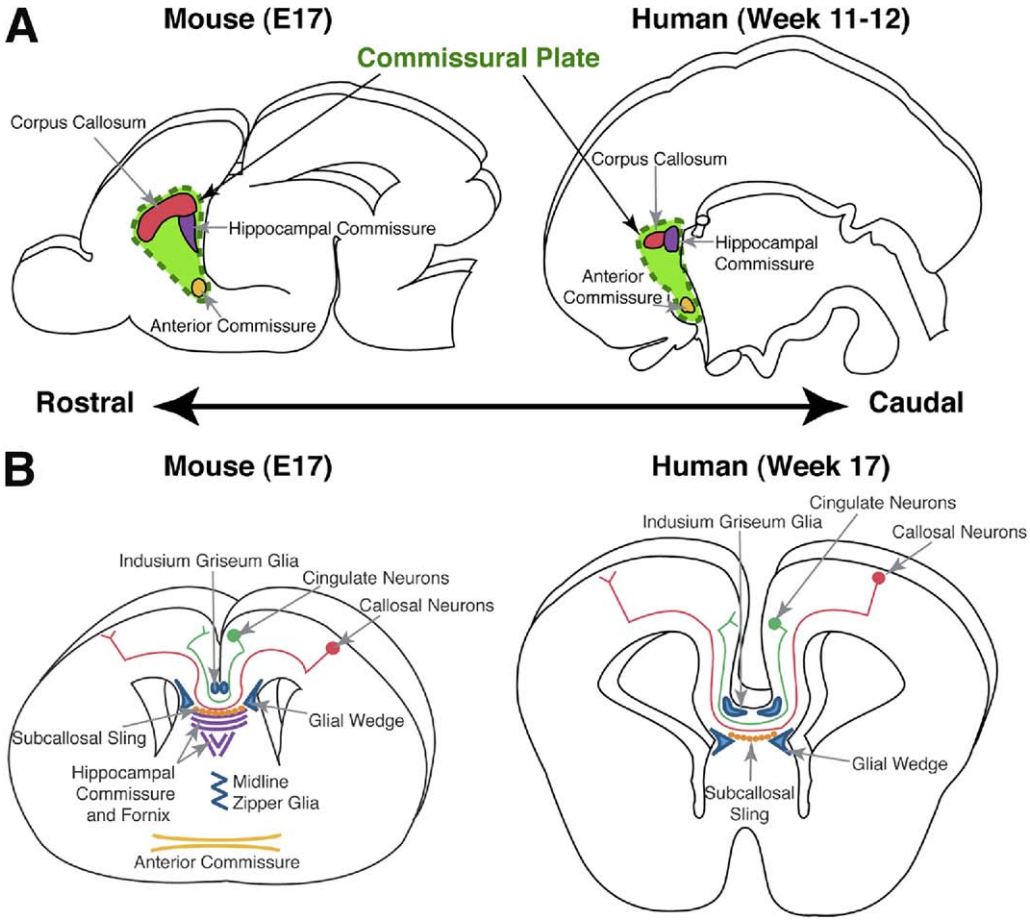

Figure 3 (A) The commissural plate and other anatomical similarities in mouse and humans. The commissural plate is the anatomical domain which all the interhemispheric commissures of the forebrain traverse during development. This is easily identified (green shading) in a sagittal view at the telencephalic midline in embryonic day (E17) mice and embryonic week 11-12 in human beings. (B) In the coronal plane, developing human fetal brains (week 17; D) have been shown to have anatomically similar cellular populations to those seen in mouse (at E17), including the glial wedge, the indusium griseum, and the subcallosal sling. (Color version of this figure is available online.)

factors can elicit many axonal responses: chemoattraction (growth toward the cue), chemorepulsion (growth away from the cue), permissive (promotes growth into a particular area), nonpermissive (represses growth into the area), or a variety of these based on other environmental cues and concentration gradients (Fig 4).

The environment along the extent of the callosal trajectory is complex, comprising multiple overlapping combinations of guidance factors. Transgenic and knockout mice studies have demonstrated the different guidance mechanisms that cooperate to achieve callosal formation, and these include both secreted proteins and membrane-bound molecules (Table 1).

Guidance cues and transcription factors are connected through an intracellular signaling network. The pathways within this network are generally activated by ligand-receptor binding (or separation) which stimulates a signal cascade inside the cell, including alterations to the phosphorylation of coreceptors and secondary messengers. Cytoskeletal molecules can then be directly altered through the signal cascade and thus can alter the morphology of the growth cone and axon to the environment.[134]

Growth-associated protein 43 (Gap-43) is a growth cone molecule widely used to label growing axons in mouse and human beings.[9,55] In the *Gap-43* mutant mouse, all forebrain commissures are malformed.[55] However, as the midline in these animals is normal, the defects in commissure formation are principally because of axon guidance and growth. Therefore, the stability and guidance of the growth cone is crucial for the formation of forebrain commissures. As the molecules involved in these processes in mouse are also conserved in human beings, it is clinically relevant to understand how these processes achieve normal development of the nervous system.

Extracellular matrix molecules are also important for stabilization of tissue integrity. In the cerebral cortex, in particular, the loss of extracellular matrix molecules leads to defects in the radial-glial basement-membrane framework. This has been shown to result in phenotypes representative of type II cobblestone lissencephaly, such as cortical lamination defects, cortical heterotopias, and midline fusion defects, such as seen in *FAK*,[53] *MARCK*,[60] *Jsap1*[57] and *β-catenin*[47] mutant mice (Table 1). In these mice, cases

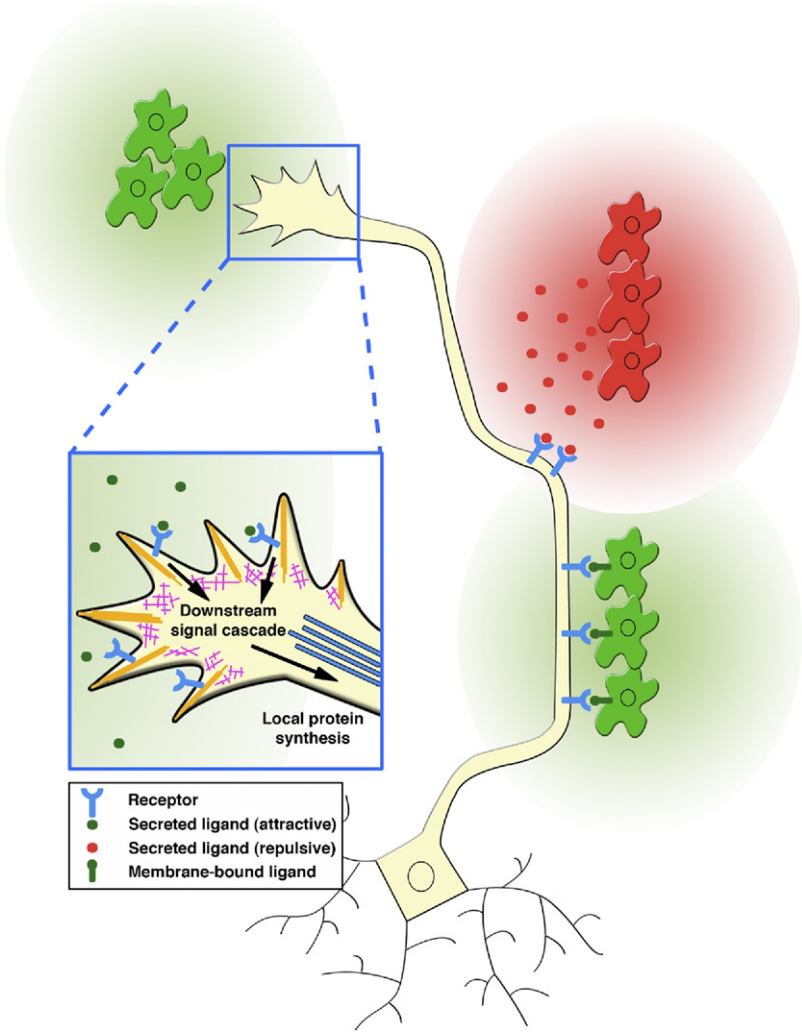

Figure 4 Mechanisms of axon guidance. To locate their target, pyramidal neurons project a specialized structure, known as an axon, into the cellular substrate where its guidance is mediated by extrinsic cues. These cues can be either attractive (green shading) and promote the growth of the axon toward the source, or repulsive (red shading), inhibiting the axons from growing near the source. Guidance cues can be secreted into the environment, or they can be expressed on the surface of another cell, enabling guidance by cell-cell interactions. The leading process of the axon (the growth cone) is extremely sensitive to different gradients of guidance cues, based on the repertoire of receptors expressed. The response of this structure to its environment involves the dynamic rearrangement of its actin cytoskeleton (yellow lines are actin bundles that comprise the filopodia, pink lines are the actin filaments within the lamelipodia), often following downstream signaling, initiated by ligand binding of the receptors expressed on the growth cone. (Color version of this figure is available online.)

of dysgenesis of the corpus callosum are thought to be secondary to the anomalies in cortical development.

Pioneering Axons Pave the Way for Later Callosal Axons

The corpus callosum comprises 2 axonal populations from different cortical areas: (1) the neocortex and (2) the medialmost part of the cortex, known as the cingulate cortex. Axons originating from the cingulate cortex are the first to arrive at the telencephalic midline [at embryonic day (E)14-15 in mice], and innervate the homotopic cingulate cortex in the contralateral hemisphere,[135,136] approximately 24 hours before callosal axons from the neocortex reach the midline (comprising the bulk of this tract).[136] Axons from the cingulate cortex are thus termed the "pioneering" axons of the corpus callosum in rats and mice.[135,136] It has been shown that a population of callosal axons also arises from the cingulate cortex in human beings[137] (Fig 3), but whether these axons pioneer the corpus callosum has yet to be determined. However, there is evidence to suggest that malformation, or injury of the cingulate cortex in human beings, can also impede callosal connectivity.[138,139]

Similar to callosal axons from the neocortex, pathfinding of the cingulate pioneering axons may be regulated cell-au-

tonomously by transcription factors, or determined by the extracellular guidance cues expressed at the midline. The extracellular guidance molecules involved in callosal development that have been identified thus far at the midline are temporally expressed during both cingulate axon crossing and neocortical crossing of the midline, suggesting that both populations of callosal axons are regulated by similar guidance cues. Possible differences in the guidance of these 2 populations may be dependent on the different expression levels of transcription factors within the cingulate cortex and the neocortex. Several transcription factors are expressed by both the cingulate cortex and the neocortex, but occur in a gradient across these areas (high medial to low lateral for Sp8,[140] Emx1, and Emx2,[141] and low medial to high lateral for Pax6[142] and Coup-TFI[143]; Fig 2). Whether the graded mediolateral expression pattern of these transcription factors is functionally relevant in the connectivity of either the cingulate or the neocortical callosal axons has yet to be determined.

Recently, the receptor neuropilin-1 (Npn-1) has been shown to be involved in corpus callosum formation.[19,20,144] Cingulate pioneering axons of the corpus callosum express Npn-1 at a crucial temporal stage for callosal development in both mouse and human beings,[9] and the axons of explants from the cingulate cortex are attracted toward the Npn-1 ligand, Sema3C (which is expressed at the midline[19]). As Npn-1 is highly expressed on axons from the cingulate cortex, it is likely to be a key regulator of cingulate axons. However, Npn-1 is also expressed (albeit a lesser extent) in the cortex,[145] and a recent study using dominant negative Npn-1 in cortical axons demonstrated a cell-autonomous affect on callosal axon guidance.[144] Thus, Npn-1 can also play a role in cell-autonomous callosal axon guidance from the neocortex. The pioneering axons are hypothesized to guide neocortical axons by providing a structural framework for callosal axons to follow by direct axon-axon contact, and this is possibly mediated through Npn-1. Evidence to support this hypothesis is that the cortical axons that were injected with a dominant-negative Npn-1 displayed defects in fasciculation.[144] Therefore, Npn-1 may play an important role in callosal formation, first by the guidance of cingulate pioneering axons to the midline, and then through the cell-cell guidance of callosal axons from the neocortex.

Midline Cellular Populations Regulate the Formation of the Corpus Callosum

Callosal axons are guided medially to the midline where they are channeled into the contralateral hemisphere. Midline cellular populations that have been shown to assist in the formation of the corpus callosum include the midline zipper glia, the glial wedge, the indusium griseum glia, and the subcallosal sling.[119,146,147] These populations have been identified in the rostral forebrain of both mouse and human beings[9] (Fig 3), suggesting that their function in formation of the human corpus callosum may also be conserved.

Midline zipper glia are positioned at the telencephalic midline and are thought to assist in the ventral fusion of the forebrain on the basis of their anatomical location.[119] However, there is currently nothing known about the molecular regulation of forebrain midline fusion.

The glial wedge is a specialized wedge-shaped glial structure positioned at the medial aspect of the lateral ventricle[148] (Figs 1 and 3). The glial wedge assists the guidance of callosal axons across the midline by preventing their ventral growth into the septum. This boundary is thought to be due to both its anatomical position and also its expression of the guidance molecule Slit2, which has been shown in vitro to act as a repellent for callosal axons expressing the Slit2 receptor, Robo.[21,22,24]

The indusium griseum is an area of neurons and glia positioned dorsal to the corpus callosum (Figs 1 and 3). Indusium griseum glia express Slit2 and are thought to act as a dorsal repulsive barrier for the axons of the corpus callosum.[24] *FGFR1* is crucial for the positioning of the indusium griseum glia.[74,75] In FGFR1 mutant mice, the indusium griseum glia do not form properly, causing callosal pathfinding defects despite the maintenance of guidance cue expression.[74] Thus, the indusium griseum glia and the glial wedge are important for the correct development of the corpus callosum, due to both their position and the expression of functional guidance molecules.[24,74,75]

The subcallosal sling (originally termed the "glial sling"[149]) comprises a transient population of neurons that arise from the medial wall of the lateral ventricle and migrate to the midline, parallel with, and immediately ventral to, the callosal axons.[150] The subcallosal sling has also been described in human beings[9,151] (Fig 3). The specific 'U' shape of this formation and the position of these cells relative to the callosal axons are suggested to be requisites for the formation of the corpus callosum.[152] However, an alternate hypothesis is that the subcallosal sling cells require the axons of the corpus callosum to direct their migration to the midline (as previously described for a postnatal glial population of cells in rat[153]). As the formation of the sling occurs simultaneously with the pathfinding of the callosal axons, determining whether callosal axons direct sling cell migration or callosal axons project across the midline guided by sling cues, will be difficult.

ACC in mouse models is often correlated with a disruption to the subcallosal sling. Interestingly, this correlation is prominent when Probst bundles have formed; in this situation the sling cells either migrate aberrantly into the septum (as in *Nfia*[32]) or accumulate lateral to these structures (as seen in *JSAP* homozygous mutants[57] and when callosal agenesis is induced by gamma-irradiation in Swiss mice[154]). In contrast, the sling is not malformed in mouse mutants that have a subpopulation of callosal axons crossing the midline and do not display Probst bundles (such as *Satb2*[36] and BALB/cCF acallosal mice[149]).

Hippocampal Commissure (Possible Differences Along the Rostrocaudal Axis?)

The environment that callosal axons project through is not consistent along the rostrocaudal axis of the telencephalon, suggesting that different mechanisms regulate different regions of callosal pathfinding. In the caudal telencephalon, the hippocampal commissure lies directly ventral to the corpus callosum and is thought to provide a guidance substrate for axons of the splenium (the caudal portion of the corpus callosum).[155-158] In many mouse strains and transgenic mutants that have malformation of the corpus callosum there is also a disruption of the hippocampal commissure (which crosses the midline approximately 24 hours before the corpus callosum[159] (Table 1). Thus, the developmental mechanisms involved in the formation of these commissures may overlap. Humans also have a hippocampal commissure (Fig 3)[160] but this projection is less developed than in rodents. The hippocampal commissure may have a structural role in callosal formation although this hypothesis has yet to be fully investigated.

Activity-Dependent Target Finding

Following crossing of the midline, callosal axons project into the contralateral hemisphere and innervate targets in a homotopic manner. Information transfer between cortical areas is dependent upon their connectivity, and as the homotopic targeting of callosal neurons is highly specific, understanding the mechanisms involved in this event is imperative to understanding function. The caudal corpus callosum axons form the splenium and originate within the visual cortex. Eye enucleation studies in cats have demonstrated a role for visual experience in callosal formation and maintenance.[161,162] A phenomenon known as exuberance and pruning during development establishes the axonal organization of the visual cortex, a process that has been widely investigated in cats.[163-165] This process involves the initial overproduction of axons that invade the contralateral hemisphere in search of a target, followed by the subsequent refinement of the connections by the removal of any unnecessary axons. Neural activity plays a crucial role in axon exuberance and pruning, but this process has yet to be fully investigated in mice.

A series of elegant studies in mice using neural activity inhibitors coupled with in vivo electroporation has highlighted that activity plays a central role in the contralateral targeting of callosal axons in both the occipital and the somatosensory cortices.[166,167] In both of these areas, the callosal fibers mis-targeted when activity was inhibited, and in some cases resulted in the aberrant innervation of other cortical layers instead. Interestingly, the dependence of axonal connectivity on activity may not be consistent across the rostrocaudal axis. In the visual cortex, reduced excitability resulted in aberrant layer positioning of axonal arbors.[166] The same procedure in somatosensory cortex also demonstrated aberrant layer positioning of axonal arbors; however, a loss of region-specific targeting was also found.[167] This suggests that activity could have different roles in different cortical areas, and this may be modulated through the expression of region-specific "positional cues" (undetermined thus far). Whether activity provides instructive cues for growing axons or whether it regulates molecular cues within the contralateral cortex has yet to be determined.

Vasculature Insult and Corpus Callosum Maintenance

Following development of the corpus callosum, the cerebral cortex requires continual metabolic nourishment from the vascular system to ensure the maintenance of this axon tract and its function. Many clinical studies in human beings have revealed a correlation between reduced cerebral blood supply and atrophy of the corpus callosum.[168,169] Symptoms that develop from reduced blood flow include a reduction in the size of the corpus callosum (likely through demyelination), disruptions to callosal connectivity, and lesions to neighboring cortical areas (in particular the cingulate gyrus). Reduction in cerebral blood flow is also correlated with deficits in cognitive function.[138,139] However, a caveat to some of these studies is that they were conducted using elderly patients, thus it is difficult to determine whether the observed cognitive decline stemmed from reduced blood flow or from age.[170,171] Lesions of the white matter are most commonly described in stroke patients, although there is increasing evidence for this occurrence in Alzheimer's disease and possibly other neurodegenerative disorders. In patients with hydrocephalus (which is commonly associated with callosal dysgenesis), there is often a reduction in cerebral blood flow.[172] Thus, the preservation of the cortical vasculature, not only during development but also throughout life, is required to maintain the homeostasis and function of the corpus callosum.

Stroke has been widely investigated, primarily using rat models[173]; however, the potential for genetic modification in this model is limited. Recently, several research groups have begun to generate mouse models of stroke enabling the removal of relevant genes implicated in cerebrovascular repair.[174,175] The removal of either matrix metalloproteinase-2 or 9, for example, reduces the severity of cerebrovascular injury (including lesions of white matter tracts) following the induction of stroke in these mice.[176,177]

There is increasing evidence that many molecular mechanisms involved in nervous system development also play a role in the development of the vascular system; in particular, there is strong overlap between molecules implicated in axon guidance and those that play a role in angiogenesis and blood vessel pathfinding[178] (Table 2). As discussed earlier, the Sema-Npn ligand-receptor pair are crucial regulators of callosal axon guidance.[19,144] In addition, Npn-1 mutant mice have deficiencies in blood vessel formation.[179,180] Npn-1 is not only a receptor for the class III Semaphorins but also for vascular endothelial growth factor (VEGF), both of which

Table 2 Guidance Molecules That Play a Role in Both Vascular and Neural Development

		Nervous System		Vascular System	
Ligand	**Receptor**	**Activity**	**Ref**	**Activity**	**Ref**
EphrinB	EphB	Axon guidance	14	Determination of arteriole or venous domains; induce capillary sprouting	186
VEGF	Npn-1	—		Blood vessel formation	179,180,187
Sema	Npn-1	Axon guidance (attraction)	19,144	—	
Sema	Plexin	Axon guidance (repulsion)	188	Angiogenesis	182
**Sema4A*	**Plexin-D1*	*Repulsion*	189	*Suppresses*	190,191
**Sema4D*	**Plexin-B1*		—	*Induces*	192-194
**Sema3F*	Npn-2		—	Suppresses tumor angiogenesis	195
?	Robo4	—		Angiogenesis	196,197,198
Slit 2	Robo1	Axon guidance (repulsion)	24	Promotes tube formation	199,200
Netrin-1	*Unc5b and neogenin?*	Axon guidance (repulsion or attraction)	18,201	Angiogenesis (promotion or suppression) attracts blood vessels	202,204,205
Netrin-4	*Unc5b and neogenin?*	—		Angiogenesis	203

have also been implicated in blood vessel development. However, Sema-Npn interactions regulate axon guidance only, and VEGF-Npn interactions specifically regulate vascular development.[181] Semas do regulate angiogenesis but through their interactions with the plexin receptors (*plexin* transgenic mice also display vascular defects[182]).

VEGF has been comprehensively characterized as an integral factor of vasculature development.[183,184] It has also recently been reported to play a role in neuro-protection and neuro-repair. However, analysis of a transgenic *VEGF-A* mouse revealed that VEGF-A regulates the development of the cerebral cortex, specifically through the vascular system (compared with a separate transgenic mouse with neuron-specific mutations to the VEGF-A receptor, Flk1, which showed a relatively normal cortical phenotype[185]). Thus, there is considerable overlap between the molecules that regulate development of the nervous and vascular systems. This could complicate the interpretation of cortical malformations (ie, cortical malformations may be secondary to an underlying vasculature deficiency). Thus, the multiple roles that these molecules play in development must be considered when analyzing phenotypes of transgenic mouse lines. Research is currently underway to develop therapies for vascular injuries, with many of these molecules as target candidates (Table 2). The interplay between the vascular and the nervous systems also needs to be considered post-development, in the prevention or protection against conditions, such as Alzheimer's disease, tumors, and stroke.

Conclusions

The formation of the corpus callosum is complex and the use of mouse models allow researchers to dissect the genes involved in molecular regulation of this axon tract. These studies help unravel the processes involved, with the ultimate goal of understanding how intrinsic and extrinsic cues act in concert to form the adult structures. The implementation of new imaging techniques, HARDI and q-ball imaging, allows a more detailed characterization of the callosal connectivity within both mouse and human patients afflicted with congenital malformations. Future use of this technique will provide insight into the processes that contribute to callosal formation. Further research to unveil the regulatory mechanisms along the rostrocaudal, mediolateral, and dorsoventral axes of the forebrain will expand our understanding of callosal formation and will bring us 1 step closer to the development for therapies and treatments for acallosal syndromes in human beings.

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