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Diverse axonal morphologies of individual callosal projection neurons reveal new insights into brain connectivity

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Abstract

In the mature brain, functionally distinct areas connect to specific targets, mediating network activity required for function. New insights are still occurring regarding how specific connectivity occurs in the developing brain. Decades of work have revealed important insights into the molecular and genetic mechanisms regulating cell type specification in the brain. This work classified long-range projection neurons of the cerebral cortex into three major classes based on their primary target (e.g. subcortical, intracortical, and interhemispheric projections). However, painstaking single-cell mapping reveals that long-range projection neurons of the corpus callosum connect to multiple and overlapping ipsilateral and contralateral targets with often highly branched axons. In addition, their scRNA transcriptomes are highly variable, making it difficult to identify meaningful subclasses. This work has prompted us to reexamine how cortical projection neurons that comprise the corpus callosum are currently classified and how this stunning array of variability might be achieved during development.

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Introduction

The mammalian neocortex is a complex, six-layered structure, parcellated into distinct areas that are responsible for motor coordination, sensory perception, learning and memory, and higher cognitive functions such as social interaction, decision-making, and executive function. These brain functions are made possible through the formation of complex networks connecting areas of the brain together in functionally appropriate circuits. Here we focus on a major subset of these circuits connecting the cerebral hemispheres, which in placental mammals occurs via the corpus callosum, the largest fiber tract in the brain.

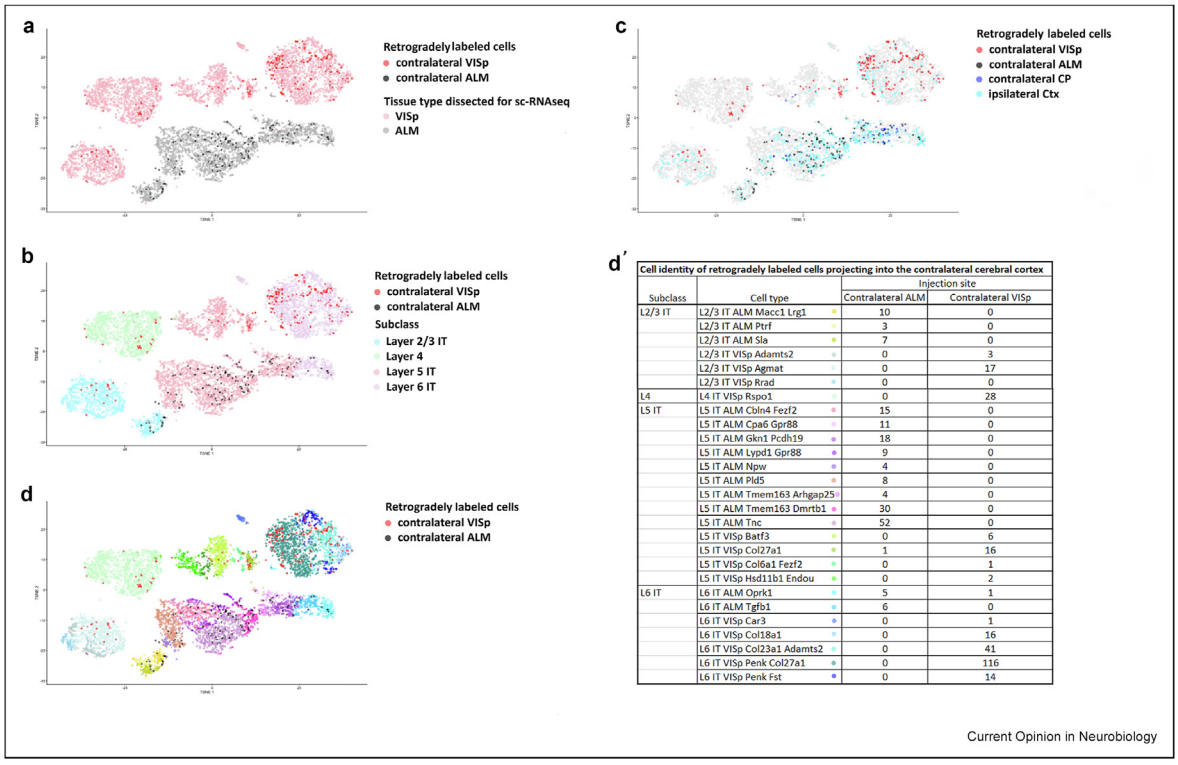
Callosal projection neurons (CPNs) primarily reside in layers 2/3 (L2/3) and L5 of the neocortex and extend long-range projections to the midline, where they cross into the contralateral hemisphere. Callosal projections then grow in the white matter of the contralateral cortex, locate their precise target area, and then either branch or turn to innervate the cortical layers and make appropriate connections with their postsynaptic partners.

Visualizing callosal projections and molecular profiling of callosal projection neurons

The earliest strategies to uniquely label CPNs involved bulk retrograde labeling by injecting horseradish peroxidase, fluorescent dyes, or microspheres into their contralateral axonal target [1–6]. Although this was a robust strategy to visualize projections to a specific target and, in later studies, to obtain a purified population of CPN cell bodies, bulk labeling precluded the identification and visualization of all axonal projections from these neurons. Nevertheless, profiling of transcripts enriched in CPN cell bodies, first conducted using microarray [5] and subsequently by bulk RNA-seq experiments [7,8], revealed key molecular determinants of CPNs and hinted at a high degree of heterogeneity in this class type [5,7,9]. In more recent years, single-cell RNA-seq analysis of the developing cortical plate has significantly advanced our abilities to genetically profile all projection neuron subtypes [10–12].

One such study by Tasic et al. (2018) profiled single cells within the adult mouse anterior lateral motor (ALM) and primary visual (VISp) cortices following retrograde labeling from various brain regions (Figure 1) [13]. This study confirmed that CPNs that formed homotopic connections within the contralateral hemisphere were molecularly heterogenous but nevertheless belonged to subclasses of intratelencephalic neurons

Figure 1



d'

Cell identity of retrogradely labeled cells projecting into the contralateral cerebral cortex			
		Injection site	
Subclass	Cell type	Contralateral ALM	Contralateral VISp
L2/3 IT	L2/3 IT ALM Macc1 Lrg1	10	0
	L2/3 IT ALM Ptrf	3	0
	L2/3 IT ALM Sla	7	0
	L2/3 IT VISp Adamts2	0	3
	L2/3 IT VISp Agmat	0	17
	L2/3 IT VISp Rrad	0	0
L4	L4 IT VISp Rspo1	0	28
L5 IT	L5 IT ALM Cbln4 Fezf2	15	0
	L5 IT ALM Cpa6 Gpr88	11	0
	L5 IT ALM Gkn1 Pcdh19	18	0
	L5 IT ALM Lypd1 Gpr88	9	0
	L5 IT ALM Npw	4	0
	L5 IT ALM Pld5	8	0
	L5 IT ALM Tmem163 Arhgap25	4	0
	L5 IT ALM Tmem163 Dmrtb1	30	0
	L5 IT ALM Tnc	52	0
	L5 IT VISp Batf3	0	6
	L5 IT VISp Col27a1	1	16
	L5 IT VISp Col6a1 Fezf2	0	1
	L5 IT VISp Hsd11b1 Endou	0	2
L6 IT	L6 IT ALM Oprk1	5	1
	L6 IT ALM Tgfb1	6	0
	L6 IT VISp Car3	0	1
	L6 IT VISp Col18a1	0	16
	L6 IT VISp Col23a1 Adamts2	0	41
	L6 IT VISp Penk Col27a1	0	116
	L6 IT VISp Penk Fst	0	14

Intratelencephalic neurons that form homotopic connections within the contralateral hemisphere have highly heterogeneous transcriptomes and potentially reside within all cortical layers except L1.

a. TSNE plot depicting intratelencephalic neurons harvested from the VISp and ALM cortex , obtained from the Tasic et al. (2018) scRNA-seq dataset. Cells are colored based on the respective cortical areas from which they were collected. Overlaid in red and black are retrogradely labeled cells that project into the contralateral VISp and ALM, respectively. This data demonstrates that these neurons project to largely homotopic areas when compared between the frontal and occipital poles of the cortex.

b. TSNE plot depicting the subclass identity of intratelencephalic cells in different cortical layers, overlaid by retrogradely labeled cells in red and black, respectively.

c. Retrogradely labeled cells identified through injections of viral tracers into the contralateral VISp, contralateral ALM, contralateral caudate putamen (CP), or ipsilateral cortex (Ctx) and overlaid on the TSNE plot of all intratelencephalic cells. Injection sites within the ipsilateral cortex included the primary motor area, primary somatosensory area, secondary somatosensory area, perirhinal area, and retrosplenial area for cells harvested from the ALM. Ipsilateral injection sites for cells harvested from the VISp consist of the anterior cingulate and retrosplenial areas. This data shows that individual cell types, as defined by their transcriptome, could project to multiple target areas both contralaterally and ipsilaterally.

d. TSNE plot depicting cell types as described in Tasic et al. (2018). Cell type labels, represented by each color, are described within the table in panel d'. Overlaid in red and black are retrogradely labeled cells that project into the contralateral VISp and ALM, respectively.

d'. Table of the number of retrogradely labeled cells within each subclass and their corresponding cell type as defined by Tasic et al. (2018). This data shows the heterogeneity of retrogradely labeled cells and different callosal subclasses observed in this dataset. Abbreviated forms indicate the following: ALM, anterior lateral motor; VISp, primary visual cortex; TSNE, t-distributed stochastic neighbor embedding.

that reside predominantly within L2/3 and L5 but also occupied L4 and L6 of the adult cortex (Figure 1a, b, d–d'). Retrograde injections into other brain regions demonstrated that these cell types could also project to cortical areas within the ipsilateral cortex as well as the contralateral caudate putamen (Figure 1c). In these instances, the labeling strategy utilized was limited in its ability to determine whether individual cells with similar transcriptomic profiles formed connections with different brain regions or whether individual cells formed axonal branches that enabled them to project to multiple brain regions. If these cells indeed formed axonal branches (as demonstrated by the single callosal neuron labeling experiments discussed below), it remains to be determined whether only specific CPN subtypes are capable of this. Therefore, while this study advanced our understanding of the transcriptome of adult CPNs, it also demonstrated a gap in our knowledge of the molecular determinants of their projection patterns.

Single neuron labeling of callosally-projecting neurons reveals connectivity to multiple targets and a high degree of variability in their projection patterns

High-throughput platforms that simultaneously enable single callosal neuron labeling, molecular identification,

and tracing of axonal projections are required to reliably identify CPN subtypes and examine the full spectrum of their postsynaptic targets. Such databases or analysis pipelines that have performed detailed axonal tracing and molecular profiling of CPNs together have not yet been generated.

Recently, advanced viral tracing and microscopy techniques employed by the MouseLight project (https://ml-neuronbrowser.janelia.org/) have brought about a major technological advance in visualizing the anatomical diversity of projection neurons. Precise labeling of cell bodies and axonal trajectory reconstruction at a single neuron level were achieved by injection of low-titer preps of adeno-associated viruses, followed by high-resolution 3-D light microscopy of optically cleared whole mouse brains [14,15]. Neurons were assigned to a given layer and area based on their anatomical location in accordance with the Allen Mouse Common Coordinate Framework. Using axonal reconstructions from about 1200 neurons, Winnubst et al. (2019) performed analysis on intratelencephalic and pyramidal tract neurons that revealed diversity in neuron projection patterns and subtypes projecting to distinct targets. This database's high-resolution axonal morphology data also enabled quantitative analysis and comparison between these different classes of neurons [16]. We performed a comprehensive analysis of this publicly available dataset, focusing on the CPNs, to examine their diversity in interhemispheric projection patterns and to categorize them into subtypes, if possible. An exhaustive analysis of the axonal projection patterns from the NeuronBrowser of the MouseLight consortium shows remarkable diversity in the targets innervated by CPNs (Figure 2). By carefully examining each neuron reconstruction, we confidently identified 43 of the 1200 projection neurons (analyzed by the MouseLight consortium) that display interhemispheric callosal projections, with their soma located in the motor, anterior cingulate, frontal, somatosensory, and visual cortices (Figure 2). This proportion of neurons (43/1200) likely reflects aspects of the viral injection strategy rather than an actual indication of interhemispheric versus noninterhemispheric projection neurons. A close examination of these 43 neurons (1 in the primary somatosensory cortex, 3 in the visual cortex, and the remaining 39 in the motor/frontal/cingulate cortices) reveals that they may belong to multiple classes of previously unknown subtypes based on their diverse projection patterns in the contralateral hemisphere (Figures 2, 3). Indeed, Figure 3 demonstrates neurons with long-range, highly-branched projections in both the ipsilateral and contralateral hemispheres. This has also been observed using FluoroGold and DiI retrograde labeling, which showed that many long-range projection neurons simultaneously project to the contralateral somatosensory cortex and the ipsilateral frontal cortex. These dual projections persist until adulthood, indicating that they may be critical for integrating information from both motor and sensory areas in both hemispheres [17]. Our observations are also corroborated by the analysis of frontal L5 callosal neurons, which display heterogeneity in physiological and morphological attributes that directly correlate with corticocortical and subcortical projection patterns [18].

Here, we highlight recent studies that investigate mechanisms that faithfully guide interhemispheric callosal projections to anatomically diverse target regions.

How is this variability in projection patterns achieved?

Sequential waves of transcription factor-regulated genetic programs in the cortical ventricular zone act to establish cortical areas [19–21] and to generate the vast diversity of projection neuron subtypes found in the neocortex [9,22,23]. Each subtype expresses a distinct set of transcription factors, has unique morphological features and electrophysiological characteristics, and subserves different functions based on their diverse anatomical projections [5–7,24–28]. Expression of key transcription factors like SATB2 and CUX1 is critical for the specification of CPNs and the establishment and stabilization of callosal projections [29–35]. Spatial expression patterns of key guidance factors acting downstream and in tandem with transcription factors provide cues for circuit formation between different areas [36–39]. The interplay between the genetic signatures of developing neurons and the sensory influences of the surrounding environment plays a major role in sculpting neuronal circuits [40]. Such mechanisms have been reviewed elsewhere [41–43]. Here, we review mechanisms that may generate axonal projection diversity after neurons are specified to a particular layer and cortical area.

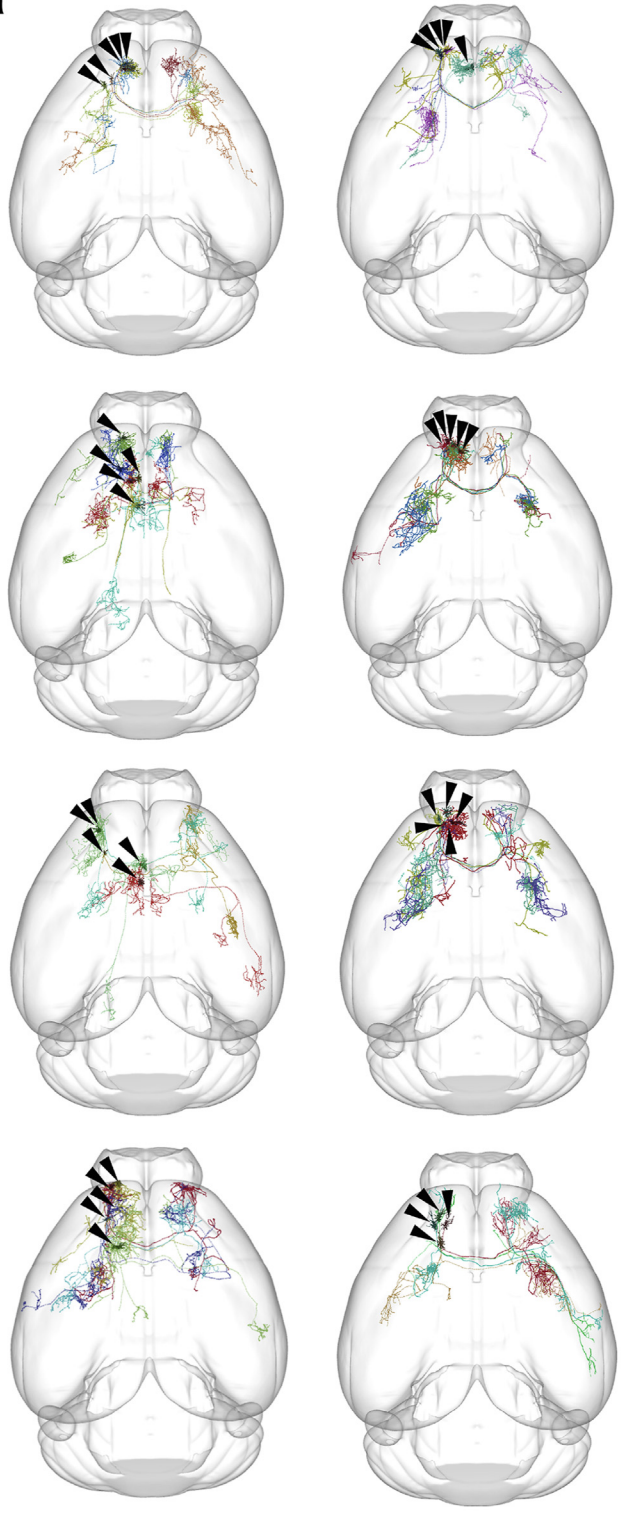
Genetic mechanisms governing axonal extension

In the last decade, new systems-level strategies integrating multiple cutting-edge techniques and high-throughput approaches have identified key genes that mediate the assembly and function of callosal circuits. Here, we provide examples of the types of experimental approaches that are revealing new insights into callosal axon targeting without providing an exhaustive review of this literature.

Flow cytometry-assisted sorting of labeled growth cones of individual L2/3 CPNs, followed by paired subcellular proteome-transcriptome analysis, has revealed the enrichment of diverse classes of proteins, including those regulating synaptic transmission as well as axonal growth and guidance [44]. This study employed an extensive approach to identify specialized sub-cellular molecular signatures of the growth cone, both at the mRNA and protein level, and compared them to those of their parent cell bodies. The approach enabled the

Figure 2

a



b

Neuron ID	Soma location	Type	Long range projections in the contralateral hemisphere	Long range projections in the ipsilateral hemisphere
AA0916	MOs2/3	Homotopic	MOs2/3; Insular cortex	MOs2/3
AA0914	MOs2/3	Homotopic	MOs2/3	Striatum
AA0905	MOs5	Homotopic	MOs2/3, 5, S1J, Striatum, Ectorhinal cortex	Striatum
AA0897	MOs2/3	Homotopic	MOs2/3; Striatum	Striatum
AA0884	MOs2/3	Homotopic	MOs2/3	Striatum
AA0873	MOs2/3	Homotopic	MOs2/3; Striatum	Striatum
AA0872	ACAv2/3	Homotopic	ACA2/3	M2, V2
AA0859	MOs2/3	Homotopic	MOs2/3, Infrailmbic cortex	MOs2/3
AA0802	MOs2/3	Homotopic	MOs2/3, MOp	MOp, Insular Cortex, Striatum, Basolateral Amygdala
AA0801	ACAv2/3	Homotopic	ACAv2/3, Striatum	Striatum, Medial Parietal Association Cortex
AA0800	ACAv2/3	Heterotopic	MOp; Cingulate cortex; Striatum	Striatum; V2
AA0795	MOs2/3	Homotopic	MOs2/3; Striatum; Entorhinal cortex	Striatum
AA0672	MOs2/3	Homotopic	MOs2/3; Striatum	Striatum
AA0663	MOs1	Homotopic	MOp, Striatum	-
AA0659	MOs2/3	Homotopic	MOp, Insular cortex	Insular cortex
AA0655	MOs1	Homotopic	MOs2/3; Striatum	Striatum
AA0594	MOs1	Homotopic	MOs2/3; Ectorhinal cortex	Striatum
AA0590	FRP1	Heterotopic	MOs2/3; Striatum	Insular cortex
AA0589	MOp1	Homotopic	MOs2/3; Striatum	Striatum; Insular Cortex
AA0534	MOs5	Homotopic	MOs2/3; Insular Cortex	Striatum
AA0475	MOs2/3	Homotopic	MOs2/3	-
AA0472	MOs1	Homotopic	MOs2/3	Striatum; Insular Cortex
AA0471	MOs2/3	Heterotopic	Medial Orbital cortex	Striatum
AA0450	MOs2/3	Homotopic	MOs2/3; Striatum	Striatum
AA0425	PL2/3	Heterotopic	MOs2/3; Insular cortex	Striatum
AA0424	MOs2/3	Homotopic	MOs2/3	Insular Cortex
AA0421	MOs5	Homotopic	MOs2/3; Striatum	Striatum
AA0419	MOs2/3	Homotopic	MOs5; Striatum	Striatum
AA0418	MOs2/3	Heterotopic	Medial Orbital cortex	
AA0416	MOs2/3	Homotopic	MOs5; Striatum	Ectorhinal Cortex
AA0411	MOs5	Homotopic	MOs2/3; Striatum; Insular cortex	Striatum
AA0409	MOs2/3	Homotopic	MOs2/3, Striatum	Striatum
AA0407	MOs2/3	Homotopic	MOs2/3; Striatum; Insular cortex	Striatum
AA0395	MOs2/3	Homotopic	MOs2/3	Ventral Pallidum
AA0329	MOs2/3	Homotopic	MOs2/3; Striatum	MOp, Striatum
AA0291	MOs2/3	Homotopic	MOs2/3; Ectorhinal cortex	Striatum
AA0289	MOs5	Homotopic	MOs5; Striatum; Ectorhinal cortex	Striatum
AA0281	MOs5	Homotopic	MOs2/3; Striatum	-
AA0274	MOs5	Homotopic	MOs2/3; Ectorhinal cortex	Striatum

c

Soma location	Number of neurons that have axon terminals in these contralateral target areas												
	Homotopic					Heterotopic							
	MOs2/3	MOs5	ACA	MOs1	MOp	Str	Ins	mOFC	Ect	Ent	IL	Cg	S1J
MOs2/3	17	2	0	0	2	10	3	2	1	1	1	0	0
MOs5	6	2	0	0	0	6	2	0	3	0	0	0	1
ACAv2/3	0	0	2	0	1	2	0	0	0	0	0	1	0
MOs1	3	0	0	5	1	2	0	0	1	0	0	0	0
FRP1	1	0	0	0	0	1	0	0	0	0	0	0	0
PL2/3	1	0	0	0	0	0	1	0	0	0	0	0	0
MOp1	1	0	0	0	0	1	0	0	0	0	0	0	0

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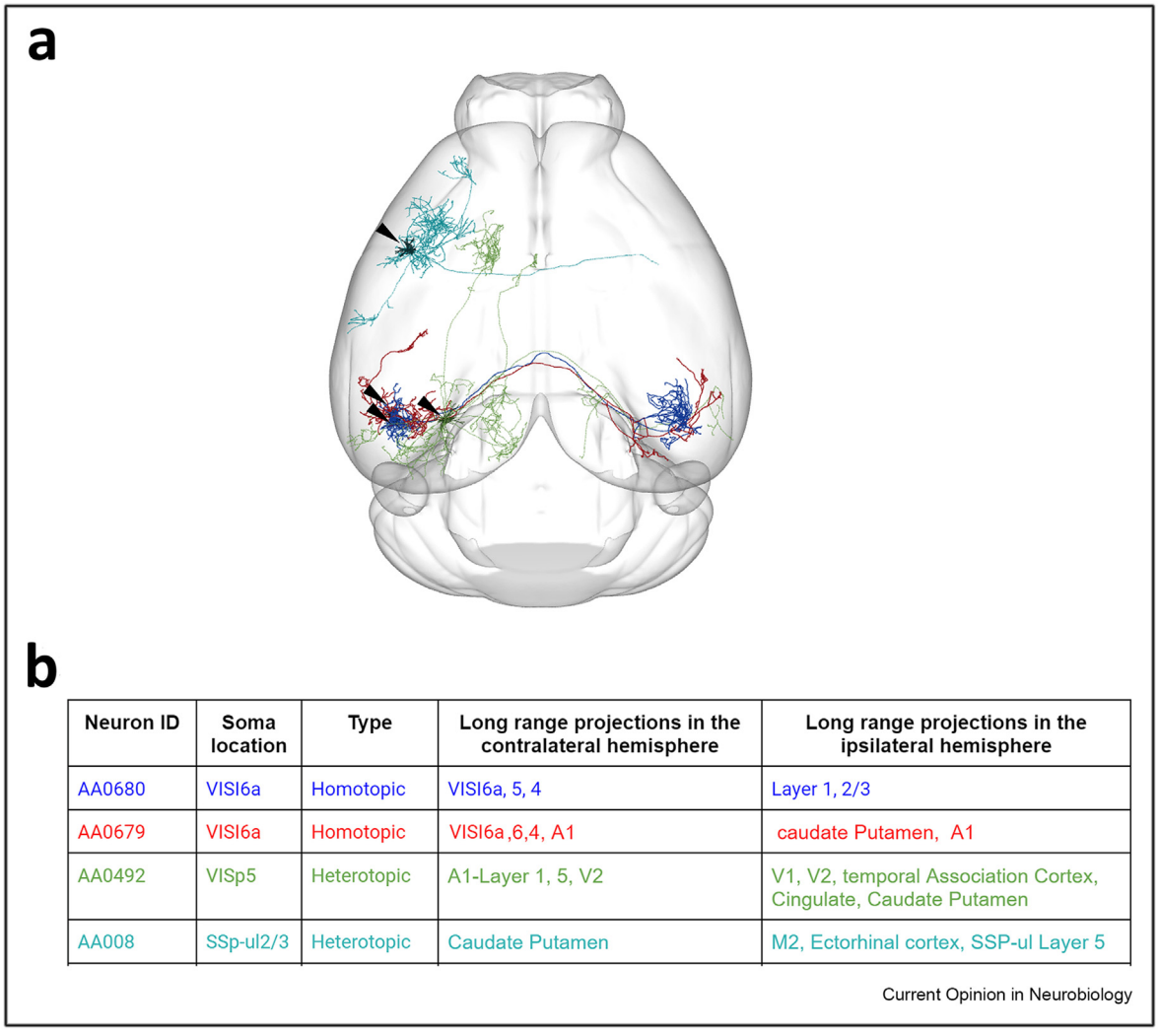
Figure 3



Neuron ID	Soma location	Type	Long range projections in the contralateral hemisphere	Long range projections in the ipsilateral hemisphere
AA0680	VISl6a	Homotopic	VISl6a, 5, 4	Layer 1, 2/3
AA0679	VISl6a	Homotopic	VISl6a ,6,4, A1	caudate Putamen, A1
AA0492	VISp5	Heterotopic	A1-Layer 1, 5, V2	V1, V2, temporal Association Cortex, Cingulate, Caudate Putamen
AA008	SSp-ul2/3	Heterotopic	Caudate Putamen	M2, Ectorhinal cortex, SSP-ul Layer 5

Diverse projection patterns of callosal neurons originating in the sensory cortices were analyzed using data available in the MouseLight database.
a. Dorsal view of callosal neurons, three originating in the primary visual cortex (VISp, VISl) and one in the primary somatosensory cortex (SSp), which extend interhemispheric and intrahemispheric projections. Each neuron is represented by a different color. Black arrowheads represent the location of the soma.
b. Analysis of the anatomically diverse targets innervated by callosal neurons originating from primary somatosensory and visual cortices.

identification of mTOR signaling as necessary for transhemispheric callosal axon growth. mTOR foci, along with mRNA transcripts controlling the translation machinery, were found to be enriched in CPN growth cones, a site of intense axonal growth and where the sensing of target-derived signals occurs. The outcomes of this analysis, for the first time, provided a strong experimental foundation for carrying out quantitative

Callosal neurons in motor, cingulate, and frontal cortices project to anatomically diverse targets in both hemispheres, analyzed using data available in the MouseLight database (https://ml-neuronbrowser.janelia.org/).
a. Dorsal view of axonal projection patterns of 39 callosal neurons, originating in MOp, MOs, ACA, and FRP1 cortices, which extend interhemispheric projections. In each individual brain, five representative callosal neurons are shown, each in a different color. Black arrowheads represent the location of the soma.
b. Tabular analysis of the diverse anatomical targets innervated by each of these 39 neurons in both the ipsilateral and contralateral hemispheres. Detailed information for each neuron has been tabulated under the following headers: neuron ID (from the MouseLight database), soma location and cortical layer, classification as homotopic or heterotopic, brain regions innervated in the contralateral and ipsilateral hemispheres.
c. Categorization of these 39 callosal neurons based on their soma location and the presence of axonal terminals in distinct brain regions. Abbreviated forms indicate the following: Str, Striatum; Ins, Insula; mOFC, medial orbitofrontal cortex; Ect, Ectorhinal cortex; Ent, Entorhinal cortex; IL, infralimbic; Cg, cingulate; S1J, primary somatosensory area. ACA, anterior cingulate area; FRP1, frontal; MOp, primary motor cortex; MOs, secondary motor cortex.

investigations of subcellular RNA-proteome mapping in developing CPNs. Together, these novel findings propose a unique concept that involves coupling cellular translational machinery to signaling components at the growth cone to facilitate axon extension of specialized projection neuron subtypes. In the growth cones of interhemispheric projections, candidates like neurexin and neuregulin were also enriched, which are known to control excitatory and inhibitory synapse development and synapse specificity [45].

Recently, techniques like BRICseq (brain-wide individual animal connectome sequencing) have also been developed, which allow correlations between transcriptomic signatures and neuronal activity during behavior [46]. This technique harnesses the power of multiplexed viral tracing, DNA barcoding, and high-throughput sequencing to extensively map neocortical region-to-region connectivity from single individuals at an affordable cost in a few weeks. This technique was used to correlate spatial expression patterns of a few genes with region-to-region connectivity that predicted neuronal activity patterns in the adult brain while the animal performed a decision-making task. The outcomes of the BRICseq corticocortical projectome mapping align with our findings based on the MouseLight dataset that corticocortical projection neurons originating in a given region project in a highly variable manner to anatomically diverse cortical regions in both hemispheres.

In addition to extrinsic axon guidance mechanisms, the ability of CPNs to extend interhemispheric projections is also contingent upon intrinsic cues such as firing responses characteristic of cortical L2/3 neurons. Acquisition of such characteristic firing patterns is achieved by the expression of master regulator genes, either in CPNs themselves or in their contralateral target area. The transcription factor CUX1, which is expressed in L2/3 CPNs, is necessary for modulating the ion channel KV1-dependent firing properties of L2/3 neurons. Down-regulation of either CUX1 or KV1 resulted in decreased callosal innervation of the contralateral hemisphere [34], suggesting transcription factor-regulated neuronal firing is critical for stabilizing interhemispheric callosal projections.

Primary callosal axons upon reaching their contralateral target undergo extensive arborization, and some of these mechanisms are disrupted in human developmental disorders. Callosal arborization is influenced by multiple factors, including mitochondrial function. Mitochondria within the axonal compartments of CPNs are shorter in length compared to dendritic mitochondria, which are long and tubular in structure. This characteristic small size of axonal mitochondria is necessary for controlling the calcium buffering capacity of presynaptic mitochondria, which in turn regulates neurotransmitter release and axonal branching of callosal neurons [47]. Callosal branching is also affected by gain-of-function mutations in the L-type Ca2+ channel, CAV1.2, in Timothy syndrome, which results in greatly reduced callosal axon arborization in the contralateral hemisphere [48].

Early neuronal activity sculpts initial callosal targeting and pruning

During development, the combinatorial expression of transcription factors plays a pivotal role in the specification, guidance, wiring, and assembly of callosal circuits [5]. As callosal neurons mature and extend projections into contralateral cortical targets, coordinated patterns of spontaneous activity and sensory-evoked neuronal activity patterns emerge that are crucial for circuit development and function [49–51]. These activity patterns may emerge within callosal neurons themselves or in target neuronal populations. Spontaneous firing of action potentials in developing cortical neurons during prenatal and early postnatal periods plays an important role in the establishment and maintenance of cortical circuits [51]. When axonal firing is compromised, for example, when exogenous expression of an inward rectifying potassium channel KIR2.1 is used to hyperpolarize L2/3 neurons in the visual cortex, it results in impaired lamina-specific axonal targeting in the contralateral hemisphere but does not affect region-specific targeting [52]. In contrast, similar perturbations introduced in somatosensory cortex L2/3 neurons resulted in slightly different outcomes by affecting both area- and lamina-specific targeting [53]. Overexpression of KIR2.1 also resulted in the slowing of axon extension along the corpus callosum, as well as during innervation of the contralateral cortex [53]. These effects could occur through KIR2.1-mediated suppression of neuronal excitation or a failure to establish proper synaptic transmission. Overexpression of the tetanus toxin light chain, TeNT-LC, that interferes with vesicular exocytosis of neurotransmitters resulted in an initial delay in the innervation of the contralateral cortex and failure in the stabilization of callosal terminals at the end of the second postnatal week. These results suggest that both intrinsic neuronal excitability and proper synaptic transmission are required for precise targeting and maintenance of callosal projections in the contralateral somatosensory cortex [53]. Further studies have uncovered that spontaneous network activity is selectively required during a critical period in the second postnatal week for the development of callosal projections [54]. Further, silencing of neuronal activity in the CPNs and in the contralateral target hemisphere indicated that both presynaptic and postsynaptic neuronal activity play a critical and differential role in axon growth, branching, and the formation of arbors during callosal axon development [55]. This indicates that the coordination of neuronal firing levels, between presynaptic and

postsynaptic neurons across both hemispheres regulates the wiring of long-range projection neurons. Such coordination of neuronal firing levels may be orchestrated by the expression of key genes encoding transcription factors, axon guidance cues, and ion channels.

Correlated patterns of spontaneous activity in small neuronal ensembles in the developing neocortex may also operate in combination with molecular cues to instruct callosal connectivity [50]. In late embryonic to postnatal mammals, distinct modality-specific spontaneous activity patterns with unique spatiotemporal properties emerge in sensory areas [56–58]. Spontaneous wave-like activity patterns emerging in peripheral sensory organs like the retina propagate to the visual cortex and the superior colliculus in a bilaterally synchronized manner [56]. This motivates the question of whether these coordinated retinal waves could facilitate interhemispheric wiring between the visual hemispheres [59]. Spontaneous activity in developing circuits also primes the nascent neuronal networks for subsequent sensory-experience-dependent fine-tuning of circuits and maturation of complex networks [59,60]. This has been elegantly demonstrated in the developing visual cortex, where callosal inputs from the contralateral hemisphere coordinate with spontaneous retinal activity to eliminate chandelier interneurons in the binocular zone prior to vision onset. This callosal-input-driven elimination is critical for developing binocular vision [61].

Over development and into adulthood, these sensory-specific spontaneous and experience-evoked patterns are replaced with more complex activity rhythms in the adult brain that engage multiple networks and propagate over different neocortical areas [62]. In the mature sensory system, spontaneous activity is regulated by behavioral states such as attention and fixation in visual or auditory tasks, and in varying degrees, anesthesia (reviewed in 63). A comparison of cortical spontaneous activity across behavioral states indicates that there is significant diversity of these activity signatures across cortical areas. Even within a specific behavioral state, area and layer diversity in cortical spontaneous activity patterns can be observed [63]. Voltage-sensitive dye imaging of adult mice in both awake and anesthetized states at millisecond temporal resolution has revealed that spontaneous oscillatory activity is highly synchronized across multiple regions and between hemispheres via transcallosal interhemispheric projections [64]. This coherence of complex local rhythms originating at several points may facilitate parallel modification of functionally linked synaptic connections in both hemispheres. This has also been exemplified in studies employing specific activation of the corpus callosum coupled with whole-brain activity imaging. Using fMRI and calcium imaging, optogenetic activation of the corpus callosum connecting the barrel cortices was found to elicit signals in the ipsilateral motor cortex and the posterior thalamus, suggesting that callosal projections are functionally connected with diverse targets [65].

Sensory-deprivation experiments reveal that unilateral sensory disruption causes greater changes in callosal targeting than bilateral disruptions of sensory input to the cortex

Experience-driven inputs relayed by peripheral sensory organs play a major role in the formation and function of developing cortical circuits. Using sensory deprivation paradigms, sensory-input-driven neuronal activity has been shown to play a crucial role in the development of callosal projections.

Visual system

In the visual cortex of adult cats, callosal connections are distributed in a restricted manner, and they are particularly enriched at the boundary of area 17 (primary visual cortex) and area 18 (secondary visual cortex). Such distribution is acquired postnatally by the progressive elimination of callosal projections from the rest of areas 17 and 18. Kittens deprived of visual inputs by binocular eyelid suturing develop a normal distribution of callosal inputs. However, monocularly deprived kittens displayed unusual retention of callosal inputs in area 17 (where they are normally eliminated) at adult stages [2]. Monocularly deprived rats also display ectopic callosal terminals in area 17, while bilateral enucleation results in a reduction of callosal projections in the border between areas 17 and 18 [66]. Overall, these observations point to the fact that monocular deprivation results in greater disruption of callosal connections as compared to binocular deprivation. These studies indicate that visual input is critical for the normal development of callosal projections, in the absence of which callosal projections are either reduced or retained in ectopic locations.

Somatosensory system

The ablation of peripheral sensory organs in other sensory circuits, like the somatosensory system, by transecting the infraorbital nerve impacts callosal projections in both hemispheres. In the rodent somatosensory cortex, unilateral transection of the infraorbital nerve results in an abnormal pattern of callosal projection terminals in the contralateral somatosensory cortex. However, anomalies in callosal projection patterns were also observed in the hemisphere ipsilateral to the nerve transection, which receives normal peripheral input from the contralateral periphery [67]. This indicates that periphery-driven activity alone is insufficient to maintain normal callosal projection patterns and that alternate mechanisms are involved. The fact that bilateral disruption of peripheral sensory organs does not

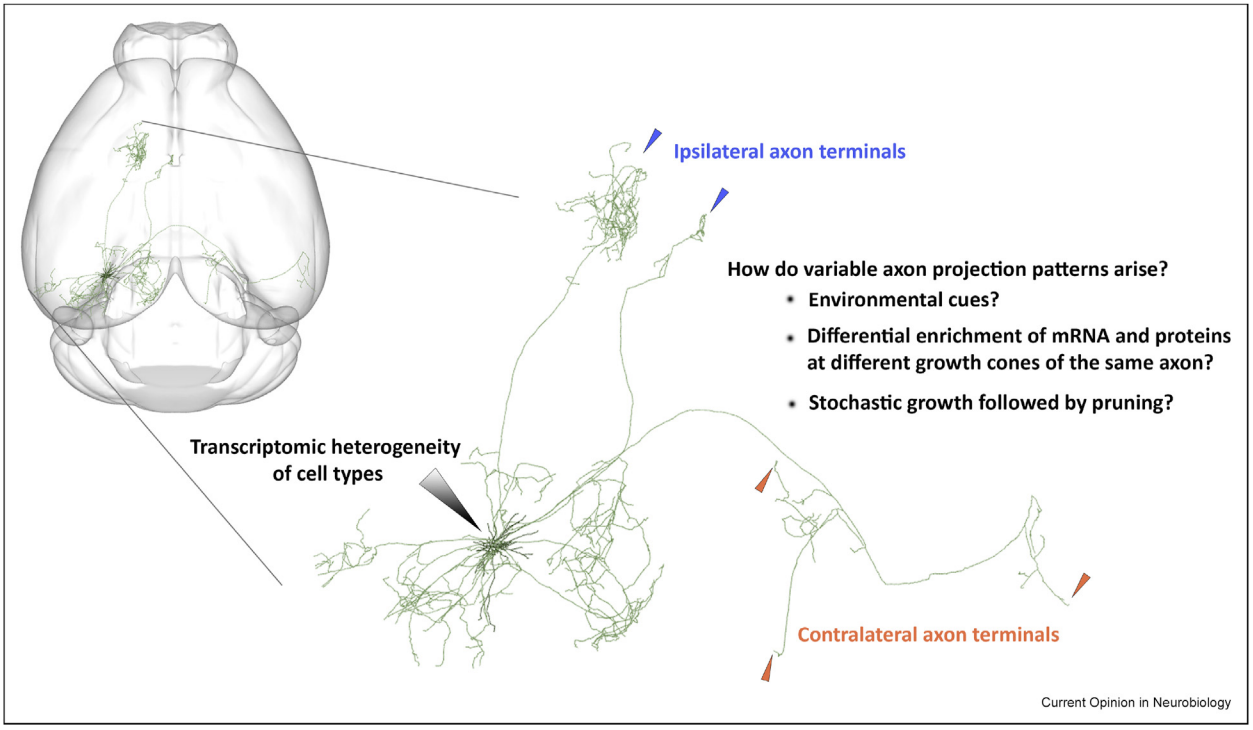
affect callosal projections provides additional evidence in favor of this argument [68,69]. Experiments involving disruption of either spatially symmetric whisker pad organization or cortical neuronal activity have demonstrated that symmetrically balanced neuronal activity in both hemispheres is necessary for correct callosal axon targeting [69]. During the targeting phase, interhemispheric axons likely match the activity of the contralateral targets with their cell bodies to faithfully establish long-range connections. This indicates that extrinsic periphery-relayed sensory inputs or intrinsic neuronal activity alone are insufficient for precise guidance of callosal projections. Correlated patterns of activity that emerge in a balanced manner in both hemispheres may act as an instructive cue for guiding callosal projections between homotopic targets. Hence, diverse mechanisms are required for the formation of callosal projections in modality-specific sensory circuits.

The influence of experience-dependent sensory input also extends to the elimination of developmentally transient exuberant callosal projections during postnatal refinement. Such sensory-input-dependent pruning mechanisms have recently been demonstrated in the somatosensory cortex for transient callosal projections that extend from L4 neurons [70].

CPNs in L2/3 also receive input relayed from the thalamus via L4. Manipulation of spontaneous neuronal activity originating in the sensory thalamic nuclei has been shown to result in the modulation of thalamocortical axon extension, branching complexity, and cortical area specification [71–74]. Using mouse models that undergo an alteration of sensory areas upon peripheral manipulations or activity silencing [73,74], it would be interesting to test whether a reorganization of thalamic projections influences the innervation pattern of callosal axons in sensory cortices.

Although most callosal projections are glutamatergic in nature, a small proportion of callosal projections also belong to L5 GABAergic neurons that express parvalbumin and modulate the gating of long-range interhemispheric inhibition [75,76]. Thus, in addition to excitatory pyramidal neurons, activity from long-range inhibitory callosal neurons can sculpt interhemispheric circuits.

Figure 4



hypothetical model depicting how diverse axonal projections may arise during callosal development.
a. Dorsal view of the axonal projection pattern of representative neuron AA0492 (MouseLight database) on the left and an enlarged version on the right. The representative neuron has five axonal terminals (2 ipsilateral, blue arrowheads and 3 contralateral, red arrowheads). Such variability in the axonal projection patterns of a single callosal neuron could occur through various mechanisms. These factors include, but are not limited to, transcriptional heterogeneity at the cell body [5,11,13], periphery- and activity-driven cues [2,52,53,66–69], growth-cone-specific enrichment of mRNA and proteins [44], or stochastic axon growth that is stabilized in a more specific manner.

Conclusion

Callosal axons display remarkably heterogeneous axonal trajectories, which often include extensive ipsilateral and contralateral heterotopic connections. Such diverse projection patterns remain hidden by bulk labeling approaches that are the basis for most histological connectomic tracing. Furthermore, MRI-based techniques such as tractography accurately produce streamlines up to the midline but are not able to trace streamlines into the contralateral gray matter. Such techniques that trace and label callosal neurons in bulk have indicated that callosal projections are largely homotopic. However, it is now unclear to what degree this is the case, given the results reviewed here from the MouseLight and BRICseq studies. A number of models could be put forward as to how such diverse axonal projections to a variety of contralateral and ipsilateral targets from a single neuron might arise. Callosal axons might follow a stereotypical growth pattern following guidance cues toward, across the midline, and into the contralateral hemisphere. However, considerable evidence presented here suggests that callosal axons also require activity, either spontaneous or evoked, to locate their target and stabilize branches in the appropriate layers. An alternative model might be that axon growth is constrained by molecular cues but largely stochastic, becoming pruned when activity patterns are encountered that can enable functional connections to be stabilized. Figure 4 summarizes factors that may be involved in driving the variability observed in callosal projection patterns. Variability between neurons is an important consideration, but it is also necessary to consider how a single neuron can project to multiple targets. For example, multiple neuronal growth cones originating from a single neuron and projecting to different targets could be presented with different guidance cues when growing toward their target, including potentially conflicting cues regulating contralateral and ipsilateral targeting. Exactly how these signals would be integrated within the same neuron suggests the compartmentalization of signals within a neuron and perhaps more integration at the local level of the individual growth cone [44], rather than being controlled by signals from the cell body. A combination of the mechanisms outlined in Figure 4, perhaps deployed in a hierarchical manner, might mediate the heterogeneity observed. Such heterogeneity may also be important for axonal plasticity. An example of this is the heterogeneity in whole-brain connectivity patterns observed between animals and people with brain wiring defects such as corpus callosum dysgenesis (for example, see [77]). Regardless of how these circuits are formed, the morphology and anatomical targeting of callosal neurons suggest an important role for the integration of information within individual neurons, not only between the hemispheres but across the entire cortex.

Methods

Reanalysis of Tasic et al. (2018) scRNA-seq dataset

The scRNA-seq data generated by Tasic et al. (2018) was downloaded from the Allen Brain Map RNA-seq data portal (https://portal.brain-map.org/atlases-and-data/rnaseq/mouse-v1-and-alm-smart-seq). The class, subclass, and cell type cluster identities that were originally assigned were obtained from the full metadata table available as Supplementary Table 10 in Ref. [13]. Similar to the original study, we used read counts that mapped to exons and further filtered the data to include only glutamatergic neurons using the following filter: class = glutamatergic. To generate t-distributed stochastic neighbor embedding (TSNE) plots, we reanalyzed this data in R (version 4.2.2) using the following R packages: SingleCellExperiment (version 1.20.0), scuttle (version 1.8.4), scater (version 1.26.1), and scran (version 1.26.2). The R script used for normalization, feature selection, and dimensionality reduction is available at https://doi.org/10.5281/zenodo.8179341. Retrogradely labeled cells were identified from the metadata table via the column 'injection_primary' following filtering for injection_exclusion_criterion = OK.

Analysis of MouseLight data

The source of the dataset used in this article is the MouseLight project (Economo et al., 2016, Winnubst et al., 2019), which is hosted online as an interactive database of 1200 neurons, available at https://ml-neuronbrowser.janelia.org/. We used the functionality of the search bar on this website to arrive at our final list of 43 neurons for analysis. In order to screen the database for neurons located in the frontal and motor cortices, the following parameters were entered into the search bar, and additional filters were applied as follows: Query type=anatomical region; source or target location=corpus callosum, "and" (isocortex + primary motor area + secondary motor area + anterior cingulate cortex + frontal pole of cerebral cortex), "not" (thalamus + primary somatosensory area + primary visual area + posterior commissure + anterior commissure); structure=any. This input yielded a list of 104 neurons. In order to check whether each of these 104 neurons satisfied the definition of a true CPN projecting across the midline, the dataset for each neuron was then manually analyzed for the presence of an interhemispheric projection that crossed the midline of the neocortex. Following this initial screening, we arrived at our final list of 39 neurons, which we analyzed for multiple parameters and categorized them as shown in Figure 2. In a similar way, in Figure 3, for neurons located in the sensory cortices, the following filters were used, and for each hit obtained, the dataset was manually analyzed for the presence of an interhemispheric projection, and 4 neurons satisfied our criteria: query type=anatomical region; source or target location=corpus callosum, "and" (primary somatosensory

area + primary visual area), "not" (thalamus + primary motor area + secondary motor area + anterior cingulate cortex + frontal pole of cerebral cortex); structure=any.

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Author contributions

Conceptualization (SP; LJR); Formal analysis (SP; JWCL; LJR); Funding acquisition (LJR); Investigation (SP; JWCL; LJR); Methodology (SP; JWCL; LJR); Supervision (LJR); Roles/Writing - original draft (SP; LJR); and Writing - review & editing (SP; JWCL; LJR)

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Publicly available datasets were used in this manuscript.

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References

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest

1. Innocenti GM, Fiore L, Caminiti R: **Exuberant projection into the corpus callosum from the visual cortex of newborn cats**. *Neurosci Lett* 1977 Apr, **4**:237–242, https://doi.org/10.1016/0304-3940(77)90185-9. PMID: 19604951.

2. Innocenti GM, Frost DO: **Effects of visual experience on the maturation of the efferent system to the corpus callosum**. *Nature* 1979 Jul 19, **280**:231–234, https://doi.org/10.1038/280231a0. PMID: 450139.

3. Katz LC, Burkhalter A, Dreyer WJ: **Fluorescent latex microspheres as a retrograde neuronal marker for in vivo and in vitro studies of visual cortex**. *Nature* 1984 Aug 9-15, **310**: 498–500, https://doi.org/10.1038/310498a0. PMID: 6205278.

4. Catapano LA, Arlotta P, Cage TA, Macklis JD: **Stage-specific and opposing roles of BDNF, NT-3 and bFGF in differentiation of purified callosal projection neurons toward cellular repair of complex circuitry**. *Eur J Neurosci* 2004 May, **19**:2421–2434, https://doi.org/10.1111/j.0953-816X.2004.03303. PMID: 15128396.

5. Molyneaux BJ, Arlotta P, Fame RM, MacDonald JL, MacQuarrie KL, Macklis JD: **Novel subtype-specific genes identify distinct subpopulations of callosal projection neurons**. *J Neurosci* 2009 Sep 30, **29**:12343–12354, https://doi.org/10.1523/JNEUROSCI.6108-08.2009. PMID: 19793993; PMCID: PMC2776075.

6. Fame RM, MacDonald JL, Macklis JD: **Development, specification, and diversity of callosal projection neurons**. *Trends Neurosci* 2011 Jan, **34**:41–50, https://doi.org/10.1016/j.tins.2010.10.002. Epub 2010 Dec 2. PMID: 21129791; PMCID: PMC3053014.

7. Molyneaux Bradley J, Goff Loyal A, Brettler Andrea C, Chen Hsu-Hsin, Brown Juliana R, Hrvatin Siniša, Rinn John L, Arlotta Paola: **DeCoN: genome-wide analysis of in vivo transcriptional dynamics during pyramidal neuron fate selection in neocortex**. *Neuron* 2015, **85**:275–288.

8. Heavner Whitney E, Shaoyi Ji, Notwell James H, Dyer Ethan S, Tseng Alex M, Birgmeier Johannes, Yoo Boyoung, Bejerano Gill, McConnell Susan K: **Transcription factor expression defines subclasses of developing projection neurons highly similar to single-cell RNA-seq subtypes**. In *Proceedings of the national academy of sciences*, **117**; 2020:25074–25084. 40.

9. Lodato Simona, Arlotta Paola: **Generating neuronal diversity in the mammalian cerebral cortex**. *Annu Rev Cell Dev Biol* 2015, **31**:699–720.

10. Loo Lipin, Simon Jeremy M, Xing Lei, McCoy Eric S, Niehaus Jesse K, Guo Jiami, Anton ES, Zylka Mark J: **Single-cell transcriptomic analysis of mouse neocortical development**. *Nat Commun* 2019, **10**:134.

11. Di Bella DJ, Habibi E, Stickels RR, Scalia G, Brown J, Yadollahpour P, Yang SM, Abbate C, Biancalani T, Macosko EZ, Chen F, Regev A, Arlotta P: **Molecular logic of cellular diversification in the mouse cerebral cortex**. *Nature* 2021 Jul, **595**: 554–559, https://doi.org/10.1038/s41586-021-03670-5. Epub 2021 Jun 23. Erratum in: Nature. 2021 Aug;596(7873):E11. PMID: 34163074; PMCID: PMC9006333.

12. Magrinelli Elia, Baumann Natalia, Wagener Robin Jan, Glangetas Christelle, Bellone Camilla, Jabaudon Denis, Klingler Esther: **Heterogeneous fates of simultaneously-born neurons in the cortical ventricular zone**. *Sci Rep* 2022, **12**:1–11.

13. Tasic B, Yao Z, Graybuck LT, *et al.*: **Shared and distinct transcriptomic cell types across neocortical areas**. *Nature* 2018, **563**:72–78, https://doi.org/10.1038/s41586-018-0654-5.

14. Economo MN, Clack NG, Lavis LD, Gerfen CR, Svoboda K, Myers EW, Chandrashekar J: **A platform for brain-wide imaging and reconstruction of individual neurons**. *Elife* 2016 Jan 20, **5**, e10566, https://doi.org/10.7554/eLife.10566. PMID: 26796534; PMCID: PMC4739768.

15. • Winnubst J, Bas E, Ferreira TA, Wu Z, Economo MN, Edson P, Arthur BJ, Bruns C, Rokicki K, Schauder D, Olbris DJ, Murphy SD, Ackerman DG, Arshadi C, Baldwin P, Blake R, Elsayed A, Hasan M, Ramirez D, Dos Santos B, Weldon M, Zafar A, Dudman JT, Gerfen CR, Hantman AW, Korff W, Sternson SM, Spruston N, Svoboda K, Chandrashekar J: **Reconstruction of 1,000 projection neurons reveals new cell types and organization of long-range connectivity in the mouse brain**. *Cell* 2019 Sep 19, **179**:268–281.e13, https://doi.org/10.1016/j.cell.2019.07.042. Epub 2019 Sep 5. PMID: 31495573; PMCID: PMC6754285.
This study established a robust pipeline for high-resolution imaging of optically cleared whole mouse brains, injected previously with viral tracers to reconstruct the axonal trajectories of 1200 long-range projection neurons in their entirety, available for easy three-dimensional visualization in an online database. Before this study, only a few studies had traced projection neurons of different subtypes in their entirety. The experimental strategy used by Winnubst et al., 2019 has enabled the identification of previously unknown projection neuron subtypes.

16. Morita K, Im S, Kawaguchi Y: **Differential striatal axonal arborizations of the intratelencephalic and pyramidal-tract neurons: analysis of the data in the MouseLight database**. *Front Neural Circ* 2019 Nov 15, **13**:71, https://doi.org/10.3389/fncir.2019.00071. PMID: 31803027; PMCID: PMC6872499.

17. • Mitchell BD, Macklis JD: **Large-scale maintenance of dual projections by callosal and frontal cortical projection neurons in adult mice**. *J Comp Neurol* 2005 Jan 31, **482**:17–32, https://doi.org/10.1002/cne.20428. PMID: 15612019.
Using tracing experiments, this study demonstrated that a large proportion of projection neurons maintain simultaneous callosal and frontal projections into adulthood. This is one of the early studies that demonstrate that callosal projection neurons have anatomically diverse targets, and such dual projections may function to mediate easy integration of motor information with higher-order association areas.

18. Otsuka T, Kawaguchi Y: **Cell diversity and connection specificity between callosal projection neurons in the frontal cortex**. *J Neurosci* 2011 Mar 9, **31**:3862–3870, https://doi.org/

10.1523/JNEUROSCI.5795-10.2011. PMID: 21389241; PMCID: PMC6622807.

19. O'Leary, Dennis DM, Chou Shen-Ju, Sahara Setsuko: **Area patterning of the mammalian cortex**. *Neuron* 2007, **56**: 252–269.

20. Cadwell Cathryn R, Bhaduri Aparna, Mostajo-Radji Mohammed A, Keefe Matthew G, Nowakowski Tomasz J: **Development and arealization of the cerebral cortex**. *Neuron* 2019, **103**: 980–1004.

21. Ypsilanti Athéna R, Pattabiraman Kartik, Catta-Preta Rinaldo, Golonzhka Olga, Lindtner Susan, Tang Ke, Ian R, Jones, *et al.*: **Transcriptional network orchestrating regional patterning of cortical progenitors**. In *Proceedings of the national academy of sciences*, **118**; 2021, e2024795118. 51.

22. Molyneaux Bradley J, Arlotta Paola, Menezes Joao RL, Macklis Jeffrey D: **Neuronal subtype specification in the cerebral cortex**. *Nat Rev Neurosci* 2007, **8**:427–437.

23. Greig Luciano Custo, Woodworth Mollie B, Galazo Maria J, Padmanabhan Hari, Macklis Jeffrey D: **Molecular logic of neocortical projection neuron specification, development and diversity**. *Nat Rev Neurosci* 2013, **14**:755–769.

24. Arlotta Paola, Molyneaux Bradley J, Chen Jinhui, Inoue Jun, Kominami Ryo, Macklis Jeffrey D: **Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo**. *Neuron* 2005, **45**:207–221.

25. Kumamoto Takuma, Hanashima Carina: **Neuronal subtype specification in establishing mammalian neocortical circuits**. *Neurosci Res* 2014, **86**:37–49.

26. Lodato Simona, Ashwin S, Shetty, Arlotta Paola: **Cerebral cortex assembly: generating and reprogramming projection neuron diversity**. *Trends Neurosci* 2015, **38**:117–125.

27. Paolino Annalisa, Fenlon Laura R, Rodrigo Suárez, Richards Linda J: **Transcriptional control of long-range cortical projections**. *Curr Opin Neurobiol* 2018, **53**:57–65.

28. Matho Katherine S, Huilgol Dhananjay, Galbavy William, He Miao, Kim Gukhan, Xu An, Lu Jiangteng, *et al.*: **Genetic dissection of the glutamatergic neuron system in cerebral cortex**. *Nature* 2021, **598**:182–187.

29. Nieto Marta, Monuki Edwin S, Tang Hua, Jaime Imitola, Haubst Nicole, Khoury Samia J, Cunningham Jim, Gotz Magdalena, Christopher A, Walsh: **Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II–IV of the cerebral cortex**. *J Comp Neurol* 2004, **479**:168–180.

30. Alcamo Elizabeth A, Chirivella Laura, Dautzenberg Marcel, Dobreva Gergana, Fariñas Isabel, Grosschedl Rudolf, Susan K, McConnell: **Satb2 regulates callosal projection neuron identity in the developing cerebral cortex**. *Neuron* 2008, **57**: 364–377.

31. Britanova Olga, de Juan Romero Camino, Cheung Amanda, Kwan Kenneth Y, Schwark Manuela, Gyorgy Andrea, Vogel Tanja, *et al.*: **Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex**. *Neuron* 2008, **57**:378–392.

32. Cubelos Beatriz, Sebastián-Serrano Alvaro, Beccari Leonardo, Calcagnotto Maria Elisa, Cisneros Elsa, Kim Seonhee, Dopazo Ana, *et al.*: **Cux1 and Cux2 regulate dendritic branching, spine morphology, and synapses of the upper layer neurons of the cortex**. *Neuron* 2010, **66**:523–535.

33. Leone Dino P, Heavner Whitney E, Ferenczi Emily A, Dobreva Gergana, Huguenard John R, Grosschedl Rudolf, McConnell Susan K: **Satb2 regulates the differentiation of both callosal and subcerebral projection neurons in the developing cerebral cortex**. *Cerebr Cortex* 2015, **25**:3406–3419.

34. Rodríguez-Tornos FM, Briz CG, Weiss LA, Sebastián-Serrano A, Ares S, Navarrete M, Frangeul L, Galazo M, Jabaudon D, Esteban JA, Nieto M: **Cux1 enables interhemispheric connections of layer II/III neurons by regulating Kv1-dependent firing**. *Neuron* 2016 Feb 3, **89**:494–506, https://doi.org/10.1016/j.neuron.2015.12.020. Epub 2016 Jan 21. PMID: 26804994.

35. Paolino Annalisa, Fenlon Laura R, Peter Kozulin, Haines Elizabeth, Lim Jonathan WC, Richards Linda J, Rodrigo Suárez: **Differential timing of a conserved transcriptional network underlies divergent cortical projection routes across mammalian brain evolution**. In *Proceedings of the national academy of sciences*, **117**; 2020:10554–10564. 19.

36. Levitt Pat, Barbe Mary F, Eagleson Kathie L: **Patterning and specification of the cerebral cortex**. *Annu Rev Neurosci* 1997, **20**:1–24.

37. Nakagawa Yasushi, Johnson Jane E, O'Leary Dennis DM: **Graded and areal expression patterns of regulatory genes and cadherins in embryonic neocortex independent of thalamocortical input**. *J Neurosci* 1999, **19**:10877–10885.

38. Grove Elizabeth A, Fukuchi-Shimogori Tomomi: **Generating the cerebral cortical area map**. *Annu Rev Neurosci* 2003, **26**: 355–380.

39. Gao Peng, Sultan Khadeejah T, Zhang Xin-Jun, Shi Song-Hai: **Lineage-dependent circuit assembly in the neocortex**. *Development* 2013, **140**:2645–2655.

40. O'Leary DD, Nakagawa Y: **Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex**. *Curr Opin Neurobiol* 2002 Feb, **12**:14–25, https://doi.org/10.1016/s0959-4388(02)00285-4. PMID: 11861160.

41. Simi Alessandro, Studer Michèle: **Developmental genetic programs and activity-dependent mechanisms instruct neocortical area mapping**. *Curr Opin Neurobiol* 2018, **53**:96–102.

42. Reyes De León, Noelia S, Bragg-Gonzalo Lorena, Nieto Marta: **Development and plasticity of the corpus callosum**. *Development* 2020, **147**, dev189738.

43. Bragg-Gonzalo Lorena, Reyes NS De León, Nieto Marta: **Genetic and activity dependent-mechanisms wiring the cortex: two sides of the same coin**. In *Seminars in cell & developmental biology*, **118**. Academic Press; 2021:24–34.

44. • Poulopoulos A, Murphy AJ, Ozkan A, Davis P, Hatch J, Kirchner R, Macklis JD: **Subcellular transcriptomes and proteomes of developing axon projections in the cerebral cortex**. *Nature* 2019 Jan, **565**:356–360, https://doi.org/10.1038/s41586-018-0847-y. Epub 2019 Jan 9. PMID: 30626971; PMCID: PMC6484835.

This study employed a novel subcellular RNA-proteome mapping approach to discover the interaction of the cellular translation machinery with mTOR signaling components at the developing callosal projection neuron growth cone, which is critical for facilitating axonal extension.

45. Favuzzi E, Rico B: **Molecular diversity underlying cortical excitatory and inhibitory synapse development**. *Curr Opin Neurobiol* 2018 Dec, **53**:8–15, https://doi.org/10.1016/j.conb.2018.03.011. Epub 2018 Apr 25. PMID: 29704699.

46. • Huang L, Kebschull JM, Fürth D, Musall S, Kaufman MT, Churchland AK, Zador AM: **BRICseq bridges brain-wide interregional connectivity to neural activity and gene expression in single animals**. *Cell* 2020 Jul 9, **182**:177–188.e27, https://doi.org/10.1016/j.cell.2020.05.029. Epub 2020 Jul 2. Erratum in: Cell. 2020 Dec 23;183:2040. PMID: 32619423; PMCID: PMC7771207.

The BRICseq pipeline is a low-cost DNA barcoding and sequencing technique that has been used to uncover correlations between spatial expression patterns of genes, connectivity, and activity patterns.

47. • Lewis Jr TL, Kwon SK, Lee A, Shaw R, Polleux F: **MFF-dependent mitochondrial fission regulates presynaptic release and axon branching by limiting axonal mitochondria size**. *Nat Commun* 2018 Nov 27, **9**:5008, https://doi.org/10.1038/s41467-018-07416-2. PMID: 30479337; PMCID: PMC6258764.

This study uncovered a novel fission-dependent regulatory mechanism for the maintenance of shorter mitochondria in the axonal compartments of callosal projection neurons that is directly linked to neurotransmitter release and axonal branching.

48. Nakagawa-Tamagawa N, Kirino E, Sugao K, Nagata H, Tagawa Y: **Involvement of calcium-dependent pathway and β subunit-interaction in neuronal migration and callosal projection deficits caused by the Cav1.2 I1166T mutation in developing mouse neocortex**. *Front Neurosci* 2021 Dec 8, **15**:

747951, https://doi.org/10.3389/fnins.2021.747951. PMID: 34955712; PMCID: PMC8692569.

49. Yamamoto N, López-Bendito G: **Shaping brain connections through spontaneous neural activity**. *Eur J Neurosci* 2012 May, **35**:1595–1604, https://doi.org/10.1111/j.1460-9568.2012.08101.x. PMID: 22607005.

50. Leighton AH, Lohmann C: **The wiring of developing sensory circuits-from patterned spontaneous activity to synaptic plasticity mechanisms**. *Front Neural Circ* 2016 Sep 5, **10**:71, https://doi.org/10.3389/fncir.2016.00071. PMID: 27656131; PMCID: PMC5011135.

51. Luhmann HJ, Sinning A, Yang JW, Reyes-Puerta V, Stüttgen MC, Kirischuk S, Kilb W: **Spontaneous neuronal activity in developing neocortical networks: from single cells to large-scale interactions**. *Front Neural Circ* 2016 May 24, **10**:40, https://doi.org/10.3389/fncir.2016.00040. PMID: 27252626; PMCID: PMC4877528.

52. Mizuno H, Hirano T, Tagawa Y: **Evidence for activity-dependent cortical wiring: formation of interhemispheric connections in neonatal mouse visual cortex requires projection neuron activity**. *J Neurosci* 2007 Jun 20, **27**:6760–6770, https://doi.org/10.1523/JNEUROSCI.1215-07.2007. PMID: 17581963; PMCID: PMC6672694.

53. Wang CL, Zhang L, Zhou Y, Zhou J, Yang XJ, Duan SM, Xiong ZQ, Ding YQ: **Activity-dependent development of callosal projections in the somatosensory cortex**. *J Neurosci* 2007 Oct 17, **27**:11334–11342, https://doi.org/10.1523/JNEUROSCI.3380-07.2007. PMID: 17942728; PMCID: PMC6673014.

54. Tezuka Y, Hagihara KM, Ohki K, Hirano T, Tagawa Y: **Developmental stage-specific spontaneous activity contributes to callosal axon projections**. *Elife* 2022 Aug 24, **11**, e72435, https://doi.org/10.7554/eLife.72435. PMID: 36001081; PMCID: PMC9402231.

55. Mizuno H, Hirano T, Tagawa Y: **Pre-synaptic and post-synaptic neuronal activity supports the axon development of callosal projection neurons during different post-natal periods in the mouse cerebral cortex**. *Eur J Neurosci* 2010 Feb, **31**:410–424, https://doi.org/10.1111/j.1460-9568.2009.07070.x. Epub 2010 Jan 25. PMID: 20105242.

56. Ackman JB, Burbridge TJ, Crair MC: **Retinal waves coordinate patterned activity throughout the developing visual system**. *Nature* 2012 Oct 11, **490**:219–225, https://doi.org/10.1038/nature11529. PMID: 23060192; PMCID: PMC3962269.

57. Mizuno H, Ikezoe K, Nakazawa S, Sato T, Kitamura K, Iwasato T: **Patchwork-type spontaneous activity in neonatal barrel cortex layer 4 transmitted via thalamocortical projections**. *Cell Rep* 2018 Jan 2, **22**:123–135, https://doi.org/10.1016/j.celrep.2017.12.012. PMID: 29298415.

58. Suárez R, Bluett T, McCullough MH, Avitan L, Black DA, Paolino A, Fenlon LR, Goodhill GJ, Richards LJ: **Cortical activity emerges in region-specific patterns during early brain development**. *Proc Natl Acad Sci U S A* 2023 May 30, **120**, e2208654120, https://doi.org/10.1073/pnas.2208654120. Epub 2023 May 22. PMID: 37216522; PMCID: PMC10235933.

59. Ackman JB, Crair MC: **Role of emergent neural activity in visual map development**. *Curr Opin Neurobiol* 2014 Feb, **24**:166–175, https://doi.org/10.1016/j.conb.2013.11.011. Epub 2013 Dec 22. PMID: 24492092; PMCID: PMC3957181.

60. Kirkby LA, Sack GS, Firl A, Feller MB: **A role for correlated spontaneous activity in the assembly of neural circuits**. *Neuron* 2013 Dec 4, **80**:1129–1144, https://doi.org/10.1016/j.neuron.2013.10.030. Erratum in: Neuron. 2014 Jan 8;81:218. PMID: 24314725; PMCID: PMC4560201.

61. • Wang Bor-Shuen, Sarria Maria Sol Bernardez, Xu An, He Miao, Nazia M, Alam Glen T Prusky, Crair Michael C, Josh Huang Z: **Retinal and callosal activity-dependent chandelier cell elimination shapes binocularity in primary visual cortex**. *Neuron* 2021, **109**:502–515.

This study demonstrated a novel role for retinal and callosal activity-driven regulation of chandelier cell distribution that is critical for developmental priming of the binocular circuit prior to the onset of experience-dependent visual input.

62. McVea DA, Murphy TH, Mohajerani MH: **Large scale cortical functional networks associated with slow-wave and spindle-burst-related spontaneous activity**. *Front Neural Circ* 2016 Dec 21, **10**:103, https://doi.org/10.3389/fncir.2016.00103. PMID: 28066190; PMCID: PMC5174115.

63. Tan AY: **Spatial diversity of spontaneous activity in the cortex**. *Front Neural Circ* 2015 Sep 24, **9**:48, https://doi.org/10.3389/fncir.2015.00048. PMID: 26441547; PMCID: PMC4585302.

64. Mohajerani MH, McVea DA, Fingas M, Murphy TH: **Mirrored bilateral slow-wave cortical activity within local circuits revealed by fast bihemispheric voltage-sensitive dye imaging in anesthetized and awake mice**. *J Neurosci* 2010 Mar 10, **30**:3745–3751, https://doi.org/10.1523/JNEUROSCI.6437-09.2010. PMID: 20220008; PMCID: PMC6632233.

65. • Chen Y, Sobczak F, Pais-Roldán P, Schwarz C, Koretsky AP, Yu X: **Mapping the brain-wide network effects by optogenetic activation of the corpus callosum**. *Cerebr Cortex* 2020 Oct 1, **30**:5885–5898, https://doi.org/10.1093/cercor/bhaa164. PMID: 32556241; PMCID: PMC7673479.

This study used fMRI imaging coupled with optogenetic activation of the corpus callosum to uncover activation of brain-wide networks, extending to the motor cortex and thalamus, underscoring the functional importance of callosal connectivity to diverse regions.

66. Olavarria J, Malach R, Van Sluyters RC: **Development of visual callosal connections in neonatally enucleated rats**. *J Comp Neurol* 1987 Jun 15, **260**:321–348, https://doi.org/10.1002/cne.902600302. PMID: 3597836.

67. Koralek KA, Killackey HP: **Callosal projections in rat somatosensory cortex are altered by early removal of afferent input**. *Proc Natl Acad Sci U S A* 1990 Feb, **87**:1396–1400, https://doi.org/10.1073/pnas.87.4.1396. PMID: 2304906; PMCID: PMC53482.

68. Huang Y, Song NN, Lan W, Zhang Q, Zhang L, Zhang L, Hu L, Chen JY, Zhao CJ, Li L, Xu L, Ding YQ: **Sensory input is required for callosal axon targeting in the somatosensory cortex**. *Mol Brain* 2013 Dec 5, **6**:53, https://doi.org/10.1186/1756-6606-6-53. PMID: 24305168; PMCID: PMC4234978.

69. • Suárez R, Fenlon LR, Marek R, Avitan L, Sah P, Goodhill GJ, Richards LJ: **Balanced interhemispheric cortical activity is required for correct targeting of the corpus callosum**. *Neuron* 2014 Jun 18, **82**:1289–1298, https://doi.org/10.1016/j.neuron.2014.04.040. PMID: 24945772.

This study demonstrated that spatially balanced activity between hemispheres, both at the peripheral and cortical levels, is critical for correct callosal axon targeting, thus uncovering novel principles that instruct axonal connectivity.

70. De León Reyes NS, Mederos S, Varela I, Weiss LA, Perea G, Galazo MJ, Nieto M: **Transient callosal projections of L4 neurons are eliminated for the acquisition of local connectivity**. *Nat Commun* 2019 Oct 7, **10**:4549, https://doi.org/10.1038/s41467-019-12495-w. PMID: 31591398; PMCID: PMC6779895.

71. Mire E, Mezzera C, Leyva-Díaz E, Paternain AV, Squarzoni P, Bluy L, Castillo-Paterna M, López MJ, Peregrín S, Tessier-Lavigne M, Garel S, Galcerán J, Lerma J, López-Bendito G: **Spontaneous activity regulates Robo1 transcription to mediate a switch in thalamocortical axon growth**. *Nat Neurosci* 2012 Jul 8, **15**:1134–1143, https://doi.org/10.1038/nn.3160. PMID: 22772332.

72. Castillo-Paterna M, Moreno-Juan V, Filipchuk A, Rodríguez-Malmierca L, Susín R, López-Bendito G: **DCC functions as an accelerator of thalamocortical axonal growth downstream of spontaneous thalamic activity**. *EMBO Rep* 2015 Jul, **16**:851–862, https://doi.org/10.15252/embr.201439882. Epub 2015 May 6. PMID: 25947198; PMCID: PMC4515124.

73. Moreno-Juan V, Filipchuk A, Antón-Bolaños N, Mezzera C, Gezelius H, Andrés B, Rodríguez-Malmierca L, Susín R, Schaad O, Iwasato T, Schüle R, Rutlin M, Nelson S, Ducret S, Valdeolmillos M, Rijli FM, López-Bendito G: **Prenatal thalamic waves regulate cortical area size prior to sensory processing**. *Nat Commun* 2017 Feb 3, **8**:14172, https://doi.org/10.1038/ncomms14172. PMID: 28155854; PMCID: PMC5296753.

74. Antón-Bolaños N, Sempere-Ferràndez A, Guillamón-Vivancos T, Martini FJ, Pérez-Saiz L, Gezelius H, Filipchuk A, Valdeolmillos M, López-Bendito G: **Prenatal activity from thalamic neurons governs the emergence of functional cortical maps in mice**. *Science* 2019 Jun 7, **364**:987–990, https://doi.org/10.1126/science.aav7617. Epub 2019 May 2. PMID: 31048552; PMCID: PMC7611000.

75. Rock C, Zurita H, Lebby S, Wilson CJ, Apicella AJ: **Cortical circuits of callosal GABAergic neurons**. *Cerebr Cortex* 2018 Apr 1, **28**:1154–1167, https://doi.org/10.1093/cercor/bhx025. PMID: 28174907.

76. Zurita H, Feyen PLC, Apicella AJ: **Layer 5 callosal parvalbumin-expressing neurons: a distinct functional group of GABAergic neurons**. *Front Cell Neurosci* 2018 Mar 6, **12**:53, https://doi.org/10.3389/fncel.2018.00053. PMID: 29559891; PMCID: PMC5845545.

77. Edwards TJ, Fenlon LR, Dean RJ, Bunt J, , IRC5 Consortium, Sherr EH, Richards LJ: **Altered structural connectivity networks in a mouse model of complete and partial dysgenesis of the corpus callosum**. *Neuroimage* 2020 Aug 15, **217**:116868, https://doi.org/10.1016/j.neuroimage.2020.116868. Epub 2020 Apr 29. PMID: 32360691.