OXFORD

Cerebral Cortex, 2023, **33**, 4752–4760
https://doi.org/10.1093/cercor/bhac377
Advance access publication date 30 September 2022
Original Article

The relevance of heterotopic callosal fibers to interhemispheric connectivity of the mammalian brain

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The corpus callosum (CC) is the largest white matter structure and the primary pathway for interhemispheric brain communication. Investigating callosal connectivity is crucial to unraveling the brain's anatomical and functional organization in health and disease. Classical anatomical studies have characterized the bulk of callosal axonal fibers as connecting primarily homotopic cortical areas. Whenever detected, heterotopic callosal fibers were ascribed to altered sprouting and pruning mechanisms in neurodevelopmental diseases such as CC dysgenesis (CCD). We hypothesized that these heterotopic connections had been grossly underestimated due to their complex nature and methodological limitations. We used the Allen Mouse Brain Connectivity Atlas and high-resolution diffusion-weighted imaging to identify and quantify homotopic and heterotopic callosal connections in mice, marmosets, and humans. In all 3 species, we show that ~75% of interhemispheric callosal connections are heterotopic and comprise the central core of the CC, whereas the homotopic fibers lay along its periphery. We also demonstrate that heterotopic connections have an essential role in determining the global properties of brain networks. These findings reshape our view of the corpus callosum's role as the primary hub for interhemispheric brain communication, directly impacting multiple neuroscience fields investigating cortical connectivity, neurodevelopment, and neurodevelopmental disorders.

Key words: axonal tracing; corpus callosum; diffusion-weighted imaging; structural brain connectivity; tractography.

The corpus callosum (CC) is the primary white matter structure of the brain and the largest commissure connecting the 2 brain hemispheres (Zhou et al. 2013; Fenlon and Richards 2015; Shen et al. 2015; Roland et al. 2017; Suarez et al. 2018; Mancuso et al. 2019; Loomba et al. 2021). Formed by axons from cortical projection neurons (Lefebvre et al. 2015) connecting interhemispheric regions of the neocortex and the paleocortex (Ebner and Myers 1965), the CC is present in all placental mammals (Suarez et al. 2014). The CC has been systematically studied for over 100 years (Probst 1901), and its connectivity has been probed multiple times (Fame et al. 2011; Zhou et al. 2013; Lefebvre et al. 2015). The typical investigative approach relies on injecting an axonal tracer into a specific brain region to reveal its interhemispheric connectivity by classical histological (axon degeneration), immunohistochemical (horseradish peroxidase), and autoradiographic (tritiated amino acid markers) staining (Ebner and Myers 1965; Wahlsten 1974). Significant technological advances in viral tracing methods now allow the precise detection of fine and sparse connectivity, as the virus infects each neuron individually and therefore conserves signal intensity to each axon, differently from traditional tracers that have to be uptaken and transported by the cell, which can lead to a bias towards tracking strong connections.

Historically, the CC has been described as a primarily homotopic structure (Raybaud 2010; Zhou et al. 2013; Fenlon and Richards 2015; Shen et al. 2015; Roland et al. 2017; Suarez et al. 2018; Mancuso et al. 2019; Loomba et al. 2021). Homotopic connections are dense, following predictable paths to well-defined targets in the contralateral cortex that usually overshadow the more sparse heterotopic targets spread out throughout the contralateral cortex. Functionally, homotopic interhemispheric cortical regions work in coordination; thus, it makes sense to expect these homotopic projections to be predominant (Raybaud 2010; Mancuso et al. 2019; Innocenti et al. 2022). However, although most investigators considered heterotopic connections as scarce or atypical, several groups have characterized heterotopic callosal connections (Di Virgilio et al. 1999; Houzel et al. 2002; Marconi et al. 2003; De Benedictis et al. 2016; Chovsepian et al. 2017; Lanz et al. 2017; Swanson et al. 2017; Velona et al. 2019). Heterotopic connections are particularly prominent in human patients and animal models with corpus callosum dysgenesis (CCD), CC malformations that cause significant rewiring of the brain (Probst 1901; Paul et al. 2007; Tovar-Moll et al. 2007; Edwards et al. 2020 ; Szczupak et al. 2020). One striking example is the sigmoid bundle, which connects the frontal lobe with the contralateral parieto-occipital lobe. The sigmoid pathway was first described in CCD patients (Paul et al. 2007; Tovar-Moll et al. 2007). Recently, we showed (Szczupak et al. 2020) that a sigmoid pathway consisting of heterotopic fibers connect these same brain regions in wild-type C57BL6/J and Balb/c mice with spontaneous CC anomalies (Szczupak et al. 2020). These findings were confirmed in other mouse models of CC malformations (Edwards et al. 2020; Szczupak et al. 2020).

Recently, Swanson and colleagues (Swanson et al. 2017) extracted a database of over 5,000 rat brain cortical projections

Received: July 4, 2022. **Revised:** August 29, 2022. **Accepted:** August 30, 2022

from the literature and used network analysis tools to investigate associative and commissural connections. Their analysis revealed an order of magnitude more heterotopic than homotopic commissural projections. Here, we aimed to add evidence to the robust existence of heterotopic callosal connections. Reasoning that a richer diversity of anatomical connections across the cerebral hemispheres is required to enable the global integration of the multiple functions of the cortex (Swanson et al. 2017), we hypothesized here that the heterotopic callosal connections might have been vastly underestimated. To test our hypothesis, we took advantage of the advanced methods for axonal tracing provided in the Allen Institute for Brain Science Mouse Brain Connectivity Atlas (Oh et al. 2014), combined with diffusion-weighted imaging (DWI) data, to assess and quantify the proportions of homotopic and heterotopic cortical connections, their specific topography, and the direct impact of heterotopic connections in brain networks in mice, marmosets, and humans. We conclude that heterotopic connections are not only abundant but predominant in the 3 species. These results require reevaluating the organization and function of commissural connectivity in mammals.

Materials and methods

Histology-based mouse brain heterotopicity map

To establish a "gold-standard" quantitative map of the number of interhemispheric connections of the mouse brain, we resorted to the Allen Institute for Brain Science Mouse Brain Connectivity Matrix (Oh et al. 2014), which compiles >1,000 experiments in which different cortical areas were injected with AAV9-GFP (adeno-associated virus 9 green fluorescent protein conjugated) as a unitary anterograde neuronal tracer and measured the signal intensity of the viral tracer in each ROI. We grouped the injection sites into the mouse brain regions of the Queensland Brain Institute (QBI) MRI atlas (Ullmann et al. 2013) to allow clear visualization of the resulting connectivity maps in the magnetic resonance imaging, MRI space. We classified the resulting target projections as intrahemispheric or interhemispheric for each region. We then computed the number of interhemispheric projections terminating in the contralateral homologous area to the injection site (homotopic connections) versus the number of interhemispheric projections (quantified by the fluorescence intensity of the fibers) terminating elsewhere in the contralateral hemisphere (heterotopic projections; Supplementary Fig. 1, see online supplementary material for a color version of this figure). Finally, we calculated a heterotopicity index, dividing the number of heterotopic connections by the number of interhemispheric projections, and mapped the heterotopicity index in the same MRI space as the QBI mouse brain atlas.

DWI MRI acquisitions

Mice

The DWI data of 17 C57BL6/J mice (10 males and 7 females) were obtained at 16.4T (Liu et al. 2016). Briefly, 100-μm isotropic high-resolution images were acquired ex vivo from brains perfused with paraformaldehyde (PFA) 4% and treated with gadolinium. The DWI data used a standard spin-echo sequence with the following parameters: time repetition (TR) = 400 ms, time echo (TE) = 20 ms, $\delta/\Delta = 2.5/12$ ms, field of view (FOV) = $18.99 \times 11.16 \times 8$ mm, matrix = $190 \times 112 \times 80$, bandwidth = 50 kHz, 30 diffusion-encoding directions with b-value = 5,000 s/mm^2, and 2 images acquired without diffusion-weighting ($b = 0$ s/mm^2).

Marmosets

The DWI data of 3 marmosets (2 females and 1 male) were obtained at 7T (Liu et al. 2018). Briefly, 150-μm isotropic high-resolution images were acquired ex vivo from brains perfused with PFA 4% and treated with gadolinium. The DWI data used standard echo-planar imaging (EPI) with the following parameters: TR = 450 ms, TE = 34 ms, FOV = $38.4 \times 28.8 \times 28.8$ mm, matrix size = $256 \times 192 \times 192$, 396 diffusion-encoding directions with 3 shells of 126 directions each, b-values = 2,400, 4,800, and 7,200 s/mm^2), and 6 b0, number of averages = 1 for the first 2 shells, and 2 for the latter.

Humans

The DWI data of 51 human subjects (21 males and 20 females) were obtained at 3T from the Human connectome project (HCP) 100 unrelated subjects database (Van Essen et al. 2013). The DWI data used standard EPI with the HCP standard protocol parameters: 200 diffusion-encoding directions with 2 shells, b-values of 1,500 and 3,000 s/mm^2, 1.5-mm isotropic resolution acquired in 2 different phase encoding directions to minimize drop-out signal and EPI distortion.

Structural connectomes

Callosal tractogram

DWI images were corrected for eddy currents and geometric distortions and denoised. Furthermore, we estimated a response function using the Dholander algorithm and calculated the fiber orientation distribution (FOD) in MRtrix software (Tournier et al. 2012), calculated the tractogram by generating tracts targeting callosal connections, seeding from the whole brain with an inclusion ROI at the CC with the following parameters: cutoff = 0.06 (stops the tractography when FOD value is lower than 0.06), select 1M (selecting a million streamlines to assure enough coverage of the tractogram). Then, we registered the mouse, marmoset, and human DWI data to the QBI (Ullmann et al. 2013), Marmoset Brain Mapping (Liu et al. 2018), and AAL 116 ROIs (Tzourio-Mazoyer et al. 2002) brain atlases, respectively, and used the command tck2connectome to calculate the connectome adjacency matrix, as previously described (Szczupak et al. 2021).

Whole-brain tractogram

For the whole-brain networks, we have used the whole-brain tractogram instead of the callosal tractogram ("callosogram") with 10M tracts, following the same tractography parameters seeding streamlines randomly from the brain mask.

Heterotopicity index maps

For each of the 3 species, we calculated the heterotopicity index for every cortical region defined as the number of heterotopic interhemispheric (callosal) connections (streamlines) divided by the total number of interhemispheric connections of the same cortical region and generated a 3D cortical heterotopicity index map rendered in Mango.

Heterotopicity callosal maps

To map the heterotopic pathways through the CC, we generated the tractography of every cortical region to all other contralateral cortical regions. We generated a tract density image, cropped it to the CC at the midline, normalized it, and performed a voxel-to-voxel operation to calculate the heterotopicity ratio for each callosal voxel. We then coregistered the callosal maps of every

subject and calculated the population-averaged map for each of the 3 species.

Network-Based-Statistics

We used the GRETNA (Wang et al. 2015) automated software to calculate the Network-Based-Statistics (NBS) of humans, marmosets, and mice based on diffusion-weighted structural connectivity. We chose to evaluate the properties of hierarchy, small-worldliness, assortativity, and efficiency. These global network features provide a better understanding of how heterotopic connections influence the whole-brain network.

Efficiency is defined as the number of different paths connecting 2 nodes and relates to efficiency itself and the network's redundancy. Small-worldliness is how the network approaches a pure small world motif (many short-range connections and few long-range integrative connections) associated with high communication efficiency and information transfer reliability. On the other hand, hierarchy comprises classifying the individual nodes (ROI) according to each node's degree (number of connections). Finally, assortativity defines if these nodes communicate with nodes of a similar class, relating to the network's pattern and type of connectivity (Sporns et al. 2005; van den Heuvel and Sporns 2011).

Non-heterotopic network generation

We calculated the non-heterotopic network by subtracting the heterotopic connections from the whole-brain connectome. This way, we could compare the whole-brain connectome with and without the heterotopic connections. We evaluated the NBS with a parametric *t*-test using GraphPad 7.0 Software (GraphPad Inc.).

Results

The Allen Institute Mouse Brain Connectivity Atlas compiles the most significant number of cortical injections with the latest anterograde neuronal tracer currently available. To better understand the topography of the cortical interhemispheric connectivity, we analyzed the Allen Institute data to quantify the number of intrahemispheric versus interhemispheric connections in the mouse brain. We further classified the interhemispheric connections as homotopic and heterotopic. These data, summarized in Fig. 1a, show an injection of AAV-9 into the right frontal pole of the mouse cortex and its projecting axons to the rest of the brain (experiment #263242463). The map shows both intrahemispheric and interhemispheric tracer profiles. Both homotopic and heterotopic projections can be easily identified within the interhemispheric connections. Figure 1b illustrates interhemispheric DWI tracts from the same cortical region. Homotopic streamlines are shown in blue and heterotopic in red and orange (see Supplementary Figs. 2–5, see online supplementary material for a color version of these figure and Supplementary Videos 1 and 2 for more examples). These data validate the use of DWI tractography to map interhemispheric connectivity.

We then grouped the connectivity matrix data of the Allen Institute Mouse Brain Connectivity Atlas according to the cortical regions of the QBI MRI atlas. We identified interhemispheric axonal projections for each region and classified them as homotopic and heterotopic to create heterotopicity index maps (see Methods). Figure 1c shows the heterotopicity map of the mouse brain. Generally, primary cortical areas have a more even balance of homotopic and heterotopic connections, whereas integrative areas are largely heterotopic. Figure 1d and e show the complete quantification of cortical connectivity. We found that 81% of the connections were intrahemispheric, whereas 19% were interhemispheric. Among the interhemispheric connections, roughly 2/3 (11.7% of the total) are heterotopic, and only 1/3 (6.9%) are homotopic. These results challenge the conventional wisdom of the CC as a homotopic structure (Mancuso et al. 2019). Instead, the data suggest that the brain connectivity across the CC is primarily heterotopic.

To investigate whether the broad heterotopicity of the CC can be generalized across different mammalian species, we used DWI to map the interhemispheric connections in mice, marmosets, and humans. First, we generated heterotopicity maps for the 3 species (Fig. 2a–c). The results were consistent for all of them, revealing that cortical interhemispheric connections are mainly heterotopic and suggesting that heterotopicity is a general organizational principle of the CC that is conserved across species. For example, all species present a lower heterotopicity index on temporal regions and a higher heterotopicity index on posterior dorsal regions and medial frontal structures (Fig. 2a–c). Interestingly, the inferior temporal lobe of the human brain (Fig. 2c, blue and cyan colors) presented lower heterotopicity than the marmoset's (Fig. 2b, green). However, the overall heterotopicity of the marmoset brain (Fig. 2b, orange and red colors) is only slightly higher than that of the human brain (Fig. 2c, yellow and orange colors).

We also leveraged the high spatial resolution of our DWI methods to map heterotopicity indices within the CC (Fig. 2d–f). We observed that the heterotopic connections are centrally located along the anteroposterior axis of the CC, whereas homotopic connections are distributed peripherally along the callosal surface. Quantifying the interhemispheric connectivity data across all 3 species shows that mice, marmosets, and humans present a similar heterotopicity ratio of 72–78%, i.e. 3/4 of the interhemispheric connections are heterotopic (Fig. 2g–i).

To probe the influence of heterotopic connections on whole-brain network properties, we calculated the NBS for C57bl6/J mice, marmosets, and humans with (full network, FN) and without the heterotopic connections (non-heterotopic, NH) in a paired manner (Fig. 3). Removal of the heterotopic connections significantly altered the network properties of hierarchy, small-worldliness, assortativity, and efficiency in mice (Fig. 3a–d), marmosets (Fig. 3e–f), and humans (Fig. 3g–h). These results demonstrate that heterotopic connections are essential integrants of brain networks.

Discussion

Is the CC homotopic or heterotopic?

The CC is the primary white matter structure connecting both cerebral hemispheres across the midline. Although many anatomical studies (Olavarria et al. 1988; Houzel et al. 2002; Garcez et al. 2007; Donahoo and Richards 2009; Fame et al. 2011; Yuan et al. 2020) have investigated interhemispheric connectivity, most employed large amounts of axonal tracers to stain many neurons within the injection site. Consequently, high-density axonal projections become easily observable within the microscope field of view, whereas the background noise often overshadows sparse connections. Moreover, histology slices are usually thin, limiting the possibility of identifying heterotopic connections throughout the whole cortex, especially when careful examination of several adjacent slices is needed to detect only a few axons. Taken together, the low sensitivity to sparse heterotopic connections intermixed with the easily detected dense homotopic connections led to the erroneous assumption that the CC is primarily a homotopic pathway (Zhou et al. 2013; Fenlon and Richards 2015; Shen et al. 2015;

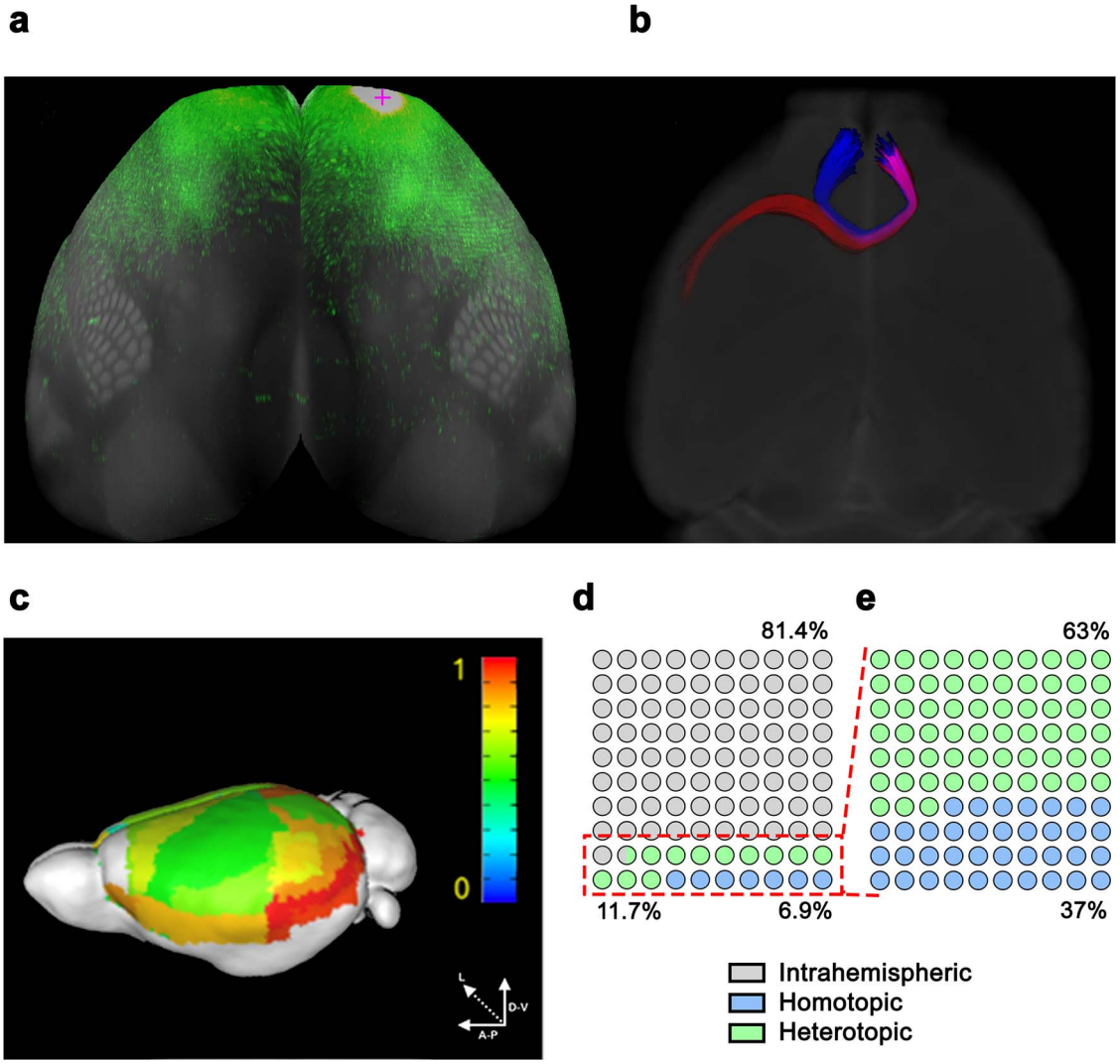

Fig. 1. Homotopic and heterotopic histological connections of the mouse CC. a) 3D dorsal surface projection of a mouse brain extracted from the Allen Institute Mouse Brain Connectivity Atlas showing the injection site of an intracellular neuronal anterograde AAV9 viral tracer into the right frontal cortex (+, experiment 263242463) and its axonal projections to both hemispheres. Interhemispheric axonal connections comprise both homotopic and heterotopic projections. b) DWI tractography of the right frontal cortex in a mouse brain reveals a robust heterotopic callosal fascicle: Two targets are shown in the contralateral (left) hemisphere, a homotopic (blue) and a heterotopic (red). c) The heterotopicity map of the mouse cortex was generated using the Allen Institute mouse brain connectivity atlas. The color bar represents the scale of heterotopicity, with cool colors showing homotopic areas and hot colors showing heterotopic areas. In general, integrative areas have higher heterotopicity indices than primary areas. d) Quantification of the total number of intra- and interhemispheric axonal connections of the mouse cortex: The vast majority (>80%) of the brain's cortical connections are intrahemispheric. e) Quantification of the interhemispheric cortical connectivity shows that the majority (63%) of the interhemispheric fibers are heterotopic.

Roland et al. 2017; Suarez et al. 2018; Mancuso et al. 2019; Loomba et al. 2021). Recently, the literature has started challenging this dogma showing substantial heterotopic callosal connectivity (Marconi et al. 2003; Swanson et al. 2017; Velona et al. 2019). Some authors identified rare heterotopic connections in rodent brains, but these connections were presumed to be minor participants of the CC (Houzel et al. 2002). Pioneering studies using Wallerian degeneration showed that heterotopic connections exist in surprisingly high numbers in the human brain (Di Virgilio et al. 1999). However, heterotopic connections were considered pathological, as they are much more numerous in developmental callosal malformations (Paul et al. 2007; Tovar-Moll et al. 2007; Tovar-Moll et al. 2014; Siffredi et al. 2019; Szczupak et al. 2021) and other midline abnormalities (Arrigoni et al. 2016) relative to normally developed brains.

Swanson et al. (2017) were the first to quantify the number of heterotopic connections. The authors collated a collection of published pathway tracing experiments that included over 5,000 axonal projections from 77 cortical regions in each hemisphere of the rat brain, of which >1,000 connections (20%) were interhemispheric. They reported that all 77 cortical regions in one hemisphere have a unique and strongly correlated set of association (intrahemispheric) and commissural (interhemispheric) input and output connections. Two-thirds of the regions send homotopic commissural connections, whereas the remaining third sends no known homotopic connections. Interestingly, the heterotopic connections outnumbered the homotopic connections by a factor of 10, with the cortical regions receiving homotopic commissural inputs having many more heterotopic inputs than regions not receiving a homotopic input (Swanson et al. 2017). However, even when outnumbered 10:1, homotopic connections make a much stronger contribution to the shortest paths linking the 2 brain hemispheres, accounting for 35.6% of the aggregate connection weight. Therefore,

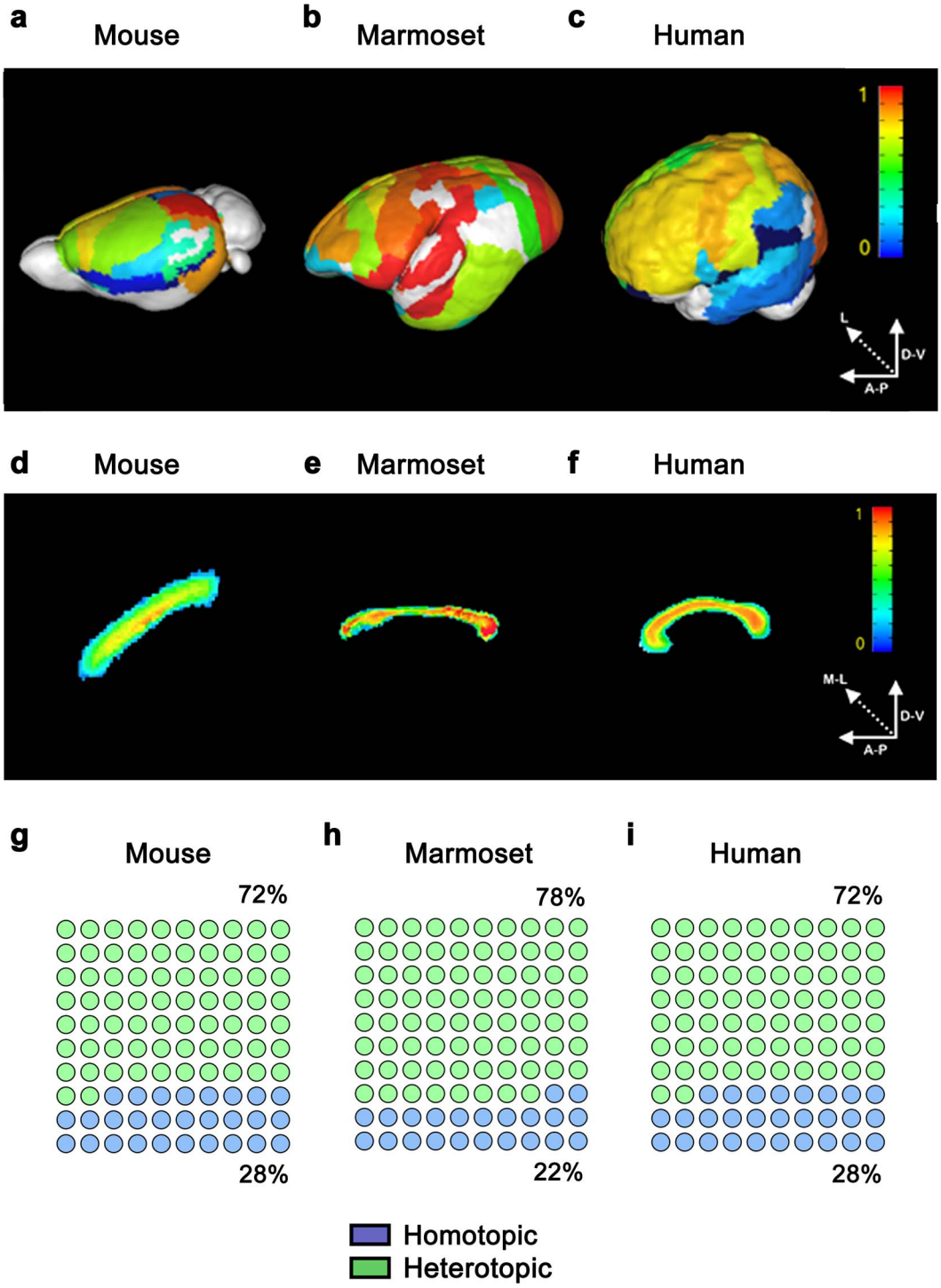

Fig. 2. DWI-based heterotopicity map in mice, marmosets, and humans. Population-averaged DWI-based cortical heterotopicity maps of C57BL6/J mice a), marmosets b), and humans c), showing a phylogenetically conserved pattern of heterotopicity. Voxel-based maps of the CC heterotopicity d–f) reveal that the heterotopic connections are centrally located along the anteroposterior axis of the CC, whereas homotopic connections are located along the periphery. The similar relative proportion of homotopic and heterotopic connections across species g–i) shows that heterotopicity is evolutionarily conserved.

Swanson et al. (2017) showed that 20% of the rat brain's cortical connections are interhemispheric and that heterotopic connections account for 64.4% of the aggregate connection weight of the commissural projections, which is identical to our findings shown in Fig. 1. Our present findings, obtained in mice, marmosets, and humans, confirm the rat brain study of Swanson et al. (2017). Heterotopicity, thus, seems to be a typical structural feature of the CC of Eutherian brains, amplifying the complexity of brain connectivity.

Recent advances in optics technology, axonal tracing methods, tissue clearing techniques, and the increasing availability of tract-tracing data over the entire brain allow the reevaluation of hidden heterotopic connectivity. For example, the Allen Brain Institute Mouse Brain Connectivity Atlas (https://connectivity.brain-map.

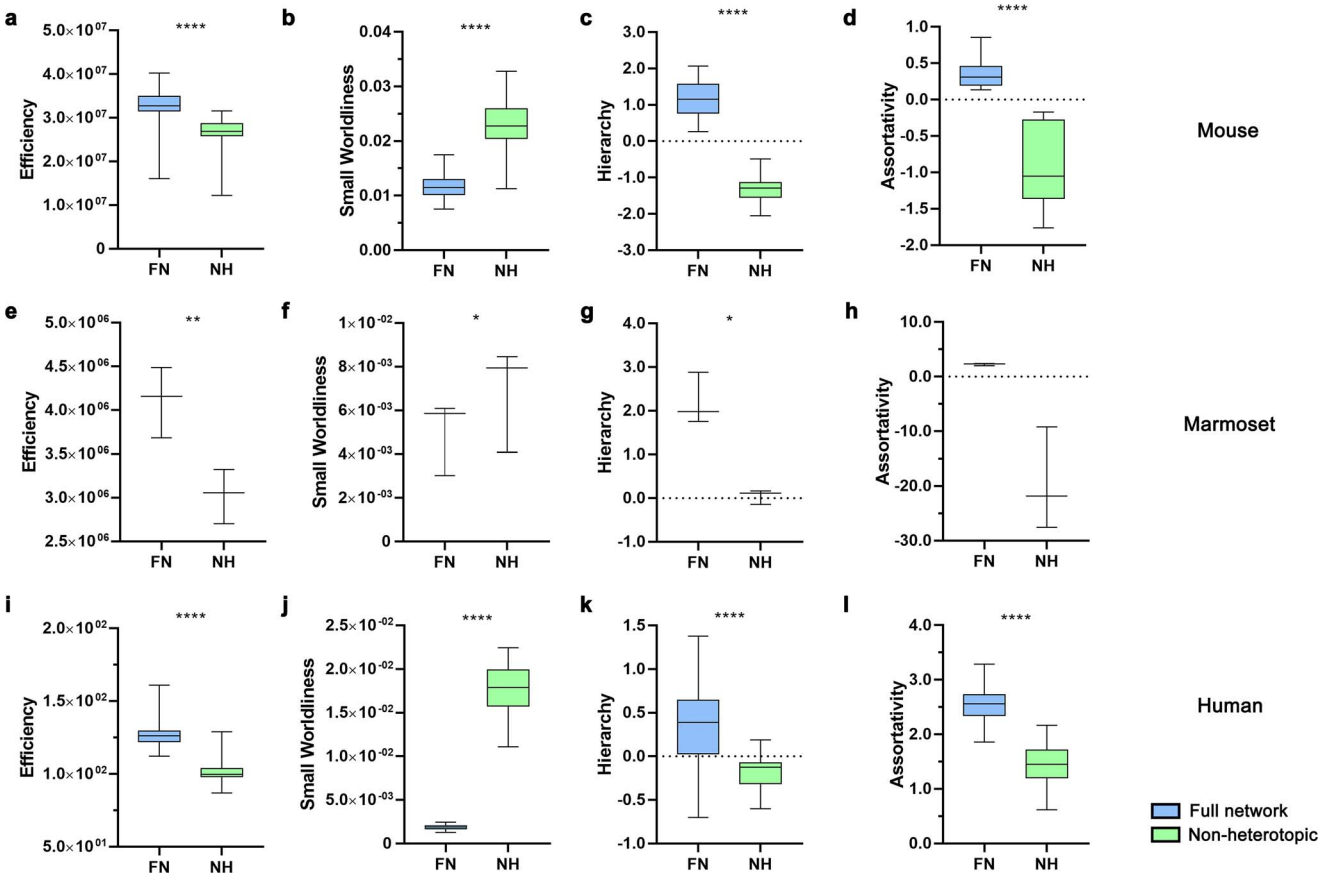

Fig. 3. Impact of heterotopic connections on NBS. Pairwise analysis of the impact of heterotopic connections on whole-brain NBS in mice a–d), marmosets e–h), and humans i–l). The network properties of efficiency, small-worldliness, hierarchy, and assortativity were computed for the full network (FN) and after removing the heterotopic connections (NH). There was a clear impact of removing the heterotopic connections on all network properties, showing the importance of such connections to understanding brain function. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$.

org) contains over 2,900 injections with the AAV9 viral tracer allowing precise quantification of projections in different brain areas (Oh et al. 2014). However, although the technique is a valuable tool for investigating brain connectivity, it was necessary to surgically manipulate and sacrifice a large number of animals to perform this extensive work. This endeavor would be challenging in nonhuman primates and impossible in humans. Therefore, the investigation of heterotopic connectivity requires using noninvasive imaging techniques. We used diffusion MRI tractography to compare with the Allen Institute Mouse Brain Connectivity Data. Relative to the neuronal tracing data, we found that DWI only slightly overestimates the number of heterotopic projections, likely due to methodological differences between the techniques. For example, diffusion imaging cannot distinguish afferent from efferent fibers. Furthermore, the spatial resolution of DWI is significantly limited compared with the resolution of histological data. These differences are natural sources of biases.

Our results in mice, marmosets, and humans establish the CC as a connectivity pathway for heterotopic communication across the hemispheres. This finding directly impacts the research of electrophysiologists, atlas creators, theoretic neuroscientists, and all approaches that currently assume that cortical connectivity can be represented as a closed system within each brain hemisphere, with the CC linking homotopic areas with the same function. The presumed traditional regularity of commissural architecture hides a much more intricate and complex organization. In addition, the finding challenges the predominant model of callosal development as performed by antiparallel axonal fasciculation across the midline. We develop these issues in greater detail below.

Structural and functional role of heterotopic connections

Homotopicity is the connectivity of a brain area to its contralateral homologous counterpart (e.g. right primary visual cortex to left primary visual cortex) in Eutherian (i.e. callosal) mammals. By definition, heterotopicity encompasses all the interhemispheric connections that are not homotopic. Note that, in general, the interhemispheric connectivity has a symmetric motif, as much as the intrahemispheric connectivity, that is mirrored in the contralateral hemisphere (see examples in Supplementary Figs. 2–5, see online supplementary material for a color version of these figure and Supplementary Videos 1 and 2). We found that cortical regions are usually connected with several other contralateral regions in addition to their homotopic area. Consistently, their heterotopicity indices tend to be high. This new finding contradicts the widespread belief that the CC is a predominantly homotopic structure (Zhou et al. 2013; Roland et al. 2017; Suarez et al. 2018; Mancuso et al. 2019; Loomba et al. 2021).

Because the heterotopic connections are numerous but sparse, connecting diverse brain regions of the contralateral hemisphere, it is unlikely that they are drivers to other brain regions. Instead, they may act as modulators of the whole-brain interhemispheric

connectivity (Innocenti et al. 2022). This form of modulation would be similar to the well-established cortico-thalamocortical circuit. This circuit is based on the information flow from the cortex, signaling simultaneously to other cortical regions and the thalamus, with the latter modulating back to the target cortical region (Sherman 2016). We hypothesize that the heterotopic connections constantly modulate the contralateral hemisphere to facilitate or inhibit the following intrahemispheric connection. For example, the ipsilateral S1 could send parallel information to the contralateral S1 (homotopic) and M1 (heterotopic), activating the right S1 and modulating the right M1 to receive the following intrahemispheric input from the right S1 to the right M1.

We showed that the heterotopicity of the CC is conserved across rodents and primates. It is conceivable, thus, that this would be a consolidated evolutionary achievement that amplifies connectivity and integration between cortical brain regions to allow more complex and multimodal functions. Future research should investigate other species from different taxonomic orders to better understand the interhemispheric heterotopic connectivity evolution.

Development of heterotopic connections

The current understanding is that the callosal tract develops mainly via a process known as fasciculation. Some bilateral pioneer cortical neurons originating in the anterior cingulate cortex project their axons towards the midline (Rash and Richards 2001; Bak and Fraser 2003) following several guidance cues (Donahoo and Richards 2009). Along this trajectory, followers adhere to pioneers and themselves (fasciculation), using one another as rails towards their cortical targets (Bak and Fraser 2003).

With the new finding that the CC is a primarily heterotopic structure, an interplay of different mechanisms becomes necessary to understand the CC development, requiring multiple sources of attractive/repulsive factors to explain heterotopic connectivity and the complex anatomy of the CC. Homotopic and heterotopic projections cross the CC in a latero-lateral direction and then split into different cortical targets (Supplementary Figs. 2–5, see online supplementary material for a color version of these figure and Supplementary Videos 1 and 2). The mirrored fasciculation of axons would hold mainly within the antiparallel sector of the CC close to the midline, supplemented by other guidance mechanisms that make fibers diverge to take different destinations. Future studies shall aim to understand if there are guidance cues at play to attract/repel these heterotopic axons to guide them towards their final destination and explain how heterotopic fibers can connect the brain in a mirror-like fashion. Heterotopic fibers may display a fingerprint that makes their trajectory different from homotopic axons.

In addition, strategies such as bifurcating axons that target different cortical regions (Garcez et al. 2007) or axons that retract their branches to find new pathways and connect specific brain areas (Olavarria et al. 1988) might be important avenues of research that can help understand CC development. Furthermore, developmental timing is a critical feature yet to be investigated, as homotopic and heterotopic connections might not form within the same time window. Moreover, it is also essential to understand the stability of these connections from birth to adulthood and their eventual pruning, as demonstrated for different cortical connections, including callosal ones (LaMantia and Rakic 1994). Another vital feature to consider is the anatomical arrangement of heterotopic axons, which seem to concentrate at the core of the CC, alongside its entire sagittal extent, whereas homotopic connections lie in the periphery. This arrangement suggests that there is an inside-out gradient that merits further investigation in 2 different domains: (i) molecular, analyzing the different receptors expressed at the growing axon surface and their relationship with the guidance cues both at the midline and on their divergency points; and (ii) temporal, investigating different developmental time courses of the homotopic as compared with heterotopic connections. This possibility would also imply that the CC has an anteroposterior and mediolateral developmental gradient (Rash and Richards 2001) and an inside-out gradient.

Heterotopic networks

Connectome graph analysis has become an essential mathematical tool for understanding complex networks, allowing estimating and quantifying computational properties of the brain, such as efficiency, small-worldliness, and others (Sporns et al. 2005). These properties enable us to directly compare interventions in complex networks, such as diseases (Zhao et al. 2016), behavior (Liu et al. 2016), and fetal imaging (Jakab et al. 2015) with the same parameters in typical brains. Here, we used the same concept to investigate the impact of heterotopic connections on overall brain connectivity. Our data show that heterotopic connections significantly weigh all tested connectivity metrics, except for marmosets assortativity ($P = 0.056$), and are an integral and essential component of brain networks. These results collectively show that the heterotopic connections are fundamental to wire the brain in a correct, high-efficiency fashion, enabling information flow across long distances and allowing brain areas to work together successfully.

Furthermore, they shine a new light on callosal dysgenesis (CCD), as the heterotopic connections can partly explain the plasticity found in the brain connectomes of mouse models of CCD and humans with CCD (Owen et al. 2013; Jakab et al. 2015; Edwards et al. 2020; Szczupak et al. 2020, 2021). For example, the sigmoid bundle connecting the frontal pole with the contralateral occipito-parietal cortex and prominently found in humans with CCD (Paul et al. 2007; Tovar-Moll et al. 2007) is formed exclusively of heterotopic fibers. Heterotopic brain connections can also help explain diseases known to have callosal involvement, such as ADHD (Luders et al. 2016) and ASD (Paul et al. 2014; Lefebvre et al. 2015). In addition to other structural biomarkers, such as the callosal shape and size, which have been used clinically to diagnose CCD (Wolff et al. 2015), the heterotopicity index might also be used as a quantitative biomarker to help evaluate disease status, progression, and prognosis. The advantage of the heterotopicity index is that it directly reflects the functional status of interhemispheric cortical connectivity instead of being a solo anatomical characteristic.

Supplementary material

Supplementary material is available at *Cerebral Cortex* online.

Acknowledgments

We thank the International Research Consortium for the Corpus Callosum and Cerebral Connectivity (IRC5, https://www.irc5.org) members and affiliates for discussions and input.

Funding

This work was supported by the PA Department of Health SAP #4100083102 and by the National Institutes of Health, National

Institute on Aging grant R24AG073190 to ACS, the Research Support Foundation of the State of Rio de Janeiro (FAPERJ), the National Council for Scientific and Technological Development (CNPq), as well as by intramural grants from D'Or Institute for Research and Education (IDOR).

Conflict of interest statement: None declared.

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