EXPERT REVIEWS

Understanding and preventing the development of post-stroke dementia

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Post-stroke dementia (PSD) is a clinical entity but it now appears that most of PSD may be categorized as vascular dementia. The well-established relationship between vascular factors and dementia provides a rationale for the implementation of intervention and prevention efforts. Larger primary prevention trials related to lifestyle factors are warranted in association with dementia. Published clinical trials have not been promising and there is meager information on whether PSD can be prevented through the use of pharmacological agents. Control of vascular disease risk and prevention of recurrent strokes are obviously key to reducing the burden of cognitive decline and dementia after stroke. However, modern imaging and analysis techniques will help to elucidate the mechanism of PSD and establish better treatment.

KEYWORDS: Alzheimer's disease • cognitive impairment • dementia • microinfarct • neuroimaging • post-stroke dementia • stroke • vascular dementia • white matter

There is increasing interest in cognitive function after stroke episodes as medical management and care improve with patients surviving long periods. The development of cognitive impairment and incident dementia or post-stroke dementia (PSD) is relatively common following stroke [1,2]. While delirium may be a frequent immediate consequence after stroke injury [3,4], cognitive decline following an index stroke could be insidious with the latent appearance of dementia. PSD is a clinical entity to define any dementia occurring after stroke, irrespective of whether it involves vascular, degenerative or mixed processes. Therefore, PSD can entail a complex etiology with varying combinations of large and small vessel disease as well as non-vascular neurodegenerative pathology. The development of PSD depends on several factors including the location and volume of the stroke, degree of related neuronal damage, presence of pre-existing cognitive impairment or cerebral pathology. At this stage, the contribution of any specific genetic factors is not clear. PSD is generally defined by dementia that occurred within 3 months after stroke onset. However, many may develop dementia well beyond 3 months after the stroke or only after recurrent stroke(s). The recognition of cognitive impairment in the acute phase after stroke may offer vital information to the clinician for early cognitive rehabilitation [5] and preventing early fatality by improved management [6].

Recent prospective studies suggest stroke survivors may unmask or trigger additional pathologies including those attributed to our current understanding of subcortical vascular dementia (VaD), multi-infarct dementia and even strategic infarct dementia [1,7,8] (FIGURE 1). Given this definition, most PSD may be described under the umbrella term of vascular cognitive impairment (VCI) [9,10], which has been introduced to describe the full spectrum of cognitive change related to all causes of vascular disease from VCI no-dementia to variable degrees of frank dementia of vascular origin. It is suggested that dysfunction of the neurovascular unit and mechanisms regulating cerebral blood flow particularly in the deep white matter (WM) are important components of the pathophysiological processes underlying VCI. The continuum of VCI is also discussed broadly under the rubric of vascular cognitive disorders (VCDs) [11], which comprise many diseases, each with varying severity and patterns of dysfunction. The categorical diagnosis

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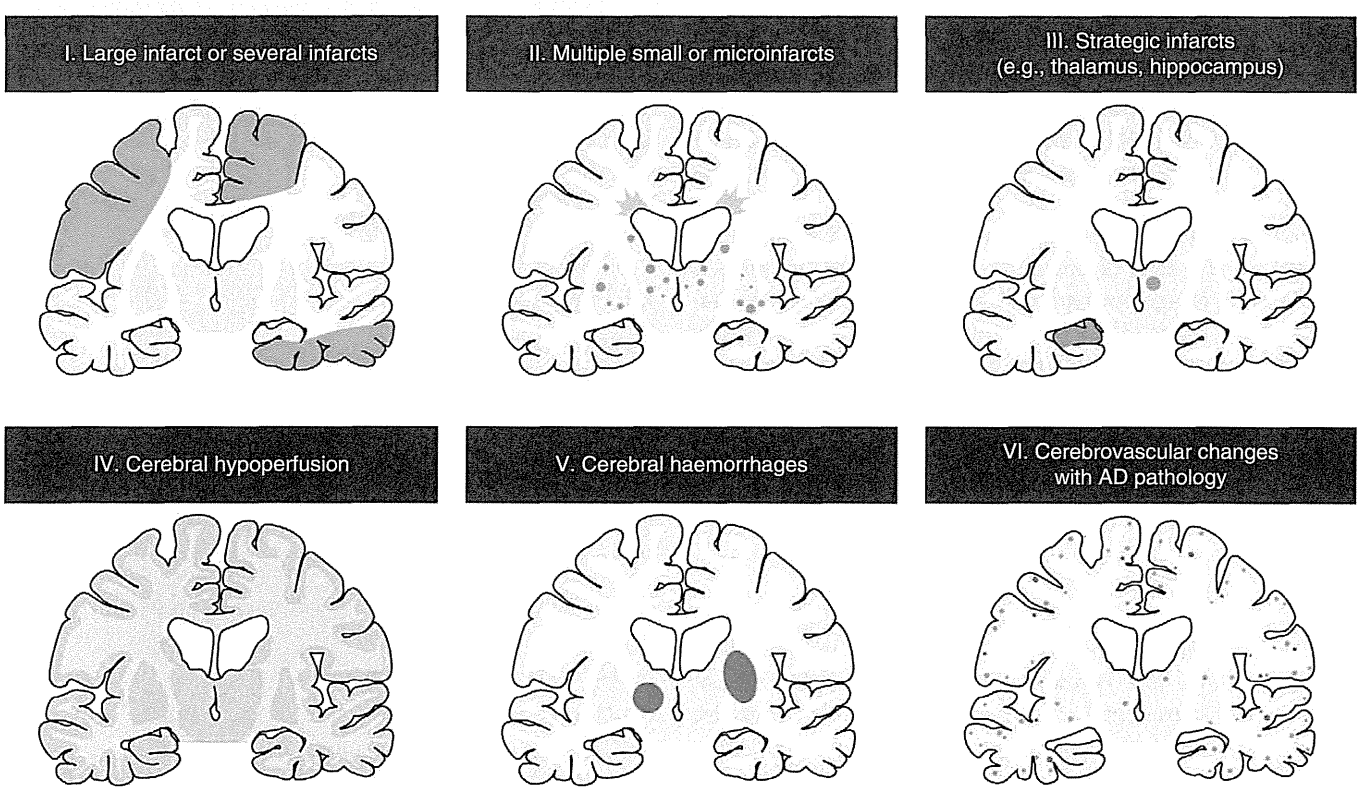

Figure 1. Dementia associated with different cerebrovascular pathologies [16]. Subtype I may result from large vessel occlusion (atherothromboembolism), artery-to-artery embolism or cardioembolism. Subtype II usually involves descriptions of arteriosclerosis, lipohyalinosis, hypertensive, arteriosclerotic, amyloid or collagen angiopathy. Subtype III is caused by infarcts in the 'strategic' areas such as the thalamus and hippocampus. Subtype IV results from hippocampal sclerosis, ischemic-anoxic damage, cortical laminar necrosis and border zone infarcts involving three different coronal levels. Subtype V follows lobar, intracerebral (hypertensive) or subarachnoid hemorrhages. Subtype VI includes so-called mixed dementia with co-existing cerebrovascular and Alzheimer changes that may be characterized by cortical or deep small infarcts, cerebral microbleeds and hippocampal atrophy. These subtypes would include dementia among post-stroke survivors who fulfill the National Institute of Neurological Disorders and Stroke and Association Internationale pour la Recherché et l'Enseignement en Neurosciences (NINDS-AIREN) criteria for probable vascular dementia.

of VCDs encompasses mild impairment, pre-dementia and dementia syndrome, and major VCD category is equivalent to dementia as adopted in the DSM-5. PSD would be better categorized as severe VCD [11].

While dementia after stroke is clinically diagnosed using the widely accepted DSM IIIR or IV criteria, it is important to note that there is great paucity of autopsy studies to confirm the clinical diagnosis. PSD may incorporate different types of dementias, but there is no pathological confirmation for most of the studies even when there may be strong contribution of neurodegenerative disease. The 'gold standard' for diagnosis of dementia has been based on the results of the extensive neuropsychological examination, clinical presentation and information from a close relative as well as the usefulness of Revised-Cambridge Assessment Test (R-CAMCOG) of the Cambridge Examination for Mental Disorders of the Elderly [12] or the Montreal Cognitive Assessment [13–15] has been reported for the clinical screening tool for PSD. Although such short screening tests are useful for both clinical and research purposes, their sensitivity is limited and there is no clear consensus as to which test is the most appropriate. Our recent study [8] incorporating pathological examination indicated that almost 75% of demented stroke survivors fit current criteria for VaD [9,16,17], whereas the rest had mixed pathology with either Alzheimer type of lesions or Lewy bodies. Thus, in large part, clinically diagnosed and neuropsychometrically assessed PSD may turn out as VaD. It is reasonable to assume that many of the cerebral changes would be consistent with the small vessel disease type of pathology (Figure 1, subtype II), while there may be high prevalence of small cortical infarcts and microinfarcts [8].

PSD is a frequent but neglected consequence of stroke compared with other neurological deficits such as sensory or motor impairment [18]. However, not all strokes result in cognitive impairment, but stroke may at least double the risk of dementia [19]. In community-based studies with adjustment for age, the prevalence of dementia in people with a history of stroke is about 30% with 3.5- to 5.8-times higher than in those who have not experienced stroke [20]. The incidence of dementia in

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older people with a longer follow-up time increases from 10% at 1 year to 32% after 5 years [1]. Recent meta-analysis indicated that pooled prevalence estimates of PSD less than 1 year after the stroke ranged from 7.4% in population-based studies of first-ever stroke, in which pre-stroke dementia was excluded, to 41.3% (29.6–53.1) in hospital-based studies of recurrent stroke in which pre-stroke dementia was included. The cumulative incidence of dementia after the first year was slightly greater (3.0%, 1.3–4.7) per year in hospital-based studies than expected on the basis of recurrent stroke alone [2]. In subjects over 75 years of age, we reported that although 41% were stable and 50% improved in cognition at 15 months post-stroke in survivors who were dementia free at 3 months post-stroke [21], a substantial proportion of these subsequently progress to delayed dementia [22]. Consistent with other studies utilizing the 3-month design [23–25] before follow-up, delayed PSD was estimated to occur in at least 25% of the subjects with various risk factors.

Risk factors of PSD

Besides older age (Table 1), risk factors for PSD include prior or recurrent stroke, and pre-stroke dependency and cognitive impairment and, low education status [1,2,24,26]. More recent studies have consistently shown that post-stroke cognitive impairment or VCI is frequent and associated with poor functional outcome, development of apathy or quality of life with lower educational status, strategic site lesion, greater severity of age-related WM changes and baseline stroke severity as key contributors to the impairment [27–31]. In addition, a growing body of evidence indicates the role of genetic factors as the risk for PSD [32]. Current data assessing the significance of specific candidate genes such as angiotensin-converting enzyme gene [33], α-1-antichymotrypsin [33,34] or *APOE* gene [33,35,36] as risk factors for PSD are not entirely in agreement. However, in older stroke patients with early cognitive impairment, the presence of an *APOE* ε4 allele was reported to be associated with greater progression of cognitive decline [37].

Studies from general community populations indicate that VaD is more frequent in males than in females, but most studies suggested no substantial gender difference for the risk of PSD. Recent findings suggest this is attributable to neuroprotective role of adiponectin because of the relatively steeper decline of serum adiponectin levels associated with aging in females than in males [38]. Epileptic seizures, sepsis, cardiac arrhythmias and congestive heart failure are listed as other risk factors of incident PSD [39]. It is not surprising that atrial fibrillation and nephropathy independently contribute to the risk in addition to older age, previous mental decline and stroke severity [23].

Brain lesion correlates of PSD include a combination of infarct features (volume, site), the presence of white matter lesions (WMLs; extent, location) as well as brain atrophy [40]. Important critical locations include dominant hemisphere and lesions affecting the prefrontal–subcortical circuit that mediates executive dysfunction [41]. In post-stroke cohorts, the presence of executive syndrome and depression is the predictor of poor long-term survival rather than depression itself [42]. It is also clear that dementia and depression interact with each other in the post-stroke period [43]. If there is preceding dementia, stroke worsens the cognitive impairment, it may be called pre-stroke dementia (see below) with possibility of co-existing neurodegenerative pathology as a cause of dementia. Progressive dementia without any symptomatic stroke but with only radiologically-proven cerebrovascular diseases may not be diagnosed with PSD because cerebrovascular lesions such as WM changes and apparently silent lacunar infarcts are common in demented elderly with mixed pathology consisting of Alzheimer's disease (AD) with cerebrovascular diseases. Since the rates of dementia after stroke may continue to rise in a relatively linear fashion [2], they illustrate that stroke, vascular risk factors or co-existing neurodegenerative changes make the brain more vulnerable to dementia in the longer term. Comorbidities that raise the risk of PSD are hypertension, atrial fibrillation, diabetes mellitus, myocardial infarction and congestive heart failure [1,39,44,45]. Consistent with this, a prospective longitudinal study [8] revealed that presence of three or more cardiovascular risk factors increased risk of dementia or death by fourfold in

Table 1. Risk factors in post-stroke dementia.

Demographic features	Odds ratios ($p < 0.05$)
Advanced age	6.6 for >65 years[†]; 1.05–1.2 per year
Low education	2.5
Stroke characteristics	
Transient ischemic attacks	1.83[‡]
Recurrent stroke	2.3
Multiple infarcts	2.5
Strategically located infarcts	NA
Stroke severity	2.5
Neuroimaging markers of brain lesions	
Silent brain infarcts	1.8
White matter lesions	2.5
Medial temporal lobe atrophy	2.69 –5.2[§]
Cerebral atrophy (global/temporal lobe atrophy)	2.6
Cerebral microbleeds	NA[¶]

[†]This study showed rather high risk with age [127].
[‡]OR in post-stroke patients with pre-stroke transient ischemic attack less than 4 weeks was determined to be 1.83 (95% CI: 1.32–2.52).
[§]ORs of post-stroke amnestic vascular cognitive impairment and mild cognitive impairment groups compared with non-amnestic vascular cognitive impairment no-dementia group [83].
[¶]Cerebral microbleeds predicted frontal-executive impairment with OR 8.4 up to 5.7 years of follow-up [128].
NA: Not available; OR: Odds ratio.
Data taken from several publications [1,2,126,127].

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Box 1. Modifiable or treatable risk factors in post-stroke dementia.

Vascular disorders and conditions

- Hypertension
- Atrial fibrillation
- Diabetes mellitus type II
- Myocardial infarction
- Congestive heart failure
- Pre-stroke cognitive status
- Obesity, metabolic syndrome

the elderly stroke survivors. These observations contrast with a systematic review [46], where the findings concluded that the effect of stroke on dementia incidence in the population was explained by recurrent stroke rather than cardiovascular risk factors. A number of studies have found significant effects of individual vascular risk factors in both early and delayed PSD, but have not examined their cumulative effect. However, metabolic syndrome, a clustering of those cardiovascular risk factors may well affect PSD through 'metabolic-cognitive syndrome' [47]. Another longitudinal study [48] showed that patients with PSD had a higher prevalence of several vascular risk factors including hypertension, diabetes, atrial fibrillation, previous myocardial infarction and history of transient ischemic attacks (Box 1). However, these associations have not been found in other studies with shorter follow-up [23,24,49–51]. Independent contribution of vascular risk factors or disorders to the development of dementia following occurrence of stroke still needs consensus [52].

Pathophysiology of PSD

PSD is caused by all-encompassing effects of vascular disease or lesions affecting cognition. It incorporates the complex interactions between vascular etiologies, risk factors and cellular changes (e.g., aging and neurodegeneration) within the brain. Furthermore, neuroinflammation and immunodepression associated with stroke, aging and infection may have a detrimental role in PSD [53–56]. The cognitive domains involved in the development of dementia after stroke may vary depending on stroke characteristics such as stroke type, volume, numbers, location and severity. However, it is apparent that frontal lobe functions comprising processing speed, reaction time, working memory and executive task measures are most affected [57]. Regarding the stroke type, patients with ischemic strokes usually have higher survival rates than do those with hemorrhagic strokes, which explains why ischemic strokes lead to psychiatric morbidity more frequently than do hemorrhagic strokes [58]. A single large cortico-subcortical brain ischemic lesion, if located in an area that is functionally critical for cognition, may present with acute cognitive deterioration. Strategic infarct dementia is attributed to locations in the angular gyrus, the medial frontal lobe and the inferomedial portion of the temporal lobe, all of which may be caused by large-vessel pathology [17]. Bilateral hippocampal or thalamic infarctions and unilateral thalamic infarctions are other examples of strategically localized infarctions that are reported to cause dementia. Strategic infarction may be caused by damage to the components of Papez (hippocampal memory loop) [59] or Yokovlev circuits.

Radiological determinants of PSD

A combination or interaction of different types of brain lesion, including neurodegenerative markers, and pre-existing underlying processing may play a more important role in the development of cognitive decline after clinical stroke. For example, the 24-year study [48] showed that the prevalence of PSD associated with lacunar stroke was seven-times higher than other types of stroke including intracerebral hemorrhage. Strategic infarction dementia as a disease entity has been challenged since the influence from other lesions is generally ignored in studies addressing strategic infarction dementia [60]. However, pre-stroke medial–temporal lobe atrophy, silent brain infarcts and microbleeds and extensive WMLs are associated with an increased risk of post-stroke memory dysfunction, a prerequisite for the diagnosis of PSD.

WM lesions

Stroke patients with more severe WMLs have an increased risk of recurrent strokes. Thus, the presence and severity of WMLs seen on MRI may be predictive of PSD [1,29,61,62]. However, WMLs may not be predictive of subsequent decline in all stroke survivors and other anatomical substrates appear more involved [61,63]. Ischemic WM changes are most prominent in the frontal lobe [64] and appear linked with frontal-subcortical disconnection [65,66]. The WM changes consist of myelin rarefaction with shrunken oligodendrocytes and axonal abnormalities resulting from vascular insufficiency and a chronic hypoxic state [67–69]. The independent effect of WMLs on dementia among stroke patients needs to be verified by simultaneously taking into account all other MRI findings as other types of brain lesions, such as cerebral atrophy and silent infarcts are strongly correlated with WMLs. Microstructural changes of normal appearing WM as evident in diffusion tensor imaging may be a better predictor of cognitive decline in patients with WM hyperintensities [70]; such new measures should be incorporated in clinical trials for treatment of PSD.

Silent brain infarcts & microbleeds

Several studies have consistently reported that cerebral silent infarcts detectable with computed tomography or MRI were independently predictive of PSD [71,72]. Silent infarcts may be more important to the delayed onset of dementia in patients with clinical stroke because presence of silent infarcts is associated with PSD detected in the 3rd year, but not in the 2nd year after the index stroke [73]. In addition, microbleeds are radiological hallmarks of cerebral amyloid angiopathy (CAA) that can be detected with the T2*-weighted gradient-echo sequence of MRI. CAA, which is correlated with WMLs and lacunar infarcts, may subsequently result in lobar hemorrhages. CAA can be also a cause of cortical microinfarcts (50–500 μm in diameter) that are invisible on conventional 1.5 and

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3 T MRI [74–76]. While observations on the occurrence of stroke and PSD in patients with microbleeds are not generally available [77], a recent study in a large cohort of patients in a memory clinic found a relatively high frequency of microbleeds in patients with VaD (65%), AD (18%) and mild cognitive impairment (MCI) (20%) [78]. This clinic-based study is consistent with the neuropathological observations, which showed that severe CAA and cerebral cortical microinfarcts are in tandem important substrates of cognitive decline [79]. Changes in the hemodynamics such as hypotension in the presence of CAA may be indicated as a key factor in the genesis of cortical watershed microinfarcts [63,80].

Cerebral atrophy

Global and medial–temporal lobe atrophy are shown to be associated with PSD [1] (Table 1). If medial–temporal lobe atrophy is considered a marker of AD, the development of PSD in subjects with medial–temporal lobe atrophy may be caused by the concomitant neurodegenerative process that was ongoing in the preclinical phase at the time of stroke occurrence. Previous studies found that in elderly stroke survivors, medial–temporal lobe atrophy was associated with shorter time to dementia [81], but it was more strongly associated with subsequent cognitive decline than were WMLs [63], which suggested a greater role for Alzheimer-type pathology than cerebrovascular lesions in the development of delayed cognitive impairment after the onset of clinical stroke. This was further supported by the findings that reductions in blood flow, assessed by arterial spin labeling, in the posterior artery territories and in volumes of the hippocampal formation were similar in PSD and AD subjects [65,82]. In accordance with these findings, another study showed that increasing severity of medial temporal lobe atrophy was associated with amnestic VCI no-dementia (odds ratio [OR]: 2.69; 95% CI: 1.21–5.99) and amnestic MCI (OR: 5.20; 95% CI: 2.41–11.23) compared with non-amnestic VCI no-dementia post-stroke survivors [83]. Moreover, the impaired post-stroke episodic memory function may be caused by reduced medial temporal lobe functionality [84]. However, WMLs in the frontal and parieto-occipital regions correlate with hippocampal atrophy, suggesting that there is a tangible link between vascular pathology and hippocampal atrophy [85]. That such a link exists, there is selective hippocampal neuronal shrinkage which not only appears to be an important substrate for AD, but also shows delayed dementia after stroke in the absence of any neurodegenerative pathology [22]. This is consistent with the findings in animal models that long-term hypoperfusion does not require co-existing neurodegenerative changes to induce hippocampal atrophy [86] and demonstrates the vascular basis for neurodegeneration as substrate of dementia.

Pre-stroke dementia

The risk of PSD is increased in patients with pre-stroke cognitive decline, with about one-third of patients meeting the criteria for AD and two-thirds meeting the criteria for VaD [87]. Pre-existing brain structural changes may have impact on the occurrence of PSD. These pre-existing changes are related to different mechanisms that result in the development of dementia. Brain atrophy and medial–temporal lobe atrophy may underlie AD pathology, while WMLs underlie VaD of the subcortical type. However, as has been emphasized [88], there is an overlap between vascular and degenerative mechanisms responsible for the pathogenesis of VaD, and the term 'pre-stroke dementia' has been coined [87]. Only one single factor cannot explain the development of dementia related to large-vessel pathology, as the etiology is probably multifactorial, where stroke characteristics (stroke type, volume, number, location and severity) as well as host factors such as comorbidities contribute to the risk independently. In the majority of the cases, several mechanisms interact to exceed the critical threshold for normal cognition and dementia subsequently occurs. Thus, even marginally increased burden of amyloid and neurofibrillary pathology above normal aging but insufficient for pathological diagnosis of AD likely adds to the tissue degenerative process leading to dementia after stroke. This important issue may be further investigated in future using PET imaging for amyloid [89] or tau [90].

Prevention & treatment

Any measure that reduces or controls vascular disease would be preventative for PSD [91]. In recent years, a variety of lifestyle factors including diet and physical activity has been highlighted for prevention of dementia (Box 1). These would all be equally applicable in the prevention of PSD. Despite its high prevalence, the treatment options for PSD are limited. As most vascular risk factors and disorders are modifiable (Box 1), understanding the role of vascular risk factors in dementia provides much potential for the treatment of PSD [92]. As dementing disorders, once established, are not curable, implementation of primary treatment of vascular disorders seems to be the most promising for reducing the burden of not only PSD, but also dementia in general.

Stroke is related to two- to ninefold increase in risks of dementia [93]; therefore, adequate primary prevention and neuroprotective intervention after stroke occurrence or recurrent events [94] will have enormous impact. Results collected between 1995 and 2011 (n = 4413) from the community-based South London Stroke Register [92] showed that, in patients with ischemic strokes without a history of atrial fibrillation, there was a significantly reduced risk of cognitive impairment associated with the use of antihypertensives (relative risk [RR]: 0.7; 95% CI: 0.57–0.82) for diuretics; RR: 0.8; 95% CI: 0.64–0.98 for angiotensin-converting enzyme inhibitors and RR: 0.7; 95% CI: 0.55–0.81 for their combination) when clinically indicated. In addition, there was a tendency of reduced risk of cognitive impairment associated with the use of a combination of aspirin and dipyridamole (RR: 0.8; 95% CI: 0.68–1.01) and statin (RR: 0.9; 95% CI: 0.76–1.06) [92]. Protective effects against cognitive impairment were also observed in patients on the combination of antihypertensives, antithrombotic agents and lipid-lowering drugs (RR: 0.55; 95% CI: 0.40–0.77).

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Antihypertensive drugs

Clinical trials provide some evidence that high blood pressure (BP) might be associated with recurrent stroke-related dementia and cognitive decline [95]. Although there exist no clinical trials of antihypertensive drugs targeting specifically PSD, there are six large randomized trials of antihypertensive drugs in which dementia and cognitive function were assessed. Four of these trials showed that antihypertensive treatment had no positive effects on the risk of dementia or cognitive function. In one study (Syst-Eur), in which calcium antagonist nitrendipine was used with the possible addition of enalapril (angiotensin-converting enzyme inhibitor) and/or hydrochlorothiazide (diuretic), there was a beneficial effect on the risk of dementia [96]. In another study (Perindopril Protection Against Recurrent Stroke Study, PROGRESS), in which perindopril was used with the possible addition of indapamide, a positive effect was shown on the risk of PSD [95]. However, no benefit was found in cognitive performance after administration of telmisartan, an angiotensin II receptor blocker, at the subacute stage (within 15 days) after stroke in the Prevention Regimen for Effectively Avoiding Second Strokes (PRoFESS) study [97]. In the PRoFESS study, excessive lowering of BP in the period with cerebrovascular autoregulatory dysfunction may have affected the cerebral circulation and neuronal function, although other factors could also be involved. However, the above-mentioned London Stroke Registry showed that antihypertensive treatment reduced the risk by 20–30% by use of diuretics and/or angiotensin-converting enzyme inhibitors [92]. Secondary prevention of small subcortical stroke trial that included 3020 patients with MRI-verified lacunar strokes within the preceding 6 months [18] showed a modest decline in the patients' cognitive function after a lacunar stroke, but a target systolic BP either of 130–149 mmHg (n = 1519) or <130 mmHg (n = 1501) did not change the trajectory of cognitive decline [98].

Antidiabetic agents

Diabetes mellitus is an independent predictor of PSD [87]. There is strong evidence for an elevated risk of both VaD and AD in patients with type 2 diabetes mellitus, albeit with strong interaction of other factors such as hypertension and dyslipidemia [99]. Intriguingly, higher glucose levels may be a risk factor for dementia, even among persons without diabetes [100]. Nevertheless, intensive glucose lowering was found to increase mortality without significantly reducing cardiovascular events including non-fatal stroke [101]. One of the reasons is that hypoglycemia requiring assistance was more frequent in the intensive-therapy group. There are several novel antidiabetic drugs such as sodium-dependent glucose cotransporter 2 inhibitors with putatively less occurrence of hypoglycemia. It should be determined whether antidiabetic therapy without hypoglycemia would reduce the burden of PSD.

Statins

There are no randomized trials of treating PSD with statins. Currently, Prevention of Decline in Cognition after Stroke Trial is ongoing to investigate whether intensive lowering lipids and/or BP lowers the risk of cognitive decline after stroke. Intensive lipid-lowering targets low-density lipoprotein-cholesterol <1.4 mmol, while guideline level is <3 mmol/l. The London Stroke Registry showed that statin use reduced the risk by 10%, although there was no statistical significance [92]. The Dijon Stroke Registry showed negative association of both hypercholesterolemia and statin use with PSD, further complicating this issue, although a meta-analysis suggests that statins are not effective for the treatment of dementia. However, the question of whether statins also have a neuroprotective effect in humans and reduce the risk of PSD remains unsettled.

Antiplatelet drugs

Studies on aspirin in terms of protection of cognitive decline are inconclusive. An earlier study [102] allocated 70 patients with multi-infarct dementia either to aspirin or no treatment. Subjects were followed annually for 3 years and those treated with aspirin showed higher cognitive performance compared with those receiving no treatment. However, this study had several limitations derived from the small sample size, the high dropout rate, the lack of placebo and the lack of true randomization. The PRoFESS trial showed no significant difference in the occurrence of cognitive decline, MCI or dementia in those treated with aspirin plus extended-release dipyridamole or clopidogrel [97]. The London Stroke Registry shows a non-significant tendency that a combination of aspirin and dipyridamole is effective to reduce risk of cognitive impairment [92]. Based on the PRoFESS study, antiplatelet regimes do not appear to differ from each other in their effect on cognition and dementia [97]; cognitive decline in patients with ischemic stroke were not different between the two antiplatelet regimens (either 25 mg aspirin and 200 mg extended-release dipyridamole twice a day or 75 mg clopidogrel once a day). The subcortical stroke trial showed that dual antiplatelet therapy (aspirin 325 mg/clopidogrel 75 mg vs aspirin 325 mg/placebo) did not modify the rate of cognitive decline [98].

Acetylcholinesterase inhibitors

Several studies have demonstrated beneficial effects of cholinesterase inhibitors (donepezil, galantamine or rivastigmine) for patients with VCI [10]. Demonstration of a cholinergic deficit in AD has led to the development of the cholinesterase inhibitors [103] such as donepezil, rivastigmine and galantamine, all of which produce a modest benefit in people with AD [104]. Early trials of cholinesterase inhibitors in VaD suggested some benefits [105], but several large studies of the above cholinesterase inhibitors have produced no significant change regarding global outcome scales, activities of daily living or behavioral features despite a small cognitive benefit. A study of donepezil in 168 subjects with a familial form of VaD (cerebral autosomal dominant arteriopathy with subcortical ischemic leucoencephalopathy) showed no significant benefit with donepezil on primary outcome, although some benefits were shown on secondary end points, which may be consistent with some cholinergic deficits in

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cerebral autosomal dominant arteriopathy with subcortical ischemic leucoencephalopathy [106]. However, a meta-analysis of cholinesterase therapies for VaD has concluded that the data are insufficient to support widespread use of the drugs [107]. However, those with mixed Alzheimer and vascular pathology do appear to benefit [105] and this is consistent with the findings from autopsy studies that cholinergic deficit is as great in those with mixed Alzheimer/vascular pathology as in AD [108].

Memantine – NMDA glutamate receptor antagonist

Little is known about the changes of glutamatergic transmission in VaD. Glutamate is one of the key molecules involved in long-term potentiation and, therefore, in learning and memory [109]. In addition, glutamate is also a critical neurotransmitter for signaling neurons to degenerate following stroke [110]. In rat cortical stroke model, NMDA receptor density is increased in the caudoputamen [111]. Clinical trials for stroke using non-competitive agent MK-801 was discontinued because of the several dose-related adverse events. However, a partial NMDA antagonist, memantine, has been indicated for mild-to-moderate AD patients [112] and has also shown benefits for VaD patients [113]. Two studies with memantine likewise showed cognitive benefit without global or functional benefit in mild-to-moderate VaD patients [114,115].

Citicoline

Citicoline, also known as cytidine-5′-diphosphate-choline, is an intermediate in membrane phospholipid synthesis. The main components of citicoline are choline and citodine, which are readily absorbed in the gut and cross the blood–brain barrier. Citicoline drew much attention as a drug to treat acute stroke, but was shown to be ineffective in treating stroke [116], although there remains a possibility that a liposome formulation of citicoline would be effective on the basis of preclinical studies [117]. Nonetheless, a recent trial starting within 24 h of stroke and lasting 12 months in patients with first-ever ischemic stroke showed that citicoline prevented cognitive decline after stroke with significant improvement of attention-executive functions and temporal orientation [118]. Large clinical trials are needed to confirm the net benefit of this therapeutic approach [118].

Immunotherapy

The inflammatory and/or immunological hypothesis of cognitive impairment is supported by neuropathological and epidemiological observational studies [119]. Since stroke induces immunosuppression [54], immunostimulation or blockade of stress pathways (e.g., sympathetic nervous system) may improve outcomes in persons with stroke by restoring immune function and preventing infections [120]. Although little is known about long-term effects of the adaptive immune response associated with stroke and their role in PSD, immunotherapy needs to be pursued as a future putative therapy to deviate the post-stroke immune response away from tissue damage and toward brain protection.

Regenerative therapy

Although not proven effective yet, regenerative therapy may hold a promise in treatment of PSD because neurovascular regeneration is an ultimate goal to restore damaged tissue after stroke. Because post-stroke cognitive impairment occurs as a result of persistently compromised blood flow to the brain, angiogenesis may enhance recovery of the brain after stroke. Using animal models, potential drugs such as angiogenic peptides [121] or cell therapies [122] have been proposed to enhance angiogenesis as a preclinical step toward developing novel treatments for dementia of vascular origin. There are several clinical trials reported using bone marrow-derived stem cells for stroke patients [123]. Intravenous injection of autologous mesenchymal stem cells has been shown to recover neurological status as assessed with the National Institutes of Health Stroke Scale and modified Rankin scores in stroke patients [124], although cognitive function is not evaluated. Compared with the acute stroke, the therapeutic time window is relatively wider in PSD cases. In future clinical trials of regenerative therapy for stroke, long-term efficacy for cognitive function should be evaluated to determine whether regenerative therapy is available for patients with PSD.

Other putative treatments

Given the well-established disease entity of post-stroke depression, serotonergic dysfunction may well be involved in pathophysiology of PSD. Trials of selective serotonin reuptake inhibitors have shown some benefit in cognitive enhancement after stroke, both as a consequence of their antidepressant effects and through other independent routes [112,113], although none of these studies has been so far definitive. According to recent evidence, concurrent cerebrovascular disease lowers the threshold for dementia due to α-synucleinopathies such as Parkinson's disease or dementia with Lewy bodies [114], which suggests that the overlap between cerebrovascular diseases and α-synucleinopathies can offer an important avenue for treatment of PSD.

The ongoing ARTEMIDA (A Randomised, Double-Blind, Placebo-Controlled Trial of Actovegin) study will evaluate the efficacy and safety of actovegin for the symptomatic treatment of post-stroke cognitive impairment and will explore whether actovegin has any disease-modifying effect by assessing whether any changes are sustained after treatment [125]. Actovegin is a deproteinized ultrafiltrate of calf blood composed of more than 200 biological substances and anticipated to have pleiotropic neuroprotective and metabolic effects.

Expert commentary

Most PSD may be described under the umbrella term of VCI, which is associated with all causes of vascular disease. However, PSD would be better categorized as severe VCD. The well-established relationship between vascular factors and dementia provides a rationale for the implementation of intervention and prevention efforts. Published clinical trials have not been promising and little is known whether PSD

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can be prevented using pharmacological agents. However, there is increasing evidence that appropriate vascular risk management is associated with a long-term reduced risk of cognitive impairment.

Five-year view

This review provides a speculative viewpoint on how the field will evolve in 5 years' time.

Modern imaging and analysis techniques such as 7T MRI and amyloid PET imaging will help to elucidate the mechanism of PSD; 7T MRI will be used to detect microinfarcts that distribute in the brain and amyloid or tau PET imaging will be useful to determine contribution of Alzheimer changes to PSD. A combination therapy may be developed to target multiple components of PSD such as vascular risk factors, neurotransmitters and neurodegeneration based on its multifactorial nature.

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Key issues

- Post-stroke dementia (PSD) is a clinical entity that encompasses all types of dementia following an index stroke.
- The type of stroke can be either ischemic, hemorraghic or hypoperfusive.
- The risk factors of PSD are multifactorial, which include older age, low education, vascular comorbidities, prior or recurrent stroke, pre-stroke dependency and cognitive impairment.
- Cognitive impairment before the index stroke is called pre-stroke dementia, which may be caused by vascular burden as well as insidious neurodegenerative changes.
- High-resolution MRI will elucidate microinfarcts, which would be important substrates of PSD.
- There is a clear need for effective treatments for PSD.
- Primary prevention trials related to lifestyle factors are greatly warranted in association with dementia after stroke.
- A combination therapy may be developed to target multiple components such as vascular risk factors, several neurotransmitters and even neuroregeneration.

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PLOS ONE

A Novel Mouse Model of Ischemic Carotid Artery Disease

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Abstract

Background: Carotid artery occlusive disease gradually develops over time, eventually leading to cerebral infarction and high mortality rate. Animal models replicating cerebral infarction resulting from carotid artery occlusive disease have thus been developed to test potential novel treatments, which could be subsequently administered clinically.

Methods: Adult C57BL/6J male mice were subjected to ameroid constrictor (AC) placement to gradually narrow the bilateral common carotid arteries. Cerebral blood flow (CBF) was measured at several time points. At 7 and 28 days post-operation, post-mortem brain samples were analyzed for ischemic changes.

Results: The mortality rate was 58.8% at 28 days post-operation. Surviving mice with AC showed continuous reduction of CBF by up to 70% of the baseline level at 28 days. Most of the mice (75%) showed multiple cerebral infarctions in the gray and white matter. Non-surviving mice showed critical CBF reduction below 20–30% of the baseline level before death.

Conclusion: The application of the AC on the bilateral common carotid arteries in mice could offer a reliable model of severe cerebrovascular insufficiency due to carotid artery occlusive disease and may thus be useful in exploring pharmacological intervention in stroke through monitoring survival rate, infarct formation, and CBF profile.

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Introduction

Number of patients with severe carotid artery diseases is gradually increasing because metabolic disorders are prevalent worldwide with the adoption of a sedentary lifestyle combined with excessive caloric intake. They are susceptible to cerebral ischemia such as cerebral infarction [1] and cognitive impairment [2]. Patients with bilateral carotid artery occlusion have a particularly poor prognosis as a result of a high subsequent stroke prevalence rate (66% in bilateral internal carotid artery occlusion and 71% in bilateral common carotid artery occlusion [3]), and high mortality rate of more than 50% over six years [4]. Therefore, animal models which can mimic natural history of severe carotid artery disease are warranted to develop novel and safer prophylactic medications to protect patients at risk from cerebral ischemia distal to severe carotid artery diseases.

Several animal models of cerebral infarctions have been established including models of transient/permanent middle cerebral arterial occlusion and embolic stroke [5], with an infarction-inducing model of severe carotid artery disease developed in Mongolian gerbils [6]. Nevertheless, the suitability of the gerbil model of cerebral infarction distal to bilateral common carotid artery stenosis has been questioned due to an incomplete circle of Willis, absence of the posterior communicating artery (PcomA) [7] in such species. Furthermore, as gerbils are seizure-prone animals, generalized tonic-clonic seizures tend to occur spontaneously or in response to environmental stimuli including cerebral ischemia and may alter cerebral blood flow after seizures [8]. Besides, all C57BL/6J and almost all of Institute of Cancer Research (ICR) and BALB/c mice subjected to ligation of bilateral common carotid arteries (CCAs) die within 24 hours [9]. Thus, a suitable mouse model of cerebral infarction by carotid artery stenosis/occlusion has not been established.

We thus propose a mouse model that successfully induces severe cerebrovascular insufficiency and multiple cerebral infarctions using ameroid constrictors (AC) placed on the bilateral CCA.

Materials and Methods

Ethical Statement

All procedures in this study were carried out in strict accordance with the guidelines for animal experimentation from the Animal Research Committee of Kyoto University and that of National Cerebral and Cardiovascular Center. The protocol was approved by the Animal Research Committee, Kyoto University (Permit Number: MedKyo13270), and National Cerebral and Cardiovascular Center (Permit Number: 13055). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Ameroid Constrictor (AC)

The AC consists of a titanium casing surrounding a hygroscopic casein material with an internal lumen (Tokyo Instruments). The

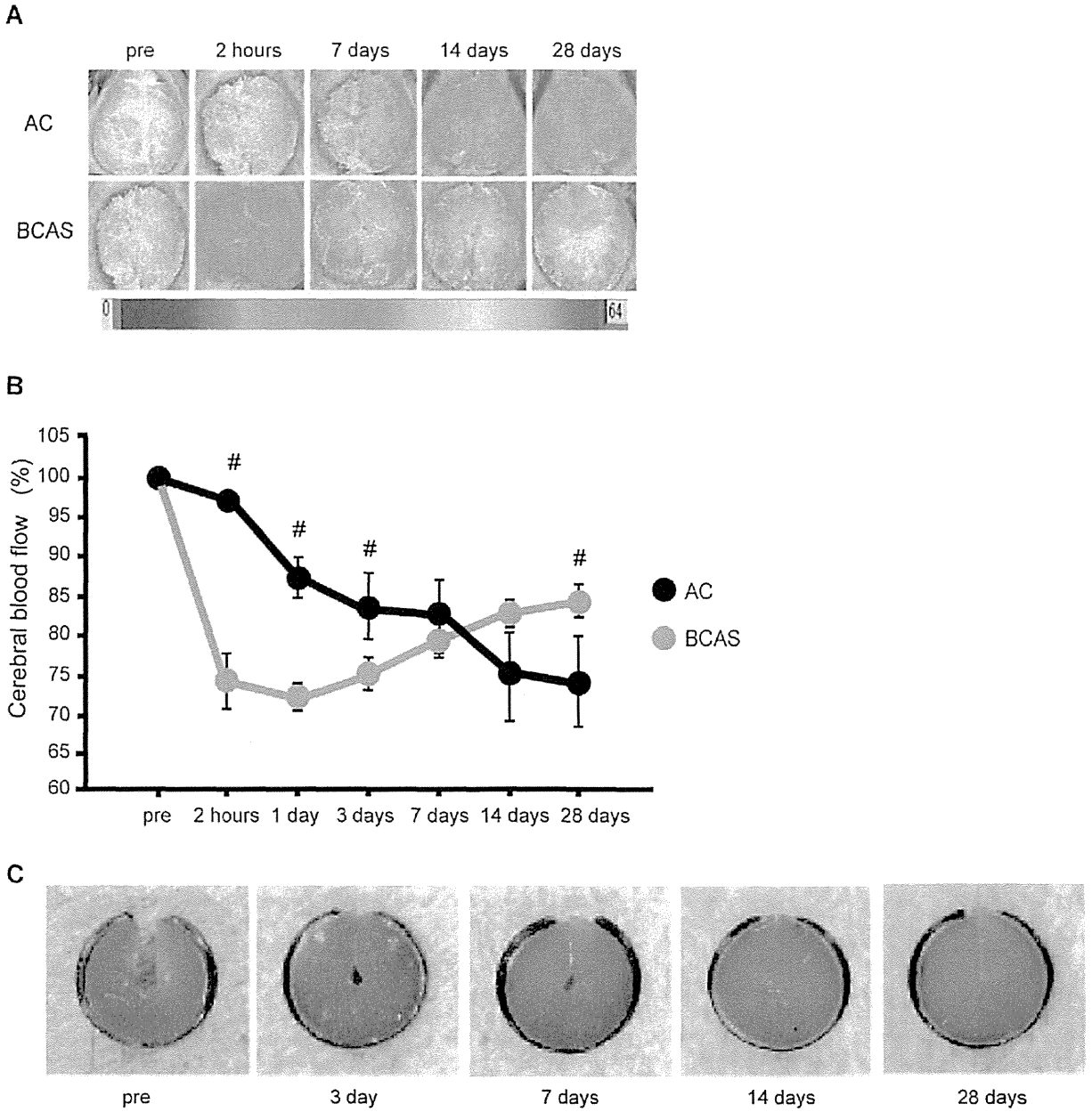

Figure 1. Cerebral blood flow (CBF) was gradually decreased in mice with the ameroid constrictors (ACs). (A) Representative CBF images of AC-implanted mice and bilateral common carotid artery stenosis (BCAS)-operated mice as assessed by laser speckle flowmetry at indicated time points. (B) Temporal profiles of CBF of the AC-implanted mice (n = 8) and BCAS-operated mice (n = 7) pre- and post-operation (2-way repeated-measures ANOVA, $p<0.05$; unpaired t-test, $\#p<0.01$ vs. BCAS group). (C) Representative images of ACs at indicated time points.
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casein component gradually absorbs water and consequently swells, leading to predictable narrowing and occlusion of the arterial lumen it encases. The inner diameter was 0.5 mm, the outer diameter 3.25 mm, and the length 1.28 mm [10].

Experimental Protocol

Male C57BL/6J mice of 10–12 weeks of age (CLEA Japan) were assigned into three groups: (1) sham-operation group (n = 6), (2) AC group (n = 29) [10] and (3) bilateral common carotid artery stenosis (BCAS) group (n = 7) [11]. Cerebral blood flow (CBF) was monitored before and at 2 hours, 1 day, 3 days, 7 days, 14 days and 28 days after operation. After 7 and 28 days following each operation, the mice were humanely euthanized by transcardial perfusion fixation with 4% paraformaldehyde after they were completely static and unresponsive to a toe pinch under anesthesia with intraperitoneal injection of 40 mg/kg pentobarbital. The brains were removed and post-mortem brains were analyzed for ischemic changes with hematoxylin and eosin (H&E) staining and

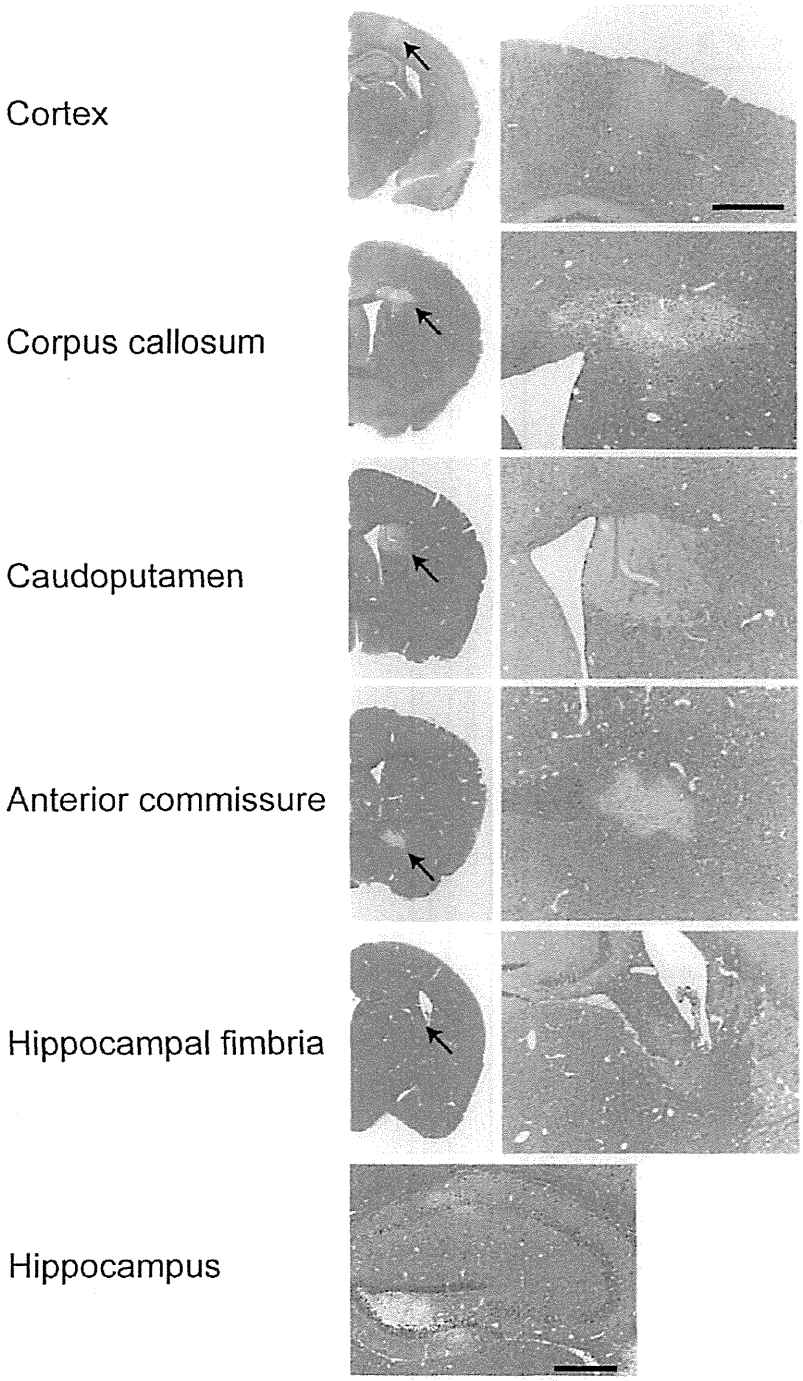

Figure 2. Multiple cerebral infarcts in mice with ACs. Hematoxylin and eosin staining showed multiple infarcts in the cerebral cortex, the corpus callosum, the caudoputamen, the anterior commissure, the hippocampal fimbria, and the hippocampus of the mice in the AC group at 28 days post-operation. Arrows indicate infarct changes. Scale bars indicate 200 μm.
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immunohistochemistry for a rabbit antiglial fibrillary acidic protein antibody (GFAP, a marker of astrocyte) and a rabbit anti-Iba1 antibody (a marker of microglia). All mice were housed in a room with a 12-hour light/dark cycle (lights on at 7:00 a.m.) and were given access to food and water *ad libitum*. We have monitored the condition of the animals every day until 14th day after operation, and twice per week after 14th day.

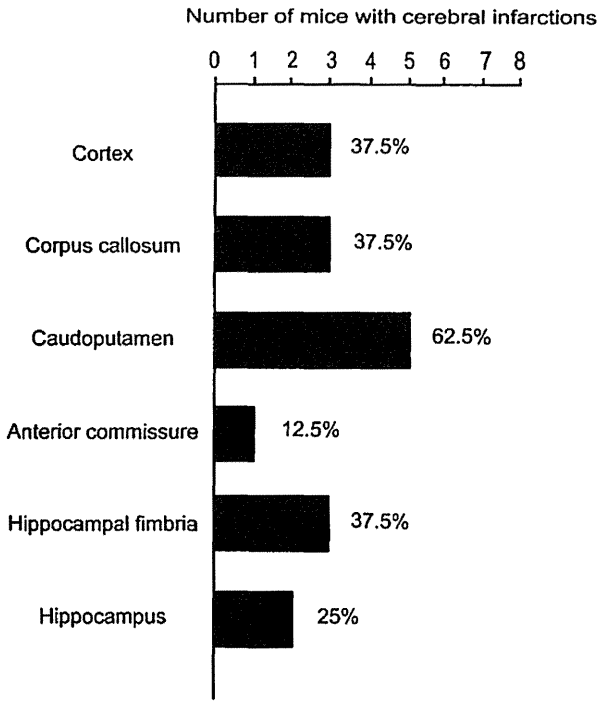

Figure 3. Percentage of AC-implanted mice with cerebral infarctions. The histogram shows the number and percentage of AC-implanted mice (n = 8) that developed cerebral infarctions in cortex, corpus callosum, caudoputamen, anterior commissure, hippocampal fimbria, and hippocampus.
doi:10.1371/journal.pone.0100257.g003

Surgical Procedure

Under anesthesia with 1.5% isoflurane, operation was conducted after confirming the mice being completely static and unresponsive to a toe pinch. Both CCAs were exposed through midline cervical incision and freed from their sheaths. In the AC group, the ACs were applied to the bilateral CCAs; while in the BCAS group, mice were subjected to surgical implantation of microcoils with an inner diameter of 0.18 mm to the bilateral CCAs [11]. Rectal temperature was maintained between 36.5°C and 37.5°C by a self-regulating heating pad.

Measured CBF by Laser Speckle Flowmetry

Relative CBF was recorded by laser speckle flowmetry (Omegazone, Omegawave), which produces high-resolution, two-dimensional imaging with a linear relationship to absolute CBF values [12]. Recordings were performed under anesthesia with 1.5% isoflurane. The scalp was removed by a midline incision so that the skull was exposed throughout the experiment. During the measurement of CBF, the skull surface was illuminated by 780 nm of laser light. The scattered light was filtered and detected by a CCD camera positioned over the head. The filter detected only scattered light that had a perpendicular polarization to the incident laser light. The raw speckle images were used to compute speckle contrast, which corresponds to the measured velocity of moving red blood cells thus approximating CBF. Signal processing was performed by an algorithm developed by Forrester et al. [13]. Color-coded blood flow images were obtained in high-resolution mode (639×480 pixels; 1 image/sec) and the sample frequency was 60 Hz. One blood flow image was generated by averaging

Table 1. Localization of cerebral infarctions of the ameroid constrictor-implanted mice.

mouse	Bregma Level				Hippocampal level					
No.	Cx	CC	CPu	ACo	Cx	CC	CPu	F	H
1	1		1		1				
2									
3	1	1	3		1			2	3
4	1				1				
5			1						
6		1	1	1		1	1	1	
7		1				1	1	2	1
8									

Cx, cortex; CC, corpus callosum; CPu, caudoputamen; ACo, anterior commissure; F, Hippocampal fimbria; H, hippocampus.
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numbers obtained from 20 consecutive raw speckle images. The recordings were initiated after the examiner confirmed that CBF did not change over 1 min, and the five recordings of blood flow image were averaged. In order to prevent the fluctuation of CBF and blood pressure during the measurement of CBF, anesthesia was induced, as described above. During the measurement of CBF, mice were held in a small plastic holder on a warming pad and thermostatically controlled at 36.5°C to 37.5°C in rectal temperature. Blood pressure was measured by the tail cuff method (Softron), which confirmed consistency throughout the experiment.

Histologic Evaluation

Paraffin-embedded coronal sections of the brain (6-μm-thick) were analyzed with H&E staining and immunohistochemistry for GFAP (1:2000; DAKO) and Iba1 (1:200; Wako) for detection of infarct areas at the level of the forebrain (bregma) and the hippocampus (+2 mm from bregma). The number of infarcts was counted in the cerebral cortex, the corpus callosum, the caudoputamen, the anterior commissure, the hippocampal fimbria, and the hippocampus. Infarcts were defined as focal areas of rarefaction that were accompanied by a group of astrocytes or microglia/macrophage proliferation.

Humane Endpoints during the Survival Study

We used moribund conditions as humane endpoints during the survival study. The moribund condition was defined as irreversible conditions leading inevitably to death. Signs of moribundity included a) lack of responsiveness to manual stimulation; b) immobility; and/or c) an inability to eat or drink. In such conditions, animals were euthanized by carbon dioxide asphyxiation.

Statistical Analysis

All values are expressed as means±standard error of the mean (SEM) in the text and figures. Differences with $p<0.05$ were considered statistically significant in all statistical analyses used, Student's t-test and two-way repeated-measures analysis of variance (ANOVA).

Results

Temporal Profiles of CBF Recorded by Laser Speckle Flowmetry

At 2 h, 1 day, and 3 days post-operation, the percent of the baseline CBF value of the AC group was significantly preserved compared with that of BCAS group (AC vs. BCAS; 2 h, 98.2±0.1% vs. 74.9±4.3%; 1 day, 87.5±2.2% vs. 73.1±1.3%; 3 days, 85.1% ±3.9% vs. 75.2±1.5%). However, the CBF continued to decrease at least up to 28 days after the operation in the AC group while CBF started to recover at 3 days after the operation in the BCAS group. Eventually, the CBF of the AC-implanted mice significantly decreased compared with that of BCAS-operated mice at 28 days post-operation (AC, 74.7±5.4% vs. BCAS, 85.1±1.4%) (Fig. 1A and B). In the sham-operation group, no apparent change of CBF was detected by 28 days post-operation (data not shown). Bilateral placement of the ACs on the CCAs produced the expected narrowing of the inner lumen and finally the inner lumen was occluded without any corrosion or infection of ACs at 28 days after operation (Fig. 1C).

Histologic Changes after Surgical Implantation of ACs

BCAS-operated mice did not show any cerebral infarction at 7 and 28 days after operation as previously reported [11]. In AC-implanted mice, cerebral infarctions did not develop at 7 days after operation, but developed in 6 of 8 mice (75%) at 28 days after operation. Infarctions occurred in the cerebral cortex, the corpus callosum, the caudoputamen, the anterior commissure, the hippocampal fimbria, and the hippocampus (Table 1 and Fig. 2, 3). The size of infarcts in the cortex tended to be smaller, which ranged from 100–200 μm in diameter while those in the other areas ranged from 300–800 μm with greater rarefaction. Some of the infarcts in the cerebral cortex were found in the arterial borderzone between anterior and middle cerebral arteries.

Furthermore, in 5 of 8 mice, hippocampal neuronal loss was observed (Fig. 4). By contrast, BCAS-operated mice did not show any hippocampal damage as previously reported [11].

Mortality Rate

The mortality rate at 28 days after implantation of ACs was 58.8% (Fig. 5A) while all BCAS-operated mice survived. Most of the non-surviving mice with ACs died during the second week; the

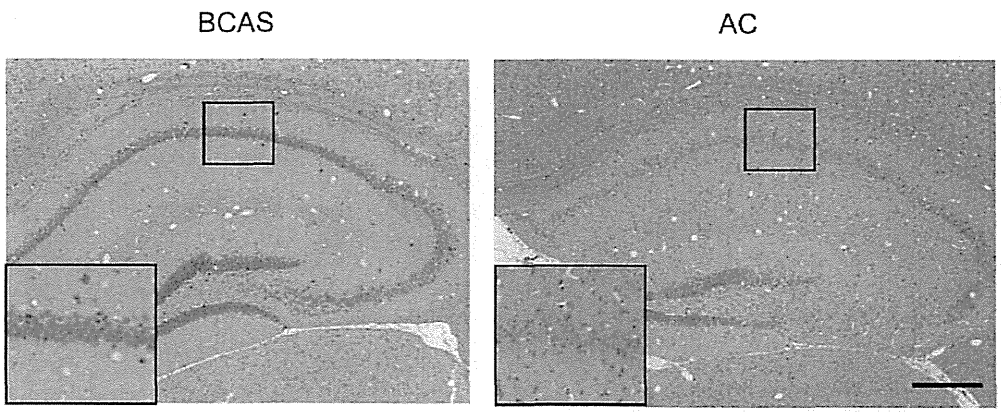

Figure 4. Hippocampal neuronal loss in AC-implanted mice. Hematoxylin and eosin staining showed intact hippocampus of the BCAS-operated mouse (left panel) and hippocampal neuronal loss of the AC-implanted mouse (right panel) at 28 days post-operation. The inset shows a magnified image of the indicated area. Scale bar indicates 200 μm.
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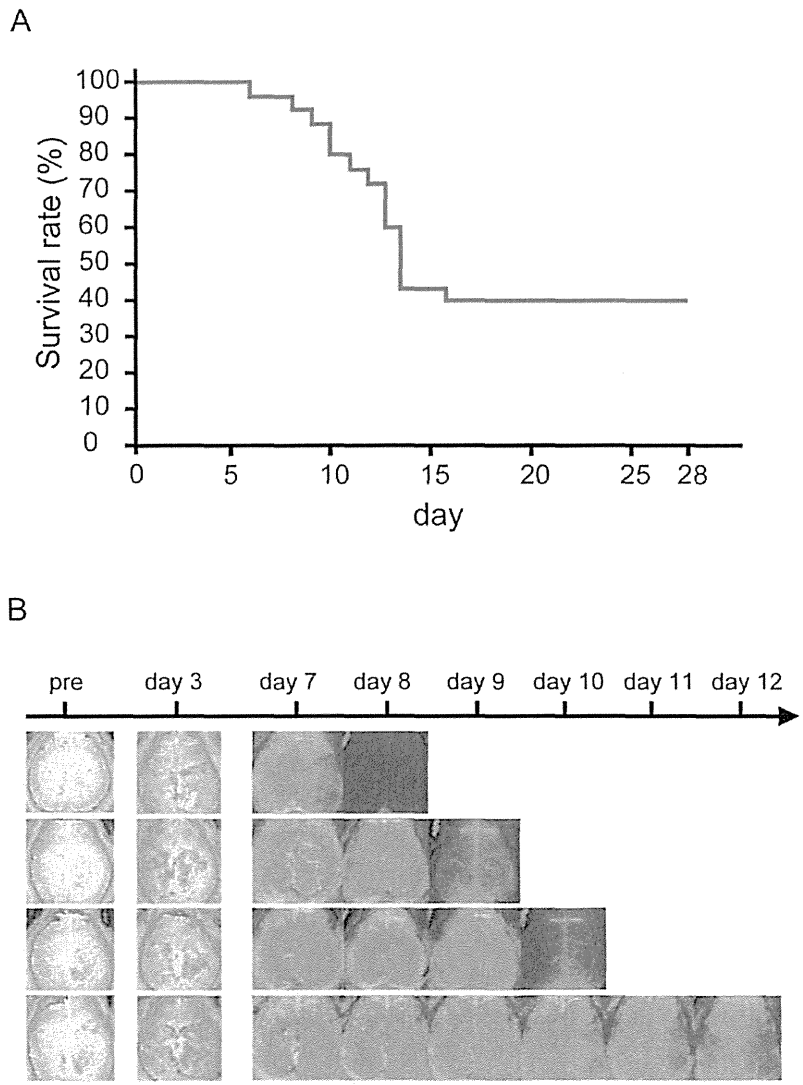

Figure 5. Survival rate in the AC group. (A) Kaplan-Meier method indicates survival rate of mice in the AC group (n = 29). (B) CBF images of the 4 non-surviving mice in the AC group which died during the second week. These mice died on the next day of last CBF measurement.
doi:10.1371/journal.pone.0100257.g005

LSF showed significant reductions of CBF below 20–30% of the baseline level reflecting massive infarctions or hemodynamic derangements (Fig. 5B).

Discussion

This study proposes a novel mouse model of carotid artery occlusive disease which showed that (1) ACs gradually narrowed and finally occluded the bilateral CCAs by 28 days post-operation; (2) CBF gradually decreased up to 28 days post-operation without acute CBF drop; (3) multiple cerebral infarctions were induced in the gray and white matter of most animals; and (4) the mortality rate was 58.8%, which may reflect severe pathophysiology of carotid artery occlusive disease like in humans in which the mortality rate is high [4].

The BCAS model is currently thought to be the most promising animal model of chronic cerebral hypoperfusion [14,15], but one of the limitations is an acute drop of CBF by approximately 30% and gradual CBF recovery due to compensatory mechanism [11]. To circumvent this limitation, models were developed by placing ACs to the bilateral CCAs of Wister-Kyoto rats, which reproduced gradual CBF reduction, chronic cerebral hypoperfusion and specific white matter changes [10]. However, because of the functioning PcomA, Wistar-Kyoto rats do not develop any cerebral infarcts even after complete occlusion of bilateral CCAs.

In the current C57BL/6J mouse model, however, we could reproduce several characteristics of carotid artery occlusive disease of humans. First, gradual narrowing of CCAs partly simulates consequences of atheromatous plaques that gradually enlarge in the carotid arteries. Second, ischemic infarcts were induced after the AC implantation as often observed in human carotid artery occlusive disease [3,4]. Compared to the rats or other mouse strains, the C57BL/6 mice have less developed PcomA and are thought to be the most susceptible to cerebral ischemia following

bilateral common carotid occlusion/stenosis [7]. Such background characteristics may have contributed to the successful modeling of carotid occlusive disease with infarcts in mice. Therefore, the current model differs from the BCAS model in terms of severity of histological changes because BCAS model induces white matter rarefaction but not infarcts while the current AC model is characterized by ischemic infarcts in the gray and white matter as a result of greater CBF reductions at later phase post-operation. Therefore, this model will be used to elucidate the mechanism of CBF autoregulation observed in humans and how this mechanism fails when systemic arterial pressure decreases below a critical point [16]. This would also be useful to investigate the dynamics of interstitial fluid and cerebrospinal fluid of the brain [17] as well as to test new therapeutic strategies for the large spectrum of neurological conditions associated with cerebral ischemia.

This study has a limitation in that the detailed mechanism of infarct formation remains unknown. In humans, cerebral infarctions distal to severe carotid artery diseases are caused by hemodynamic impairment and/or artery-to-artery embolism [1]. In the current mouse model without atheromatous lesions, although hemodynamic mechanism may be more plausible, detailed mechanisms remains unclear. Further analysis of the underlying mechanism of infarct formation in carotid artery occlusive disease can be conducted by using this novel animal model.

Another limitation is the relatively high mortality rate of the current model raising an issue of survival bias when future intervention study will be conducted with histological or behavioral assessment. However, since severe hemodynamic derangement itself seems to be a direct cause of mortality, death may become an endpoint and survival rate will be compared between the experimental groups to overcome the inherent limitation of the survival bias.

In summary, we have successfully established a mouse model of severe cerebrovascular insufficiency which almost invariably induce cerebral infarctions by placing ACs on the bilateral CCAs. We anticipate that pharmacological intervention for stroke will be investigated using this model by evaluation of survival rate, infarct formation, and CBF profile after the operation.

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Author Contributions

Conceived and designed the experiments: YH MI. Performed the experiments: YH. Analyzed the data: YH AK KN. Contributed reagents/materials/analysis tools: MI. Wrote the paper: YH MI.

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In Vivo Imaging of the Mouse Neurovascular Unit Under Chronic Cerebral Hypoperfusion

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Background and Purpose—Proper brain function is maintained by an integrated system called the neurovascular unit (NVU) comprised cellular and acellular elements. Although the individual features of specific neurovascular components are understood, it is unknown how they respond to ischemic stress as a functional unit. Therefore, we established an in vivo imaging method and clarified the NVU response to chronic cerebral hypoperfusion.

Methods—Green mice (b-act-EGFP) with SR101 plasma labeling were used in this experiment. A closed cranial window was made over the left somatosensory cortex. To mimic chronic cerebral hypoperfusion, mice were subjected to bilateral common carotid artery stenosis operations using microcoils. In vivo real-time imaging was performed using 2-photon laser-scanning microscopy during the preoperative period, and after 1 day and 1 and 2 weeks of bilateral common carotid artery stenosis or sham operations.

Results—Our method allowed 3-dimensional observation of most of the components of the NVU, as well as dynamic capillary microcirculation. Under chronic cerebral hypoperfusion, we did not detect any structural changes of each cellular component in the NVU; however, impairment of microcirculation was detected over a prolonged period. In the pial small arteries and veins, rolling and adhesion of leukocyte were detected, more prominently in the latter. In the deep cortical capillaries, flow stagnation because of leukocyte plugging was frequently observed.

Conclusions—We established an in vivo imaging method for real-time visualization of the NVU. It seems that under chronic cerebral hypoperfusion, leukocyte activation has a critical role in microcirculation disturbance. **(*Stroke*. 2014;45:3698-3703.)**

Key Words: astrocytes ■ leukocytes ■ microcirculation ■ microscopy, fluorescence, multiphoton ■ pericytes

The neurovascular unit (NVU) is a conceptual framework that integrates responses in all cell types, including neuronal, glial, inflammatory, and vascular elements.[1–5] This cell–cell integrated response is an indispensable factor used to maintain brain function and homeostasis. In fact, dysfunction of the NVU is the basis for many diseases.[2–4] The concept of NVU emphasizes that maintenance of integrated cellular function is more important than just salvaging an individual cell alone. Although the intricate molecular pathway of neuronal death has been dissected in detail, the mechanisms of how the entire NVU responds to cerebral ischemia are not completely understood. Understanding this concept may provide a comprehensive framework for investigating mechanisms and therapies of ischemic brain damage.[5]

The NVU is a dynamic structure assembled by endothelial cells, basement membranes, perivascular astrocytes, pericytes, and neurons. Therefore, it is difficult to understand the whole structure (including the anatomic relationship between cells) and the dynamic changes that occur within a single specimen. To understand the NVU more easily, an imaging method that can detect the whole NVU component at one time in vivo should be introduced. Thus, the aim of the present experiments was (1) to establish an in vivo imaging method for the NVU, which has extraordinarily spatial and time-dependent features. A spatial feature would require in-detail, 3-dimensional (3D) observation of the intricate NVU structure, whereas a time-dependent feature would require repeated longitudinal observation to capture real-time events, such as dynamic microcirculation in capillaries and (2) to clarify the stress response of the NVU in the bilateral common carotid artery stenosis (BCAS) model of chronic cerebral hypoperfusion.

Materials and Methods

Animal Model

All procedures were performed in accordance with the guidelines for animal experimentation from the ethical committee of Mie University. Male green fluorescent protein transgenic mice (C57BL/6

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The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.114.005891/-/DC1.

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TgN [b-act-EGFP] Osb) were used in this experiment (aged 9–14 weeks; Japan SLC, Inc, Shizuoka, Japan).[6]

Two-Photon Laser-Scanning Imaging Experiment

Mice were initially anesthetized with isoflurane (1%–2%), and a custom-made attachment device for holding the head in place was fixed to the skull. A closed cranial window was made by removing part of the skull (4 mm in diameter) over the left somatosensory cortex while leaving the dura intact, and the exposed cortex was sealed with a cover glass (Figure 1A).[7] These animals were then allowed to recover from anesthesia and were housed in a cage with free access to food and water. Mice were also kept in the cage in-between scheduled experiments. For plasma labeling, sulforhodamine 101 (SR101) dissolved in saline (0.01 mol/L) was injected intraperitoneally (8 μL/kg body weight) just before beginning the imaging experiment.[8] Each animal was placed on a custom-made apparatus under 1.2% isoflurane, and imaging was conducted with a 2-photon laser-scanning microscope (FV1000-2P; Olympus, Japan; For more detailed methodology see in the online-only Data Supplement).

Surgical Procedure of Chronic Cerebral Hypoperfusion

To replicate chronic cerebral hypoperfusion, mice were subjected to BCAS using microcoils.[9] In brief, under anesthesia with 1.3% isoflurane, the common carotid arteries (CCA) were exposed through a midline cervical incision, and a microcoil (inner diameter: Rt CCA, 0.18 mm; Lt CCA, 0.16 mm) was applied to the bilateral CCA. We applied a 0.18 mm×0.16 mm combination coil (purchase from SAMINI Co, Ltd., Japan) to elicit neurovascular response as much as possible. This combination has been selected because that of 0.16 mm×0.16 mm results in many mice dying over the observation period.[9] Sham-operated animals underwent bilateral exposure of the CCA without applying the microcoil.

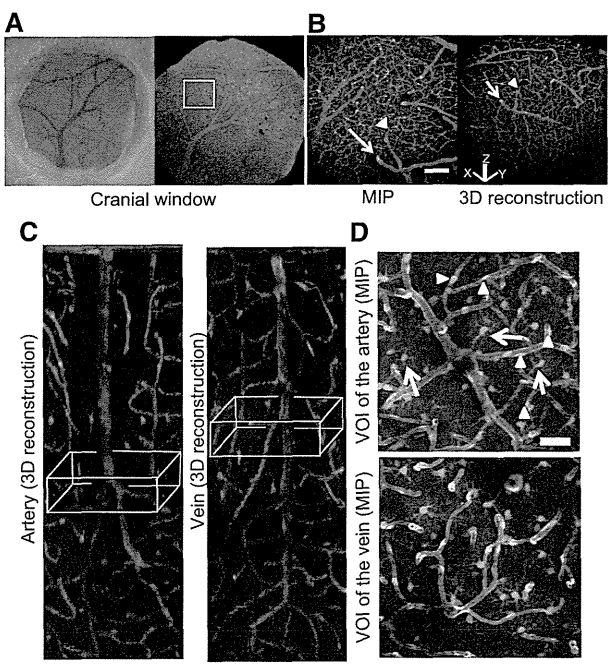

Figure 1. Identification method of whole vascular architecture ranging from the small vessels to capillaries. Stereoscopic and 2-photon images of a cranial window (**A**). Green fluorescence indicates the actin-green fluorescent protein and red fluorescence indicates the SR101 plasma labeling. The square in **A** indicates the volume of interest (VOI) in this brain. **B**, The maximum intensity projection (from surface to 550 μm) and the 3-dimensional (3D) reconstruction (view from diagonally upward) for the VOI. The arrows in **B** indicate a selected artery in this brain, whereas the arrowheads indicate a selected vein. Scale bar, 100 μm. **C**, Three-dimensional reconstructions (lateral view) of the selected artery and vein. Each of cuboid indicates arteriole branching portion (VOI of the artery) and venule branching portion (VOI of the vein). **D**, The maximum intensity projections for the VOI of the artery and VOI of the vein. The arrows indicate astrocytes and arrowheads indicate pericytes. Scale bar, 30 μm. MIP indicates maximum intensity projection.

Measurement of Cerebral Blood Flow

Cerebral blood flow (CBF) was determined by a laser speckle flowmetry (Omegazone, laser speckle blood flow imager, Omegawave), which obtains high-resolution 2D images in a matter of seconds, as previously described.[10] Briefly, we measured CBF through the cranial window in the same physical condition in terms of anesthesia and body temperature as during 2-photon laser-scanning. A 780-nm laser semiconductor illuminated the area of interest, and light intensity was accumulated in a charge-coupled camera device and transferred to a computer for analysis. Image pixels were then analyzed to produce average perfusion values.

Experimental Design

To exclude the effect of damage from the procedure involved in creating the cranial window, mice were allowed to rest for 1 week after the surgery. Laser speckle flowmetry and 2-photon laser-scanning were then performed as part of the preoperation period (Pre). Mice were randomly assigned to a sham (n=7) or BCAS (n=7) group, and 3 to 5 days after the preoperation period, sham or BCAS operations were performed (3 mice from the Sham group and 2 mice form the BCAS group were excluded because the cranial window became dim during the observation period.). Laser speckle flowmetry and 2-photon laser-scanning were then performed 1 day after BCAS or shame operations, and repeated again after 1 and 2 weeks. Anatomic morphology of the NVU (vessel structures, astrocytes, and pericytes) and microcirculation profiles (kinetics of erythrocytes and leukocytes) were evaluated at each time point for both sham and BCAS groups (for more detailed Methods see in the online-only Data Supplement).

Statistical Analysis

Results are expressed as the mean± SD. The Mann–Whitney *U* test was used to evaluate differences between groups, and an ANOVA followed by a post hoc Tukey–Kramer test was used to evaluate differences over time. Two-sided $P<0.05$ was considered statistically significant.

Results

NVU in the Normal Brain

In the operated cranial window, we were able to observe and clearly identify small pial vessels (see Figure 1A for a representative image). We identified volumes of interest and were then produced a maximum intensity projection from the surface of the brain to an imaging depth of 550 μm (Figure 1B). We 3D reconstructed these volumes of interests and then visualized whole vascular compartments (from pial small arteries to pial small veins) as is demonstrated in Figure 1B. In addition, zoom scanning of each vessels allowed us to identify the shapes of entire vessels (Figure 1C), and the NVU compartment from arterioles to capillaries and from capillaries to postcapillary venules (Figure 1D). In the transgenic mouse, only 4 cell types including astrocytes, pericytes, leukocytes, and platelets could be detected as cells positive for green fluorescent protein fluorescence under 2-photon laser-scanning microscope; therefore, each cell type could be identified based on their morphology (Methods and Figure I–III in the online-only Data Supplement).[6,11] Thus, astrocytes,