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**Citation for published version:**

Duncombe, J, Kitamura, A, Hase, Y, Ihara, M, Kalaria, RN & Horsburgh, K 2017, 'Chronic cerebral hypoperfusion: a key mechanism leading to vascular cognitive impairment and dementia. Closing the translational gap between rodent models and human vascular cognitive impairment and dementia', *Clinical science*, vol. 131, no. 19, pp. 2451-2468. <https://doi.org/10.1042/CS20160727>

**Digital Object Identifier (DOI):**

[10.1042/CS20160727](https://doi.org/10.1042/CS20160727)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Peer reviewed version

**Published In:**

Clinical science

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**Chronic cerebral hypoperfusion: a key mechanism leading to vascular cognitive impairment and dementia (VCID)**

**Closing the translational gap between rodent models and human VCID.**

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Running Title: Rodent Models of Cerebral Hypoperfusion

Abbreviations list:

Alzheimer's disease (AD)

Amyloid (A $\beta$ )

Amyloid precursor protein (APP)

Bilateral carotid artery stenosis (BCAS)

Blood brain barrier (BBB)

Cerebral amyloid angiopathy (CAA)

Cerebral blood flow (CBF)

Extracellular matrix (ECM)

Intercellular adhesion molecule-1 (ICAM-1)

Matrix metalloproteinase (MMP)

Mild cognitive impairment (MCI)

Small vessel disease (SVD)

Spontaneously hypertensive stroke prone rat (SHRSP)

Vascular cell adhesion molecule-1 (VCAM-1)

Transgenic mice with amyloid precursor protein mutations (TgAPP)

Vascular cognitive impairment and dementia (VCID)

## **Abstract**

Increasing evidence suggests that vascular risk factors contribute to neurodegeneration, cognitive impairment and dementia. While there is considerable overlap between features of vascular cognitive impairment and dementia (VCID) and Alzheimer's disease (AD), it appears that cerebral hypoperfusion is the common underlying pathophysiological mechanism which is a major contributor to cognitive decline and degenerative processes leading to dementia. Sustained cerebral hypoperfusion is suggested to be the cause of white matter attenuation, a key feature common to both AD and dementia associated with cerebral small vessel disease. White matter changes increase the risk for stroke, dementia and disability. A major gap has been the lack of mechanistic insights in the evolution and progress of VCID. However, this gap is closing with the recent refinement of rodent models which replicate chronic cerebral hypoperfusion. In this review, we discuss the relevance and advantages of these models to elucidating the pathogenesis of VCID and explore the interplay between hypoperfusion and the deposition of amyloid  $\beta$  protein, as it relates to AD. We use examples of our recent investigations to illustrate the utility of the model in pre-clinical testing of candidate drugs and life-style factors. We propose that the use of such models is necessary for tackling the urgently needed translational gap from preclinical models to clinical treatments .

Key words: Alzheimer's disease; animal models; cerebral hypoperfusion; cognitive impairment; dementia; small vessel disease; stroke; vascular dementia

Summary statement:

Vascular cognitive impairment and dementia (VCID) is an important contributor to the global burden of disease. While there are no perfect animal models to recapitulate all the features of VCID, current laboratory rodent models which simulate cerebral hypoperfusion allow some aspects of VCID to be explored. Despite their limitations, rodent models are still useful to evaluate specific mechanisms for testing drug targets and close the translational gap between animal models and VCID.

## Introduction

Vascular disease has been invariably linked to cognitive impairment. There is increasing evidence that vascular risk factors contribute to neurodegeneration and dementia. Recent analysis on a large sample, as part of the Alzheimer's Disease Neuroimaging Initiative, surprisingly revealed that early vascular dysfunction plays a role in Alzheimer's disease (AD) (1). However, one of the most common causes of vascular cognitive impairment and dementia (VCID) is cerebral small vessel disease (SVD), which affects small arteries, arterioles, venules and capillaries in the brain leading to arteriolar occlusion, lacunes and white matter changes. The main clinical features of VCID may include pure motor, sensorimotor, pure sensory, ataxic hemiparesis or gait impairment, dysarthria, cognitive dysexecutive slowing and depression (2). Cerebral amyloid angiopathy (CAA), another form of SVD, is found in almost all AD patients and more than 50% of the elderly over 90 years old (2, 3). CAA mostly leads to lobar haemorrhage, white matter damage and cortical microinfarcts (4). Moderate to severe CAA is also considered an independent risk factor for dementia (5).

It is now recognised that there is considerable overlap between VCID and AD. Several previous reports, including recent ones from the AD research centres in the USA, suggest that some form of brain vascular pathology exists in up to 80% of sporadic late onset AD (6). Moreover, cerebrovascular lesions increase the clinical expression of AD syndrome. Traditional risk factors for stroke and cardiovascular disease (e.g. hypertension, diabetes, hyperlipidaemia) are recognised as risks for both VCID and AD with salt intake, chronic inflammation and gut infection now emerging as additional risk factors (7,8). Although the mechanisms by which these different factors may impact on VCID and AD are currently ill defined, considerable evidence, including that derived from neuroimaging and pathology studies, indicates that endothelial dysfunction is pivotal to the pathophysiology (see reviews 9, 10, 11). It is proposed that risk factors may alter vascular haemodynamics and impact on endothelial cell function. Endothelial dysfunction can in turn reduce vasomotor reactivity and impede cerebral hemodynamic changes. Related to this vascular factors may impair neurovascular coupling, leading to transient or chronic cerebral hypoperfusion which exacerbates small vessel pathology including white matter damage. Alternatively, it is proposed that the blood brain barrier (BBB) is initially compromised in VCID leading to a chronic hypoxic state and hypoperfusion (see reviews 9, 10, 11).

Cerebral hypoperfusion is emerging as a major contributor to cognitive decline and degenerative processes leading to dementia. Reduced cerebral perfusion correlates with the

severity of dementia and also predicts which individuals with mild cognitive impairment (MCI) will progress to develop dementia (12, 13). Cross-sectional studies show that low cerebral blood flow (CBF) is related to the severity of white matter hyperintensities upon T2 weighted MRI (14-16). Neuropathological investigations have revealed marked reductions in myelin density in the white matter in AD but particularly in VaD compared to age-matched controls (17-19) with evidence that this may be related to reduced white matter perfusion (20, 21). It is unclear whether CBF changes are causal or secondary to white matter changes. Nonetheless, imaging studies suggest the extent and presence of these white matter changes, particularly in the frontal lobe are important determinants of cognitive function and impact on dementia (22, 23). Thus it is proposed that understanding the earliest events leading to white matter changes could provide vital opportunities to prevent brain damage at the earliest stages and ameliorate its impact on cognitive decline and precipitation of dementia (23).

In addition to correlative pathological and imaging studies in human, there is a need to provide mechanistic insight of white matter changes through the development of relevant animal models and translate these findings to the clinic (24). Over the past five decades, various animal models have been described to examine the pathophysiology of global and focal ischaemic injury (25, 26) (Figure 1). Several of these models were developed to primarily test the acute effects of compounds administered within the therapeutic window, after occlusion, to reduce the effects of focal stroke injury and salvage the hypoperfused region or penumbra peripheral to the ischaemic core (25). Several models have since been developed to mimic specific aspects of the pathology relevant to VCID or genetic causes (most recently reviewed in 27). In this current review, we focus on rodent models which have been developed and refined over the last few years to mimic the chronic hypoperfusive state in VCID and which are used as a basis to probe mechanisms related to reduced perfusion of the brain. We have focussed on models of bilateral common carotid artery occlusion in rats (often referred to as 2 vessel occlusion), bilateral common carotid artery stenosis (BCAS) model in mice and the newly developed bilateral common carotid artery gradual occlusion model in rats and mice. We also explore the interplay between cerebral hypoperfusion and amyloid beta protein (A $\beta$ ), which is currently being evaluated as a model in which to explore mixed dementia. Finally, we discuss approaches currently underway to close the translational gap between the rodent models of cerebral hypoperfusion and human VCID.

## **What have we learned from the rodent models of chronic cerebral hypoperfusion?**

### **Cerebral blood flow alterations in models**

In order to study early pathological events that may lead to VCID, rodent models of chronic cerebral hypoperfusion were first established using occlusion or ligation of both common carotid arteries in rats (2 vessel occlusion) (see review 28). The resulting reductions in blood flow are severe, whereby cortical blood flow drops by over 70% in the days immediately following surgery, recovering to a 40% reduction by one month (29). In C57Bl/6J mice, complete occlusion of both carotid arteries leads to death due to poor collateral flow through the Circle of Willis and as an alternative the carotid arteries are temporarily occluded for durations lasting no more than 30 minutes (30). This approach leads to transient global ischaemia with blood flow reduced by 80-90% (31, 32). As such, these occlusion models do not accurately recapitulate the more modest reductions in blood flow seen in chronic hypoperfusive conditions related to VCI where blood flow may only be reduced by ~20-30% (33-35).

A refinement of rat models of cerebral hypoperfusion was introduced to more faithfully represent the subtle reductions in flow in VCI. Bilateral common carotid artery stenosis (BCAS), by application of microcoils, reduces luminal diameter to approximately 50% in young adult C57Bl/6J mice (36). This approach takes advantage of the poor Circle of Willis in C57Bl/6J mice and results in blood flow reductions of 30-40% immediately following surgery using microcoils of 0.18mm diameter (36, 37). With increasing time there is a recovery of blood flow in young mice to 15-20% baseline levels at 1 month when measured by laser Doppler ultrasound or laser speckle imaging (36, 37). These changes have been attributed to vascular remodelling in young mice (38) but as yet the effects in older (>1 year) mice remain unknown. Blood flow measures are normally conducted using laser speckle or Doppler flowmetry and constrained by limited depth of penetration and the restriction of these techniques to the cortical surface vasculature. Hattori et al. (39) performed arterial spin labelling MRI to measure blood flow in cortical and subcortical brain regions following BCAS in mice. From 1 day up to 14 days following surgery, blood flow was shown to be reduced to 50% of baseline in both cortical and subcortical regions, however at 28 days blood flow had recovered to 70% of baseline. Boehm-Sturm et al. (40) also utilised arterial spin labelling to demonstrate global flow reductions of 50% at 24 hours following BCAS surgery, recovering to ~75% of baseline at 4 weeks. Despite the recovery of blood flow, these studies importantly highlight that hypoperfusion is not restricted to the cortical vasculature and

supports BCAS as a model for chronic cerebral hypoperfusion. Thus, the studies emphasise the utility of arterial spin labelling as a quantitative and non-invasive tool for assessing global and regional blood flow changes in models of hypoperfusion.

A limitation of the BCAS model is the acute reduction in CBF that occurs on application of the microcoils (36). To overcome this a new gradual stenosis model was developed which uses ameroid constrictor devices applied on both common carotid arteries in rats and mice (41, 42). These ameroid devices absorb extracellular fluid over time and as a result gradually expand to constrict the arteries resulting in a slower, more gradual onset of hypoperfusion. In rats, a maximum blood flow reduction of 30% is observed at 3 days following surgery followed by recovery of blood flow to 85% of baseline when assessed for 28 days similar to levels reached in the 2 vessel occlusion rat model (41). In mice however, blood flow progressively reduces over 28 days, without recovery, to reach 70% of baseline levels after the application of the constrictor cuffs (42). The differing patterns of blood flow reductions between the microcoil induced stenosis and gradual stenosis were illustrated by Hattori et al. (42), in which BCAS mice were compared to those with ameroid constrictor devices applied to the common carotid arteries (Figure 2). A gradual reduction in CBF can clearly be seen in the gradual stenosis mice whereas an acute drop in CBF is detected in the BCAS mice. In another model an ameroid constrictor is applied to the right common carotid artery resulting in gradual occlusion of the vessel over 28 days, whereas placement of a microcoil to the left common carotid artery induces ~50% arterial stenosis (43). To our knowledge, there are no published data available on blood flow reductions at longer time points (>1 month) in ameroid constrictor models, and whether these devices eventually go on to completely occlude the carotid arteries remains to be confirmed.

## **Neuropathological changes**

### ***Parenchymal alterations***

As indicated previously white matter alterations, a prominent feature of VCID, contribute to cognitive impairment and serve as a potential target to ameliorate the burden of dementia (23). Rat 2 vessel occlusion models have been extensively studied since they were first found to develop white matter rarefaction similar to that in humans (28). However, these pathological changes occur very quickly in conjunction with the severe and sudden drop in cerebral perfusion after occlusion (29). Furthermore, in the 2 vessel occlusion model ischemic neuronal damage may be present in the cerebral cortex and hippocampus 1-3 days and infarctions in the striatum at 7 days (29) albeit this can vary considerably between studies and groups (see review, 28). In parallel with white/grey matter pathology, microglia

markedly increase in number which can be detected from as early as 1 day after occlusion in the white matter and remain significantly elevated at 28 days (44). A prominent astrocytic response is also evident in this model but appears delayed compared to the other pathological changes (44).

A refinement of these 2 vessel occlusion rat models was undertaken to overcome the severe reduction in blood flow with the development of the BCAS microcoil models in mice. Investigation of this model led to the discovery that white matter is damaged in the absence of overt ischaemic neuronal perikaryal damage. Shibata et al. (36) using the BCAS model in mice first reported white matter rarefaction and vacuolation (detected using Klüver-Barrera), which is evident from 2 weeks alongside a prominent glial response that evolves with time. Others, including our group, have shown that BCAS using microcoils causes an anatomically widespread but diffuse damage to myelinated axons in white matter tracts detectable at one month (36, 45-47). These changes can be observed throughout the forebrain (such as corpus callosum, fimbria, internal capsule, optic tract) and can be visualised using immunohistochemical approaches in the form of degraded myelin basic protein and myelin debris with less pronounced axonal damage. Hypoperfusion also leads to selective disruption of key proteins within the paranodal axon-glia junctions, which are critical to the stability and functions of myelinated axons and white matter function (46). Paranodal septate-like junctions are damaged and axon-glia integrity is disrupted, as determined by spatial distribution of myelin-associated glycoprotein staining (46). Ultrastructural alterations comprise loss of septate junctions at paranodal regions (46) but that there is no overt demyelination in the model (37). Such subtle white matter pathology in the absence of severe focal disruption can be detected by diffusion tensor (DT) or magnetization transfer (MT) MRI first in the corpus callosum at one month (47), that then progresses over 6 months with more pronounced alterations in the corpus callosum, internal capsule, fimbria and subcortex (48). One of the prominent features of hypoperfusion models is a robust increase in microglial number. In response to increasing durations of hypoperfusion, microglia gradually augment in parallel with the evolving damage to myelinated axons, resulting in a marked and sustained increase in microglial number, particularly in the white matter (36, 37, 45-47). Astrogliosis can also be observed but these changes appear to occur later than microglial alterations in BCAS models with diffuse white matter injury (48). However, in severely damaged white matter GFAP-positive cisternodendrocytes (irreversibly damaged astrocytes) can be readily detected (40, 49).

Pathological changes in the gradual stenosis models are similar to the microcoil model but as expected these progress more slowly. In the rat gradual stenosis model, acute inflammatory responses subsequent to acute CBF reduction observed in the 2 vessel occlusion rats are eliminated (50). Myelin pathology (assessed by Klüver-Barrera, degraded MBP and GST-pi staining), is less severe in the gradual compared to the 2 vessel occlusion model, and disruption of axon-glia integrity is apparent at 28 days (50). Selective white matter changes are induced with relatively preserved neurovascular coupling and substantially less metabolic and histological derangements in the grey matter including the hippocampus in the gradual compared to 2 vessel occlusion model (50). Moreover, in the mouse gradual stenosis model, activation of astrocytes and microglia with loss of oligodendrocytes were found in the white matter at 32 days post-operation (39).

Most of the mild hypoperfusion models do not exhibit overt white matter lesions or infarcts. However, using histological approaches and T2\* MRI, after long-term BCAS subcortical microinfarcts and haemorrhages were evident (48). As yet the longer-term effects (>1 month) have not been studied in the gradual stenosis models.

### ***Small vessel and BBB changes***

In the rat 2 vessel occlusion model, BBB disruption is observed as early as 3 hours post-occlusion most likely as a result of the sharp and severe CBF reduction in this model (51). However as CBF restores from day 7, the BBB changes appear less prominent (51). In the BCAS model, it is not clear if the BBB is compromised but this could be explained by variations in CBF reductions across different models. In our BCAS model, overt BBB disruption was not observed until 6 months after hypoperfusion when fibrinogen was detected in the parenchyma and the levels of the tight junction protein claudin-5 were markedly reduced (48). Other studies, in which white matter damage appears more severe, showed earlier BBB disruption at 3 and 7 days after BCAS (52). An ultrastructural study suggested that subtle alterations in the BBB occur early whereby at two hours post BCAS, irregularities in the endothelium include opening of tight junctions (53). A systematic study of the BBB, including tight junction proteins, across a range of times post-hypoperfusion in the different models is required to assess the dynamics of the BBB and whether these changes may be transient or sustained.

Sustained hypoperfusion can also induce morphological small vessel changes such as increased thickening and fibrosis of capillary walls, which are one of the characteristic features of human SVD (2). These features have been identified after 12 weeks

hypoperfusion in a rat 2 vessel occlusion model (28) and after 6 months hypoperfusion in a mouse BCAS model (48). Fibrin deposition or accumulation of hyaline-like substance, key neuropathological findings in human SVD, are also recognized after 6 months hypoperfusion in the BCAS mice (48). In the gradual stenosis models, small vessel and BBB changes are yet to be described.

### ***Brain atrophy***

Clinical imaging studies have demonstrated both whole brain and regional brain atrophy in VCID (54, 55). Brain atrophy, in particular medial temporal lobe atrophy and subcortical atrophy, is associated with cognitive decline and can potentiate the effect of white matter lesions on cognition (54, 56). Longitudinal progression of brain atrophy and white matter lesions are strongly correlated, with white matter lesions recently suggested to drive cortical grey matter atrophy (57). Despite the prevalence of brain atrophy in VCID and its relation with cognition, there is a relative paucity of evidence regarding brain atrophy in preclinical models of cerebral hypoperfusion. Nishio et al. (58) reported no apparent change in cortex or corpus callosum at 8 months following BCAS surgery, however the hippocampal volume was found to be significantly reduced in hypoperfused mice. In agreement with this finding, hippocampal glucose uptake was also reduced when assessed with <sup>18</sup>F-FDG PET. Holland et al. (48) showed reductions in brain volume at 6 months following BCAS but not at 1 month using T2\*MRI, atrophy was also correlated with burden of ischaemic and haemorrhagic lesions. Together, these findings suggest that brain atrophy occurs at later time points following hypoperfusion, and are secondary to neuronal loss and white matter damage. Future studies may utilise preclinical in vivo imaging where possible, in order to measure volumetric changes longitudinally over the entire volume of the brain, so as to definitively describe the pattern of atrophy following hypoperfusion. White matter alterations have been suggested to contribute to cortical atrophy (57) and thus the use of larger animal models with greater volume of white matter, for example non-human primates, may enable better understanding of this mechanism. Since brain atrophy is the major substrate of cognitive decline, atrophic change may be a useful endpoint when incorporated into preclinical intervention studies.

### ***Cognitive impairments***

It was proposed many years ago that chronic cerebral hypoperfusion leads to cognitive impairment (59) but human studies have shown at best a moderate association. Animal models have since provided compelling evidence that chronic cerebral hypoperfusion can

lead to cognitive impairments (see 28, 60, 61 for reviews). Most studies have utilised the Morris water maze to assess spatial reference learning or the radial arm maze tasks to assess spatial working memory. Impaired spatial learning has been widely reported in the 2 vessel occlusion rat model (28, 60, 61) and in some studies deficits have been detected as early as 7 days post-occlusion (62). These behavioural changes reflect pathological changes in the hippocampus with proliferation of astrocytes and neuronal cell loss in the CA1 area (62, 63). At later time points impaired spatial working memory (28, 60) has been observed and correlates well with the development of white matter pathology. Interestingly, poor performance in the odour discrimination task suggests that cognitive functions related to olfaction are also impaired (64), which is also seen as an early sign of cognitive impairment in patients with various neurodegenerative diseases, indicating the relevance of this model to human VCID.

The development of the BCAS mouse model demonstrated that chronic cerebral hypoperfusion causes deficits mainly in spatial working memory (45, 65-67) using a conventional 8-arm radial maze or Y-maze tests. We highlighted that 1 month after BCAS, spatial working memory is impaired while reference memory remains intact, probably due to the select disruption of frontal-subcortical circuits (45). Notably, disruption of axon-glia integrity and proliferation of microglia are indicated to be strongly associated with the impairment in working memory (68). Recent reports also confirm that BCAS causes impaired working memory assessed by an innovative three dimensional 9-arm radial maze as well as impaired nesting ability at four months after surgery (69). After long-term i.e. 6 months of hypoperfusion after BCAS, both spatial working memory and spatial reference memory were impaired (48). The emergence of deficits in both working and reference memory likely reflects the presence of white and grey matter pathology including whole brain and hippocampal atrophy (48, 70). Thus, long-term hypoperfusion induces a pronounced cognitive impairment coincident with pathological alterations and more accurately replicates features of human SVD. In the gradual stenosis mouse model, impaired spatial working memory is again reflective of white matter pathology whereas hippocampal-dependent reference learning and memory is preserved, probably due to a lack of hippocampal changes compared with other mouse models (42). Similarly, in the rat gradual occlusion model spatial working memory is impaired (41). In general, impaired spatial working memory is a consistent and robust finding in models of chronic cerebral hypoperfusion. This highlights the relevance of these models to human VCID in which the frontal-subcortical circuits are disrupted.

## **Mechanisms of the hypoperfusion models**

As indicated above, rodent models of hypoperfusion recapitulate some pathological features observed in VCID, such as disruption of white matter integrity, microvascular alterations and atrophy. These alterations are related to cognitive deficits. However, the precise molecular and cellular mechanisms that lead to such changes are currently being unravelled as outlined below:

### ***Hypoxia-induced white matter damage***

Following vessel occlusion or carotid stenosis, cerebral perfusion is demonstrably reduced (28, 37) but it is less clear whether these changes affect tissue oxygen tension and whether there are differences between white matter and grey matter to account for their differential vulnerability. Tissue levels of oxygen (pO<sub>2</sub>), can be measured using precalibrated sensors. We have used this approach to measure pO<sub>2</sub> levels in the corpus callosum of anaesthetised mice in the BCAS model. Levels of pO<sub>2</sub> are profoundly decreased in the corpus callosum at 3 days, 1 week and 6 weeks following BCAS surgery (see Figure 3) to levels consistent with hypoxic conditions. The levels of pO<sub>2</sub> in normal appearing white matter are less than those reported within grey matter (71) and may suggest that white matter is predisposed to additional reduction in oxygen caused by BCAS. Dong et al. (72) also reported an increase in hypoxia at 3 weeks following BCAS surgery, although regional differences were not assessed.

At 3 days following hypoperfusion surgery in the BCAS model, we demonstrated expression of hypoxia-related genes in the white matter (46). Oligodendrocytes are sensitive to hypoxia (73, 74) and a rapid loss of oligodendrocytes occurs in the different models of hypoperfusion. At 3 days post-BCAS we demonstrated there is a loss of mature oligodendrocytes and oligodendrocyte precursors at a time when axon-glia integrity is compromised (37). Early hypoxic signalling could result in degeneration of oligodendrocytes and a loss of axonal trophic support, resulting in mis-localisation of key axonal proteins (46) that may impair signal conduction. In human post-mortem brain, disruption of nodal proteins has also been reported in white matter adjacent to lacunar infarcts (75) and white matter lesions express characteristic markers of a hypoxic environment, including hypoxia inducible factor 1 (HIF-1), HIF-2 and matrix metalloproteinase (MMP)-7 (19).

Hypoxia, in addition to deleterious effects on oligodendrocytes, can also directly induce changes in BBB permeability (76). Intriguingly, oligodendrocytes and oligodendrocyte precursor cells have also been demonstrated to modulate BBB function by altering tight

junction protein expression (77). In the mouse BCAS model oligodendrocyte precursors were shown to be responsible for BBB opening, via increase MMP and contribute to white matter damage in hypoperfusion models (52). Blood-brain barrier dysfunction is also observed in white matter hyperintensities and normally appearing white matter in SVD that is predictive of cognitive decline (78). Disruption of oligodendrocyte-endothelial signalling may also allow entry of toxic blood products into brain contributing to the pathophysiology of VCID (79).

The described molecular changes in hypoperfused animal models occur at a time when microglia number increase. Microglia, resident macrophage cells in the brain, are sensitive to changes in the local brain environment (80). Through the release of pro-inflammatory cytokines/chemokines microglia could also contribute to hypoperfusion-induced white matter damage. In support of this, as early as 3 days after hypoperfusion inflammatory-related genes, such as those associated with the Jak-STAT signalling pathway and cytokine-cytokine receptor interaction, are significantly up-regulated in white matter (46). Similarly activated microglia have been identified in white matter lesions in aged post-mortem human brain (81) with transcriptome analysis indicating alterations in genes related to the immune pathway (82). In the aged primate brain, microglial reactions predominate in the white matter and correlate with cognitive impairment (83). Notably, we have now determined that the number of microglial cells is significantly associated with nodal gap length and axon-glia disruption after hypoperfusion (84). Higher numbers of microglial cells correlate with decreased nodal gap length and increased paranodal axon-glia disruption, supporting the idea that the structural alterations observed in response to hypoperfusion could be secondary to a pro-inflammatory environment. With progressive myelin breakdown at increasing durations of hypoperfusion, phagocytosis of myelin debris may also result in microglial dysfunction (85) and the activation of a neuroinflammatory phenotype that contributes to further degenerative changes.

### ***Microvascular inflammation***

Endothelial dysfunction is considered to be one of the pivotal mechanisms of the structural and functional cerebral vessel alterations in SVD (10, 11) leading to VCID. Early endothelial failure and subsequent BBB breakdown are hypothesised to be major precipitants of sporadic SVD (9-11). Cerebral hypoperfusion upregulates the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), markers of endothelial cell activation (68, 86). In the rat 2 vessel

occlusion model, markers of endothelial activation increase as early as one day post-occlusion up to 28 days with a peak at 3 days (86). Our findings in the BCAS model, indicate mild hypoperfusion gradually induces upregulation of ICAM-1 and significantly at 3 months post operation (68). Increased expression of adhesion molecules on the endothelial surface functions to facilitate the attachment and extravasation of leukocytes across the BBB (87). In support of leukocyte activation post-hypoperfusion, an elegant in vivo 2-photon imaging study revealed leukocyte rolling and adhesion in the pial vessels occurs within 24 hours in the BCAS model (88). These changes take place in the absence of notable alterations in vessel structure, astrocytes or pericytes, suggesting this is an early mechanism. However there is no evidence that monocyte or T cells infiltrate the brain parenchyma post-hypoperfusion (84, 89) but a detailed study of the myeloid cell population post-hypoperfusion is required.

Opening of the blood-brain barrier and entry of blood products such as fibrinogen into the brain (as demonstrated in the BCAS model (48) is also likely to initiate an inflammatory response from resident microglia. An increase in microglial cell number following hypoperfusion is a consistent finding in preclinical studies (36, 37, 44-47, 49, 84) and activation of microglia in hypoperfused mice and rats is associated with release of matrix metalloproteinase, MMP2 (81), inflammatory cytokines, such as tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6, and the progression of white matter lesions (90-93). Increased MMPs have been consistently shown in hypoperfusion models (90-92) and increased expression has been shown in the white matter (91, 92) localised to microglia and the endothelium (91). MMPs are proteases that degrade the extracellular matrix as well as tight junctions between endothelial cells and have been implicated in BBB breakdown in neurodegenerative diseases (52). Furthermore increased MMPs can also degrade myelin (94). Increased production of reactive oxygen species by activated microglia may also drive endothelial dysfunction through disruption of nitric oxide signalling (95). 8-hydroxy-deoxyguanosine, a marker of oxidative stress, is increased by hypoperfusion in the endothelial cells in the BCAS model (64, 96). Thus a pro-inflammatory cascade is likely to further damage the BBB through degradation of the extracellular matrix by MMPs and promoting oxidative damage.

Microvascular inflammation, as outlined previously, is a common feature of hypoperfusion models with markers of chronic inflammation and endothelial activation associated with progressive changes. It is not yet known whether inflammation is a primary driver of VCID and if this is triggered by intrinsic or systemic processes. Certainly from clinical observations

there appears to be an interplay between CNS and peripheral inflammation that contributes to VCID (97, 98). Additionally, age, a key risk factor for VCID, is also associated with alterations in microglial phenotype and function (99). There is an indication from recent paper (100) that white matter, glial and cognitive changes are more pronounced in older mice with BCAS as compared to younger mice. The mechanistic impact of advanced age remains to be fully explored in the different hypoperfusion models and this will be important to evaluate in future studies.

### ***Neuro-glio-vascular function***

As outlined above, no single cell type is likely to be solely responsible for the pathophysiology of VCID. Instead, disruption of the finely tuned interplay between cells of the neuro-glio-vascular unit (oligodendrocytes, endothelial cells, astrocytes and end-feet contacts, pericytes, microglia and neurons) (for review see 101) likely contributes to the pathophysiology. Breakdown of the neuro-glio-vascular unit disrupts the BBB, impairs the exchange of substances between blood and brain and alters the immune system. Critically, the efficient communication between the cells within the neuro-glio-vascular unit ensures that cerebral vessel diameter is finely tuned to neuronal activity to maintain cerebral perfusion and meet metabolic demands, a process known as neurovascular coupling. Nishino et al. (102) report that 7 days following unilateral common carotid artery stenosis, vascular responses to neural activity evoked by whisker stimulation are impaired. We have also observed impaired neurovascular coupling at 1 month following BCAS in mice (see Figure 4). Other studies have shown impaired vascular responses to hypercapnia (103) and pharmacological vasodilators (104) following hypoperfusion. In a clinical study, Sam et al. (105) also observed poor cerebrovascular reactivity in normally-appearing white matter that preceded the appearance of white matter lesions.

Astrocytes contact synapses and the vasculature via end-feet processes. They are ideally located to mediate neurovascular coupling, facilitated by astrocytic calcium signalling and release of vasoactive substances from the endfoot terminus (106). Astrocytic activation has been detected in white matter at 7 days following BCAS surgery in mice (107) and has the potential to disrupt the contact between astrocytes and blood vessels. Indeed, chronic cerebral hypoperfusion has been shown to induce AQP4 displacement and redistribution after 3months BCAS (48, 68). This widespread gliovascular disruption may impair the regulation of blood flow, reducing the ability of the cerebral vessels to respond to dynamic demands in perfusion and exacerbating hypoxia. In humans, this may be further compounded by age-related increases in vessel stiffness and microvascular rarefaction

(108). However, a direct causal link between impaired neurovascular coupling and white matter lesion development has yet to be proven, as disrupted neurovascular coupling may reflect reduced tissue metabolic demand as a result of other ongoing pathological processes. The displacement of astrocytic endfeet or mislocalisation of AQP4 from vascular endfeet contacts could further impact on glymphatic clearance pathways, as has been elegantly demonstrated by Iliff et al. (109). As yet, the impact of hypoperfusion on glymphatic or perivascular drainage pathways has not been explored. Astrocytes have also been shown to support oligodendrogenesis through secretion of brain derived nerve growth factor (BDNF) in order to promote repair of white matter damage following BCAS in mice (110). This protective coupling between astrocytes and oligodendrocytes may also fail with age (111) or in neuroinflammatory conditions, and exacerbate white matter injury. Thus, the cellular interactions within the neuro-glio-vascular unit may be critically important for maintaining tissue health.

Pericytes are contractile cells that ensheath capillary endothelial cells. They have also been suggested to regulate blood flow in capillaries and may irreversibly constrict vessels following ischaemic injury (112). Although pericytes may be important regulators of BBB function remarkably their function in hypoperfusion models remains largely unknown. Perivascular macrophages have also not been studied in hypoperfusion models, but have been shown to mediate vascular dysfunction in hypertensive mice through production of reactive oxygen species (113), and may therefore be an important cell type for future study.

Central to maintaining neuro-glio-vascular integrity is the basement membrane (BM)/extracellular matrix (ECM) complex, a key interface between endothelial cells, mural cells and astrocytic end-feet that provides essential structural and functional stability to the neuro-glio-vascular unit through a complex meshwork of ECM proteins (114). As has been indicated previously, hypoxic and inflammatory changes including the release of MMPs (90, 91) may damage the ECM and compromise its functional integrity. Mutations in ECM-related proteins result in familial forms of SVD (114). It is plausible therefore that the ECM may also be an important target in sporadic SVD.

Taken together, hypoperfusion is likely to drive key pathways related to hypoxia, inflammation and BBB disruption, resulting in progressive deterioration of the neuro-glio-vascular unit (see Figure 5). The loss of integrity is likely to affect diverse functions including brain clearance pathways and regulation of CBF, potentially resulting in the accumulation of toxic waste products and ischaemic damage. Additionally, coexisting vascular risk factors

such as age, CAA and hypertension may exacerbate neuro-glio-vascular dysfunction and their impact is only starting to be explored.

### **Interplay between cerebral hypoperfusion and co-morbidities**

Post-mortem studies in brains from the elderly indicate the presence of a variety of pathological lesions or mixed pathologies (such as white matter changes, infarcts, microbleeds and amyloid pathology) (115). The accumulation of these changes is likely to be influenced by a number of factors such as age, lifestyle and genes in addition to vascular risk factors. Thus in selecting appropriate animal models the study of co-morbidities should be considered. Surprisingly few studies have attempted to look at interactions between different factors. This would be important in identifying drug targets and as a basis for testing treatments.

Of the few studies conducted, these have shown that the presence of more than one risk factor can exacerbate structural and functional changes in the hypoperfusion models. The induction of hypoperfusion in genetic or dietary models of VCI can exacerbate reductions in blood flow or tissue oxygen deficit, potentially through impairment of cerebrovascular autoregulation or modulation of tissue oxygen extraction fraction (116). Weaver et al. (116) demonstrated the utility of electron paramagnetic resonance oximetry to study white matter pO<sub>2</sub> reductions longitudinally in a mixed SHRSP/Japanese Permissive Diet model with unilateral common carotid artery occlusion. In this SHRSP model, white matter lesions were also exacerbated in the presence of occlusion and dietary factors (116).

It has been estimated that approximately 50% of AD risk is explained by traditional vascular risk factors (117) and most of AD cases at post-mortem have some form of vascular pathology (1, 118). Amyloid  $\beta$  protein deposition is a key hallmark of AD and believed to be a key driver of the pathophysiology of AD. Since rodents do not naturally accumulate A $\beta$ , most studies use transgenic models that harbour mutations in the amyloid precursor protein associated with rare familial forms of AD (TgAPP). Critically these TgAPP mice deposit amyloid in the brain parenchyma and the vasculature and have provided a powerful basis for the study of amyloid dynamics (see summary of models on Alzforum (<http://www.alzforum.org/research-models>)). The TgAPP mouse models in combination with BCAS have demonstrated profound effects caused by cerebral hypoperfusion on amyloid dynamics and neurodegenerative changes. In the absence of other factors, blood flow reductions in TgAPP mice may be explained by the potent effects of soluble amyloid (A $\beta$ )

on vascular function by acting as a potent vasoconstrictor (119). In another TgAPP model of CAA, Okamoto et al. (120) showed that blood flow reductions at 12 weeks following BCAS were greater in TgAPP than in wild type mice. Hypoperfusion also promoted CAA and the development of microinfarcts. We have similarly shown that BCAS alters the pools of amyloid precursor protein and amyloid peptides to promote CAA and degenerative changes (121). Others have similarly shown BCAS promotes amyloid deposition and exacerbates cognitive changes in TgAPP mice (122). CAA can also impede vascular reactivity and drainage pathways (123, 124) that may further increase the accumulation of amyloid  $\beta$ . Oxidative stress and in particular the superoxide-producing enzyme NADPH oxidase (NOX) 2 plays a key role in mediating amyloid induced vascular dysfunction (125, 126). Thus, conceivably there may be an interplay between increased NOX and amyloid following hypoperfusion that exacerbates neurovascular dysfunction. Interestingly a study using the GCAS model has demonstrated that cerebral hypoperfusion in TgAPP mice leads to prominent neurovascular unit damage that appears to be cholinergically mediated, since galantamine treatment reduces the degenerative changes (127).

A pronounced pro-inflammatory environment including activated microglia and increased release of inflammatory cytokines are features of hypoperfusion models. In TgAPP models and human AD brain a pro-inflammatory environment is closely related to the increasing accumulation of A $\beta$  protein (128, 129). Studies also support an interaction between hypoperfusion and amyloid whereby pro-inflammatory protein levels are markedly elevated and cognitive impairment is greatly exaggerated (130). The interpretation of these studies however is somewhat limited as most TgAPP models are confounded by the overexpression of APP and presence of APP fragments that may themselves impact on neuroinflammation. Recently single *App* knock-in mouse (TgAPP-KI) models of AD have been developed that produce physiological levels of APP (131). One of these, *App*<sup>NL-F/NL-F</sup> harbours the Swedish and Beyreuther/Iberian mutations in the APP gene to overproduce A $\beta$ 42 without overexpressing APP. These mice display typical A $\beta$  pathology, neuroinflammation and memory impairment in an age-dependent manner (131). Collectively, these observations suggest a vicious cycle whereby vascular factors (of which hypoperfusion is a common mechanism) promote the accumulation and/or impedes clearance of amyloid leading to impaired vascular reactivity, promotion of hypoperfusion, oxidative stress, microvascular inflammation and further production of amyloid (Figure 5).

### **Pre-clinical investigation of drug targets**

Assessment of pipeline drugs can be evaluated in preclinical models before clinical trials are initiated providing a translational opportunity. However, in reality there has been a major translational block with few agents showing promise in human VCID (see suggestions below for ways to overcome this). We provide examples that currently appear to be of potential value. One compound that has shown promise is cilostazol, a potent phosphodiesterase III inhibitor, which has been widely used as an antiplatelet agent for the prevention and treatment of peripheral arterial disease. Cilostazol normally does not cross the BBB but increases cyclic AMP in vascular cells and can exert multiple beneficial effects on the vasculature such as endothelial protection (132, 133), maintenance of microvascular integrity (134), vasodilatory, anti-oxidant, anti-inflammatory effects and regulation of smooth muscle cells (135). Moreover, in vitro experiments showed that cilostazol promoted differentiation of oligodendrocyte precursor cells (OPCs) (110). In the BCAS model, pre-treatment and post-treatment of cilostazol reduced endothelial activation, suppressed microglial proliferation and improved cognitive function without affecting resting CBF and white matter integrity (68). After three months of hypoperfusion, BBB structure is preserved, suggesting cilostazol restored gliovascular disruption via endothelial protection without affecting white matter directly (68). These reports suggest that cilostazol has beneficial effects on the gradual progression of VCID, particularly in the early stages. In the rat 2 vessel occlusion model, administration of cilostazol showed a remarkable protection against hypoperfusion-induced white matter damage including demyelination leading to improvement of cognitive impairment. In the 2 vessel occlusion model, cilostazol appears to afford greater effects potentially by crossing the disrupted BBB and directly affecting the white matter (64, 136). In experimental studies relevant to AD, cilostazol may protect against amyloid induced cognitive deficits and oxidative damage (137). Cilostazol has also been shown to reduce vascular amyloid by drainage mechanisms that promote clearance (138). In a pilot study of patients with moderate AD (139) and in a larger retrospective study of patients with mild dementia (140), receiving donepezil, cilostazol add-on treatment demonstrated significantly increased cognitive scores in comparison to baseline.

Minocycline is another compound that has shown preclinical utility in several models relevant to VCID. Minocycline is a semi-synthetic tetracycline with FDA-approval for the treatment of acne vulgaris. It is highly lipophilic and readily able to cross the BBB. The drug is shown to be a potent inhibitor of inflammatory responses in a number of vascular conditions where microglia are activated, including hypertension (141), stroke (141, 142) and cerebral hypoperfusion (84, 143, 144). In particular, administration of minocycline for 3 months in the

BCAS model restored the hypoperfusion-induced impairment in white matter function related to a reduction in the number of microglia (84). Other studies, in which white matter is damaged by cerebral hypoperfusion via unilateral occlusion (143), two vessel occlusion (144) and in hypertensive stroke prone rats with two vessel occlusion (141) have also convincingly shown protective effects of minocycline. Minocycline has also been shown in animal models of cerebral amyloidosis to improve cognition (145) via inflammatory/oxidative stress mechanisms (145, 146) independently of amyloid levels. Interestingly, additional beneficial effects of minocycline include reduction in gelatinase activity and in spontaneous haemorrhage (146, 147). These observations suggest minocycline may be a promising candidate for use in VCID, whilst it is currently already being trialled for mild AD (see [http://www.kcl.ac.uk/ioppn/depts/oldage/research/Medication-studies/Clinical-Trials/minocycline-in-alzheimers-disease-\(MADE\).aspx](http://www.kcl.ac.uk/ioppn/depts/oldage/research/Medication-studies/Clinical-Trials/minocycline-in-alzheimers-disease-(MADE).aspx)).

As indicated above increased MMPs are closely related to the pathophysiology of white matter damage and have also been shown to be increased in human VCID (148). Genetic deletion of MMP2 is protective against the effects of BCAS in the mouse model (90). In a rat model, of two vessel occlusion, inhibition of MMP2 protects against hypoperfusion white matter damage and reduces the extent of glial activation (90). A broad spectrum MMP inhibitor was also shown to protect against BBB opening in the mouse BCAS model and against the ensuing white matter pathology and cognitive deficits (52). MMP inhibitors, whilst potentially protective in pre-clinical models of hypoperfusion, their use needs to be carefully considered in human VCID where MMP effects are likely to be complex and involved in both the contribution to the damage but also in the repair processes (see 149 for review).

Life-style factors, including environmental enrichment and physical activity have been proposed as strategies to alleviate cognitive impairment in VCID. A recent report by our groups showed beneficial effects of environmental enrichment in a mouse model of chronic cerebral hypoperfusion by BCAS particularly in the white matter (49) that attenuated working memory deficits (150). These effects included a dramatic reduction in astrocyte damage and activation and microgliosis (49). It was found that limited rather than full-time exposure to environmental enrichment was more beneficial. Therefore, the implementation of even limited environmental enrichment may be beneficial for patients diagnosed with VCID.

Collectively the experimental studies provide pre-clinical support for drug and lifestyle modification which may hold potential clinical value in SVD and VCID. Although further experimental studies are needed, moderate environmental enrichment appears a safe and

effective future interventional strategy for cerebrovascular diseases, especially for patients with VCID.

**Important considerations of the models; How can we close the gap between rodent models of chronic cerebral hypoperfusion and human VCID?**

In order to effectively translate information generated from animal models such as cerebral hypoperfusion, we need to consider the general design, methodology and reporting of preclinical animal studies. Since the basis of the models are dependent on the extent of reduction of cerebral perfusion, it would be critical to monitor blood flow in each study. MRI with arterial spin labelling or similarly sensitive methods would be ideal to assess regional alterations in blood flow, particularly in subcortical areas. It is also important to recognise that blood flow is mostly measured in anaesthetised animals and may differ from that obtained in awake animals, particularly when vasodilatory anaesthetics such as isoflurane are used. Additionally, monitoring blood flow alterations in the white matter would be important. This measure is seldom reported in rodent models or in VCID, presumably relating to technical challenges regarding access and the inherently low blood flow in white matter, where small changes in flow may be difficult to detect reliably.

The outcome measures in preclinical models, including those relating to extent of white matter damage, may be influenced by operator differences, background strain of the mice (cerebrovascular differences), size of constrictor devices, anaesthesia, time of application of coils and environment (pathogen status, temperature). In some studies animals are allowed to recover between the placement of the first and second coils to allow restoration of cerebral haemodynamics (40, 44, 46-48). Furthermore, the use of markers to assess white matter pathology can also vary. For example histological approaches such as Luxol fast blue and histology, whilst useful for detection of overt white matter lesions are not sufficiently sensitive to detect diffuse white matter changes. Instead, immunohistochemical approaches are required and one of the most sensitive markers of damage is myelin associated glycoprotein which is now used for detection of hypoxia-induced white matter pathology in human brain (18). Arguably, a further refinement would be to conduct ultrastructural analysis of white matter to assess myelin and axonal alterations, albeit these approaches are challenging within the corpus callosum. In addition to pathological changes induced by reduced blood flow itself, it is important to recognise that induction of hypoperfusion in animal models may result in alterations to the vasculature, independent of perfusion deficits that could also be detrimental. For example, application of microcoils or ligation of vessels may alter autoregulation, vascular stiffness or pulsatility and impact on the dynamics of

cerebrospinal fluid circulation. Each of these mechanisms are proposed to contribute to VCID (9) and thus it would also be of interest to determine whether they are altered in the hypoperfusion models. It also needs to be considered that localised cellular inflammation may also be increased in the tissue surrounding the microcoils or constrictor devices and contribute to the pathological and behavioural outcomes (48). Critically age is a major risk factor for development of VCID and yet most studies continue to use relatively young rodents in which endogenous repair mechanisms will be more efficient. In older animals, white matter lesions are indicated to be more pronounced (100) and it may be predicted that these would be less amenable to intervention. Age and additional co-morbidities (such as systemic inflammation) need to be carefully factored in to preclinical testing of future drug targets if we are to enable meaningful translation from models to the clinic.

In summary, it must be recognised that there are no animal models which perfectly recapitulate all of the features of VCID. However, several rodent models reflect aspects of human VCID and are pertinent to tease out specific questions that are impossible to readily address in human studies. To close the gap between rodent models and human VCID, it is therefore important to understand the pathological and cognitive features of preclinical models in order to select the appropriate model for the purpose.

### **Acknowledgements**

Prof Horsburgh, Kalaria and Ihara would like to acknowledge the many talented post-graduate students and post-doctoral researchers who have contributed to the work in their groups over the years and that form the basis for the work as described within this review. KH research gratefully acknowledges the grant support from the Alzheimer Society (152 (PG-157); 290 (AS-PG-15b-018); 228 (AS-DTC-2014-017)), Alzheimer Research UK (ART-PG2010-3; ARUK-PG2013-22; ARUK-PG2016B-6), and The University of Edinburgh Centre for Cognitive Ageing and Cognitive Epidemiology, part of the cross council Lifelong Health and Wellbeing Initiative (G0700704/84698). RK acknowledges grant support from Alzheimer's Research UK (ARUK- PG2013-022) and the Medical Research Council (G0500247 and G0700718). MI acknowledges grant support from Grant-in-Aid for Scientific Research (B) (15H04271). JD was the recipient of an ARUK PhD studentship ARUK-PhD2013-18. AK was the recipient of Japan Heart Foundation / Bayer Yakuhin Research Grant Abroad.

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## Figure legends:

### Figure 1

Multiple experimental models have been developed in order to study disruption of cerebral blood flow. Focal ischaemia models, largely developed to study stroke pathophysiology, can be achieved through targeted occlusion of a specific vessel, leading to reductions in flow (>70%) that are spatially restricted to the vessel's territory. Alternatively, intra-arterial injection of emboli leads to multiple infarcts. In one model, SHRSP, strokes develop spontaneously. Global ischaemia models recapitulate severe (>90%) blood flow reductions to the forebrain. Due to the severe reductions in flow typically observed in models of ischaemia and resultant ischaemic neuronal damage, new models have since been developed and refined in order to mimic the subtle yet chronic reductions in blood flow relevant to vascular cognitive impairment. Stenosis or occlusion (rats only) of the common carotid arteries induces moderate blood flow reductions (~30-50%) without acute ischaemic damage. Abbreviations: BCAS, bilateral common carotid artery stenosis; SHRSP, spontaneously hypertensive stroke prone rat

### Figure 2

Adapted from Hattori et al. [J Am Heart Assoc.](#) 2016;5(2). A. Temporal profile of cortical surface CBF in mice subjected to gradual occlusion of both common carotid arteries (GCAS) (n=12) and bilateral common carotid stenosis with microcoils (bilateral common carotid stenosis (BCAS); n=7). The levels of cortical surface cerebral blood flow (CBF) estimates at indicated time points (before, and 1, 3, 7, 14, and 28 days after each surgery) are shown as percentage of the baseline CBF. Two groups were not significantly different in 2-way repeated-measures ANOVA. \* $P < 0.01$ , GCAS vs BCAS at indicated each time point. B. Regions of interest (ROIs) used for measurement of CBF images obtained from arterial spin labelling magnetic resonance perfusion imaging. The CBF values in cerebral cortical area were calculated from the 6 circular ROIs in blue and those in the subcortical area from the 2 circular ROIs in red. C. Representative multislice coronal CBF images obtained from arterial spin labelling at the Bregma and hippocampal levels pre-GCAS surgery and at 14 and 28 days after GCAS surgery.

### Figure 3

A. Fibre-optic oxygen sensors were implanted stereotactically into the corpus callosum of anaesthetised sham and hypoperfused mice (n=6/gp) at different times post-surgery. The measurement of tissue oxygen tension (pO<sub>2</sub>) provides a measure of oxygen availability at the cellular level. B. At all times post-hypoperfusion, pO<sub>2</sub> levels were significantly reduced to hypoxic levels (<10mmHg) \*\*\*\* $p < 0.0001$

#### Figure 4

Laser speckle imaging was used to assess vascular responses to whisker stimulation in anaesthetised sham (n=7) and hypoperfused mice (n=5). (A) Neurovascular coupling was significantly impaired in hypoperfused mice compared to sham, \*p<0.05. (B) Representative responses to whisker stimulation from sham and hypoperfused mice. The onset and end of whisker stimulation are highlighted by arrows.

#### Figure 5

Summary of proposed pathways by which chronic cerebral hypoperfusion may lead to cognitive impairment. Cerebral hypoperfusion reduces tissue oxygen levels (pO<sub>2</sub>) leading to oxidative stress and endothelial injury. The ensuing microvascular inflammation (ECM disruption, increased MMPs and ROS; inflammatory cytokines) is associated with activated microglia and astrocytes resulting in disruption of axon-glia integrity and neurovascular coupling. These inter-related pathways may lead to cognitive impairment. Cerebral hypoperfusion can also increase amyloid which may potentiate these mechanisms and promote further degenerative changes and cognitive impairment. Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteinase; ROS, reactive oxygen species; BBB, blood brain barrier

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