Neurovascular coupling and oxygenation are decreased in hippocampus compared to neocortex because of microvascular differences

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The hippocampus is essential for spatial and episodic memory but is damaged early in Alzheimer’s disease and is very sensitive to hypoxia. Understanding how it regulates its oxygen supply is therefore key for designing interventions to preserve its function. However, studies of neurovascular function in the hippocampus in vivo have been limited by its relative inaccessibility. Here we compared hippocampal and visual cortical neurovascular function in awake mice, using two photon imaging of individual neurons and vessels and measures of regional blood flow and haemoglobin oxygenation. We show that blood flow, blood oxygenation and neurovascular coupling were decreased in the hippocampus compared to neocortex, because of differences in both the vascular network and pericyte and endothelial cell function. Modelling oxygen diffusion indicates that these features of the hippocampal vasculature may restrict oxygen availability and could explain its sensitivity to damage during neurological conditions, including Alzheimer’s disease, where the brain’s energy supply is decreased.
Brain processes that depend on the hippocampus (HC) become dysfunctional early in several disease states, including Alzheimer’s disease and vascular dementia, as well as during aging and after lifestyle changes like the adoption of a high fat diet\textsuperscript{1–3}. Changes in vascular structure and function are often early features of these conditions\textsuperscript{2}. In Alzheimer’s disease, blood–brain barrier dysfunction and inadequate cerebral perfusion promote A\textsubscript{β} accumulation and neurofibrillary tangles\textsuperscript{4,5}, and patients with mild cognitive impairment show lower local blood volume in the HC\textsuperscript{2}. Indeed, reduced hippocampal perfusion in the absence of other pathology is itself associated with impaired cognition\textsuperscript{7}, and CA1 pyramidal cells are particularly susceptible to death after a hypoxic insult\textsuperscript{8}. Thus, maintenance of an adequate oxygen supply may be particularly vital for the HC.

Physiologically, the brain finely regulates its oxygen supply by a process called neurovascular coupling whereby active neurons signal to dilate local blood vessels, increasing blood flow and the supply of oxygen and glucose to these active brain regions. In the neocortex, neurovascular coupling produces an increase in blood flow that more than compensates for the increase in oxygen consumed by active neurons, generating an overall increase in blood oxygenation in the active brain region. The associated decrease in levels of deoxygenated haemoglobin underlies the positive blood oxygen level-dependent (BOLD) signal. In the neocortex, this increase in BOLD correlates with neuronal activity, enabling it to be used as a surrogate measure of brain activation in functional magnetic resonance imaging (fMRI) studies. In the HC, however, the BOLD signal does not seem to reliably correlate with neuronal activity\textsuperscript{9}, as local field potential changes occur without measurable BOLD signals\textsuperscript{10}. This not only suggests that fMRI studies measuring hippocampal BOLD are less-sensitive than cortex to increases in neuronal activity, but also that the HC may be physiologically less able to increase its oxygen supply, and this may underlie its sensitivity to damage at the onset of pathophysiological conditions.

However, because the HC lies beneath the neocortex, and is, therefore, less accessible, previous studies have not been able to directly measure neurovascular coupling in this region in vivo, and therefore no direct evidence exists whether the HC is indeed less able to regulate its energy supply. By removing the overlying cortex\textsuperscript{11}, we could implant a cranial window over the HC and record neuronal and vascular activity using two-photon imaging, laser doppler flowmetry, and haemoglobin spectroscopy to compare neurovascular coupling in dorsal CA1 to that in the visual cortex (V1). This cranial window allowed us to record from different layers across both regions, which in the V1 comprised of layers I–IV (Fig. 1a), and in CA1 of stratum oriens, stratum pyramidale, stratum radiatum and stratum lacunosum–moleculare (Fig. 1b). In V1, neuronal cell bodies are more dispersed throughout the layers, although slightly more concentrated in layer IV, whereas in CA1 the cell bodies are densely packed in stratum pyramidale and send their long, apical dendrites into stratum radiatum. We studied both resting haemodynamics and neurovascular coupling in HC and cortex, as both factors are likely to be important for maintaining neuronal and cognitive health. As discussed above, decreases in overall perfusion have been associated with brain damage\textsuperscript{2,7}, whereas decreases in neuronal activity-driven vessel dilations correlated with reduced oxygenation and preceded cell death in neocortex\textsuperscript{12}.

We found that the HC had lower resting blood flow and blood oxygenation compared with V1, which was due both to a lower capillary density and reduced RBC velocity and flux in individual capillaries than in V1. We then studied how well blood vessels could respond to increases in local excitatory neuronal activity and found that not only did blood vessels in the HC dilate less frequently to local increases in activity than in V1, but when responses did occur, the dilations were smaller. Pericyte morphology and vascular expression of proteins that mediate dilation were significantly different in the hippocampal capillary bed compared with cortex, suggesting that the hippocampal vasculature is less able to dilate to increases in neuronal activity. To understand how these vascular differences impact neuronal oxygen availability, we modelled oxygen diffusion from vessels. Our results indicated that oxygen becomes limiting for ATP synthesis in tissue furthest from blood vessels in the HC, but not the V1. We propose that this decreased neurovascular function in the HC contributes to its vulnerability to damage in disease states.

**Results**

We measured neurovascular coupling in the dorsal CA1 region of the HC and in V1 of head-fixed mice expressing GCaMP6f in excitatory (glutamatergic) neurons\textsuperscript{13}. Mice could run on a running cylinder or remain stationary, whereas visual stimuli (drifting gratings or a virtual reality environment) or a black screen were presented (Methods, Fig. 1c–f). Throughout, resting baseline measurements refer to those taken when the animal is immobile and in the dark. In some experiments, combined laser doppler flowmetry/haemoglobin spectroscopy was used to record levels of deoxy-Hb and oxyhaemoglobin (HbO) and cerebral blood flow (CBF) (oxy-CBF probe; Fig. 1g), allowing us to calculate regional blood oxygen saturation (SO\textsubscript{2}) and the cerebral metabolic rate of oxygen consumption (CMRO\textsubscript{2}; Fig. 1g, h). In other experiments, individual neurons and blood vessels were imaged using two-photon microscopy.

**Blood flow and oxygenation are lower at rest in HC than V1.** We first compared haemodynamics in HC and V1 in the absence of visual stimulation when the mouse was stationary. CMRO\textsubscript{2}, reflecting energy use by summed neuronal activity, was not different between regions (Fig. 2a). However, despite similar energy demands, the resting CBF and SO\textsubscript{2} were significantly lower in HC (Fig. 2b, c). In part, these differences in net blood flow and oxygenation arise from a lower capillary density in HC than cortex (Fig. 2d, e\textsuperscript{14,15}). However, when we measured red blood cell (RBC) flux, haematocrit and RBC velocity in individual blood vessels, after loading them with fluorescent dextran (Fig. 2g), we found that, despite the sampled vessels themselves being of equal size (Fig. 2f), RBC velocity and haematocrit were also significantly lower in the HC than V1 (Fig. 2h–j). This combination of a lower capillary density and RBC velocity and flux in the HC explains both the observed lower net flow and, because fractionally more oxygen is extracted from capillaries with lower flow rates\textsuperscript{16}, the lower blood oxygenation we measured in HC.

**Blood vessels in CA1 dilate less to local neuronal activity than those in V1.** Because fMRI studies suggest neuronal activity might be less well-matched to blood flow in HC than cortex, we investigated the capacity of blood vessels to respond to local excitatory neuronal calcium events in vivo by capturing movies of neurons and nearby blood vessels (including arterioles, pre-capillary arterioles and capillaries) in HC and V1 using two photon microscopy (Fig. 3). Calcium events occurred across environmental conditions (i.e., in the dark or when stimuli were presented on the screens, and when the mouse was at rest or running). In both regions, vessels dilated shortly after neuronal calcium events (Fig. 3d, e, g, h), however, the frequency and size of dilations were significantly greater in V1 than in HC (Fig. 3c, e, h), whereas the average size of calcium peaks was larger in HC (Fig. 3d, g). This suggests that, relative to the amount of neuronal activity, hippocampal vessels dilate less than those in V1,
supplying less oxygen. Most of our dilations are related to calcium events and not due to random vasomotion because when traces were shuffled so that vessel dilation data were no longer aligned to calcium events, dilations occurred less frequently (V1: 5.2%, HC: 5.7%; Fig. 3f, i). Even when vessels did dilate in HC, responses were smaller than in V1, despite larger triggering calcium events (Fig. 3j–m). Therefore, smaller dilations across all vessels in HC were due to both a decrease in responsiveness of vessels, and to dilations being smaller in HC when they did occur.

We next looked at vessels with diameters <7 µm to explore the effect of neuronal calcium activity specifically on capillaries. The smaller dilations observed in HC corresponded to smaller increases in RBC velocity in HC capillaries following local calcium activation, as assessed using fast line scanning of capillaries and nearby neuronal soma (Supplementary Figure 1), though RBC velocity was equally likely to increase in the two regions. Whilst these same HC capillaries captured by fast line scanning were less likely to dilate than V1 capillaries, if they did dilate, their responses were the same size in both regions. When we split our xy movie data in Fig. 3 into vessels smaller and larger than 7 µm, we confirmed that both groups of vessels were less likely to dilate in HC than V1. When they did dilate, vessels larger than 7 µm also had smaller dilations in HC, whilst dilations in smaller vessels were the same size in both regions (Supplementary Figure 2). Dilations of HC vessels larger than 7 µm were 51% of those in V1, however, so similarly scaled responses in smaller HC vessels would be undetectable (and classed as non-responders) as they would be <60 nm, or only 0.5 SDs above baseline.

To characterise the relationship between each individual calcium event and corresponding vessel response, we calculated a neurovascular coupling index (NVCindex) by dividing responding vessel diameter peaks by the corresponding neuronal calcium peak, so a large NVCindex represents a large dilation in response to a given change in calcium. NVCindex was significantly higher in V1 than HC (Fig. 3n), suggesting that the hippocampal vasculature increases blood flow less in response to increased oxygen use compared with cortex. The reduced matching of blood supply to changing energetic demands in the HC could be due to a decrease in the instruction from the neurons to tell blood vessels to dilate, or a decreased ability of HC blood vessels to respond to this signal.

HC neurons do not express lower synthetic enzymes for vasodilatory signalling pathways. HC neurons could be less able to instruct the vasculature to dilate if HC neurons and astrocytes are less able to produce vasodilatory messengers than similar cells in V1. We tested for differences in expression levels of vasodilatory second messenger pathways in neurons and astrocytes between regions, using an open access single-cell RNA-Seq data set from neocortex (S1) and HC (CA1)17. There were no differences in levels of mRNA transcripts in individual pyramidal cells,
interneurons and astrocytes of the synthetic enzymes for prostaglandins, epoxyeicosatrienoic acids (EETs) and nitric oxide (Supplementary Tables 1–3), suggesting that cells in HC are as capable of producing these vasoactive molecules as those in cortex. In fact, there were significantly higher levels of Nos1 (Fig. 4a) and Ptges3 (Fig. 4b) expression in HC pyramidal neurons, and of Ptges3 (Fig. 4c) in HC astrocytes, which might predict that, if anything, the production of vasodilatory molecules (prostaglandin and nitric oxide) would be greater in HC than V1.

**HC neurovascular coupling is not lower because neuronal firing is less synchronous.** Alternatively, HC neurons could be less effective at signalling to blood vessels if their firing is less synchronous than in V1, so that levels of dilatory second messengers (e.g., nitric oxide or prostaglandin) summate less, meaning their concentration and potential effect on the vasculature is reduced. Indeed, coding in HC is sparser and distributed, whereas that in V1 is retinotopic, so it might be predicted that neuronal firing in HC would, indeed, be less synchronised. We tested this by imaging across a wide field-of-view to capture large numbers of excitationary cells (Fig. 5a). We tested for synchronous firing in two ways: (1) by investigating whether the cells fired at the same time, using cross-correlation of the activity trace from each cell with that of each other cell (Fig. 5c), and (2) by measuring whether peaks in the average population calcium trace across all cells (i.e., bursts of synchronous activity) were larger in V1 than in HC (Fig. 5b, d). There were no significant differences in either the correlation of firing, or the average size of population calcium peaks between the regions.

An alternative measure of net neuronal activity is provided by the CMRO$_2$ signal. We detected peaks in CMRO$_2$, alongside corresponding regional increases in cerebral blood volume due to vascular dilation (reflected in the total blood volume, Hbt; Fig. 5e). The sizes of the peaks in the CMRO$_2$ signal (Fig. 5f, h) and the associated cerebral blood volume changes (Fig. 5g, i) were both smaller in HC than V1, but in HC, these blood volume increases were smaller relative to the change in CMRO$_2$ (Fig. 5j; NVC$_\text{index}$ = CMRO$_2$/Hbt). Thus, measurements of single vessels
**Fig. 3 Vessel responses to local neuronal calcium events.**

a Texas Red dextran-filled vessel (red) and GCaMP6f-positive pyramidal neurons (green) from one recording in V1 before (top) and during (bottom) increases in neuronal calcium (to represent the 87 vessels with local neuronal calcium imaged). Yellow outlines show vessel before calcium event. Arrows indicate the largest dilation. Scale bars represent 5 μm.

b Neuronal calcium averaged over all cells in the field-of-view of one imaging session and the corresponding vessel diameter. Red dots mark calcium events.

c Vessel responses to preceding calcium events were more frequent in V1 than HC (Chi-square test).

d Average calcium response and e diameter change in HC (purple, N = 779 trials, 46 vessels, 6 animals) and V1 (orange, N = 1238 trials, 41 vessels, 7 animals).

f Diameters when vessel traces were shuffled 100 times so no longer aligned to calcium events (HC N = 77900 events, V1 N = 123800).

g The calcium events were larger in HC compared with V1.

h Vessel dilations were larger in V1 than HC when aligned to calcium events, but not when shuffled (statistical comparisons were made on individual calcium/dilation/shuffled dilation events, N specified in d/e & f).

i Calcium events that led to dilations in HC (N = 120 events, six mice) and V1 (N = 313 events, seven mice).

k Corresponding diameter changes.

l Calcium peaks leading to dilations were higher in HC (N = 120, V1 N = 313).

m Diameter peaks were significantly larger in V1 than HC.

n A neurovascular coupling index (NVCindex) was calculated by dividing each dilation peak by its corresponding calcium peak. NVCindex was lower in HC than V1. P values are from Mann–Whitney U tests, unless stated (see Statistics Report Tables SR2a–b). Averages and shaded error bands show mean ± SEM. Horizontal lines on violin plots show median and interquartile range. Source data are provided as a Source Data file.
and summed regional responses both suggest weaker neurovascular coupling in HC.

We wondered whether the cellular or laminar organisation of HC and V1 could explain the different neurovascular coupling properties, perhaps if second messengers released from different neuronal compartments have differential effects on the vasculature. To this end, we tested whether neurovascular coupling was different in vessels in response to calcium signals in the neuropil (NP) or nearby somas (Supplementary Figure 3). We found that, although vessels in V1 were more likely to respond to calcium signals from the NP, vessel responsiveness in HC did not distinguish between the different neuronal compartments. The NVCindices between the different neuronal compartments were no different within each region, with a significantly greater NVCindex in V1 than HC for both NP and soma. We also investigated whether there were laminar differences in neurovascular coupling that could explain our results. We found some differences in responses between layers in both regions (Supplementary Figure 4), however across all layers, the NVCindex was significantly greater in V1 than HC.

**HC neurovascular coupling is not lower because of vascular deficits arising from the surgical preparation.** The surgery to create a cranial window over CA1 is more invasive than for V1, because it requires aspiration of some of the overlying cortex and the implantation of a cannula with glass overslip. We tested for vascular network damage in CA1 in the surgical versus non-surgical hemisphere. We found that the aspiration and cannula implantation did not cause significant compression to dorsal CA1, nor alter the overall vascular density of the region (Supplementary Figure 5). Furthermore, signs of inflammation were limited to tissue <100 µm from the window and did not affect the responses recorded (Supplementary Fig 6).

**Pericytes have a less-contractile morphology in HC than V1.** In the absence of clear differences in neuronal firing properties or expression of neurovascular coupling signalling molecules, we next investigated whether our results could reflect differences in vascular structure or function. The architecture of the vasculature is well established in neocortex, where pial vessels run along the brain’s surface before penetrating the tissue and branching into a dense capillary network (e.g., Figure 6a). The hippocampal vascular network is less well characterised, but is known to be inverted compared with that in neocortex, with large arteries and veins emerging in the hippocampal sulcus and sending their arch-like branches up into CA119. Our in vivo recordings confirm this vascular organisation (Fig. 6a–b, Supplementary Figure 7) with the large (>15 µm) diameter perfusing vessels located ~300 µm below the dorsal surface of layer SO. Our imaging depth was on average 70 µm (range: 1–308 µm), so the vessels we sampled in HC were generally further from their source (HC vessels averaged 227 µm from their source, versus 135 µm in V1), but the distance to the perfusion source did not alter resting RBC velocity or calcium-dependent blood vessel dilations in HC (Supplementary Figure 7). In V1, the distance from the pial arteries did not affect the size of dilations, but RBC velocity was higher in vessels nearer the source arteries (Supplementary Figure 7).

Next, we examined microvascular anatomy for differences that could help explain the functional deficits in NVC in HC. Vascular anatomy in neocortex is often described in reference to branching order from either the pia or the penetrating arteriole, but this is not possible in HC, due both to the less-stereotyped anatomy of the hippocampal vasculature, and the depth at which the largest vessels occurred (sometimes below our imaging plane). Instead, we compared vessels with similar vascular mural cell morphologies (from NG2-DsRed labelling of vascular mural smooth muscle cells (SMCs) and pericytes). The morphology of vascular mural cells is linked to their ability to constrict and dilate blood vessels18. Broadly, more contractile cells are shorter in length, with a denser spacing of cells along vessel walls, and more circumferential processes that cover the vessel more completely.

To better understand these morphological differences, we categorised mural cells as being SMCs, or ensheathing, mesh or thin-strand pericytes, based on the morphology of their processes (Fig. 6i)18. SMCs have a banded morphology, whereas pericytes have distinct soma and processes, with a morphology that changes along the vascular tree19. Ensheathing pericytes express αSMA and are strongly contractile with a shorter length and higher vessel coverage than the less-contractile mesh and thin-strand pericytes, which are sited on smaller vessels18.

We first studied the number of main arteries and veins, and the length and diameters of arterioles, pre-capillaries and venule branches in imaging stacks recorded in vivo. Arterioles were defined as having a continuous layer of banded SMCs surrounding the vessel, pre-capillaries as being covered in ensheathing pericytes18, and venules as large vessels proceeding from the mid-capillary bed and lacking continuous SMCs or substantial pericyte coverage. The artery/vein ratio was no different in HC and V1 (0.69 ± 0.25 in V1 and 0.53 ± 0.1 in HC, t test, p = 0.53, Supplementary Table 7). We measured the length of arteriole and venule branches, and of the pre-capillaries, as well as vessel diameters. The lengths and initial diameters of arterioles, venules and precapillary arterioles were equivalent in HC and V1 (Fig. 6d, f, g), but at the transition between arteriole and precapillary (the boundary between the last SMC and the first ensheathing pericyte), vessel diameters were significantly smaller in HC than
CMRO2 peaks was larger in V1 than HC, and HC (left, 1 of 10 independent recordings) and V1 (right, 1 of 19 independent recordings). The spacing of pericytes across pre-capillaries and capillaries (intersoma distance: ISD) was also greater in HC than V1 (Fig. 6h).

We were better able to trace the origin of feeder arterioles deep in the HC using in vivo image stacks than confocal images of brain slices, but our resolution (when imaging whole stacks) using published descriptions, as vessel diameter decreased and cell length increased from ensheathing to mesh to thin-strand pericytes (Fig. 6k, l). The diameter of vessels at pericyte locations was not significantly different between regions, but blood vessel diameter was smaller in HC than V1 at thin-strand pericytes on the smallest capillaries, whereas pericyte processes were longer for both mesh and thin-strand capillaries in HC than V1. Because pericyte contractility is strongest nearer the cell body12,21, HC pericytes may be less contractile than their counterparts in V1, which could underlie the weaker neurovascular coupling in HC.

Functional differences between vascular cells in HC and V1 suggested by different mRNA expression profiles. In order to test for more general functional differences between vascular cells, we examined the mRNA expression profile of mural (n =...
Fig. 6 Vascular morphology across brain regions. a Example in vivo Z-stacks of vasculature in V1 (left) and HC (CA1, right, as used in b). b Left panel: all data points for vessel diameters by depth from the pia in V1 (48458 vessels collapsed from nine stacks/nine animals, orange) or layer SO in HC (21175 vessels collapsed from 8 stacks/6 animals, purple). Right panel: histogram of average ±SEM of these vessel diameters by depth. The overlaying lines are a smoothed trace of the average diameter per bin. c Example arteriole (left) and venule (right) from an in vivo Z-stack of CA1 in a NG2-DsRed mouse with FITC dextran-filled vessels (representative image from N = 3 stacks from different mice per region). Scale bars represent 100 μm. The termination point of an arteriole was the final smooth muscle cell before an ensheathing pericyte (blue arrow). Venule termination points were the final branch before the capillary bed, identified by the presence of distinct pericytes. Dots represent individual vessels (N = 3 stacks from different mice per region). The diameter and length of d arteriole branches were not different between HC (purple, N = 24) and V1 (orange, N = 25). The diameter of the vessel at the final SMC was significantly smaller in HC (N = 7) vs V1 (N = 8), despite similar vessel lengths. f The precapillary arterioles (with ensheathing pericytes, HC N = 10, V1 N = 9) and g venule branches (HC N = 16, V1 N = 12) showed no differences in length or diameter between regions. h Intersomata distances (ISD) between neighbouring pericytes (taken from fixed tissue) were longer in HC than V1 (89 vessels in HC and 127 in V1, from six mice). Confocal stacks of FITC-gel-filled vessels of NG2-DsRed mice were taken in HC and V1 from six mice. i Examples of ensheathing (EP), mesh (MP) and thin-strand (TSP) pericytes. Scale bars represent 5 μm. j The distribution of cell types was not different across regions (Cochran–Mantel–Haenszel 3D variant of Chi-square test). Numbers inside bars are numbers of vessels. k There were regional differences in vessel diameter, although post hoc comparisons revealed that these were specific to TSP locations in HC. l Ensheathing pericyte lengths were similar between regions, but both mid-capillary pericyte categories were longer in HC than V1. P values are from one-way ANOVAs with Welch’s correction to test for effects of cell type and brain region. P values above the bars are from unpaired t tests, Mann–Whitney U tests or post hoc Mann–Whitney U tests with Holm–Bonferroni correction for multiple comparisons (see Statistics Report Tables SR4a–e). Bar charts are mean ± SEM, and statistical comparisons for d–h & k–l compared data from the individual vessels represented by the dots. Horizontal lines on violin plots show median and interquartile range. Source data are provided as a Source Data file.

83, 20 in HC) and endothelial (n = 137, 10 in HC) cells using the same single-cell mRNA data set as above15 across three broad categories associated with neurovascular function: contractile machinery, ion (potassium and calcium) channels and neurovascular signalling pathways (Supplementary Tables 4 and 5). The latter category included recently identified EET and 20-HETE receptors22,23 and, given the recent finding that endothelial NMDA receptors can control vascular tone24, NMDA receptor subunits.

Several genes showed differential expression between V1 and HC, all of which pointed to the vasculature in V1 being more contractile or responsive than in HC (Supplementary Figure 8; Supplementary Tables 4 and 5). Mural cells showed higher expression in V1 of the calcium channel beta subunit Cacnb4,
whereas levels of transcripts for several other ion channel subunits were significantly higher in V1 before correction for multiple comparisons, including stargazin (Cacng2, which can reduce calcium channel activation)\(^2\), a slowly activating potassium channel (Kcnh3), and contractile proteins such as the skeletal muscle actin (Acta1) and regulators of myosin light chain phosphatase (Ppp1r12c) (Supplementary Table 4, Supplementary Figures 8a–e). In endothelial cells, several more transcripts were upregulated in V1 compared with HC (Supplementary Table 5, Supplementary Figures 8f–i), most notably the inwardly rectifying potassium channel Kir2.1 (Kcnj2), which has been shown to mediate the propagation of dilation from the capillary bed to upstream arterioles\(^2\), the NMDA receptor subunit Grin2c, the NO receptor Gcy1a2 and prostaglandin E synthase (Ptgs). These transcripts would all be expected to promote dilation of the microvasculature, and their lower expression in HC than V1 may mediate the weaker neurovascular function we observed physiologically. mRNA extraction from HC was not simply lower across all genes, as there was no difference between HC and V1 in the average number of mRNA molecules detected per cell across all genes investigated (Supplementary Table 6).

Thus, pericytes have a less-contractile morphology in HC than V1, whereas HC mural and endothelial cells are less equipped to promote vasodilation via regulation of contractility, glutamate or NO sensing, prostaglandin production or by activation of potassium channels.

**Lower oxygenation in HC may limit function.** To understand how weaker HC neurovascular functioning could affect neuronal oxygen supply, and thus ATP production, we modelled oxygen diffusion and consumption in HC and V1 (Fig. 7a). We first worked out how far brain tissue was, overall, from the nearest blood vessels in vivo (Fig. 7b, c). We then calculated the steady-state oxygen concentration in an average capillary in each region. Because the oxygen level between rather than within RBCs better reflects tissue oxygen levels\(^2\), we used the pO\(_2\) between two RBCs as our estimate of capillary pO\(_2\) (see Methods). InterRBC pO\(_2\) was 15 mmHg (21 μM) in V1 and 10 mmHg (14 μM) in HC. Oxygen diffusion into the tissue was then simulated (Fig. 7d), assuming varying rates of neuronal oxygen consumption corresponding to values reported in rodent tissue\(^2\). Because in some tissue, the oxygen gradient between the interRBC pO\(_2\) and the tissue is flat (within measurement error), and equals the interRBC pO\(_2\), we also ran simulations with a slightly higher capillary pO\(_2\) (18 μM in HC, 22 μM in V1), which yielded the predicted interRBC pO\(_2\) at the median distance between two capillaries (Supplementary Figure 9). This allows for some O\(_2\) delivery directly from RBCs themselves and is still consistent with published tissue O\(_2\) gradients.

The combination of the lower capillary oxygen concentration ([O\(_2\)]\(_{\text{cap}}\)) and capillary density meant tissue oxygen levels in HC were lower than in V1 for all conditions simulated (Fig. 7e, f, h, i, k, m). To determine whether oxygen became limiting for ATP production in the tissue, we then calculated the oxygen consumption rate (VO\(_2\)) as a proportion of the maximum rate of oxygen consumption (V\(_{\text{max}}\)) (Fig. 7g, j, l, m). In V1, VO\(_2\) (and therefore the rate of ATP generation) occurred at over 90% of the V\(_{\text{max}}\) even far from a capillary, and at the upper range of V\(_{\text{maxes}}\) tested, suggesting [O\(_2\)] barely limited ATP synthesis. In HC, however, whereas VO\(_2\) was sustained at over 90% of the V\(_{\text{max}}\) in tissue at the median distance from a capillary, in the tissue furthest (95th centile) from a capillary, [O\(_2\)] dropped to concentrations that limited VO\(_2\) even at low oxygen consumption rates (≥1 mM/min). In our simulations with a larger capillary pO\(_2\), the decrease in O\(_2\) was slightly smaller, but still limited VO\(_2\) far from the capillary to 70% of V\(_{\text{max}}\) when V\(_{\text{max}}\) = 2 mM/min (Supplementary Figure 9, vs. 60% of V\(_{\text{max}}\) with capillary pO\(_2\) fixed at interRBC pO\(_2\), Fig. 7l). Thus, our results suggest that HC ATP production through oxidative phosphorylation is restricted in tissue furthest from a capillary.

The effect of weaker HC neurovascular coupling can be estimated by considering what happens when oxygen consumption increases but capillary oxygenation does not. Typically, neuronal activation increases net CMRO\(_2\) by 20–60%\(^2\). In HC, increasing the V\(_{\text{max}}\) for oxygen consumption from 2–3 mM/min, caused [O\(_2\)] in tissue furthest from vessels to almost halve, reducing VO\(_2\) from 59% to 31% of V\(_{\text{max}}\) (Fig. 7k, l), whereas in V1, VO\(_2\) reduced only from 93% to 90%. Thus, the decreased SO\(_2\) and increased capillary spacing in HC reduce tissue [O\(_2\)], limiting oxidative phosphorylation and ATP synthesis in the tissue furthest from a capillary, and this effect is exacerbated by weaker HC neurovascular coupling.

To estimate the impact of these levels of O\(_2\) on tissue respiration (and by extension, function), for a V\(_{\text{max}}\) of 2 mM/min (in the centre of the range of published values\(^2\)), we estimated how much of the tissue is subject to rate-limiting [O\(_2\)] by calculating profiles for [O\(_2\)] and VO\(_2\) when capillaries were separated by intermediate distances between the median and 95th centile values used above (Fig. 7m). This analysis suggested that low [O\(_2\)] inhibits VO\(_2\) by at least 10% in 30% of HC tissue, and by at least 20% in 10% of HC tissue.

**Discussion**

Our data reveal, for the first time, the properties of neurovascular coupling in mouse HC in vivo and demonstrate that hippocampal vascular function is different from that in neocortex in two major ways. First, despite equivalent resting levels of oxygen consumption in HC compared with the neocortex, resting blood flow in the HC is lower than in neocortex, due both to a lower vascular density and lower RBC velocity and flux in individual capillaries. This lower energy supply leads the HC to have a lower resting blood oxygen saturation than neocortex. A simple model predicts that lower blood oxygenation and vascular density in the HC drive tissue oxygen to levels that readily become limiting for ATP generation. Second, increases in neuronal activity in the HC cause fewer and smaller dilations of local blood vessels and a smaller increase in overall blood volume, suggesting energy supply in the HC is less well-matched to fluctuations in energy demand than in neocortex. Our data suggest that neurovascular coupling is weaker in the HC because of differences in its vasculature rather than neuronal signalling properties, its pericytes having a less-contractile morphology and its vasculature showing a pattern of mRNA expression that may be less able to promote and propagate dilation. Our model predicts that these differences in vascular physiology matter for sustaining neuronal function: the lower oxygenation and vascular density in HC interact with the decreased ability to match increased oxygen use with increases in supply. This limits tissue oxygenation in the furthest regions from blood vessels so much that ATP generation, and therefore neuronal function, are more much more readily restricted in HC compared with V1.

**Lower vasodilatory capacity of hippocampal vasculature limits neurovascular coupling.** Our conclusion that differences in microvascular function between HC and V1 underlie the weaker neurovascular coupling observed in HC stems from the observation that neuronal firing and neuronal and astrocytic expression of vasoactive messengers were no different between the brain areas, whereas differences were observed in the anatomy and expression profile of key proteins of the microvasculature.
First, end-arterioles and mid-capillaries were smaller in diameter in HC than V1, suggesting the microvasculature may be more resistant to flow in HC than V1, contributing to the decrease in net CBF and lower RBC velocity observed. The flow characteristics of the two vascular beds were also different. In V1, RBC velocity was faster nearer the arterial source, consistent with a shorter, lower resistance path being taken by RBCs passing through superficial neocortical layers. HC flow did not show this dependence on distance from the perfusion source, suggesting the absence of these shorter, lower resistance paths in HC.

Ensheathing pericytes initiate the dilatory response to sensory stimulation in the olfactory bulb, and are among the earliest to respond in the neocortex. We found no regional morphological differences in ensheathing pericytes, but vessels they likely covered (>7 μm in diameter) dilated less frequently and when they did respond, dilations were smaller. Capillary (mesh and thin-strand) pericytes were longer in HC than V1, suggesting...
functional differences that may include lower contractility, because pericyte contractility is greatest near the soma\(^3\), and because the small capillaries where these mid-capillary pericytes are located dilate less frequently in the HC.

Dilations of these small capillaries are observed in several\(^2\), but not all studies\(^3\), and whether they are active or passive remains controversial\(^1\). Nevertheless, these dilations seem important. First, at least some of these mid-capillary pericytes express the contractile protein αSMA (though in a form that is less stable than in SMCs and ensheathing pericytes\(^3\)). Second, their level of intracellular calcium decreases in response to neuronal activity (consistent with a relaxation of contractile machinery\(^3\)). Finally, their dilations in olfactory bulb and neocortex seem to mediate a large proportion of the overall increase in blood flow\(^2\).

Dilation signals propagate upstream up the vascular network\(^2\), such that dilations in upstream vessels presumably reflect summed activity in downstream vessels. Our analyses of published single-cell RNA-Seq data\(^1\) supported vascular functional differences between HC and V1 including a lower hippocampal expression of Kir2.1, which is critical for upstream propagation of dilation signals\(^9\), as well as several other ion channels and neurovascular enzymes that can modulate dilation. Therefore, our data showing smaller and less-frequent dilations in larger capillaries and small arterioles in HC compared with V1 may be in part due to the decreased frequency of mid-capillary dilations (producing fewer signals summing to drive upstream dilation), a failure of upstream propagation of dilation through endothelial cells, and possibly also reduced capability for dilation in the ensheathing pericytes on these larger vessels.

mRNA data show consistent reduced vascular function in HC compared with V1. The mRNA data set comprises many more neurons and astrocytes than vascular cells, and more cortical than hippocampal cells. Thus, our power to detect differences at \(p = 0.05\) in neuronal or astrocytic expression patterns was high (>88% with an effect size of 0.4), but much lower for endothelial (33%) or mural cells (47%). Our findings should therefore be treated with some caution and should be replicated in a dedicated experiment with larger sample sizes to verify all identified targets. Nevertheless, because all the positive results indicated a lack of expression in HC of proteins expected to promote dilation, despite similar levels of mRNA transcripts being detected overall in the two regions, we conclude that there is a difference in physiology between the two vascular beds.

Regional differences in neurovascular coupling are not caused by the cranial window. Our use of a chronic cranial window over HC allows us to measure CBF, blood oxygenation and individual vascular and neuronal signals in a region that is normally inaccessible to two-photon imaging. It requires aspiration of a column of neocortex and insertion of a cannula through which dorsal CA1 can be imaged. We have tested whether surgery affected HC function or neurovascular coupling properties (Supplementary Figure 6), or altered the structure and size of CA1 and its vascular network (Supplementary Figure 5). There was a higher expression of inflammatory markers (GFAP and Iba1) near to the cranial window in HC than in V1, but this difference disappeared 100 μm from the window. There was no difference in neurovascular coupling properties in vessels above and below 100 μm, and spatial memory was unimpaired in HC mice. The vascular density was not compromised in the surgical hemisphere, and the overall size of CA1 was also equivalent between surgical and control hemispheres. We, therefore, find no evidence that the more invasive HC cranial windows are driving the regional differences we observe.

Low tissue oxygenation and weak neurovascular coupling: a perfect storm underlining HC vulnerability to disease. Because the vascular density in HC is lower, each capillary supplies oxygen to a larger volume of tissue than in V1. This means more oxygen has to be extracted from each capillary to maintain the same rate of tissue oxygen consumption in HC compared with neocortex. This increase in oxygen extraction could come from an increased supply of oxygen to HC (by an increased flux of RBCs in individual HC capillaries), or by extracting more oxygen from each RBC. An increased flux would better maintain oxygen levels in the blood, but our data suggest that in fact oxygen consumption is sustained by increasing oxygen extraction from individual slower-moving RBCs, decreasing blood oxygen saturation in HC.

To our knowledge, this is the first time hippocampal and neocortical vascular oxygenation have been directly compared, and the first time hippocampal oxygenation has been measured in a sealed skull (so the tissue is not exposed to atmospheric oxygen) in awake animals. Our estimates of capillary \([\text{O}_2]\) are at the lower end of previously observed values from anaesthetised preparations (14 μM in HC and 21 μM in V1, compared with 16 μM in HC\(^8\) and 56 μM in cortex\(^9\)), which is as expected because oxygen consumption rates are higher and \(\text{PO}_2\) values are lower in awake animals\(^10,41\), and are within the range reported in the sensory cortex of awake mice by directly measuring \([\text{O}_2]\) with a phosphorescent probe\(^1\). As we are not able to measure oxygen in individual capillaries, our results reflect average blood oxygen levels. In the neocortex, individual capillary oxygen levels, and corresponding tissue supply radii, vary quite widely, such that upstream vessels with higher oxygen levels can supply oxygen to tissue that is physically closer to capillaries with lower blood oxygenation\(^11\). The impact of this underlying heterogeneity of oxygenation on our results is not clear: our use of an average may be underestimating microvascular \(\text{sO}_2\)\(^12\), but any such error is likely to be smaller in HC than V1, as the lower vascular density will reduce the chance that an upstream vessel’s supply radius will overlap with that of a downstream, less oxygenated capillary. Our model predicts very different tissue \([\text{O}_2]\) in V1 and HC. In V1, \([\text{O}_2]\) gradients are very shallow, and the oxygen consumption rate by oxidative phosphorylation (i.e., the rate of ATP synthesis) is maintained at >90% of the maximum throughout the tissue. However, in HC the lower vascular density and lower oxygenation mean that \([\text{O}_2]\) readily becomes limiting for ATP synthesis for physiological \(V_{\text{max}}\) values (1–3 mM/min\(^9\)). An inhibition in the rate of oxidative phosphorylation of at least 20% likely occurs in around a tenth of HC volume. Furthermore, when oxygen use outstrips supply, oxygen consumption is barely affected in V1 but is reduced to as little as 30% of the \(V_{\text{max}}\) in 5% of HC tissue. Physiologically, HC seems adapted to deal with these conditions: it can sustain neuronal function despite oxygen levels that could be considered hypoxic. Decreases in synaptic function occur in vitro after induction of hypoxic conditions using higher \([\text{O}_2]\) than we estimate to be normoxic for HC (e.g., 20 mmHg/28 μM\(^3\)) compared with <14 μM here) but, in fact, such responses to hypoxia are likely to be caused by the decrease in, rather than the absolute concentration of \(\text{O}_2\)\(^4,16\). But while HC may cope with low \([\text{O}_2]\) physiologically, its lower oxygenation and weaker ability to match oxygen demand to supply may contribute towards its vulnerability to hypoxia and conditions where cerebral blood flow decreases, such as ischaemia and Alzheimer’s disease. Further decreases in oxygen availability caused by these conditions will produce a larger reduction in ATP synthesis over a larger volume of tissue in HC than in neocortex, because it already exists in a state where \(\text{O}_2\) levels are limiting. However, interventions that boost HC oxygenation may therefore prove particularly beneficial. Indeed, a drug that boosts hippocampal blood flow (nifedipine\(^3\)) benefitted a subgroup of patients with mild...
Alzheimer’s disease, suggesting that insufficient oxygen delivery to HC might be a key early factor in Alzheimer’s disease in some individuals, and that improving oxygen delivery may be therapeutic.

Interpreting BOLD. Our results also illuminate why BOLD signals in HC were found to be unreliably coupled to neuronal activity. In HC, smaller and less-frequent vascular dilations in response to local changes in neuronal activity will produce smaller positive BOLD signals. BOLD signals are therefore a less-sensitive measure of neuronal activity in HC than in neocortex. Simple experimental designs that compare the degree of activation across the brain could therefore erroneously conclude lower HC activation than cortex even when activity levels are the same. Analyses that test for specific patterns of activity, such as correlations of voxels with a behavioural measure or cognitive model of interest, will be less affected by the different neurovascular coupling properties in HC and V1, but the relative insensitivity may nevertheless lead to more failures to detect subtle effects in HC than in neocortex.

Our work suggests HC physiological and pathophysiological functioning is shaped (and limited) by its vasculature, an insight that will aid understanding of disease states and human imaging studies. Further work should directly test in which situations hippocampal oxygen availability limits its function and whether boosting oxygen availability by increasing blood flow in these conditions can preserve hippocampal function. This will help guide therapeutic strategies in conditions such as Alzheimer’s disease, where boosting hippocampal blood flow in some, but not all, conditions, can be therapeutically effective.

Methods

Animal procedures. All experimental procedures were conducted in accordance with the 1986 Animal (Scientific Procedures) Act, with the approval of the UK Home Office and the University of Sussex animal welfare ethical review board. All experiments used male and female mice unless stated otherwise. Mice were either wild types (six in total, four males, two females) or expressed GCAMP6f under the control of the Thy1 promoter (C57BL/6J-Tg(Thy1-GCaMP6f) nals in HC were found to be unreliably coupled to neuronal activity by increasing blood flow in these conditions can preserve hippocampal function. This will help guide therapeutic strategies in conditions such as Alzheimer’s disease, where boosting hippocampal blood flow in some, but not all, conditions, can be therapeutically effective.

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fluorescein Dextran dissolved in saline (70 kDa via tail vein, Fisher Scientific). All blood vessels recorded local to neuronal calcium activity were capillaries, presumably post-arteriolar capillary as vessel diameter trace and shrunken vessel diameter trace were classified as being responsive or not. Responsive diameter traces were those where dilations occurred for >0.5 s, within 5 s of the calcium event, that were >1 standard deviation above baseline.

**Behavioural testing:** Animals that had previously undergone HC or V1 surgery for in vivo imaging underwent a simple hippocampal-dependent object location memory task to test spatial memory (Supplementary Figure 6). The mouse was presented with two objects in a training environment for 10 min, before being removed from the environment. After 5 min in the home cage, the mouse was then tested in the same environment, to the objects, and asked to point to a novel location. The time spent exploring both the familiar and novel object locations was assessed by a blinded observer hand-scoring the time per object. Any mice which spent less than total 5 s exploring the test environment were excluded from the analysis.

Ex vivo imaging. Transcardial perfusion and gel-filling of the vasculature: Adult mice were anaesthetised with ketamine/xylazine and transcardial perfused using 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer solution (PBS). In cases where the vasculature was labelled, this was also followed by injection of 0.2% fluorescein isothiocyanate (FITC)- conjugated albumin. Mice were then chilled on ice for at least 30 min, before brains were extracted and fixed in 4% PFA overnight. The brains were subsequently transferred to a 30% sucrose solution in PBS with 0.1% sodium azide for a minimum of 3 days, after which they were sliced (200 μm slices) on a vibratome (Leica), and stored in the fridge (at 4 °C) in PBS with 0.1% sodium azide, before immunohistochemical labelling and slicing.

**Immunohistochemical labelling and slice mounting:** Adult NG2-DsRed mice were used for pericyte morphology and vascular network analyses (Fig. 2 and 6), as pericytes and SMCs were transgenically labelled in red. Vessel type could therefore be identified based on the smooth muscle or pericyte cell morphology. Adult wild-type or NG2-DsRed mice, which had previously undergone CA1 surgery for two-photon imaging, were used for vascular network analysis on the surgical versus the control hemisphere, and adult Thy1-GCaMP6f mice, which had also previously undergone CA1 surgery, were used for the analysis of CA1 area on surgical and control hemispheres (i.e., following cannula insertion/possible compromise, Supplementary Figure 5).

For analysis of inflammation (Supplementary Figure 6) mice that had previously undergone HC or V1 surgery for in vivo imaging were used, and active astocytes were labelled for GFAP and microglia labelled for Iba1 using immunohistochemistry. First, brain slices were washed in 1x PBS whilst being shaken for three cycles (10 min per wash). The slices were then blocked in 5% normal goat serum and 0.3% Triton X-100 in 1x PBS for an hour. Slices were incubated in the relevant primary antibodies (chicken anti-GFAP primary antibody; Abcam, ab4674, 1:500 dilution or rabbit anti-Iba1, WAKO, 019-19741, 1:600 dilution) for 36 h at 4 °C. Slices were washed three times in PBS, before being incubated in the relevant secondary antibody for 24 h at 4 °C. Anti-rabbit and anti-goat antibodies (Abcam, 1:1000 dilution; Abcam, 1:470 anti-rabbit, Abcam, 1:100 dilution). Gel-filled and/or immunohistochemically labelled slices were washed for a final three cycles in PBS, before being mounted onto slides in Vectashield Hardset mounting medium and stored at 4 °C before imaging.

**Vascular imaging:** Imaging was performed on a confocal microscope (Leica SP8, collected using LAS X software) using a ×20 objective (HC PL APO CS2, 20x/0.75, dry). Continuous wave lasers with ~488, ~543 and ~633 nm excitation wavelengths were used for FITC, DsRed and Alexa 647, respectively. Images were collected with dimensions of 1024 by 1024 pixels (with a lateral resolution of 0.57 μm per pixel), averaged 4–6 times per line, and were viewed and analysed in ImageJ software.

**Analysing pericyte and vessel morphology:** For the analysis of pericyte and vessel morphology, images stained with anti-NG2 or anti-α-smooth muscle actin, respectively, were used for measuring the length and surface area of the vasculature. The length of the vessel was measured by using an automated script in MATLAB. In brief, two-photon recordings were binarized. The vessel was skeletonised, meaning its length was eroded to generate a single-pixel vessel was used to calculate the speed at which they travelled over successive 40 ms blocks with them to be counted within a 250 ms time window. The accuracy of the automated code was evaluated. In short, the angle of the shadow cast by the RBCs travelling through the vessel was used to calculate the speed at which they moved through the vessel. The RBC velocity, flux and haematocrit from capillaries, and sometimes the line scan path also passed through a nearby neuron for concurrent calcium readings. Vessels and local calcium traces were identified from each of the distinct layers of CA1 (stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum-moleculare) and V1 (L1, 12/3, L4). The layers in CA1 were clearly distinguishable by the changing morphology of the pyramidal neurons over increasing depths within this region (Fig. 1b). The layers of V1 were distinguished based on the distance of the imaging site from the pial vessels. Two-photon imaging experiments typically lasted 1–3 h.

**Analysis of two-photon microscopy data:** Excitatory calcium activity was first registered in ImageJ to correct motion artefacts, before being extracted using the CellSort package in MATLAB. CellSort identified regions of interest (ROIs) over multiple active individual cells from which soma and from which the same cell was either merged or removed, as appropriate. For all analyses, an ROI was considered to be active if its calcium fluorescence was above the mean value of the baseline. Vessel traces were extracted across the times of these calcium events, or after bootstrapping by shuffling each of the vessel traces over 100 iterations across time using the MATLAB function randperm, so they were no longer aligned to calcium events. For every calcium event, both the corresponding vessel diameter trace and shrunken vessel diameter trace were classified as being responsive or not. Responsive diameter traces were those where dilations occurred for >0.5 s, within 5 s of the calcium event, that were >1 standard deviation above baseline.

ΔF/F = F − Fmin Fmax − Fmin

where Fmin is the baseline fluorescence intensity, and F is the maximum fluorescence intensity.

ΔF/F = F − F0 ab(F0)

where F0 represents the baseline fluorescence intensity.
Analysing the area of CA1 in surgical and non-surgical hemispheres: Wide-field images were taken of the entire brain slice by tile scanning (Supplementary Figure 5). In brief, two pairs of grids were marked around the outer edges of the brain slice, one in the upper left corner and one the lower right corner. A rectangle with multiple grids was created between these two positions, and a motorised stage was used to move the sample and create a tiled scan of the whole slice. 2D images were captured at the focal point for each grid, with blending (statistical and linear) between grid images. An ImageJ plugin was then used to stitch the grid images together in post-processing.

For analysis of the area and aspect ratio of CA1 in surgical and control hemispheres, the freehand tool was used to draw around CA1, and calibrated area and bounding rectangle measurements were generated. The aspect ratio was calculated by dividing the height of the selection by its width, meaning larger values represented a taller/narrower shape, and smaller values a wider/shorter shape.

Single-cell RNA-Seq data. The raw single-cell RNA-Seq data were taken from a freely available online database (http://linnarssonlab.org/cortex/). The authors used quantitative single-cell RNA-Seq to perform a molecular census of cells in somatosensory cortex (S1) and HC (CA1) and categorise cells based on their transcriptome. We compared expression of components of neurovascular signal-transductions, ion channels or contractile machinery in vascular cells (pericytes, SMCs, endothelial cells; Supplementary Figure 8) and vasodilatory signalling pathways, ion channels or contractile machinery in vascular cells (pericytes, SMCs, endothelial cells; Supplementary Figure 8) and vasodilatory signalling pathways in pyramidal cells, interneurons, and astrocytes (Figure 4). The levels of target transcripts in HC and cortex were compared using multiple t tests in RStudio with Holm–Bonferroni correction for multiple comparisons (see Supplementary Tables 1–4).

Modelling oxygen concentrations in tissue. Estimating capillary oxygen concentrations: Our in vivo oxy-CBF probe measurements give us the saturation of oxygen (SO2) in the blood. From SO2, we calculate the partial pressure (pO2) of oxygen in RBCs using the haemoglobin oxygen dissociation curve for C57/BL6 mice (the background of our experimental mice), with a Hill coefficient h of 2.59 and a PSO of 40.2 mmHg64. (4).

\[ \frac{pO_2}{pO_2 + PSO} = \frac{SO_2}{Hb_SO_2} \]  

(4)

This yielded RBC pO2 values of 30 mmHg (42 μM at 37 °C) in HC and 43 mmHg (60 μM) in V1. Micromolar concentrations of oxygen in tissue at 37 °C were calculated from partial pressures using a Henry’s Law constant of 1.3 × 10⁻⁵ mol/(m³ Pa) at standard temperature and pressure, and a temperature conversion factor of 1500 K⁻¹. Our calculated RBC oxygen levels were consistent with those measured by phosphorescent probe in somatosensory cortical tissue obtained from a bulb RBCs in an awake mouse (30–100 mmHg)24, in experiments that also measured pO2 between RBCs; interRBC pO2. We then estimated the interRBC pO2 for our experiments by using those interRBC phosphorescent probe measurements that had the same pO2 as we observed27.21. This yielded an estimate of interRBC [O2] in V1 of 15 mmHg/21 μM and in HC of 10 mmHg/14 μM. Because tissue pO2 equilibrates with interRBC pO2 rather than RBC pO2,27, we used these estimates of interRBC [O2] as the capillary [O2] in our model. Indeed if we ran our simulations using RBC pO2 as the capillary [O2], tissue [O2] estimates were impossibly higher than the expected interRBC [O2] values (reaching 37 μM in HC and 58 μM in V1 with a Vave of 3 mM/min), confirming that interRBC, rather than intra RBC [O2] levels are likely to be the main drivers of tissue [O2].

Calculating capillary spacings: Using ImageJ (Exact Euclidean Distance Transform 3D plugin) we calculated a 3D distance map from a smoothed and binarized in vivo z-stack of the vascular network in each brain region. We extracted five substacks of 100 × 100 × 100 μm from each distance map. The distribution of the distances of pixels from the nearest vessel was then extracted and averaged across all substacks from a given larger z-stack (giving an average distance per animal). Histograms of these data were averaged across five stacks per brain region (Fig. 7c) and the 50th, 95th and intermediate centile distances from a capillary were calculated (Fig. 7e–l, m). For a given bar in the histogram, contributing pixels are either at a midpoint between two capillaries, or are closer to one capillary than another. Those pixels above the 95th centile must lie more-or-less at the midpoint between vessels, whereas many pixels at the median distance from the vessel will be nearer to one vessel than another. However [O2] at each distance from a vessel would be larger if that point is at the midpoint between two vessels compared with being nearer to one vessel than another (and therefore oxygen from that vessel is having to feed a larger tissue volume). For the diffusion model below, we therefore used the distribution of distance intervals from a capillary as a conservative estimate of capillary separation, meaning that our estimates of levels of oxygen in the tissue are, if anything, slightly overestimated.

Diffusion model: We used the Heat Transfer functions in the Partial Differential Equation Toolbox of MATLAB to numerically solve Fick’s diffusion equation for radial geometries with Michaelis–Menten consumption of oxygen through oxidative phosphorylation (Eq. (5)): 

\[ \frac{dC}{dr} = \frac{1}{r} \frac{d}{dr} \left( D \frac{dC}{dr} \right) - \frac{V_{max} C}{C + K_m} \]  

(5)

where r is the distance from the centre of a capillary (of radius 2.5 μm; Fig. 2f), C is the concentration of O2, D is its diffusion coefficient in brain at 37 °C (9.24 × 10⁻⁸ m²/min⁶⁵), K_m = 1 μM is the EC₅₀ for O₂ activating oxidative phosphorylation⁶⁴, and V_max is the maximum rate of oxidative phosphorylation at saturating [O₂]. The initial conditions were C(r) = 0, and the boundary conditions fixed [O₂] at the edge of the capillary (r = 0) to be the calculated interRBC [O₂] for each brain region and at the midpoint point between two capillaries (r = 0.25 mm) because the basement membrane of the vessel wall may form a diffusion barrier for oxygen supply to the tissue, reducing the effective diffusion coefficient by up to 40%⁶⁶. D was reduced by 40% lower value within a capillary wall of 200 nm (the maximum of the reported range⁶⁷, r = 2.5–2.7 μm).

The equation was solved over values for r from 2.5 μm up to the average tissue distances from a capillary as calculated above (median, 95th or intermediate centile for each brain region).

Oxygen consumption: Values for Vₐer (0.5–3 mM/min) were chosen based on the range of brain oxygen consumption rates previously reported⁶⁸. Of this range, values of 1–2 mM/min may be the most physiological, being reported from recent experiments measuring the oxygen gradient using phosphorescent lifetime imaging, e.g., ²⁸ (rather than an invasive electrode) and matching the whole brain averaged oxygen consumption rate⁶⁸. However, previous measurements in rodents were done under anaesthesia, so rates may be higher in our awake system.

Statistical analysis. The sample size was determined by power analysis using G*Power. All data are presented as mean ± SEM or individual data points unless otherwise stated. Data were tested for equal variance between groups by Kruskal–Wallis or Mann–Whitney test. Two-group comparisons were evaluated by independent sample Student t tests, or in cases of unequal variance between groups either Welch’s independent sample t tests (for mRNA data sets) or Mann–Whitney U tests (for two-photon experiments). Paired comparisons were evaluated using the Wilcoxon signed-rank test. Data were compared between surgical and control hemispheres). Multiple comparisons were assessed using one-way analysis of variance (ANOVA) to compare means, multifactorial ANOVAs to assess for interactions, and in cases where the data violated the assumptions of the ANOVA due to uneven variance between groups, we ran one-way ANOVAs with Welch’s correction. Post hoc comparisons were compared with Bonferroni tests when data had equal variances and Games–Howell tests in cases of unequal variance. For the multiple t test comparisons, a procedure equivalent to the Holm–Bonferroni correction was applied to calculate an adjusted p value. This correction consisted of ranking the p values outputted from t tests in ascending order and then multiplying the lowest p value by the number of comparisons divided by the number of comparisons minus one, and so on, until comparisons were no longer significant at p = 0.05. Simple linear regressions were used to compare the slope through a data set to a flat (zero) line, and an analysis of covariance to compare the intercepts and slopes between two fitted regression lines. Chi-square tests (2 × 2 contingency with Fisher’s exact significance test, or 3D Cochran–Mantel–Haenszel tests with Pearson’s R multiple post hoc comparisons) were used to compare p values from a contingency table. The threshold for statistical significance was set at p ≤ 0.05, and for a trend at p = 0.1. Statistical analyses were conducted using SPSS, RStudio or GraphPad Prism and figures were created using GraphPad Prism. For further detail, a statistics report is also provided.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The mRNA data presented in Fig. 4 and Supplementary Figure 8 were taken from: http://linnarssonlab.org/cortex/. For all main figures, and the supplementary figures with unique data sets, the extracted traces from the raw image files are available as .xls and .mat files on Figsshare⁶⁹. The raw image files are stored in our Dropbox owing to their large size, and are available from the corresponding author upon request. Source data are provided with this paper.

Code availability

The custom code used for data analysis is available as a Github repository, and has been published via Zenodo⁷⁰.

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K.S., L.B., K.B., D.M.G. and D.C. collected data for the studies, K.S, H.C. and C.N.H. designed and analysed the studies. K.S, D.M.G., O.B., D.C. and C.N.H. wrote scripts to analyse the data. K.S. and C.N.H. wrote the manuscript with feedback from all authors.

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There are no competing interests to declare.

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