Development and plasticity of the corpus callosum

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ABSTRACT

The corpus callosum (CC) connects the cerebral hemispheres and is the major mammalian commissural tract. It facilitates bilateral sensory integration and higher cognitive functions, and is often affected in neurodevelopmental diseases. Here, we review the mechanisms that contribute to the development of CC circuits in animal models and humans. These species comparisons reveal several commonalities. First, there is an early period of massive axonal projection. Second, there is a postnatal temporal window, varying between species, in which early callosal projections are selectively refined. Third, sensory-derived activity influences axonal refinement. We also discuss how defects in CC formation can lead to mild or severe CC congenital malformations.

KEY WORDS: Agenesis, Axonal guidance and plasticity, Callosal projecting neurons, Corpus Callosum, Cortex, Development, Interhemispheric

Introduction

The corpus callosum (CC) is the major axonal tract of the mammalian brain and the principal path transferring information between hemispheres (Fame et al., 2011; Fenlon and Richards, 2015) (Fig. 1). It facilitates higher-order functions of the cerebral cortex such as multidimensional representation of information, associative and executive tasks, coordination of sensory-motor responses, intellectual processing, and management of social and emotional stimuli (Paul et al., 2003; Brown et al., 1999). It is of particular importance in humans, as several cortical functional areas, like those of language, are not symmetrically represented.

The development of the CC has mostly been inferred from animal models and from individuals with CC anomalies. These studies have revealed that CC development begins at embryonic stages and continues during a protracted period of postnatal life; as such, the CC takes longer than other circuits to develop. First, after the fusion of the hemispheres, midline structures generate a route for interhemispheric axons. Then, cortical neurons of the cingulate cortex are the first ones to elongate their axons and cross along this path following specific guidance cues. These pioneer axons serve to guide the subsequent axons of the bulk of cortical neurons. Although some developmental callosal projections are maintained, a significant amount are refined. Finally, along with increasing myelination, contralateral projections that have invaded the cortical areas, like those of language, are not symmetrically represented.

The next challenge was to address how adult CPN distribution arises during development. To investigate this, researchers performed retrograde tracer injections in young developing animals. They found that, although there are more CPNs in young animals, they distribute following adult-like patterns; the same areas and layers (e.g. L4) always appear devoid of retrotracing signal (Dehay et al., 1986; Fame...
et al., 2011; Innocenti and Clarke, 1983; Innocenti et al., 1977; Meissirel et al., 1991; O’Leary et al., 1981). The logical interpretation was that some neurons never project callosally and that CPNs are born as such. But do developing CPNs project elsewhere? The groups of Dennis O’Leary, Henry Kennedy and others, explored this question using retrograde tracers. They found that the adult and developing L5 contains both CPNs and subcerebral projecting neurons (SCPNs). Importantly, the two subpopulations were clearly segregated at all developmental stages (Stanfield et al., 1982; O’Leary and Koester, 1993). Hence, this suggested that CPNs never reach subcerebral targets, not even transiently. Associative neurons and CPNs were also found as separated subpopulations from early developmental stages (Meissirel et al., 1991). These findings supported a model in which programs determining the cortical projection fates set early major restrictions of axonal development and guidance (Lamantia and Rakic, 1990b; Fame et al., 2011; Fenlon and Richards, 2015; Meissirel et al., 1991; Dehay et al., 1988; Koester and O’Leary, 1993). However, it has been long known that there are cortical neurons with dual contralateral and ipsilateral (cortical or subcortical) projections (Wilson, 1987; Mitchell and Macklis, 2005; Sohur et al., 2014; MacDonald et al., 2018; Economos et al., 2016), indicating that some neurons are able to guide their axons through the midline without disabling subcortical or local wiring fates.

The exuberance and refinement of callosal projections during development

O’Leary, Kennedy, Dehay, Innocenti, Clarke and others not only identified distinct neuronal subpopulations but also uncovered a crucial phenomenon in the central nervous system (CNS): developmental refinement (Stanfield et al., 1982; Innocenti and Price, 2005; Innocenti et al., 1977; O’Leary and Koester, 1993; Meissirel et al., 1991; Dehay et al., 1986; O’Leary, 1987). Injections in different animal models demonstrated that regions containing CPNs always show a higher number of labeled cells at early stages than in adults. This implicated that significant axonal elimination of exuberant callosal projections follows the early pre-sorting of CPNs. This refinement did not involve the death of CPNs (Innocenti et al., 1977; O’Leary et al., 1981; Innocenti and Clarke, 1983, 1984a,b; Clarke and Innocenti, 1986; Meissirel et al., 1991; Dehay et al., 1986; O’Leary and Koester, 1993). Recent work has delved deeper into this refinement process. As explained below, novel studies have further revealed that there is not an early CPN pre-sorting event; on the contrary, sending transient callosal projections is a developmental mechanism that generally involves most neurons in all cortical layers and areas, including L4 neurons (De León Reyes et al., 2019).

In the adult cortex, L4 neurons represent the paradigm of local-only connected neurons. They are the main target of thalamocortical innervation (Feldmeyer et al., 1999). In the primary somatosensory cortex (S1), L4 neurons are ‘hubs’ of intracolumnar connectivity and their wiring is largely restricted to the barrel column (Feldmeyer, 2012). Early studies of cortical injections using retrograde tracers indicated that L4 neurons never project outside their cortical hemisphere (Fame et al., 2011; Innocenti and Clarke, 1983; Innocenti et al., 1977; Meissirel et al., 1991; O’Leary et al., 1981), which led to the assumption that they harbor intrinsically locally constrained connectivity. However, recent investigations demonstrated that all L4 neurons develop transient interhemispheric axons early in development. Their local connectivity emerges only after postnatal refinement of their early callosal projections (De León Reyes et al., 2019). The key point that led to the discovery of these transient projections was a change in the injection strategy: retrograde tracers were injected directly into the CC instead of targeting the gray matter (Fig. 2B). These injections in the white matter (WM) ensure the labeling of all callosal axons, independently of their behavior in the contralateral hemisphere. Thus, while developing axons that never invade the gray matter or those that remain there for a short time are unlikely to be detected by cortical injections, they are efficiently labeled by CC injections. In fact, the early studies of Innocenti already noticed that more neurons were labeled when injections were placed closer to the WM (Innocenti and Clarke, 1984b; Clarke and Innocenti, 1986). Notably, L4 transient callosals could also be visualized by in utero electroporation (IUE) (Fig. 2C) in a mouse transgenic line in which these neurons are specifically marked with green fluorescence protein (GFP). The combination of both techniques allowed the developmental dynamics of L4 refinement to be described. At postnatal day 3 (P3), all L4 neurons display a callosal axon. These axons are gradually eliminated from P3 until P21, when adult local connectivity is achieved and less than 3% of S1L4 remain as CPNs. This refinement is due to the elimination of transient axons and does not involve the death of CPNs. Distinct refinement ratios are also observed, leading to different proportions of callosal L4 neurons in separated functional areas, such as in the secondary somatosensory (S2), primary and secondary visual (V1 and V2), and auditory areas (A1 and A2). Remarkably, CC retrotracing showed that most neurons in the rest of the cortical layers also develop transient exuberant callosal projections (De León Reyes et al., 2019).

Hence, the majority of cortical neurons appear to acquire their adult connectivity based on a common developmental mechanism: sending exuberant callosal projections that are differentially refined.
at postnatal stages (De León Reyes et al., 2019). This argues against the early sorting of callosal versus non-callosal projecting fates. Recent work demonstrating the reprogramming of L4 neurons into L2/3-like neurons relied on their acquired ability to project callosally (Hou et al., 2019; Vitali et al., 2018). However, transient callosal axons are shared by both developing L2/3 and L4 subpopulations, and the presence of callosal axons as proof of an identity shift might not be an adequate criterion for demonstrating layer reprogramming. The existence of transient callosal axons indicates that an early callosal projection program coexists with the potential for local connectivity. This apparent contradiction, which impacts our understanding of early identities and reprogramming, might simply reflect the intrinsic molecular plasticity of young developing neurons.

**Activity-dependent refinement: the choice between elimination or terminal connectivity**

It is known that sensory cortices are shaped by activity-dependent mechanisms and CPNs are no exception (Antón-Bolaños et al., 2019; Moreno-Juan et al., 2017). The refinement of callosal axons can be influenced by activity-dependent changes at three different levels: (1) sensory alterations from the periphery; (2) alterations in thalamic inputs; and (3) alterations in the firing response or synaptic transmission of pre- and postsynaptic CPNs. In all cases, the severity of the effect strongly depends on the temporal windows of these manipulations; these temporal windows during which callosal connections demonstrate sensitivity to sensory alterations are known as ‘critical periods’.

**Sensory alterations from the periphery**

Studies of the visual cortex have revealed that postnatal visual experience largely influences CPN selection. For example, in monocularly deprived cats and rats, or in squinted kittens, there is an increase in CPNs (Fig. 3A). These ectopic CPNs are found in V1 (area 17), which is normally a non-callosal area (Innocenti and Frost, 1980, 1979; Olavarria et al., 1987). In contrast, bilateral eye enucleation leads to a reduction of CPNs in the typically callosal-rich area, the border between V1 and V2 (17/18 border) (Fig. 3A) (Innocenti and Frost, 1980, 1979; Olavarria et al., 1987).

Studies of the rodent somatosensory (SS) cortex confirm the importance of sensory-derived activity for CPN selection but with some opposite outcomes (Fig. 3B). Early unilateral whisker cauterezization or sectioning of the infranorbital nerve (ION) eliminates about half of the L2/3 callosal contralateral branches (Suarez et al., 2014a; Huang et al., 2013). CC retrotracing injections revealed that this is because only 15% of L2/3 neurons remain as CPNs in this context (De León Reyes et al., 2019). Interestingly, the number of L2/3 CPNs, or their contralateral columns, is not altered if manipulations of the whiskers or ION are performed bilaterally (De León Reyes et al., 2019; Suarez et al., 2014a; Huang et al., 2013; Koralek and Killackey, 1990). However, S1L4 CPN number increases up to 20%, resembling the increases in visual CPNs observed upon unilateral deprivations (Fig. 3B) (De León Reyes et al., 2019).

Altogether, any attempt to establish a unidirectional relationship between sensory activity and refinement, or to extract general rules for callosal decisions, appears simplistic. For example, while balanced activity between hemispheres is a crucial requirement for some callosal neurons, it seems dispensable for others, such as visual CPNs or the remaining S1L2/3 populations of unilaterally cauterized mice (De León Reyes et al., 2019; Suarez et al., 2014a; Huang et al., 2013; Koralek and Killackey, 1990). In addition, none of the studies discussed above provide the precise mechanisms by which activity influences the selection of CPNs. Thus, understanding how the thalamus orchestrates callosal circuit assembly requires further investigation.
Sensory thalamic alterations

Functional areas of the cortex are each innervated by distinct thalamic nuclei and they also exhibit very different proportions of CPNs. S1L4, which is principally innervated by the ventral posterior nucleus (VPM), contains almost no CPNs in adult mice. In contrast, many S2L4 neurons, innervated by the posteromedial nucleus (Pom), display callosal projections (23% of S2L4) (De León Reyes et al., 2019). These differences appear to be due to different refinement dynamics that depend on distinct thalamic inputs. The genetic ablation of VPM, which causes an innervation switch of S1L4 by ectopic Pom thalamocortical axons (Pouchelon et al., 2014), reduces refinement and increases the number of S1L4 CPNs (20% of S1L4) (De León Reyes et al., 2019) (Fig. 3C). An independent study that aspirated the dorsal thalamus of rats at birth also reported ectopic callosal branches innervating S1 (Koralek and Killackey, 1990). In the visual system, it was found that the ablation of thalamic axons innervating V1 duplicates the number of CPNs in all cortical layers, causing functional interhemispheric hyperconnectivity (De León Reyes et al., 2019) (Fig. 3C). This agrees with previous thalamic lesion studies performed in rats with expanded callosal innervation of V1 (Cusick and Lund, 1982). Thus, thalamic inputs determine at least three aspects of callosal assembly: (1) refinement rates and CPN numbers; (2) CPN distribution across layers; and (3) contralateral postsynaptic targets.

The firing response and synaptic transmission modulate CPN projection

The relevance of the firing response of a neuron during the establishment of callosal connections has been examined using Kir2.1, an inward rectifying potassium channel that lowers the resting membrane potential of the neuron. In the mouse visual system, overexpressing Kir2.1 in L2/3 neurons, and thus lowering their firing threshold (i.e. making it less likely to fire upon a given input), decreases contralateral innervation (Mizuno et al., 2007). Kir2.1 overexpression affects callosal axons but not neuronal identity, migration or the general capacity to project axons (Mizuno et al., 2010, 2007). Similar results were obtained in the mouse SS cortex, where the overexpression of Kir2.1 in L2/3 was shown to reduce the contralateral axonal column formed at the S1/S2 border without impairing ipsilateral branching (Suarez et al., 2014a;
Wang et al., 2007; Hand et al., 2015). Interestingly, the combination of Kir2.1 expression together with the blockade of synaptic transmission in the presynaptic CPNs—via tetanus toxin expression—causes a more severe reduction in contralateral projections than Kir2.1 expression alone (Mizuno et al., 2007). This demonstrates the importance of synaptic vesicle release for axonal connectivity. It was further shown that, in L2/3 callosal axons, mitochondrial capture dictates the points of axonal branching, and mitochondrial size determines presynaptic release during callosal refinement (Courchet et al., 2013; Lewis et al., 2018).

Callosal connectivity also appears to be coupled with the acquisition of mature and specific firing modes through the expression of certain transcription factors (TFs). In L2/3, the TF Cux1 facilitates the upregulation of the ion channel Kv1 (initiated at P8 and preserved later), which is responsible for producing the typical reliable L2/3 firing patterns. The acquisition of such mature firing patterns is necessary for callosal stabilization; downregulation of either Cux1 or Kv1 in L2/3 CPNs causes the elimination of contralateral branches (Rodríguez-Tornos et al., 2016). Another element that has a strong influence on the development of callosal connections is the activity of their postsynaptic neuronal counterparts in the opposite hemisphere. Non- altered CPNs are unable to stabilize their callosal projections when Kir2.1 is overexpressed in their postsynaptic targets (Mizuno et al., 2010). Moreover, postsynaptic activity regulates local versus long-range circuits. In L2/3 neurons, sensory activity upregulates the TF MeF2c, diminishing their long-range contralateral inputs, which in turn boosts local connectivity. Contrary, postsynaptic deletion of MeF2c increases contralateral S1 long-range responses and decreases local afferents (Rajkovich et al., 2017). This highlights the importance of neuronal-intrinsic activity and input processing in both the pre- and postsynaptic neurons for proper callosal connectivity.

Guidance cues during CC formation

During CC development, cortical neurons route their projections through the callosal pathway towards precisely defined contralateral areas and layers. CPN axons and cells located along their way express a variety of guidance ligands and receptors, ensuring proper navigation. The role of several of these guidance cues has been addressed using knock out (KO) and transgenic animal models (summarized in Table 1). From these data, we can extract some general conclusions. First, the most frequent phenotype among all models is one in which cortical axons approach the midline but fail to cross to the opposite hemisphere; or if they do, they follow aberrant interhemispheric routes, as in the case of Slit2 or Robo KO (Bagri et al., 2002; Lopez-Bendito et al., 2007; Unni et al., 2012). Second, most KO mice models are lethal at birth, and E17 or P1 are the latest stages analyzed. As callosal axons from upper cortical layers do not reach the midline until postnatal stages (De León Reyes et al., 2019), the effect of these guidance cues in the later-crossing axons remains to be addressed. Third, many genes described as necessary for callosal axon guidance, such as those encoding Slit2, netrin 1 or Eph/ephrin, are also implicated in other biological processes such as the proper development of the cells forming midline structures (Unni et al., 2012; Shu and Richards, 2001; Andrews et al., 2007). This has to be taken into account when interpreting phenotypes of KO models.

As an example, the guidance molecules Slit2 and Robo1. Slit2 is a chemorepulsive molecule secreted by glial cells at the midline and its receptor, Robo1, is located on callosal axon growth cones. Slit2-Robo1 interaction causes cortical axons to avoid both ventral and dorsal territories, forcing them to cross the midline (Shu and Richards, 2001). In Slit or Robo KO models, some CPN axons cross to the contralateral hemisphere, but the majority turn to navigate into the septum or within the rostrocaudal plane next to the midline, forming the so-called Probst bundles (Bagri et al., 2002; Andrews et al., 2007; Shu and Richards, 2001). Interestingly, in these and other KO models (such as netrin 1 or DCC KOs) an ectopic ventral commissural pathway appears (Bagri et al., 2002; Fazeli et al., 1997; Serafini et al., 1996; Lopez-Bendito et al., 2007; Unni et al., 2012). This raises the hypothesis that callosal guidance cues do not act as a midline barrier for cortical axons; rather, they might have appeared as an evolved mechanism favoring midline crossing at the corticoseptal boundary (Suarez et al., 2014b).

A closer look at these studies also reveals that only a few have tested the cell-autonomous effect of guidance molecules. For example, neurogenin 2 (Ngn2) KO, which is lethal at birth, causes partial to severe AgCC. However, when an shRNA-Ngn2 is electroporated into L2/3 neurons, no defects in midline crossing are seen. Instead, electroporated L2/3 neurons project ectopically to cortico-cortical and subcortical targets (Hand and Polleux, 2011). Similarly, in the KO of the neuropilin 1 subunit that mediates semaphorin interaction, AgCC with incomplete penetration is observed (Gu et al., 2003), but the specific downregulation of this receptor in L2/3 does not impair midline crossing (Wu et al., 2014). Hence, many guidance-related phenotypes in KO models might not be caused by cell-autonomous restrictions of midline crossing. Indeed, the primary etiology of complete AgCC is due to defects in interhemispheric remodeling by midline glial populations that provide a substrate for callosal axons to cross the midline (Gobius et al., 2016). In addition to the requirement for a fused interhemispheric glial substrate, the timing of callosal axon crossing is regulated non-cell-autonomously (Choe et al., 2012).

In agreement with all of the above, a molecule that acts specifically as a stop signal for CC axons at the midline has not yet been identified. It was thought that the acquisition of CPN identity was governed by the capacity of a neuron to cross the midline following specific guidance cues. However, previous and recent findings demonstrate that most cortical neurons cross over to the contralateral hemisphere, supporting the lack of axonal restrictions for midline crossing (De León Reyes et al., 2019; Innocenti and Price, 2005). As such, it is likely that guidance cues are not responsible for the selection of commissural neurons but fundamental for proper innervation of specific targets.

The molecular identity of CPNs and the plasticity of young cortical neurons

Cortical neurons have traditionally been classified according to their morphotype, laminar location, connectivity and neurotransmitter expression (Peters and Jones, 1984; Ramón y Cajal, 1995; Rakic, 1995). More recently, the ‘-omics’ revolution has refined this taxonomy by revealing genes, transcriptomes and proteins that identify neuronal subpopulations even at single-cell resolution (Nowakowski et al., 2017; Molyneaux et al., 2015; Lodato and Arlotta, 2015; Pouchelon et al., 2014; Azim et al., 2009a; Mayer et al., 2018; Joshi et al., 2008; Frangeul et al., 2016). However, despite these breakthroughs, we are still unable to define the precise molecular identity of CPNs.

A significant number of studies have investigated TFs as potential identity determinants by reporting connection failures after their loss of function (Molyneaux et al., 2007). Yet only a few genes have demonstrated themselves to be sufficient to promote specific connectivity, i.e. their gain of function implies the acquisition of a given projection pattern. Such is the case of Fezf2, the overexpression of which in L2/3 is sufficient to generate ectopic subcortical
Table 1. Penetrance of partial or severe AgCC in knockout mouse models

| Mutant  | Age at analysis | CC phenotype                                                                 | Penetrance | Other commissural phenotypes                                                                 | References |
|---------|-----------------|-------------------------------------------------------------------------------|------------|---------------------------------------------------------------------------------------------|------------|
| Slii2<sup>−/−</sup> | E17             | Few axons cross the midline; some are staked at the midline forming Probst bundles. Most reach the midline but ectopically enter the septum or rostral regions. | Full (n=5) | Eclectic HC projections to rostral and lateral targets. HC and CC axons are mixed. Presence of an ectopic ventral commissure located above the AC. | Bagli et al. (2002), Unni et al. (2012) |
| Slii3<sup>−/−</sup> | E17             | Rostral and medial: few axons cross the midline, most ectopically enter the septum or form Probst bundles | 33% (n=4/12) | -                                                                                           | Unni et al. (2012) |
| Slii1<sup>+/−</sup> Slii3<sup>−/−</sup> | E17             | Rostral: Probst bundles Medial: few axons cross, most form Probst bundles | Caudal: no visible defects 42% (n=5/12) | -                                                                                           | Unni et al. (2012) |
| Rbo1<sup>−/−</sup> | E17-E18         | Few axons cross the midline. Most reach the midline but ectopically enter the septum. | Full (n=7) | Eclectic HC projections to rostral and lateral targets. HC and CC axons are mixed. Presence of an ectopic ventral commissure located above the AC. | Andrews et al. (2007), Unni et al. (2012) |
| Rbo2<sup>−/−</sup> | E18             | Most reach the midline but ectopically grow into the septum or form Probst bundles | Full (n=3) | -                                                                                           | Lopez-Bendito et al. (2007) |
| Ntn1<sup>−/−</sup> | E17-E18         | Most reach the midline but ectopically grow into the septum or form Probst bundles | Full (n=11) | Absence of the HC. The AC is reduced. A large aberrant commissure is found in the roof of the fourth ventricle. | Seppajärvi et al. (1996), Fothergill et al. (2014) |
| Dct1<sup>−/−</sup> | E17             | Most reach the midline but ectopically grow into the septum or form Probst bundles | Full (n=3) | Absence of the HC. The AC is reduced. A large aberrant commissure is found in the functional region between hindbrain and midbrain. | Fazeli et al. (1997), Fothergill et al. (2014) |
| Ephb1<sup>−/−</sup> | P1              | Variable phenotypes: mild to severe AgCC | 87% (n=47) | -                                                                                           | Yue et al. (2002), Hu et al. (2003) |
| Ephb2<sup>−/−</sup> | P1              | Variable phenotypes: mild to severe AgCC | 61% (n=23) | Reduced AC                                                                                   | Yokoyama et al. (2001), Mendes et al. (2006) |
| Ephb3<sup>−/−</sup> | P1              | No effect on CC | 0% (n=22) | Reduced AC                                                                                   | Orioli et al. (1996) |
| Ephb3<sup>−/−</sup> | P0-P1           | AgCC with variable severity | 37.5% (n=3/8) | -                                                                                           | Orioli et al. (1996) |
| Ephb3<sup>−/−</sup> | P0-P1           | AgCC with variable severity | 89% (n=8/9) | Reduced AC                                                                                   | Orioli et al. (1996) |
| Ephb4<sup>−/−</sup> | P1              | AgCC with variable severity | 88% (n=22) | -                                                                                           | Yokoyama et al. (2001), Mendes et al. (2006) |
| Ephb4<sup>−/−</sup> | P1              | AgCC with variable severity | 90% (n=20) | -                                                                                           | Yokoyama et al. (2001), Mendes et al. (2006) |
| Ephb4<sup>−/−</sup> | P1              | AgCC with variable severity | Full (n=7) | -                                                                                           | Yokoyama et al. (2001), Mendes et al. (2006) |
| Ephb1<sup>−/−</sup> | E17             | AgCC. Axons ectopically project entering the ipsilateral septum. | Full (n=4) | -                                                                                           | Bush and Sotani (2009) |
| Ephb3<sup>−/−</sup> | P1              | AgCC with variable severity | 84% (n=37) | -                                                                                           | Yokoyama et al. (2001), Mendes et al. (2008) |
| Ephb3<sup>−/−</sup> | P1              | AgCC with variable severity | Full (n=29) | -                                                                                           | Yokoyama et al. (2001), Mendes et al. (2008) |
| Ephb3<sup>−/−</sup> | P1              | AgCC with variable severity | 89% (n=16) | -                                                                                           | Yokoyama et al. (2001), Mendes et al. (2008) |
| Fzd3<sup>−/−</sup> | E18             | AgCC with variable severity | - | Loss of the thalamocortical and corticothalamic tracts, and the AC | Wang et al. (2002) |
| Aplp2<sup>−/−</sup> | P0-P1           | AgCC with variable severity | - | -                                                                                           | Wang et al. (2017) |
| Daxin<sup>−/−</sup> | P0-P1           | AgCC with variable severity | Severe (n=7/12) | Reduced HC and AC. Variable severity. | Islam et al. (2009) |
| Pten<sup>−/−</sup> | P0              | AgCC in the anterior part of the CC. | Mild (n=9/12) | Reduced HC and AC. Variable severity. | Hossain et al. (2019) |
| Sema3c<sup>−/−</sup> | E18             | Partial to severe AgCC in the anterior part of the CC. Reduced elongation of post-crossing callosal axons. | - | -                                                                                           | Niquille et al. (2009), Mire et al. (2018) |
| Sema3a<sup>−/−</sup> | E17             | Slight reduction of pioneer axons Disrupted axon order in the CC and in the contralateral hemisphere | - | -                                                                                           | Catalano et al. (1998), Piper et al. (2009), Zhou et al. (2013) |
| Npn1 Naga<sup>−/−</sup> | E17             | AgCC with variable severity | - | -                                                                                           | Gu et al. (2003) |
| Npn2<sup>−/−</sup> | E18             | AgCC with variable severity | Severe (n=2/13) | -                                                                                           | Hand and Polleux (2011) |
| Npn2<sup>−/−</sup> | E18             | AgCC with variable severity | Partial (n=1/11) | -                                                                                           | Hand and Polleux (2011) |
| Ryk<sup>−/−</sup> | E18             | Axons cross the midline but are unable to reach the contralateral hemisphere. After midline crossing some axons grow back towards the midline. | Severe (n=5/22) | No visible alterations in other commissures | Kebbel and Cooper (2006) |
| Daxin<sup>−/−</sup> | P1              | AgCC with variable severity | Weak (60% n=3/5) | Agenesis of the AC | Hossain et al. (2013) |
| Tsku<sup>−/−</sup> | P1              | AgCC with variable severity | Severe (40% n=2/5) | -                                                                                           | Hossain et al. (2013) |

AC, anterior commissure; AgCC, agenesis of the CC; CC, corpus callosum; E, embryonic day; HC, hippocampal commissure; P, postnatal day; -, not analyzed or not mentioned. Rostral refers to the anterior part of the CC. Medial refers to the medial part of the CC. Caudal refers to the caudal part of the CC. When not specified, the defects in the CC are present all along the rostro-caudal axis.
projections (Rouaux and Arlotta, 2013), or Tbr1, the expression of
which in L5 converts corticospinal into cortico-thalamic neurons
(Mckenna et al., 2011). However, many other TFs do not fulfill the
criteria for being master instructors. For example, Cux1 deletion in
L2/3 CPN impairs callosal stabilization but Cux1 is also expressed in
other non-callosal populations and its overexpression does not
drive callosal fate (De Leon Reyes et al., 2019; Rodriguez-Tornos
et al., 2016).

Perhaps the most important example of a TF often mistaken as a
definitive callosal determinant is Satb2. E18.5 Satb2 KO brains show
very few axons crossing the midline and exhibit increased subcortical
projections, which led to the conclusion that Satb2 loss reprograms
CPNs into subcortical neurons (Alcamo et al., 2008; Britanova et al.,
2008). However, as Satb2 KO mice die at birth, conditional deletions of
Satb2 in the cortex were generated to overcome this lethality and test
its role in upper layers (Zhang et al., 2019; Leone et al., 2015).

Surprisingly, a significant number of neurons have callosal
connections in these mice. Furthermore, retrograde injections in the
thalamus and peduncle revealed that ectopic corticothalamic and
corticospinal axons originate exclusively from deep layers. This
argued against the conversion of Satb2-deficient L2/3 CPNs to a deep
layer fate, as proposed by the initial investigations (Zhang et al., 2019;
Leone et al., 2015). More recent studies showed that IUE-based elimination of Satb2 in L2/3 does not impair callosal connectivity.
Nevertheless, L2/3 axons are visible in the internal capsule,
indicating that they also develop subcortical projections. The same
experiment in either L5 or L6 induces a significant reduction of
contralateral connections (Paolino et al., 2020). Molecularly, Satb2
regulates distinct gene networks in layer- and time-dependent
manner (Paolino et al., 2020; Leone et al., 2015). In summary, it
seems that Satb2 expression is involved in the establishment of
callosum projections of deep layers but has a different role in L2/3.
Indeed, cumulative reports conclusively show that Satb2 expression, although developmentally regulated, is broad and not restricted to
CPN or late-born upper layer neurons (Alcamo et al., 2008; De Leon
Reyes et al., 2019; Britanova et al., 2008; Harb et al., 2016; Huang
et al., 2013; Jaitner et al., 2016).

Therefore, so far there is no common molecular program regulating
all CPNs, nor a ‘master gene’ whose expression commits neurons to a
callosum fate. But why is it so difficult to find a CPN molecular
fingerprint? This could be due to the fact that CPNs locate in all layers
and are born from different precursors during an extended
developmental window (from E12 to E15); however, this is just a
partial explanation. It has been demonstrated that CPNs are a much
more heterogeneous population than anticipated, and that their RNA
expression profiles change dynamically during development (Arlotta
et al., 2005; Molyneaux et al., 2009, 2015; Lodato and Arlotta, 2015).

If we combine this information with the notion of early transient
callosum projections and cortical epigenetic modifications, it seems
plausible that an initially unspecified molecular program allowing
axonal exuberance is subsequently overwritten by molecular
instructions added gradually during postnatal differentiation
(Rouaux et al., 2012; De Leon Reyes et al., 2019; Molyneaux et al.,
2007, 2015; Lodato and Arlotta, 2015; Pouchelon et al., 2014; Azim et al., 2009b; Mayer and Fishell, 2018; Joshi et al.,
2008; Frangeul et al., 2016; Nowakowski et al., 2017). Context-
dependent postnatal genetic editing might be responsible for
discarding a default callosal fate, triggering refinement. In
agreement with the idea of such a non-committed early program,
it has been shown that young neurons can co-express TFs that are
always segregated in the adult, and there are also reports of some
dual ‘hybrid’ subpopulations, such as those with dual interhemispheric and subcortical axons that co-express Sox5 and
Lmo4 (Sohur et al., 2014; Azim et al., 2009b).

Overall, axonal exuberance could be the natural phenotypic
consequence of early-unspecified molecular programs that are
gradually crafted during development (Fig. 4). The elusive
molecular identity of CPNs might simply reflect the existence of
multiple developmental molecular trajectories that drive stabilization
of callosal axons. This permissive scenario might explain the
plasticity of young callosal neurons and their potential to generate
diverse CC circuits in context- and activity-dependent manners
(Pouchelon et al., 2014; Su et al., 2017; Yap and Greenberg, 2018;
West and Greenberg, 2011).

The behavior of transient callosal axons

Transient axons sample multiple territories that will not be integrated
into adult circuits under normal conditions, but that can be innervated
if context changes occur, generating non-canonical circuits (Olavarria
et al., 1987; Huttenlocher and Raichelson, 1989; Uematsu et al., 1996;
Rouaux et al., 2012; De Leon Reyes et al., 2019; Innocenti and Frost,
1979). Neurons with broader exploratory behavior likely harbor a greater plasticity (Fig. 5),
and there seem to be differences in the behavior of exuberant
projections and refinement processes in different brain territories. In
the thalamus, exuberant efferent collaterals from first-order (FO)
thalamic nuclei, such as the LGN, VPM or the ventral part of the
MGN, confine their projections to the cortical area that they will
innervate in the adult (de Venecia and McMullen, 1994; Nuegele et al.,
1988; Rebsam et al., 2002; Catalano et al., 1996). In contrast,
young axons of higher-order (HO) thalamic nuclei, such as Pom

![Fig. 4. Schematic illustration of the refinement of early axonal projections.](image-url)
thalamocortical projections, navigate broadly throughout non-adult territories. They non-specifically invade barrel columns, supplementary SS and motor areas (Deschenes et al., 1998; Lu and Lin, 1993), which may explain why VPM ablation results in an expansion of Pom axons within S1L4 (Pouchelon et al., 2014). The HO visual nucleus (lateral posterior nucleus; LP) also innervates more territories than the LGN; it targets not only V1 and V2 but also A2 (Ji et al., 2016). Adult corticospinal (CS) neurons also explore multiple territories prior to refinement (Stanfield et al., 1982; Ribeiro Gomes et al., 2020; Stanfield and O’Leary, 1985; Bates and Killackey, 1984; Cabana and Martin, 1984). In the pyramidal tract, most adult CS axons decussate contralaterally and only minor populations project ipsilaterally. In young cats and monkeys, however, the proportion of ipsilateral and contralateral projections is similar (Ribeiro Gomes et al., 2020; Li and Martin, 2000). Importantly, these ipsilateral projections can survive into the adult when lesions in the sensorimotor cortex or CS tract occur early (Reinoso and Castro, 1989; Huttenlocher and Raichelson, 1989; Hicks and D’Amato, 1970; Uematsu et al., 1996).

In the CC, both transient and to be terminal callosal axons cross the midline and follow indistinguishable paths through the WM. However, the behavior of transient axons once they reach the contralateral hemisphere is more obscure, and potential targets of plastic connectivity are more difficult to predict. It was reported that, in the cat visual cortex, only some of the V1 transient axons traced at P9 barely invade the homotopic V1L6 (Innocenti, 1981; Aggoun-Zouaoui and Innocenti, 1994). However, similar experiments performed at P6 revealed a significant amount of V1 axons climbing to upper layers (Ding and Elberger, 1994). Hence, the WM might act as a barrier for some transient axons, but it is not a common impediment. Interestingly, both studies described that V1 transient CC axons preferentially explore the contralateral V1/V2. Somatosensory transient callosal axons invade the gray matter but only invade contralateral homotopic regions (De León Reyes et al., 2019; Chalupa and Killackey, 1989). In contrast, in the auditory cortex of newborn cats (P1-P4), transient callosals behave less restrictedly. They not only innervate the contralateral auditory but also transiently scan V1 and V2 cortices (Innocenti and Clarke, 1984a), which perhaps explains why auditory circuits are expanded in visually impaired individuals (Collignon et al., 2011, 2013). In sum, the fact that cortical neurons explore non-adult targets gives them the possibility to generate non-canonical circuits under altered situations. The exploratory capacity of CC transient exuberant projections is yet to be characterized but it seems to vary between areas and CPN subpopulations.

**The biological significance of axonal exuberance in the cortex**

Refinement of transient projections is a common developmental process within the CNS (Sohur et al., 2014; Catalano et al., 1996; Naegele et al., 1988; Rebsam et al., 2002; O’Leary, 1987; De León Reyes et al., 2019; Innocenti et al., 1988; O’Leary et al., 1981; Chalupa and Killackey, 1989; Stanfield and O’Leary, 1985; Stanfield et al., 1982; Galea and Darian-Smith, 1995; O’Leary and Stanfield, 1985, 1983; Innocenti and Price, 2005). Moreover, the overproduction of neuronal cells and synaptic densities is commonly observed in the nervous system (Denaxa et al., 2018; Wong et al., 2018; Duan et al., 2020). Yet spending cellular energy in producing massive numbers of transient axons that will be later eliminated might seem as an apparent contradiction. Such initial overabundance may have simply evolved as one of the viable mechanisms for building connections. Alternatively, it might have offered selective advantages during brain evolution. In this section, we elaborate on the possible benefits of developmental axonal exuberance. When discussing the biological significance of axonal exuberance in the CC, it is useful to compare it with other systems. As mentioned above, not all neuronal types enjoy the ‘permissibility’ to explore multiple targets in both hemispheres. In the retina, for example, differential Zic2 expression in RGCs directs axons exclusively to the ipsi- or contralateral hemisphere (Drager, 1985; Sretavan, 1990; Erskine and Herrn, 2014). Young vertebrate motor neurons also exhibit high muscle group specificity (Lance-Jones and Landmesser, 1981; Tosney and Landmesser, 1984, 1985). In these stereotyped systems, early molecular identities restrict axonal pathfinding. However, the cortex is built of highly complex networks that initiate their wiring according to a priori unknown environmental stimuli. If young cortical neurons were to project exclusively to their final targets, they would require very complex initial genetic codes that, in turn, could impair the adaptability of cortical networks. Axonal overproduction might therefore be the evolutionarily selected mechanism for wiring circuits that optimally respond to the external world, without requiring the early and complex genetic coding of their future connections (Cowan et al., 1984). In support of this hypothesis, theoretical modeling has shown that, for distributed networks like the brain, algorithms based on hyper-connectivity followed by pruning provide a more efficient and robust way of building circuits than a model of increasingly growing networks (Navlakha et al., 2015). For the CC, it allows the activity-dependent sculpting of specific callosal circuits required in each functional area. In summary, it is likely that the advantages – reduced initial coding, efficiency, robustness and plasticity – outweigh the energy spent in building non-definitive projections. Thus, axonal exuberance and refinement is likely the reflection of self-assembling, context-instructed and highly plastic wiring of complex networks.

**Corpus callosum development in humans**

The study of the human CC (hCC) relies on noninvasive imaging approaches that allow structural analysis, such as PET, MRI or DTI (Fig. 2E), and fMRI, which measures neuronal activity within brain regions of living subjects (Herve et al., 2013; Dennis and Thompson, 2013). However, these techniques do not have the resolution to determine the number and specific location of CPNs. Hence, current knowledge about the number, layer location and refinement dynamics of human CPNs is mostly inferred from postmortem analyses or primate models (LaMantia and Rakic, 1990a; Chalupa and Killackey, 1989; Chalupa et al., 1989; Dehay et al., 1986, 1988; Meissner et al., 1991). General descriptions of the hCC, both developmental and pathological, are also based on postmortem analyses, as well as non-invasive MRI and tractography (Fig. 2E). However, although gross callosal dysgenesis such as partial or complete AgCC can be reasonably easily diagnosed by imaging techniques, subtle CC circuit defects might be underestimated.

The human cortex is organized into neuronal layers and, as in other mammals, the CC establishes bilateral connections between cortical areas (Wahl et al., 2007, 2009). The adult hCC is thought to contain more than 200,000,000 fibers (Aboitiz et al., 1992; Tommasch, 1954) and can be subdivided into seven regions from rostral to caudal: the rostrum, genu, rostral body, anterior midbody, posterior midbody, isthmus and splenium (Fig. 6A). As most of the CC fibers connect homotopic regions, the organization of callosal fibers correlates with the rostro-caudal distribution of functional territories (Fig. 6B) (Caminiti et al., 2013).

In regard to human CC development, postmortem immunostaining of coronal sections from human fetal brains revealed that, as in mice (Ren et al., 2006; Koester and O’Leary, 1994; Rash and Richards,
(2001; Piper et al., 2009), human pioneer axons originate from the cingulate cortex (Ren et al., 2006). Following this, CC formation is thought to occur from rostral (genu) to caudal (splenium) regions, except for the rostrum, which is the last to appear (Ren et al., 2006; Rakic and Yakovlev, 1968; Byrd et al., 1978; Hewitt, 1962; Barkovich and Norman, 1988). Although some studies argue that the CC might not follow such a rigorous rostro-caudal developmental dynamic (Paul, 2011; Kier and Truwit, 1996; Huang et al., 2006; Huang, 2009), there is consensus that all CC structures are visible at gestational week (GW) 20 (Ren et al., 2006; Rakic and Yakovlev, 1968; Clarke et al., 1989; Kazi et al., 2013; Raybaud, 2010) (Fig. 6C). After GW20, CC thickness increases until GW30 (Barkovich and Norman, 1988; Clarke et al., 1989). Then, during the second postnatal month, approximately 21% of the total cross-sectional thickness of the CC is reduced (Clarke et al., 1989). This indicates that, as in other mammals, the human CC exhibits significant refinement (Fig. 6C). From this point on, developmental myelination causes CC thickening, which complicates addressing the successive amount and temporal dynamics of axonal refinement.

Myelination occurs in a gradual and spatially organized manner from the second postnatal month until 9 years of life (Krupa and Bekiesinska-Figatowska, 2013). Contrary to axonal elongation, myelination occurs from caudal to rostral territories (Krupa and Bekiesinska-Figatowska, 2013). Interestingly, its progression correlates with the functional maturation of neuronal circuits (Pujol et al., 2006; Nickel and Gu, 2018). Indeed it has been described that vocabulary acquisition in 1-year-old children is related to rapid myelination in language areas (Pujol et al., 2006). Therefore, the CC caudal to rostral myelination likely reflects earlier bilateral processing in visual and auditory regions, compared with frontal areas (social skills).

Functional changes continue to occur until adulthood, placing the human CC among the last structures to complete postnatal maturation (Keshavan et al., 2002; Pujol et al., 1993). Growing evidence suggests that activity-dependent CC maturation also plays a role in cognitive learning (Sampaio-Baptista and Johansen-Berg, 2017). During this process, environmental factors can produce long-lasting changes in the human CC. As an example,
musicians that begin musical instruction before the age of 7 have an enlargement in the CC posterior midbody and perform better on sensorimotor synchronization tasks (Steele et al., 2013; Schlaug et al., 1995).

Neurodevelopmental defects in the human corpus callosum

During development, defects in neuronal/gliai proliferation and migration, axonal growth, guidance cues, refinement or myelination can interrupt CC formation and lead to mild or severe CC congenital malformations, which are generally classified as dysgenesis. Such defects can be caused by genetic mutations (Edwards et al., 2014) or by early environmental insults, such as alcohol exposure or preterm deliveries (Riley et al., 1995; Sepulveda et al., 2011; Spadoni et al., 2007; Barnes-Davis et al., 2020). Insults during the first stages of CC development (GW11) can lead to the complete absence of CC (cAgCC), while insults occurring later (from GW12 to GW30) can interrupt CC formation and lead to mild or severe CC congenital malformations, which are generally classified as dysgenesis. Such defects can be caused by genetic mutations (Edwards et al., 2014) or can occur as a phenotypic manifestation of Anderman, Fryns and DeMorsier syndromes (Krupa and Bekiesinska-Figatowska, 2013; Edwards et al., 2014). Interestingly, animal models show that early developmental insults can result in a reduced number of callosal projections due to an imbalance in the refinement/stabilization ratio (De León Reyes et al., 2019; Innocenti and Frost, 1979; Olavarria et al., 1987), raising this as a possible cause in humans.

CC malformations are frequently related to reduced CC size. However, in rare instances, individuals show thickening of this
structure. CC enlargement has been reported in individuals with neurofibromatosis type I or as an associated clinical feature of other syndromes, such as Cohen and MMC (megalecephaly, mega CC) (Agarwal et al., 2013; Bindu et al., 2010). A recent study reported that mutations in the gene encoding MAST1 cause mega CC due to an increased number of callosal fibers and not because of an increased myelination. Such findings point to a reduction in refinement as a possible origin for mega CC (Tripathy et al., 2018).

CC defects are also present in individuals with schizophrenia, ASD, bipolar disorder and with social function impairments (Chinnasamy et al., 2006; Symington et al., 2010; Motomura et al., 2002; Kumar et al., 2010; Barnea-Goraly et al., 2009; Koshiyama et al., 2020, 2018). The neuropyschiatric features are usually described as overlapping, and neurological screening shows that they can be intermingled (8.5% of individuals with AgCC also have autism) (Edwards et al., 2014). We are grateful to L. J. Richards, L. Fenlon and IRC5 members, and to J. García-Marques for critical reading.

Conclusions
As we have highlighted here, there are two major stages of CC development. First, there is an early period of growth of non-restricted, massive callosal projections. Following this, sensory-dependent refinement occurs during specific and restricted temporal windows in each layer and area. Thus, acquiring an adult callosal identity seems to be dictated by the capacity to stabilize an early immature callosal projection, possibly via postnatal editing of neuronal-specific molecular programs. Importantly, exuberance appears to be a major contributor to plasticity. It helps to explain circuit diversity, and how genetic malformations and insults can lead to non-canonical circuits. Aonaxal exuberance might also have conferred an evolutionary advantage for the development of higher-order complex circuits. The CC is fundamental for proper human cognition and is affected in multiple neurodevelopmental disorders. However, while current imaging techniques can be used to characterize gross CC structure, they likely underestimate more subtle circuit alterations.

We therefore require further development of non-invasive imagining and tractography techniques in order to obtain a deeper understanding of CC structure and the mechanisms of its wiring, and to allow for earlier and more accurate diagnosis and prognosis. These will lead to a better understanding of neurotypical CC development, inter-individual variability, and the causes and consequences of CC dysgenesis. Overall, these approaches will hopefully promote the development of treatments that might exploit the intrinsic plasticity of the CC.

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