Diffusion MRI Anisotropy in the Cerebral Cortex is Determined by Unmyelinated Tissue Features

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Abstract

The diffusion of water molecules through the brain is constrained by tissue and cellular substructure, which imposes an anisotropy that can be measured through diffusion magnetic resonance imaging (dMRI). In the white matter, myelinated axons strongly shape diffusion anisotropy; however, in gray matter the determinants of dMRI signals remain poorly understood. Here we investigated the histological tissue properties underlying dMRI anisotropy and diffusivity in the cerebral cortex of the marmoset monkey. We acquired whole brain ex vivo dMRI data designed for high signal-to-noise at ultra-high (150 μ m) resolution. We compared the MRI to myelin- and Nissl-stained histological sections obtained from the scanned brain. We found that dMRI anisotropy corresponds most strongly not with cortical myelin content, but rather with the microscale anisotropy of tissue features, most notably those unmyelinated features highlighted by Nissl staining. The results suggest that dMRI anisotropy in gray matter derives from the organization of unmyelinated neurites, which are known to be affected by neurodegenerative diseases.

Introduction

Recent advances in high resolution diffusion MRI (dMRI) methods have revealed significant diffusion anisotropy in the cerebral cortex gray matter¹⁻⁷. This finding provides an opportunity to extend the use of dMRI beyond its well-established application for characterizing white matter projections. Determining the cellular and histological features that give rise to this anisotropy is important, because this could offer a way for MRI methods to detect and measure the detailed cytoarchitectural properties of gray matter, both in healthy tissue and in disease states. Cytoarchitecturally grounded MRI contrasts might link foundational histological studies in the medical field⁸ to *in vivo* studies in humans at the level of individual subjects, and could aid in the detection of neuropathology. For example, linking dMRI variation to tissue structure could be applied to track the progression of cortical pathology during the early stages of Alzheimer's disease⁹, or to track tissue changes in conditions like PTSD¹⁰.

The histological basis of diffusion anisotropy in gray matter is not yet well explored¹¹. Based on principles derived from the analysis of white matter, where the majority of previous histological comparisons have been carried out^{12,13}, it seems likely that both the density and arrangement of myelinated fibers contribute to cortical dMRI patterns^{2,14-20}. Large caliber myelinated axons are one component of gray matter, which for example include extrinsic projections emanating from layer II/III into white matter^{21,22}, and afferent axons projecting into cortex. Other myelinated axons in gray matter, however, differ from those in the white matter, being less densely packed, having a much smaller caliber, or having matted structure rather than forming coherent bundles²³.

The spatial distribution of cortical myelin density varies substantially by horizontal location (anatomical region) and by vertical (laminar) position. The myelin content of voxels may be assessed using a wide variety of MRI contrasts^{12,13,24} for example the T1w/T2w or magnetization transfer ratio (MTR). Such assessments are the principal means by which anatomical regions of the gray matter are identified in-vivo at present²⁵. Quantitative validation of these contrasts in both white and gray matter is ongoing, but they tend to show more reliable correlations to histologically measured myelin content than do measures of dMRI anisotropy or diffusivity^{12,13,24}. There is good evidence^{3,26} that the organization, rather than the density, of myelinated fibers drives dMRI anisotropy in a way that is not reflected in contrasts like T1w or MTR, although this has not yet been demonstrated quantitatively².

There are significant variations in myelinated axon organization between cortical anatomical regions^{23,27} and these differences may be reflected in diffusion anisotropy.

However, in contrast to white matter, the microstructure of gray matter is dominated by non-myelinated substructures and so it is possible that these shape the diffusion signal more than myelinated axons^{28,29}, or do so in a different way³⁰. Previous work in the field of dMRI indicates that lipid cell membranes are more important determinants of diffusion anisotropy than myelin per se³⁰. In white matter, these elements covary because the overwhelming majority of neurites are myelinated axons, so the distinction between myelinated and non-myelinated neurites has not been of great importance³¹. This distinction may be much more important in gray matter, and some qualitative evidence^{2,3,32} suggests that non-myelinated tissue components could be linked to dMRI anisotropy.

At a more detailed level, previous dMRI studies have found that the dominant direction of diffusion anisotropy in the cortex is nearly always perpendicular (henceforth, “vertical”) to the pial surface, with the exception of thin zones directly adjacent to the pial and white matter boundaries^{3,33–35}. Just as the diffusion orientations in white matter reflect the organization of axonal projections into directed bundles, the pronounced vertical bias in gray matter diffusion orientations may reflect the organization of cell bodies, axons, dendrites and other tissue components into columnar structures^{2,3}. Vertically oriented structures in gray matter include some myelinated axons^{21,22}, but they also include, for example, apical dendrite bundles projecting vertically from deep to superficial layers^{3,32,36,37}, as well as the neurites of many other more regional and layer specific cell types^{22,36,38}.

In this study we investigate the extent to which measures computed from histological stains for myelinated fibers and Nissl bodies reflect dMRI anisotropy and diffusivity in the gray matter. In comparison to white matter, gray matter exhibits significant variation in myelin density and other material composition within small areas of space. We therefore emphasize high SNR and high spatial resolution in our dMRI acquisition, and spatially precise co-registration in our analysis. We use the foundational diffusion tensor model of the dMRI signal³⁹ due to its mathematical simplicity, its minimal assumptions about tissue composition and its conceptual similarity to the structure tensor model of histological image anisotropy⁴⁰. We report that dMRI fractional anisotropy (dMRI-FA) in the marmoset cerebral cortex best matches histological anisotropy (HA), rather than myelin content. Myelin content has a comparatively poor correspondence with dMRI-FA, and is much better reflected in a different MRI measure, namely the magnetization transfer ratio (MTR). We then show that the anisotropy of Nissl-

stained tissue (Nissl-HA) has the strongest correspondence with MRI diffusion, including dMRI-FA as well as its underlying diffusivity components. Together, the findings suggest that dMRI-FA in the cerebral cortex gray matter is determined principally by unmyelinated components of the tissue, such as dendrites, glial processes and unmyelinated axons.

Results

To assess which histological features of the gray matter best matched the spatial variation of dMRI-FA, we compared the dMRI data to histological sections from the same brain. Analysis focused on the brain of a marmoset (case P) that was first scanned *ex vivo* using high resolution and signal-to-noise MRI and then sectioned and stained for histological analysis.

The brain was scanned for 58 hours on a 7-T Bruker preclinical scanner with 450mT/m gradient coils. Six whole-brain volumes at 150 μ m isotropic resolution were averaged for each of 60 diffusion encoded directions and two B0 volumes. This extensive averaging resulted in a dMRI dataset with a signal-to-noise ratio of approximately 85 in gray matter. In addition, magnetization transfer ratio (MTR) volumes were acquired in a separate session from the same brain at an isotropic spatial resolution of 75 μ m. The MTR sequence is known to be sensitive to myelin content⁴¹. The dMRI volumes were resampled to the higher MTR resolution for analysis using a spline interpolation.

After MRI scanning, the brain's left and right hemispheres were sectioned into coronal and sagittal sections, respectively, at 50 μ m on a freezing microtome. Adjacent sections were stained for Nissl bodies using the Thionin stain, and for myelin using the Gallyas silver stain. For quantitative analysis, nine strips of gray matter in the dorsal frontal and parietal lobes were chosen for this comparison based on their low curvature, since cortical curvature can affect dMRI-FA values⁴². Non-linear registration

Figure 1. Comparison of dMRI FA to corresponding myelin histology and MTR MRI data obtained from the same tissue in the right hemisphere of marmoset monkey case P. **a)** Sagittal sections of dMRI-FA (**top**), Myelin histology (**center**) and MTR MRI (**bottom**). The spatial variation of dMRI-FA in vertical (laminar) and horizontal direction showed minimal correspondence with the pattern of myelin intensity, but the variation of the MTR MRI closely mirrored the myelin variation. In some regions of high myelination, the dMRI-FA was high (red arrow) whereas in others it was low (blue arrow). **b)** Normalized average laminar profiles of FA, MTR and myelin intensity through the cortical thickness in the brain's left hemisphere, derived from cortical parcellation (see **Methods**, **Figure 3**). **c)** Histogram of correlation coefficients between MRI variables and histological myelin intensity over the nine sections of coronal histology used in this study. MTR MRI shows much higher correspondence with myelin intensity than dMRI-FA does.

allowed for a direct and precise pixel to pixel comparison of diffusion and histological parameters at 150µm spatial scale (see **Methods**, **SI Figure 1**).

dMRI fractional anisotropy does not reflect myelin content

We began our analysis with a simple pixelwise correlation of MRI and histology within the nine registered sections. Our first question was whether the patterns of dMRI-FA matched the distribution of myelin intensity, as might be expected based on some previous studies in white matter regions^{12,13}. We

found spatial variation of dMRI-FA was a poor match for the myelin intensity measured through histological staining. This difference was evident both across laminae (henceforth, *vertically*) and along the cortical sheet (henceforth, *horizontally*), as illustrated in a sagittal example section in **Figure 1a**. Comparing the dMRI-FA to the myelin stain intensity, there are areas in which they appear to match (blue arrow) and others in which they clearly do not (red arrow). In the vertical direction, myelin intensity declines monotonically from the white matter to the pia⁴³. dMRI-FA behaves differently, being strongest in the middle cortical layers, and lowest near the white matter and pial boundaries (**Figure 1b**)^{4,44}. This example also shows that, in contrast to dMRI-FA, the MTR MRI contrast closely matches the laminar pattern of myelin staining, following from the known sensitivity of the MTR MRI sequence to large macromolecules⁴¹. Across all sections, the correspondence of the myelin intensity was much higher to the MTR MRI contrast than to the dMRI-FA contrast (**Figure 1c**).

Fractional anisotropy of dMRI matches that of histology

Having established that myelin stain intensity is only modestly correlated to dMRI-FA, we next turned our attention to a comparison of dMRI parameters with tissue organization as reflected in the cell body and myelin histology. In each voxel, the diffusion tensor provides a principal orientation, and scalar indices of anisotropy such as dMRI-FA. For direct comparison, we sought to generate parameter maps from the histology at the same spatial resolution and with equivalent information as the dMRI. To achieve this, we computed the structure tensor (ST) of the cell body and myelinated fiber histology at microscopic scale, weighted over a mesoscopic spatial area that matched the dMRI acquisition resolution of 150 μ m (see **Methods**). We then resampled the resulting parameter maps to the MRI display resolution of 75 μ m (**Figure 2**). Like the diffusion tensor, the structure tensor provides both vector and scalar data, in the form of mean orientations and ST coherence⁴⁵. For both the myelin- and Nissl-based sections, the principal ST orientation was strikingly uniform and nearly always perpendicular to the pial surface (i.e. vertical) closely matching the orientation map obtained from the dMRI diffusion tensor (**Figure 2a**). While it was not the focus of the present study, we also found that the tractography computed from the histological and dMRI data yielded qualitatively nearly identical results (**SI Figure 3**). Only in thin laminar zones directly adjacent to the pia and the white matter did this principal orientation deviate significantly from the vertical and differ across measurements.

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163 ST coherence is similar to dMRI-FA in that it reflects the degree of alignment of objects. However,
 164 unlike dMRI-FA^{2,15}, ST coherence does not reflect object density⁴⁵ within that area. We therefore created
 165 measures of histological anisotropy (Nissl-HA and myelin-HA) by adjusting the histological ST coherence
 166 values to incorporate a pixelwise approximation of neurite density. To derive myelin-HA, we used the
 167 product of normalized histological myelin density and myelin ST coherence. For Nissl data we used the
 168 product of Nissl ST coherence and the Nissl derived “neurite fraction”⁴⁶ (see **Methods**). These measures
 169 served as summary statistics whose variation could be compared systematically with that of the dMRI-

Figure 2. Properties of directional MRI and histology data. For comparison with MRI data, the weighted structure tensor of the myelin and Nissl histology images was computed over a $150\mu\text{m}$ area (see **Methods**). **a)** Orientation data derived from Nissl and myelin histology and from dMRI are similar, showing columnar, vertically oriented vectors. The overall pattern of orientations in the gray matter is remarkably similar for the data computed from histology and from dMRI. **b)** The right column shows dMRI-FA and non-linearly registered histology anisotropy maps which show the degree of edge dispersion from the mean in $75\mu\text{m}$ tiles (see **Methods**).

FA across the gray matter (**Figure 2b**). While the inclusion of neurite density into the HA measure was deemed appropriate for this comparison, qualitatively similar results for all figures were obtained using maps of the histological coherence alone (**SI Figure 2**).

For each section, we quantitatively compared the spatial variation of dMRI-FA to the Nissl-HA, myelin-HA, and myelin stain intensity scalar parameter maps (see **Methods**). For completeness, we also compared the MTR MRI data to the same histological parameters. To accommodate anatomical variety and potential inconsistencies in histological stain intensity, we employed a parcellation approach in our analysis of the gray matter. In each of the nine low-curvature sections, we compared the spatial variation in the MRI and histological parameter maps by computing the correlation over multiple parcellated subdivisions spanning the thickness of the cortex (example red trapezoids in **Fig. 3**, see **Methods, SI Figure 4**). We focused analysis on a horizontal parcellation width of 300 μm , though the results of the analysis were robust to parcel sizes ranging over a hundred-fold in area (see **SI Figure 5**). Within a given parcel, a pixel-by-pixel correlation of variation within each column revealed different levels of correspondence for the different histological and MRI parameters. This difference can be seen in the example parcel in **Figure 3 (bottom row)**, the strongest spatial correspondence with the dMRI-FA measure was the anisotropy in the Nissl-stained sections, Nissl-HA, while the strongest correspondence with the MTR was the myelin intensity.

Figure 3. Example correlation of Histology and MRI variables sampled in columnar parcels. Top row: non-linearly co-registered histology parameter maps and MRI at 75 μm resolution. The red trapezoids indicate a columnar parcel example. Bottom row shows representative scatter plots and Pearson correlation coefficients between histological variables and MRI variables.

We evaluated such correlations across all 393 parcels in the nine sections for the different MRI and histological parameter maps (**Figure 4a**). Across the cortex, the strongest correlation with dMRI-FA was found in the Nissl histological anisotropy (Nissl-HA) (median 0.76, mean 0.72). Significantly less correlated were the myelin anisotropy (myelin-HA, median 0.63, mean 0.57) and myelin intensity (MI, median 0.47, mean 0.39). Nissl-HA and myelin-HA were also correlated moderately to one another (median 0.60, mean 0.55) (**SI Figure 6**). The difference in correlation profile between Nissl-HA and myelin-HA was even more pronounced using a measure of in-plane dMRI anisotropy⁴⁰ in place of dMRI-FA (**SI Figure 7**), yielding correlations with Nissl-HA (median 0.77, mean 0.74), myelin-HA (median 0.64, mean 0.56) myelin (median 0.43, mean 0.37) with a smaller interquartile range for Nissl-HA and larger for myelin-HA. For comparison, the MTR MRI contrast had a very strong spatial covariation with the myelin intensity (**Figure 4b**, median 0.94, mean 0.93).

The shared variation of dMRI-FA with Nissl-HA suggests that the cortical dMRI-FA is strongly shaped by unmyelinated components such as cell bodies, dendrites and unmyelinated axons. Its much weaker

Figure 4. The distribution of correlation coefficients between histological parameters and dMRI-FA over all 393 parcels of cortex. Each point in the distribution is the Pearson correlation between the variables sampled in a vertical parcel (see **Methods**). Red dot shows median value, blue line shows mean and vertical gray bars show lower and upper quartiles. **a)** Correlation of Histological variables to dMRI-FA **b)** Correlation of MTR to myelin intensity.

relationship to the myelin-stained sections, and particularly to the myelin intensity distribution, suggests that myelinated axons may have a relatively minor contribution to dMRI anisotropy in the cerebral cortex.

Laminar and tangential variation in anisotropy

Given that the laminar (vertical) and tangential (horizontal) dimensions of the cortex differ substantially in their architectural variation, we performed additional analyses to consider these dimensions

Figure 5. Average laminar profiles of FA, Nissl-HA and myelin-HA across all parcels, and the results of a spatial randomization condition to test the regional specificity of parcel-wise histology to MRI correlations. The results show Nissl-HA more closely matches the laminar profile of dMRI-FA, and is more sensitive to shuffling of horizontal MRI locations **a)** Normalized laminar profiles of dMRI-FA, Myelin-HA and Nissl-HA through the cortical thickness, computed from the average of all columnar parcels (see **Methods**). WGB indicates the white/gray matter boundary, PIA indicates the pial surface (see **Methods**, **Figure 3**) **b)** The change in mean correlation coefficient when the cortical parcels of the histology parameters and those of the dMRI-FA are shuffled between random horizontal locations. Bars indicate standard error. The star indicates significance under a paired t-test.

separately. First, we analyzed the mean laminar variation of the dMRI-FA and compared it to the histological anisotropy (**Figure 5a**). Collapsing the data across all horizontal cortical positions (see **Methods**) revealed a pronounced laminar profile for each of the measured parameters. The dMRI-FA profile had its highest responses in the middle or lower-middle cortical layers, with a decline in anisotropy in both upper layers near the pia, and lower layers near the white gray boundary (WGB). This profile was consistent with results from human dMRI⁴, although our higher resolution data revealed significantly more underlying variation in the laminar profiles of individual horizontal locations (**SI Figure 8**, **SI Figure 9**). The average laminar profile across horizontal regions in **Figure 5a** resembled the observed histological anisotropy, with somewhat stronger correspondence to the Nissl-HA measure. As described earlier, the profile of dMRI-FA differed markedly from the more monotonic profiles of myelin

intensity and MTR (**Figure 1c**). The average laminar Nissl-staining intensity exhibited nearly the inverse profile to the myelin intensity, having its highest intensity in the superficial layers (**SI Figure 10**).

Given these conspicuous laminar profiles, we next asked to what extent the observed correspondence between dMRI-FA and the two measures of histological anisotropy could be ascribed to laminar variation alone. To address this question, we performed an analysis in which we shuffled the horizontal locations of the columnar parcels in the histological parameter maps relative to the dMRI-FA maps. If such shuffling were to have no effect on the observed correlation, then we could conclude that the parcel correlation results in **Figure 4** are fully explained by laminar variation that follows a stereotypical pattern across the data. We found that shuffling strongly affected the correlation of the Nissl-HA with the dMRI-FA but, had no significant effect on the other histological/MRI correlations (**Figure 5b**). For the Nissl-HA, tangential (horizontal) shuffling causing the mean correlation to drop significantly, from 0.72 to 0.52 (t-test, $p < 10^{-10}$). These results indicate that, while the average laminar profile (**Figure 5a**) generally determines the correspondence of dMRI with myelin-HA and myelin intensity, the Nissl-HA measure was unique matching the horizontal variation of dMRI-FA.

Histological correlation with dMRI diffusivity components

We next investigated relationships between histological anisotropies (Nissl-HA, myelin-HA) and the specific directional diffusivities that underlie diffusion anisotropy in gray matter. dMRI-FA is a composite measure that relates to the ratio of axial and radial diffusivities. Axial diffusivity (AD), $\lambda_{\parallel} \equiv \lambda_1 > \lambda_2, \lambda_3$, describes the mean rate of diffusion along the principal diffusion tensor axis. As described earlier (**Figure 2a**), in gray matter the principal tensor axis for both dMRI and histology is overwhelmingly oriented in the vertical direction. Radial diffusivity (RD), $\lambda_{\perp} \equiv (\lambda_2 + \lambda_3)/2$, refers instead to diffusion perpendicular to the principal axis, in the horizontal direction in the gray matter.

Applying the same strategy as above in **Figure 4**, we computed the parcel-wise spatial correlation of MRI diffusivity parameters AD and RD with that of dMRI-FA (**Figure 6a**). We also computed the spatial correlation of AD and RD with the two measures of histological anisotropy (**Figure 6b**). We found that the diffusivity correlation profile of Nissl-HA was qualitatively similar to that dMRI-FA, whereas that of myelin-FA was not. Specifically, of the two histological measures, only Nissl-HA exhibited any positive correlation to AD. We also noted that myelin-HA exhibited a very strong anti-correlation to RD, and we

found that laminar variation in myelin-HA closely matched laminar variation in RD (SI Figure 11). MTR and myelin intensity both show a strong anti-correlation to RD (SI Figure 12). dMRI-FA in gray matter

Figure 6. Distribution of correlation coefficients between histological and dMRI anisotropy on one hand and directional diffusivities on the other. Nissl-HA exhibits a profile that is more similar to dMRI-FA than that of myelin-HA. **a)** dMRI-FA correlates to increases vertical diffusivity (AD) and decreases in horizontal diffusivity (RD). **Top Right** Nissl histological anisotropy (Nissl-HA) exhibits modest positive correlation to AD and negative correlation to RD, indicating that Nissl-HA and dMRI are associated with a similar mechanism of diffusion anisotropy. **Bottom Right** myelin histological anisotropy exhibits no mean correlation to vertical diffusivity and very strong correlation to horizontal diffusivity, as predicted from previous studies¹⁸

appears to be caused both by increases in axial diffusivity and decreases in radial diffusivity relative to the isotropic case. Spatial variation in these diffusivity parameters are similarly reflected in Nissl-HA parameter, while myelin-HA and myelin intensity appear primarily to affect only RD.

The exception of granular layer 4

While the Nissl-HA was generally a reliable marker for dMRI-FA variation across gray matter voxels, there was a notable exception. In layer 4 of granular regions of the cortex, the Nissl-HA and dMRI-FA did not match as expected, since the Nissl-HA was low but the dMRI-FA was high, driven by elevated axial diffusivity (Figure 7a, green). While above we interpreted Nissl-HA to be an indicator of oriented neurites, this assumption likely does not hold true in layer 4, where the Nissl-staining is dominated by round and unoriented, granular neurons (see SI Figure 13), and there is a pronounced gap in

256 myelination (**Figure 7b**). While most cortical neurons outside of layer 4 are pyramidal in shape⁴⁷, both
 257 excitatory and inhibitory layer 4 cells have a stellate morphology with rounded cell bodies. While these
 258 cell bodies are unlikely to elevate dMRI-FA, it is known that they project highly branched, vertically
 259 oriented, unmyelinated axons⁴⁸. Furthermore, layer 4 is a site where many thalamic afferents lose their
 260 myelin sheath and branch heavily, including into vertically oriented collaterals (**Figure 7b**, right)⁴⁹. Taken

Figure 7. In granular cortex, layer 4 is identifiable in Nissl histology by many small cell bodies and in myelin histology by a local decrease in stain intensity. In dMRI data layer 4 is associated with a local increase in Axial diffusivity (AD) and in MTR data by a band of low intensity. **a)** dMRI, MTR and histologically derived contrasts. **b) Left** Illustrative detail of the Nissl histology from an ROI of granular cortex. The internal granule layer is visible as a band of small, round cells. **Middle** the same region in myelin histology exhibits a gap in myelination. CLAHE contrast enhancement was applied uniformly across both myelin and Nissl images for display **Right** The highly branched, unmyelinated layer 4 terminals of axons from 8 thalamic afferent cells in rat Barrel cortex, reproduced from⁴⁹.

together, the elevated MRI axial diffusivity in granular layer 4 may be thus hypothesized to result from these and other unmyelinated axons, which cannot be observed in either the Nissl or myelin-stained sections.

Discussion

The results of this study suggest that diffusion MRI in cortical gray matter can capture important, hitherto inaccessible cytoarchitectural tissue properties in vivo. We analyzed the basis of dMRI-FA in the cerebral cortex gray matter of the marmoset monkey, scanned at very high signal-to-noise and spatial resolution. We focused on the diffusion tensor as a straightforward and well understood model of diffusion with minimal prior assumptions about tissue properties⁵⁰. We systematically compared dMRI-FA to finely co-registered histological parameter maps obtained from the same brain. To accommodate anatomical variety and potential inconsistencies in histological stain intensity, we used a cortical parcellation approach to conduct a mesoscale analysis of gray matter histological features that link to the spatial variation in dMRI properties. For comparison we also analyzed an MTR MRI data set, which is sensitive to large macromolecules such as myelin⁴¹.

Our principal finding was that fractional anisotropy correlated strongly with histological anisotropy derived from the structure tensor, and much less so with histological myelin density. However, myelin density did correlate very strongly with the MTR MRI contrast obtained from the same brain. We found that the dMRI-FA was particularly strongly correlated with the Nissl histological anisotropy, a result that identifies unmyelinated neurites as the principal determinant of dMRI-FA. Our second main finding was that Nissl-HA and myelin-HA correlated differently to the diffusivity components of dMRI-FA. Like dMRI-FA, Nissl-HA positively correlated to axial diffusivity - but myelin-HA did not. These two distinct correlation profiles indicate that myelinated and non-myelinated neurites in gray matter have qualitatively different contributions to diffusion anisotropy. An exception to the general pattern was found in granular layer 4, where high dMRI-FA and high axial diffusivity were not accompanied by elevated Nissl-HA or myelin-HA. We hypothesize that this exception stems from unmyelinated axons and other neurites known to penetrate that layer in great numbers⁴⁹, but which are undetectable with either Nissl or myelin staining.

The finding that the cell body anisotropy (Nissl-HA) had the strongest correlation to dMRI-FA was somewhat unexpected. This association is probably not due to the properties of cell bodies

themselves^{2,3,7,32}; however, the arrangement of cell bodies in Nissl data may be a reliable marker for the arrangement of neurites and glial processes^{22,38,51,52}, and this has previously been used to detect and quantify the columnar neurite architecture of the cortex⁵¹. The relative area of the unstained portion of Nissl stained sections has previously been used to quantify the neuropil fraction, including non-myelinated neurites⁴⁶, and we used this quantity in deriving our Nissl-HA measure (see **Methods**).

Pyramidal cells constitute 70-80% of cells in the cortex⁴⁷. Due to their shape, pyramidal cell body orientations are reflected in the ST anisotropy measure (**SI Figure 14**). The orientations of apical dendrites are strongly associated with the orientation of pyramidal cell bodies⁵³, and these non-myelinated neurites are prominent structural features of gray matter which likely influence local diffusion^{22,36,38,52}. The arrangement of smaller neural cell bodies into minicolumns along apical dendrite bundles is reflected locally in each cortical layer by our structure tensor methods^{22,36–38,52}. It is likely that other accompanying tissue features, such as unmyelinated axons, glial processes, epithelial cells and microvasculature play a role in shaping the correspondence between Nissl-HA and dMRI-FA, and these may all be indicated by patterns in cell body orientations.

Nissl-HA may also partly reflect a subset of vertically oriented myelinated axons^{22,38,46} in addition to non-myelinated tissue components. The Nissl derived “neuropil fraction”⁴⁶ includes some myelinated axons, especially in deep layers^{21,22,36–38,46,52}. We observed a median correlation of 0.6 between Nissl-HA and myelin-HA (**SI Figure 6**). However, non-myelinated substructures are more plentiful in gray matter than myelinated^{21,22,28,36–38,52}. The lower average correlation (**Figure 4**) and clear differences in the locations of peak anisotropy in the laminar profiles of myelin-HA and dMRI-FA (**Figure 5, SI Figure 9**) suggest that myelin is not the primary driver of dMRI-FA or of Nissl-HA, even in deep layers where myelin is very dense.

The specific combination of tissue features that shape cortical dMRI-FA cannot be fully known at this point, and thus warrants further investigation. The strong spatial covariation (median 0.76) of Nissl-HA to dMRI-FA holds promise that the structural basis of diffusion MRI in the cortex can be well understood. Future research using more specific stains will be able to assess more details of the relevant structural features, for example using immunohistochemical stains for proteins such as MAP 2^{36,37}, MAP τ ⁵⁴, SMI-312⁵⁵, SMI-311⁵⁵, MBP⁵⁶ and GFAP⁵⁷ which are variously specific to dendrites, neurites, axons, myelin and glial processes.

Previous work has shown that when a white matter voxel is organized as a single bundle⁵⁸, myelination and axon integrity are associated with radial and axial diffusivity, respectively^{12,13,18}. The present results of Nissl-HA and myelin-HA in the gray matter indicate that myelinated and unmyelinated neurites affect these two components of anisotropy in different ways, consistent with recent theoretical work¹¹ (**Figure 6, SI Figure 11**). Such information might potentially be used in biophysically motivated tissue models of the diffusion signal^{11,59,60}. In dMRI models of white matter, it is sometimes assumed that intra-neurite axial diffusivity – i.e. the intrinsic rate of diffusion in the axoplasm parallel to the membrane – is constant^{60,61}. However, in the more biologically heterogeneous environment of gray matter, this assumption may not hold¹¹. It may be that the cytoplasm of cell bodies, axons, dendrites and glial processes each have slightly different, characteristic intrinsic diffusivities³⁰. The differing anatomy of particular tissue features may also hinder diffusion in specific ways, for example diffusivity in cell bodies may be low due to the high lipid content of cell organelles⁶². Recent modelling efforts in white matter have sought to simultaneously measure both variation in intra-axonal diffusivity, and variation in axon orientation dispersion⁶⁰. Related models may prove especially important in gray matter. Models that include information about myelin levels, cortical curvature and location in the cortical depth, as well as different diffusion times, we may be able to gather very detailed information about the human cortex in-vivo.

In order to develop such models, our understanding of the diffusion signal in gray matter should be grounded in strong empirical data. In the present study, we see significant variations in the laminar profiles of dMRI-FA between gray matter regions (**SI Figure 8, SI Figure 9**). This variation may reflect valuable information such as cytoarchitectural differences between cortical regions, or degradation of tissue due to disease. The factors influencing the diffusion signal are likely to differ in different horizontal locations and at different cortical depths. Measurements taken in-vivo at low resolution will represent the influences not only of cyto and myeloarchitecture, but also of cortical curvature⁴². Work at high resolution in ex-vivo animal models is important in order to understand the more ambiguous signals from the more complex human brain at lower resolution. The ex-vivo marmoset model has considerable advantages for this endeavor. As a primate, the marmoset's cortical thickness and cytoarchitectural complexity is comparable to the human case, but its small size means many cortical regions with diverse properties are available at high resolution and with minimal folding confounds. The

larger, more complex and folded rhesus brain may form an intermediate subject at somewhat lower spatial resolution, before tackling the much more complex human case at low resolution in-vivo.

Diseases such as Alzheimer's are believed to involve subtle changes to tissue properties especially in their early stages, and the ability to gather specific cytoarchitectural information separately from myeloarchitectural in-vivo may be crucial⁹, particularly since relatively little post-mortem information is available regarding the early tissue changes. The early stages of Alzheimer's and other diseases of the gray matter neuropil can manifest as cellular damage and inflammation rather than demyelination⁶³. Changes in mean diffusivity in gray matter have been associated with Alzheimer's pathology⁶⁴. The present findings may inform the development of dMRI models of the gray matter, perhaps to specifically target neuroinflammation⁹ or other aspects of Alzheimer's pathology in the earliest stages⁶⁵.

Our findings may also be useful in the growing field of comparative anatomy^{66–70}. MRI methods lack the spatial precision and biochemical specificity of histology. However, the whole brain can be studied at once, non-destructively and in three dimensions. This permits sophisticated computational analysis of the geometric and topological layout of the brain⁶⁶, of the spatial trajectories of white matter pathways⁶⁷ and of how these may be related between species^{68,70}. At present however, analysis of the gray matter structural properties in such studies tends to be limited to myelin levels⁶⁶. The present results may add important information that may help explain differing cognitive abilities between species. For example, the fraction of cortical tissue occupied by neurites differs between humans and chimpanzees⁴⁶. Using dMRI measures of cortical gray matter, we might model not only the quantity, but organization of specifically non-myelinated neurites in gray matter, which may be a marker for the intrinsic connectional sophistication of local gray matter regions. We may be able to compare these tissue properties across whole brains and in many species, in a quantitative way that can be linked to patterns of long-range connectivity via white matter bundles. Such studies might shed light on cortical evolution, the relationships between cortical regions, disease states, the interpretation of the BOLD signal and differing cognitive capabilities between species.

Methods

We describe below the methods used in this study, sample preparation and MRI acquisition, sectioning and staining, registration and finally statistical analysis.

376 *Animals and materials*

377 The brain of one adult marmoset (female, 5-year-old, “Case P”) was used in this study.

378 *Ex vivo high-resolution MRI Acquisition*

379 Before MRI scanning, the formalin-fixed marmoset brain was soaked with 0.15% gadopentetate
380 dimeglumine (Magnevist, Bayer Leverkusen, Germany) for 3 weeks to reduce the T1 relaxation time. In
381 order to improve SNR, brain P was cut through the midline, and each hemisphere was scanned
382 separately with a 25-mm birdcage volume coil.

383 For the left hemisphere Diffusion MRI was collected with a 3D diffusion-weighted spin-echo EPI
384 sequence with a spatial resolution of 150µm isotropic voxel size. The 3D diffusion EPI sequence
385 parameters were: TR = 400 ms, TE = 28.4 ms, flip angle = 90°, FOV = 38.4 x 24 x 21 mm, matrix size = 256
386 x 160 x 140, resolution = 0.15 mm isotropic. Sixty two DWI volumes (2 at b=0, and 60 directions with
387 b=4800) were acquired using 3 averages. The same 62 volumes were collected a second time with
388 identical settings. The total DWI acquisition time was about 58 hours. The pulse width for the diffusion
389 weighting gradient is 6.4 ms and pulse separation is 14 ms.

390

391 The right hemisphere was scanned with 100µm resolution with the 25 mm coil. The parameters for the
392 3D diffusion-weighted spin-echo EPI sequence were: TR = 400 ms, TE = 35 ms, flip angle = 90°, FOV =
393 38.4 x 20 x 25 mm, matrix size = 348 x 200 x 250, resolution = 0.10 mm isotropic, a total of 132 DWI
394 images (6 at b=0, and 126 directions with b = 4800), and total acquisition time was about 55 hours.

395 The left and right hemispheres separately underwent scanning for MTR at 75µm and 150µm
396 respectively.

397 The MTR images were collected with a 3D FLASH sequence. For the resolution of 150µm, the
398 parameters were: TR = 21.5 ms, TE = 3.6 ms, flip angle = 20°, FOV = 38.4 x 24 x 21 mm, matrix size = 256
399 x 160 x 140, number of averages = 8 and number of repetitions = 6, total acquisition time about 13 hour
400 For the resolution of 75µm acquisition, the parameters are: TR = 21.9 ms, TE = 3.8 ms, flip angle = 20°,
401 FOV = 34.8 x 20.4 x 20.1 mm, matrix size = 464 x 272 x 268, number of averages=5 and number of

repetition = 14, total acquisition time about 60 hours. The magnetization transfer was achieved (in M_{sat} scans) by a Gaussian shaped pulse at either +2000 or -2000 Hz offset frequency; the duration of pulse is 12.5 ms and flip angle is 540° and the pulse is present in every TR. This magnetization transfer pulse is turned off in M_{off} scans. The MTR value is calculated as $100 \times (1 - M_{sat}/M_{off})$.

MRI Processing

Diffusion Tensor Estimation dMRI data was preprocessed using TORTOISE v3⁷¹ which includes code for denoising⁷². After rotation to the histology slice plane (see *Registration of histology and MRI data* below), the diffusion tensor was estimated using FSL 5.0.11 DTIFIT. AD, RD and the in-plane anisotropy⁴⁰ were calculated using FSL 5.0.11 fslmaths.

Registered dMRI tensor parameter maps and MT data were split into separate coronal 32 bit quantitative tif images and grouped according to slice number for registration.

Histological Processing

Sectioning and Staining Following scanning, the brain's left hemisphere was coronally sectioned at 50 μ m on a freezing microtome, while the right hemisphere was sectioned sagittally. Alternate sections were stained for Nissl bodies and for myelin. The Gallyas silver stain for myelin was selected to stain individual axons in high detail⁷³. We used the Thionin stain for Nissl bodies. This is a traditional staining method that identifies cell bodies and some dendrites proximal to the soma, but very few axons⁵³. Each histological section was scanned at 0.88 μ m resolution (10x magnification) using a Ziess Axioscan microscope slide scanner. Higher resolution 0.44 μ m microscopy was not shown to produce significantly different ST results using this data. Histological sections were excluded due to damage or poor stain properties.

Registration of histology and MRI data

Rigid body rotation to histology slice plane. For histology to MRI registration, we first performed an initial rigid body alignment of the 75 μ m MT_{sat} data (**SI Figure 1**) to match the histology slice plane for each of P's hemispheres. This transformation was estimated by-eye using the mango MRI editor/viewer⁷⁴ and the manually transformed MT volume was used as the target for FSL 5.0.11 FLIRT⁷⁵ to transform the remainder of the MRI data.

Registration of MT and dMRI. The 150 μ m dMRI was rigid body registered to the 75 μ m MT volume using FSL FLIRT, so that the dMRI was upsampled to 75 μ m using spline interpolation. The b-vectors were rotated using the FAST transform and custom MATLAB code.

MIND nonlinear registration of local histology ROI. After performing ST computations on the histology to produce ST anisotropy parameter maps (see *Structure Tensor Analysis of Histological Orientations and Anisotropy* below), we performed careful non-linear alignment of nine local frontal and parietal ROI from the histology to the MRI (**SI figure 1**). 75 μ m resolution stain intensity maps of nine coronal sections of each of Nissl and myelin histology were first binarized using a threshold adjustment in imageJ⁷⁶ and then registered to their corresponding MRI slices to correct for histology shrinkage and deformation, first using manual 2D alignment in imageJ⁷⁶ and then MIND MATLAB code⁷⁷ ($\alpha=0.1$). To avoid registration between MRI and histological features whose anatomical meanings and relationships were unknown, the registration source and target images were featureless masks of the GM derived from the MT data (**SI figure 1**). Custom MATLAB code was written to store and apply these transforms to ST maps (see *Structure Tensor Analysis of Histological Orientations and Anisotropy* below) and other parameter maps derived from the histology. We selected regions of frontal and parietal cortex for non-linear alignment whose minimal gyrification ensured that the diffusion MRI signal was largely unaffected by cortical curvature⁴². This registration process allowed us to directly compare the cortical variation of diffusion and histological parameters from the same brain.

Structure Tensor Analysis of Histological Orientations and Anisotropy

To estimate the anisotropy and orientation content of the histology we employed the structure tensor method⁴⁰. The 0.88 μ m per pixel resolution histology images were processed using the structure tensor as implemented in the “OrientationJ” ImageJ package⁴⁵, using a local gaussian window of $\sigma=80$ pixels and image gradients computed with a cubic spline. This was applied to the color channel of the histology that exhibited the strongest image intensity gradients. This was the red channel for Nissl data, or blue channel for Gallyas myelin data. Orientation and anisotropy information were obtained from these data according to

$$S_0[p] = \begin{bmatrix} I_x[p]I_x[p] & I_x[p]I_y[p] \\ I_x[p]I_y[p] & I_y[p]I_y[p] \end{bmatrix}$$

Equation 1. Pixelwise structure tensor

where S_0 is the structure tensor at pixel p and I_x , I_y are the partial derivatives of the 2D image intensities at pixel p . Since the dMRI data contains diffusion information gathered over a $150\mu\text{m}$ cubic area, the histology structure tensor at each pixel was a distance weighted function $S_w[p]$ of the structure tensors S_0 within a radius of $75\mu\text{m}$. This was achieved for pixel p by multiplying with gaussian window with a sum of 1, with sigma 85 pixels ($75\mu\text{m}$), shown in **Equation 2**. The ST angle is shown in **Equation 3** and anisotropy in **Equation 4**

$$S_w[p] = \sum_r w[r] S_0[p - r]$$

Equation 2. Weighted structure tensor

$$\theta[p] = \frac{1}{2} \arctan \left(2 \frac{I_x[p] I_y[p]}{I_y[p] I_y[p] - I_x[p] I_x[p]} \right)$$

Equation 3. ST orientation

$$S_{\text{Anisotropy}} = \frac{\lambda_1 - \lambda_2}{\lambda_1 + \lambda_2}$$

Equation 4. Structure tensor anisotropy

A pipeline was implemented in bash and ImageJ macro language to process the Zeiss axioscan histology output files in CZI format into 32 bit tif format with resolution $0.88\mu\text{m}$, and process these with orientationJ. The histology stain intensity data and ST data at $0.88\mu\text{m}$ were then down sampled into maps of $75\mu\text{m}$ tiles for comparison and registration to MRI data using MATLAB R2018a. The mean ST angle within $75\mu\text{m}$ was the circular mean of the $0.88\mu\text{m}$ angles within that area.

Histological Anisotropy Because diffusion anisotropy is sensitive to both the density and coherence of tissue features, but ST coherence is sensitive only to the latter, we defined composite measures of histological anisotropy. **Myelin-HA** was defined as the product of myelin stain intensity and the ST coherence of the myelin data.

For the Nissl-HA we defined the neurite fraction (NF) as 1-nissl intensity, where 1 was defined as the highest gray matter value and 0 was defined as black. Nissl-HA was defined as the product of NF and the ST coherence of the Nissl data.

2D dMRI and Histology ST Tractography

The ST analysis of the myelin and Nissl histology yielded 2D vector fields at 75 μ m resolution representing the mean 0.88 μ m ST angle within a tile. For comparison, we projected the 3D dMRI principle eigenvector at the same spatial resolution onto the 2D plane (**Figure 2**).

The vector fields of the 75 μ m weighted structure tensor permitted tractography based on histological image properties within areas equivalent to the MRI voxels. We ran identical tractography through the Nissl and myelin ST vector fields. We used a MATLAB package “even streamlines” v 1.1.0.0 to ensure the streamlines were evenly spaced in the horizontal direction⁷⁸, modified to use a fixed integration step of 0.1 . **SI figure 3** shows tractography through the 2D projected components of the first eigenvector of the diffusion tensor, overlaid on high resolution histology images. We note all three modalities yield very similar results.

Cortical Parcellation and Statistical Analysis

To sample the gray matter for analysis, we took advantage of the fact that cortex consists of vertically oriented tissue components^{21,33,36,39} to develop a matrix representation whose columns were derived from data sampled along the vertical dMRI tractography streamlines (**SI Figure 4a**) (see *2D dMRI and Histology ST Tractography* above), and whose rows spanned the horizontal direction. We first masked the registered gray matter ROI by thresholding the MT image (M_{sat}), and eroding this mask by 2 75 μ m pixels to avoid the regions where the diffusion tensor orientations were known not to be vertical (see **Figure 2**). We then ran tractography through the 2d projected principle eigenvectors of the diffusion tensor in the masked region. This produced a set of vertical, non-intersecting streamlines (**SI Figure 4a**) which were numbered from in the horizontal direction.

For each histology and MRI parameter map, we sampled a vector of unique pixels whose coordinates intersected one streamline’s coordinates. We then resampled each resulting vector of pixels to a fixed

length using bilinear interpolation, to yield a simple matrix representation of the gray matter (**SI Figure 4a**). This operation was invertible such that data in matrix form could be displayed in image space (**SI Figure 4b**).

Our statistical analysis proceeded by parcellating the matrix representation of the non-linearly registered histology and MRI data. We used parcellation in our analysis partly to separate out regions of potentially differing stain intensity, and partly because different anatomical regions of cortex have different constituent myelo- and cytoarchitectural elements^{21,37} and may therefore exhibit different relationships between histology and dMRI. For each parcel we took the Pearson correlation between sample variables. Examples are shown in **Figure 3**. **SI Figure 4b** shows parcels of matrix columns of different sizes, represented in image space and colored by the correlation coefficient of histology ST anisotropy to dMRI-FA.

Figure 4 and **Figure 6** show the distribution of correlation coefficients using a parcel size of 5 streamlines which is roughly 300µm in horizontal width. **SI Figure 5** shows that the correlation results are stable for different parcel sizes. For **figure 5** we concatenated matrices from all sections and displayed the mean of all rows. **SI figure 9** shows the mean of rows in each individual section.

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Author contributions

C.R. Initiated the study; C.R., F.Q.Y, R.B.M and D.A.L. designed research; F.Q.Y. acquired data; C.R. analysed data; D.A.L. and F.Q.Y. refined the analysis strategy; C.R. wrote the initial manuscript draft; D.A.L., F.Q.Y. and R.B.M. edited the manuscript; All authors discussed results.

Competing Interests

The authors declare no competing interests.

Figure Legends

Figure 1. Comparison of dMRI-FA to corresponding myelin histology and MTR MRI data obtained from the same tissue in the right hemisphere of a marmoset monkey brain. a) Sagittal sections of dMRI-FA (top), Myelin histology (center) and MTR MRI (bottom). The spatial variation of dMRI-FA in vertical (laminar) and horizontal direction showed minimal correspondence with the pattern of myelin intensity, but the variation of the MTR MRI closely mirrored the myelin variation. In some regions of high

myelination, the dMRI-FA was high (red arrow) whereas in others it was low (blue arrow). b) Normalized average laminar profiles of FA, MTR and myelin intensity through the cortical thickness in the brain's left hemisphere, derived from cortical parcellation (see **Methods, Figure 3**). c) Histogram of correlation coefficients between MRI variables and histological myelin intensity over the nine sections of coronal histology used in this study. MTR MRI shows much higher correspondence with myelin intensity than dMRI-FA does.

Figure 2. Properties of directional MRI and histology data. For comparison with MRI data, the weighted structure tensor of the myelin and Nissl histology images was computed over a 150 μ m area (see **Methods**). a) Orientation data derived from Nissl and myelin histology and from dMRI are similar, showing columnar, vertically oriented vectors. The overall pattern of orientations in the gray matter is remarkably similar for the data computed from histology and from dMRI. b) Diffusion MRI anisotropy and histology-derived anisotropy maps. (see **Methods**).

Figure 3. Example correlation of Histology and MRI variables sampled in columnar parcels. Top row: non-linearly co-registered histology parameter maps and MRI at 75 μ m resolution. The red trapezoids indicate a columnar parcel example. Bottom row shows representative scatter plots and Pearson correlation coefficients between histological variables and MRI variables.

Figure 4. The distribution of correlation coefficients between histological parameters and dMRI-FA over all 393 parcels of cortex. Each point in the distribution is the Pearson correlation between the variables sampled in a vertical parcel (see **Methods**). Red dot shows median value, blue line shows mean and vertical gray bars show lower and upper quartiles. a) Correlation of Histological variables to dMRI-FA b) Correlation of MTR to myelin intensity.

Figure 5. Average laminar profiles of FA, Nissl-HA and myelin-HA across all parcels, and the results of a spatial randomization condition to test the regional specificity of parcel-wise histology to MRI correlations. The results show Nissl-HA more closely matches the laminar profile of dMRI-FA, and is more sensitive to shuffling of horizontal MRI locations a) Normalized laminar profiles of dMRI-FA, Myelin-HA and Nissl-HA through the cortical thickness, computed from the average of all columnar parcels (see **Methods**). WGB indicates the white/gray matter boundary, PIA indicates the pial surface (see **Methods, Figure 3**) b) The change in mean correlation coefficient when the cortical parcels of the

histology parameters and those of the dMRI-FA are shuffled between random horizontal locations. Bars indicate standard error. The star indicates significance under a paired t-test.

Figure 6. Distribution of correlation coefficients between dMRI or histological anisotropy and directional diffusivities. Nissl-HA exhibits a profile that is more similar to dMRI-FA than that of myelin-HA. **a)** dMRI-FA correlates to increases vertical diffusivity (AD) and decreases in horizontal diffusivity (RD). **Top Right** Nissl histological anisotropy (Nissl-HA) exhibits modest positive correlation to AD and negative correlation to RD, indicating that Nissl-HA and dMRI are associated with a similar mechanism of diffusion anisotropy. **Bottom Right** myelin histological anisotropy exhibits no mean correlation to vertical diffusivity and very strong correlation to horizontal diffusivity, as predicted from previous studies¹⁸

Figure 7. In granular cortex, layer 4 is identifiable in Nissl histology by many small cell bodies and in myelin histology by a local decrease in stain intensity. In dMRI data layer 4 is associated with a local increase in Axial diffusivity (AD) and in MTR data by a band of low intensity. **a)** dMRI, MTR and histologically derived contrasts. **B) Left** Illustrative detail of the Nissl histology from an ROI of granular cortex. The internal granule layer is visible as a band of small, round cells. **Middle** the same region in myelin histology exhibits a gap in myelination. CLAHE contrast enhancement was applied uniformly across both myelin and Nissl images for display **Right** The highly branched, unmyelinated layer 4 terminals of axons from 8 thalamic afferent cells in rat Barrel cortex, reproduced from⁴⁹.