Post-Stroke Learning Impairment is Augmented in Hypertensive Mice

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Abstract

Cognitive impairment is an aging-related disorder that can arise as a result of cardiovascular pathology or cerebrovascular injury. Considering the aging of our population, the incidence of cognitive impairment is expected to rise. Hypertension is a major modifiable risk factor for stroke and cognitive impairment, but it is unclear whether it may affect post-stroke cognitive outcomes. This study aims to assess the effects of hypertension and/or stroke on the brain and its effect on cognitive impairment. C57BL/6J mice (n=117) aged 3-5 months were randomly assigned to receive infusion of either saline or angiotensin II (for 14 d at 0.7 mg/kg/day s.c.; or for 28 d at 0.28 mg/kg/day s.c.). 7 d after minipump implantation mice received either sham surgery or photothrombotic stroke surgery targeting the prefrontal cortex. The Barnes maze (28 d) was utilised to assess spatial reference memory and learning. Blood pressure was measured via tail-cuff plethysmography. Angiotensin II increased blood pressure (normotensive, NT: 118±1 mmHg vs. hypertensive, HT: 149±2 mmHg; $P<0.05$). In the Barnes maze, HT mice that received stroke surgery had increased latency to enter the escape hole when compared to other groups (day 3 escape latency: HT + stroke=166.6 ± 6.0 s vs. HT + sham=122.8 ± 13.8 s vs. NT + stroke=139.9 ± 10.1 s vs. NT + sham=101.9 ± 16.7 s), consistent with a greater learning impairment. Interestingly, we observed 5 incidences of haemorrhage within the HT + stroke group. These findings indicate that the combination of hypertension and stroke resulted in greater learning impairment and brain injury.

Statement of Authorship

Except where reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgment in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

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29th January 2021

David Edward Wong Zhang

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Abbreviations

˚C	Degrees Celsius
CO_2	Carbon dioxide
ACE	Angiotensin converting enzyme
AD	Alzheimer's Disease
ANOVA	Analysis of variance
ARB	Angiotensin receptor blockers
ATP	Adenosine triphosphate
AT1R	Angiotensin type 1 receptor
AT2R	Angiotensin type 2 receptor
BBB	Blood-brain barrier
BP	Blood pressure
Ca^{2+}	Calcium
CBF	Cerebral blood flow
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
CNS	Central nervous system
DAMPs	Damage associated molecular patterns
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acid
GFAP	Glial fibrillary acidic protein
HT	Hypertension/ hypertensive
IgG	Immunoglobulin type G
IFN-γ	Interferon gamma
IL	Interleukin
K^+	Potassium
LSCI	Laser speckle contrast imaging
LTP	Long-term potentiation
MCAO	Middle cerebral artery occlusion
MCI	Mild cognitive impairment
MLC	Myosin light chain
MPI	Mean pixel intensity
Na^+	Sodium
Na^+/K^+-ATPase	Sodium-potassium adenosine triphosphate enzyme
NADPH	Nicotinamide adenine dinucleotide phosphate

NO	Nitric oxide
NT	Normotension/ normotensive
O_2	Oxygen
OLT	Object location test
PBS	Phosphate-buffered saline
RAAS	Renin-angiotensin-aldosterone-system
ROCK	Rho-associated protein kinase
RPM	Revolutions per minute
rt-PA	Recombinant tissue plasminogen activator
SBP	Systolic blood pressure
s.c.	Subcutaneous
SHR	Spontaneous hypertensive rats
TNF-β	Tumour necrosis factor- beta
TLR	Toll-like receptors

Introduction

Dementia is an impending pandemic, as the number of people with dementia has more than doubled in the last few decades[1]. Without the development of new therapies, the incidence of this progressive neurodegenerative disease is expected to rise[1-4]. Key bodies such as G8 and the World Health Organisation (WHO) have set out an action plan to heighten awareness and outline strategies for the prevention of dementia, indicating the urgency for response[5]. Our current understanding of dementia is still in its infancy due to a lack of consensus on proper diagnosis and limited comprehension of disease mechanisms[6-8]. Although definitions vary, dementia generally describes a collection of conditions associated with cognitive impairment[9-11]. Cognitive impairment is characterised as one or more deficits within the four cognitive domains: language, memory, executive and visuospatial functioning[12]. Pathologies such as Alzheimer's disease (AD), vascular dementia, Parkinson's disease dementia, Lewy body and frontotemporal dementia amongst others are subtypes of dementia which burdens millions of individuals worldwide[13]. Many subtypes of dementia have a vascular component which contributes to its pathology. For example, AD is characterised by the aggregation of amyloid-β plaque due to the lack of vascular-mediated clearance, often as a result of cerebrovascular dysfunction[6, 14-16]. Current efforts with pharmacological and non-pharmacological treatments in improving cognition in dementia have been marginally successful at best, with no evidence of efficacy over alternate strategies such as exercise[10, 17]. Many will have to rely on pharmacological therapies which mainly treat symptoms and thus, are not disease-modifying and far from being a cure[18, 19].

Highly prevalent cardiovascular diseases such as hypertension and stroke are known contributors to the development of cognitive impairment and subsequent decline[20, 21]. Moreover, hypertension and stroke are not only important risk factors for the early onset of dementia but are themselves major causes of premature mortality and morbidity[22, 23]. Hypertension is an insidious disease where anomalously high blood pressure persists while symptoms and comorbidities arise overtime[24]. Known to have pro-fibrotic, pro-inflammatory and pro-thrombotic features, hypertension can lead to alterations in cerebrovascular function and contribute to the development of stroke[25]. Additionally, stroke can arise independently of hypertension as a result of cardiovascular risk factors[26]. The acute neurological disturbances caused by a stroke disrupts delicate cerebral blood vessels responsible for the delivery of essential blood supply. If not treated early, the damage from a stroke worsens over time. This increases the risk of post-stroke cognitive

impairment and the subsequent development of dementia[20, 27]. The complex interplay between dementia, hypertension and stroke are associated with a deteriorating quality of life[28, 29]. In animal models of hypertension and stroke, the assessment of cognitive function through neurobehavioural testing has been used to further our understanding of cognition[7, 30, 31]. However, despite their intimate ties with dementia few studies have sought to test for evidence of the combined effects of hypertension and stroke on cognition.

1.1 Hypertension

In Australia, hypertension is defined as the chronic elevation of blood pressure to greater than 140 mmHg systolic (SBP) and/or 90 mmHg diastolic (DBP)[32]. More than 90% of hypertension incidence is idiopathic and has been proposed to have multifactorial aetiology[33]. Idiopathic hypertension, also known as essential or primary hypertension, arises in the absence of other conditions or mendelian inheritance. Although the exact cause(s) of essential hypertension is not known, there are indications of genetic, lifestyle and environmental factors which can influence an individual's susceptibility to developing hypertension[34]. The likely heterogeneity of causes in hypertension makes it difficult to effectively treat this disease[35], and despite improvements in the management of high blood pressure over several decades, hypertension still remains one of the most prevalent non-communicable chronic diseases world-wide[36]. With an estimate of 1 in 3 adults affected, many individuals are undiagnosed due to the asymptomatic nature of this disease. With the use of therapeutics, 22% of individuals will have controlled hypertension, 17% will have uncontrolled hypertension, whilst 61% are untreated[37]. The long-term ramifications of unmanaged hypertension often involve the development of end-organ damage[38]. Observed in hypertension, changes to vascular wall dynamics such as arterial stiffening reduces arterial compliance[39]. The reduced capacity for arteries to distend in response to high blood pressure decreases the storage capacity of blood, thereby reducing blood flow to organs which leads to hypoxia[40]. The brain, in particular, is highly susceptible to injury due the lack of energy reserves and is in constant need for blood supply. The downstream effects of insufficient blood flow can lead to the loss of neuronal metabolic functions thereby damaging cerebral vascular function and brain parenchyma[41, 42]. Further, hypertension-induced cerebral hypoxia may damage structures associated with motor and cognitive functioning resulting in neurological deficits[30, 43-45]. The next section reviews the pathophysiological mechanisms underlying hypertension, the brain structures affected and its implications on cognitive impairment.

1.1.1 Pathophysiology of Hypertension

Hypertension arises from the disruption of various systems involved in regulating normal levels of blood pressure. The complex interplay between the brain, heart, kidneys and blood vessels affects cardiac output, vascular resistance and blood volume, all factors that determine the pressure that blood exerts on vessel walls[46, 47]. The renin-angiotensin-aldosterone system (RAAS) is an example of a system that involves the aforementioned organs. Crucial to the physiological maintenance of blood pressure, the RAAS may play a potential role in the pathogenesis of hypertension[48]. Angiotensin II, the primary effector peptide of the RAAS, is a vasoconstrictor that exerts pro-thrombotic, pro-fibrotic and pro-inflammatory actions critical to the pathological development of many cardiovascular diseases[49, 50]. Stimulated by reduced blood pressure or a decrease in Na^+ delivery to the renal tubules the kidneys releases renin. Renin then acts on angiotensinogen to form angiotensin I and subsequent conversion to angiotensin II via the action of angiotensin converting enzyme (ACE). Angiotensin II primarily binds to a class of G protein-coupled receptors, such as the angiotensin type 1 receptor (AT1R), which is associated with vasoconstrictor, fibrotic, apoptotic, oxidative and proinflammatory pathways[51]. Angiotensin II may also bind to the angiotensin type 2 receptor (AT2R) which is associated with vasodilator, anti-fibrotic, anti-apoptotic, anti-oxidative and anti-inflammatory pathways[52]. Evidence suggests that through the AT1R in vascular smooth muscle cells angiotensin II activates intracellular protein kinases and induces the generation of reactive oxygen species. Angiotensin II signalling in endothelial cells dysregulates nitric oxide (NO) signalling and induces endothelial dysfunction. As the endothelium serves as an interface between the blood and vascular wall, in addition to regulating blood flow, it acts as a cellular barrier layer and can mediate the exchange of fluid and nutrients. It also influences vascular myogenic tone, regulates cell growth via the synthesis of growth factors, responds to mechanical and chemical stimuli, is involved in thromboregulation and interacts with circulating immune cells[53-55]. Prolonged hypertensive stimuli such as angiotensin II may have inflammatory consequences that can cause maladaptive arterial remodelling and irreversible damage resulting in endothelial dysfunction. When organ systems involved in the regulation of blood pressure are impaired, the endothelium is commonly affected, often accelerating the pathogenesis of hypertension[56, 57].

Components of the RAAS are expressed in various tissues and cells that contribute to the development of prehypertensive conditions and elicit an innate and adaptive immune response[58-60]. Harrison *et al* proposed that modest increases to blood pressure, from hypertensive stimuli, sets the conditions for neoantigen formation which has the potential role for T cell activation[60]. Coppo *et al* showed that angiotensin II activates an autocrine loop to further potentiate concentrations of angiotensin II, increase mRNA expressions of the AT1R and elevate T cell gene expressions[61]. Inflammation as a result of T cell activation in organs involved in the RAAS can lead to vascular dysfunction resulting in further increases in blood pressure. For example, Trott *et al* found the accumulation of $CD8^+$ T cells in the kidneys contributed to Na^+ retention and vascular rarefaction that contributed to the development of hypertension[62]. Silva-Filho *et al* observed enhanced $CD4^+$ and $CD8^+$ T cell activation following AT1R activation which increased cytotoxicity with secretion of TNF-α and IFN-γ, both of which are associated with intimal hyperplasia, cellular proliferation, and atherosclerotic lesion formation[63, 64]. These findings are consistent with the concept that T cells and AT1R may play role in the pathogenesis of angiotensin II-induced hypertension.

The role of B cells in adaptive immunity associated with hypertension has not been as extensively studied as that of T cells, but there is evidence that B cells also play an important role in hypertension[65]. Circulating immunoglobulin type G (IgG) antibodies produced by B cells have been shown to be elevated in essential hypertension[66, 67]. Normally seen during the protection against infection, IgG in hypertension has been seen to target and activate the AT1R, α-adrenoceptors, β_1-adrenoceptors and L-type voltage gated Ca^{2+} channels, all known to regulate blood pressure[68]. Chan *et al* found that B cell deficiency resulted in the reduction of blood pressure. Further, reduced IgG and cytokine levels protected against angiotensin II-induced collagen deposition and fibrosis[69]. The specific function of IgG in hypertension is not yet known; however, it may have utility as a predictor for cardiovascular disease risk. Guzik *et al* showed that the removal of both T and B cells blunted angiotensin II-induced hypertension. However, unlike T cells, the adoptive transfer of B cells had minimal effect on blood pressure and vascular function[70], which may suggest that B cells play a limited role in hypertension. This discrepancy may be explained by the manner in which B cell activation is dependent on specific interactions with T cells and therefore in the absence of T cells, B cells would not become activated[71].

Toll-like receptors (TLRs), found in vascular smooth muscle cells and expressed in T and B cells, have been suggested to represent a link between hypertension and inflammation[72-74]. Target organs and vasculature release damage associated molecular patterns (DAMPs) as a response to cell injury leading to activation of the innate immune system and TLRs to promote and facilitate tissue repair. Long-term release of DAMPs and sustained TLR activation due to chronic injury contributes to prolonged inflammation mounting an adaptive immune response[75]. Following this, circulating cytotoxic T cells release cytokines that contribute to vascular dysfunction which may further potentiate the deleterious effects of hypertension[76, 77]. There is a substantial amount of literature to suggest that whether hypertension arises from non-immune or immune mechanisms, inflammation may play a central role in the pathogenesis of this disease leading to unintended consequences other than the elevation of blood pressure (Figure 1).

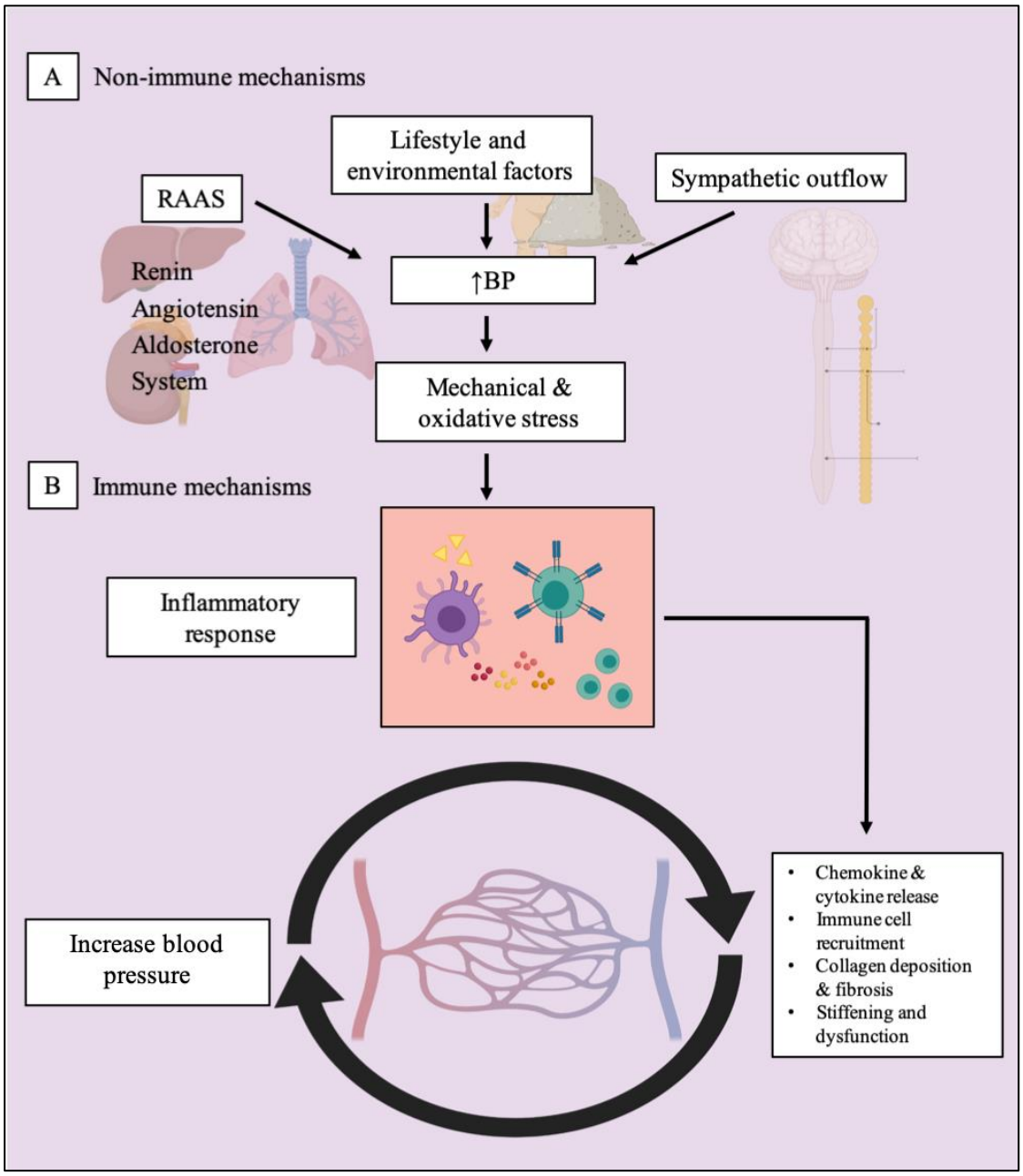


Figure 1. Schematic of Mechanisms Contributing to Hypertension.

(A) Non-immune mechanisms such as the renin-angiotensin-aldosterone system (RAAS), lifestyle and environmental factors, and sympathetic outflow can contribute to a moderate increase in blood pressure. **(B)** Immune mechanisms, neoantigens and damage associated molecular patterns (DAMPs) induced by oxidative injury can cause a cascade of inflammatory events affecting vascular, cardiac, and renal organs further perpetuating the increase of blood pressure.

1.1.2 Regulation of Cerebral Blood Flow

The cerebral circulation has several mechanisms (autoregulation, neurovascular coupling and chemoregulation) that contribute to the regulation of cerebral blood flow (CBF). Autoregulation works to maintain overall CBF within a relatively constant range despite changes in mean arterial pressure, thus protecting the brain from detrimental changes in perfusion[78-82]. A more dynamic regulation of CBF is known as neurovascular coupling or functional hyperaemia; this mechanism redistributes CBF proportional to neural activity, areas of increased neural activation lead to increased regional CBF[83]. This is to ensure that adequate nutrient delivery is meet by the energy demands of the brain and the removal of metabolic by-products[84]. As the brain lacks an energy reserve, it is highly dependent on this neurovascular exchange. Chemoregulation is involved in both general and local regulation of CBF. Vasoactive molecules such as NO, synthesised in the endothelium by constitutive NO synthase[85, 86], affect tone without interrupting cerebral autoregulation[87, 88]. NO is a vasodilator that has anti-inflammatory and anti-thrombotic properties[89, 90]; interactions with reactive oxygen species in hypertension reduce NO bioavailability, contributing to the continuation of endothelial dysfunction[91-93]. NO signalling is an area of great interest in stroke and hypertension research as it plays an important role in regulation of CBF and protection of neural tissue.

1.1.3 Neurovascular Unit and the Blood-Brain Barrier in Hypertension

The neurovascular unit is the anatomical and functional connectivity between pericytes, astrocytes, microglia, neurons and cerebral blood vessels[94]. Formed by endothelial cells, the blood-brain barrier (BBB) has been mainly regarded as a physical barrier that actively and passively prevents nonlipid-soluble molecules, foreign substances and inflammatory cells from traversing into the brain. However, unlike the endothelium in other parts of the body, the BBB highly expresses drug metabolising enzymes and transporters that prevent certain substances from entering the brain[95]. The adhesion molecules between endothelial cells are tighter and reduces vesicular transport which contributes to the highly selective semipermeable membrane of the BBB[96, 97]. It allows for the free movement of oxygen and water; and facilitates the transport of glucose and other highly lipid-soluble molecules through endothelial cells (Figure 2). The BBB is integral to the protection of the hypothalamus and medulla areas associated with central autonomic control and thus crucial for blood pressure regulation[98]. In hypertension, the effects of inflammation, reactive oxygen species and other modulating vasoconstrictors (such as angiotensin II) can contribute to the disruption of the BBB[99-102]. This disruption leads to aberrant trafficking of inflammatory cells, which have been implicated in the pathogenesis of vascular cognitive impairment[12, 103, 104]. Fan *et al* found that long-term hypertension contributed to the disruption of tight junctions in the BBB via the gradual loss of integral membrane proteins zonula occludens-1 and occludin which was associated with the development of cognitive impairment and neural atrophy[41].

An important aspect of the neurovascular unit and BBB are astrocytes which are the most abundant cell type within the brain and have a critical role in maintaining CNS homeostasis[105]. Astrocytes have been shown to play an important role in the regulation of extracellular concentrations of K^{+}[106], formation of synapses[107] and elimination of synaptic debris via phagocytosis[108]. The glia limitans is a barrier that further limits movement across the BBB. This structure is formed by the astrocytic end feet processes and the endothelial cells of the parenchymal arterioles. During chronic inflammation, found in hypertension, astrocyte phenotypes are modified within the brain parenchyma[109]. This has shown to result in degeneration of pericytes which cause the perivascular astrocytes to swell. Thus, opening the interendothelial junction increasing BBB permeability. Disruption to cellular metabolism caused by increased BBB permeability may result in energy failure of glial cells and subsequent neuronal dysfunction[110, 111].

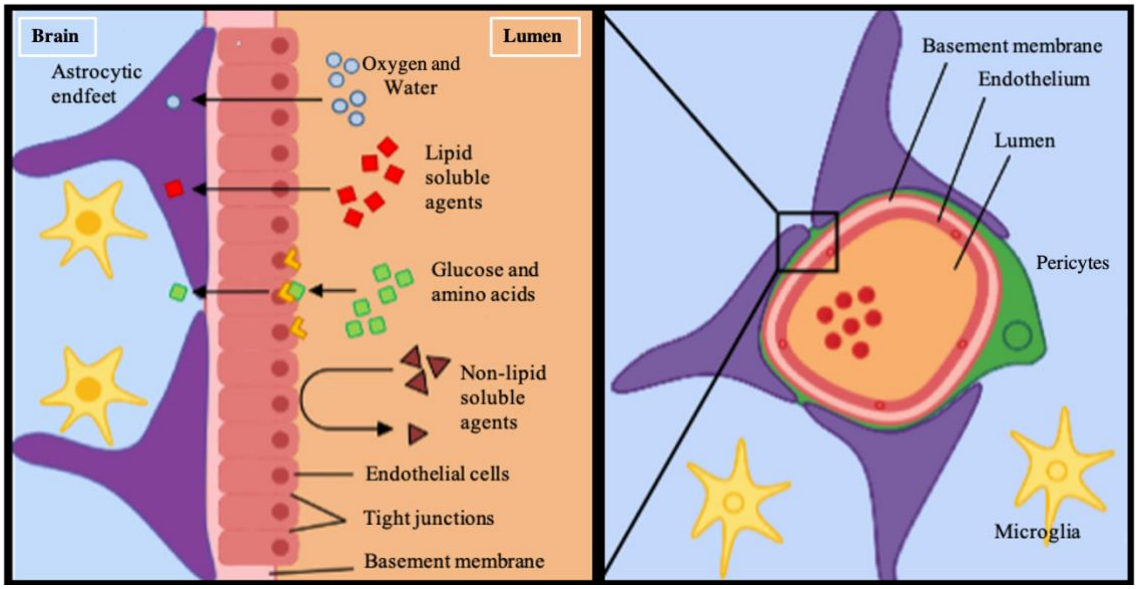


Figure 2. Schematic of the Blood-brain Barrier and the Neurovascular Unit.
The neurovascular unit constitutes astrocytes, pericytes, microglia and endothelium. This relationship between the neural and vascular structures creates a semipermeable border known as the blood-brain barrier. This barrier allows for the free movement of oxygen and lipid soluble agents through tight junctions, and delivery of glucose and amino acids via protein transporters in the endothelial cells. It also prevents foreign substances and nonlipid-soluble agents from traversing into the brain[112].

1.1.4 Cognitive Impairment as a Result of Hypertension

Cognitive impairment may arise during vascular-related diseases such as hypertension[113] in which there is also an increased risk of developing dementia later in life[114-116]. Kadish *et al* investigated chronic hypertension induced by application of an aortic stainless steel clip in Sprague Dawley rats, and found no impairment on spatial learning and memory[117]. This suggests that factors other than elevated blood pressure contributes to the development of cognitive impairment. In contrast, Tota *et al* made intracerebroventricular injections of angiotensin II 1 h prior to subjecting Sprague Dawley rats to neurobehavioural testing and found impairment of spatial learning and memory[118]. One of the effects of hypertension in the brain is the development of white matter lesions. Elevated systolic blood pressure has been associated with white matter lesion progression, and individuals with uncontrolled hypertension had significantly more white matter lesions compared to their treated counterparts[119, 120]. Delano-Wood *et al* showed mild cognitive impairment to be strongly correlated with deeper white matter lesions, which predicted poorer executive and visuospatial function[121]. It has been suggested that hypertension has a global effect on cognition, and studies with more robust neurobehavioural assessments have found that hypertension had a significant impact on memory formation, executive function, motor processing speeds and attention[122-124]. These aspects of cognition are associated with subcortical structures of the brain and susceptible to white matter lesions in hypertension.

Impairment of the neurovascular unit as a result of hypertension prevents the local increase of CBF in areas of increased neuronal activity which has been associated with neurodegeneration and cognitive impairment. Lefferts *et al* found that prefrontal cortex oxygenation was comparable in middle aged adults with controlled hypertension and those without hypertension, indicating that the neurovascular coupling was functional in controlled hypertension[125]. However, evidence suggests that the use of anti-hypertensive therapeutics have been ineffective in improving cognition indicating other factors other than blood pressure are implicated in cognitive impairment[113, 126]. There are reports of AT1R blockers (ARBs) having a neuroprotective effect as they ameliorate peripheral inflammation and maintain BBB function[127, 128]. However, treatment only reduces the risk of developing cognitive impairment as a result of hypertension rather than reversal of cognition impairment. While supporting evidence demonstrates that lowering blood pressure reduce the consequences of vascular disease and death, it may not be an effective method for treating cognitive impairment[129-132].

Cytokine-mediated interactions between neurons and glial cells have been recognised to contribute to the development of cognitive impairment. The disruption of the BBB as a result of hypertension leads to brain infiltration of proinflammatory cytokines. One such cytokine in hypertension is interleukin-6 (IL-6)[133]. Regions of the brain associated with memory such as the prefrontal cortex and hippocampus are known to express IL-6 receptors. Once activated, complex cognitive processes such as synaptic plasticity, neurogenesis and neuromodulation may be affected[134]. Furthermore, evidence suggests that high concentrations of plasma IL-6 was associated with an increased risk of cognitive decline[135-137]. A study completed by Bialuk *et al* had shown IL-6 deficient mice to have attenuated learning capability; moreover, IL-6 KO mice displayed better performance in behavioural tests assessing reference memory[138]. These findings further support IL-6 as a biomarker for cognitive decline and its involvement in cognitive processes. Thus, a current area of interest investigates potential targets of inflammation for therapeutic interventions, as it plays a key pathophysiological role in hypertension[139].

1.2 Stroke

Stroke is one of the leading causes of long-term morbidity and the second leading cause of mortality[140-142]. Most preventative strategies aim to minimise the modifiable risk factors (e.g. cardiovascular comorbidities, a sedentary lifestyle, diet, obesity, metabolic syndrome, alcohol, and tobacco use) to reduce the prevalence of stroke. Non-modifiable risk factors such as age, sex, race and genetics also contribute to its prevalence[143]. Described as when the brain is acutely deprived of sufficient blood supply, stroke can be broadly categorised as either ischaemic or haemorrhagic. Approximately 80 % of strokes are ischaemic in nature (Figure 3A), where a cerebral artery is occluded by a thrombus or embolus leading to localised hypoxia of the brain[144, 145]. Areas with insufficient collateral blood supply, and therefore no alternate route for blood delivery, are affected more severely when CBF is reduced [146]. Approximately 20 % of strokes are haemorrhagic in nature (Figure 3B) arising from the rupture of a cerebral blood vessel and may be caused by a weakened cerebral artery due to an underlying vascular disease. Haemorrhages lead to blood leaking into the brain or subarachnoid space, thus increasing intracranial pressure and reducing CBF[147]. Haemoglobin in red blood cells, the heme portion in particular, is toxic to brain parenchyma and likely contributes substantially to haemorrhage-induced brain injury[148]. Following stroke, the activation of excitatory glutamatergic receptors augment oxidative stress which can lead to protein oxidisation, enzyme inhibition and DNA damage resulting in cell death[149]. Depending on the affected brain region, neurological and functional deficits may arise within the first few minutes following stroke[150]. Acute brain injury may not resolve and become a chronic condition in the form of cognitive impairment, which is a common consequence of stroke. This may lead to the development of post-stroke dementia and worsened outcomes[151-153]. The next section discusses ischaemic strokes, the underlying mechanisms for their development and their implications for cognitive impairment.

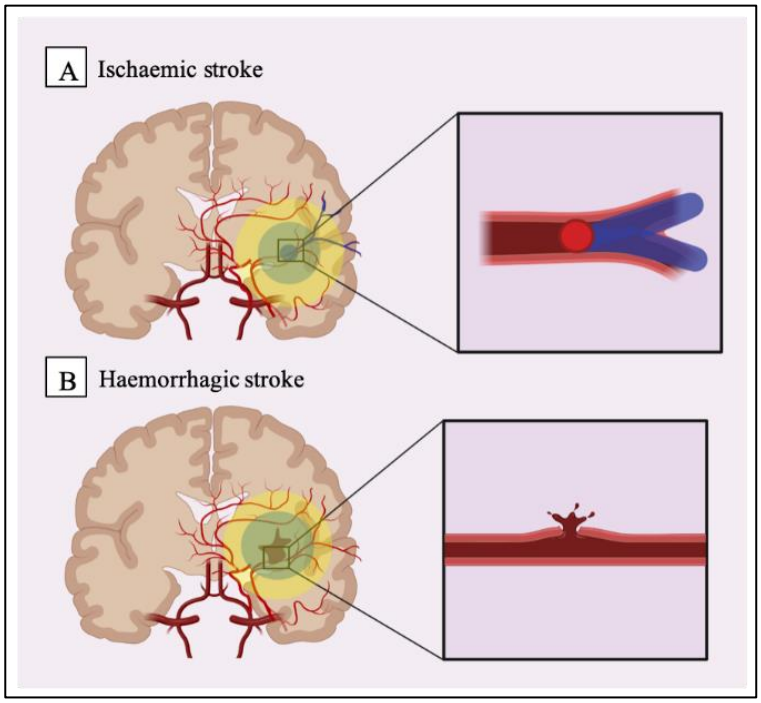


Figure 3. Schematic of the Types of Stroke.

(A) Ischaemic stroke and (B) haemorrhagic stroke. The infarct core (highlighted in green) can increase in size by absorbing the surrounding penumbra (highlighted in yellow).

1.2.1 Pathophysiology of Ischaemic Stroke

During an ischaemic event, the occluded region of the brain is initially structurally intact. However, there is an immediate cessation of function due to a reduction of O_2 and nutrient perfusion leading to the attenuation of synaptic transmission[154]. As energy levels deplete, energy-dependent Na^+/K^+-ATPase pumps fail, and membrane potential is lost. As a result, neurons depolarise, and intracellular glutamate stores are released. As a major excitotoxic neurotransmitter, excessive glutamate levels result in an influx of Ca^{2+} ions which activate several proteases, kinases, lipases and endonucleases leading to activation of intrinsic apoptotic pathways and thus cell death[155]. Furthermore, K^+ ions are released from the dying neurons, while reactive oxygen species and inflammatory mediators are released resulting in DNA damage causing further apoptosis and necrosis[144, 154, 156-158]. This process leads to the formation of an infarct and its surrounding ischaemic penumbra[159]. An ischaemic penumbra is part of the ischaemic brain where residual perfusion and O_2 persist[160]. The threshold for neuronal bioenergetics determines the progression of infarction as brain parenchyma is highly reliant on collateral blood vessels for blood supply, this region of the brain is functionally silent and potentially salvageable[161, 162]. Prolonged durations of hypoperfusion in the ischaemic penumbra contribute to the increasing size of the infarct core[163]. Therefore, early recognition of symptoms and intervention is key to the outcome of the ischaemic event.

Early signs of a stroke may include the acute onset of unilateral or bilateral weakness, blurred vision, slurred speech or paralysis of one or more limbs[145]. In the treatment of an ischaemic stroke, a thrombolytic enzyme known as recombinant tissue-type plasminogen activator (rt-PA) may be injected intravenously to break down the blood clot. This may be performed concomitantly with a mechanical thrombectomy. The window of efficacy for the administration of rt-PA is typically within 4.5 - 6 hours[164]. The narrow therapeutic window for rt-PA administration is due to the risk of developing an intracranial haemorrhage and this risk increases with later administration of rt-PA[165]. Early intervention with rt-PA may ameliorate post-stroke cognitive impairment[166]. However, for individuals ineligible to receive rt-PA (>75%), there is no therapy available. Inflammation is of particular interest as a potential therapeutic target as it is prominent throughout the development of the infarct. Known to exacerbate cell death and contribute to the breakdown of the BBB, further investigation into the role of inflammation may elucidate key pathophysiological mechanisms crucial for the treatment of stroke in the following days and weeks (Figure 4)[167-170].

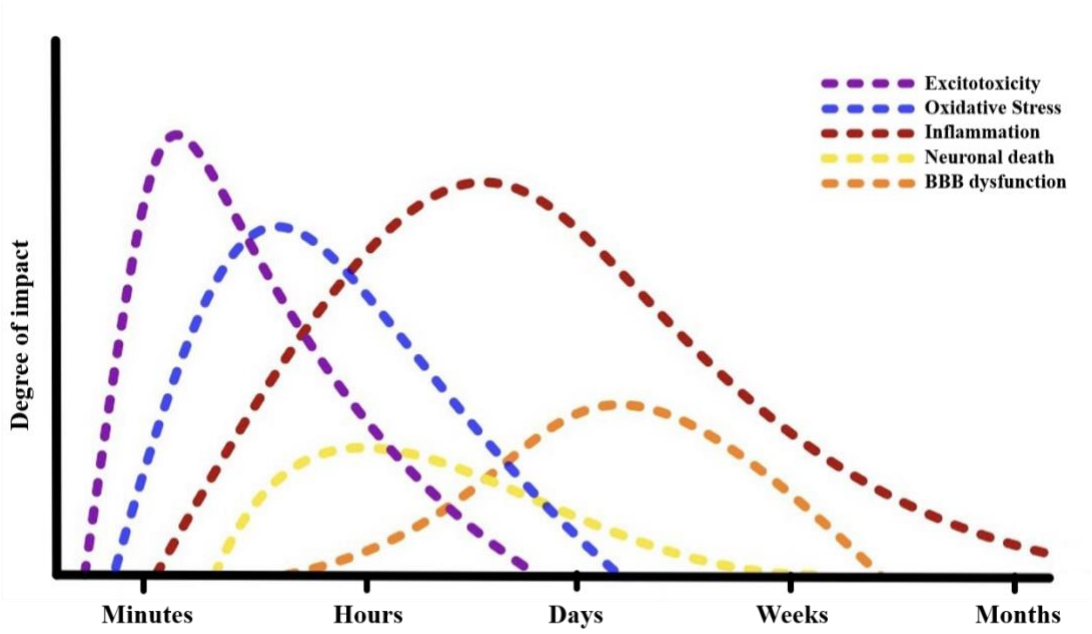


Figure 4. The Time Course of Events Following an Ischaemic Stroke.
Onset of an ischaemic stroke results in increased levels of excitotoxicity, oxidative stress, inflammation, BBB dysfunction and neuronal death in the affected brain tissue. In response, pro-inflammatory cytokines and chemokines are secreted leading to upregulation of endothelial adhesion molecules.

1.2.2 Reactive Astrocytes in Ischaemic Stroke

Astrocytes are the most abundant glial cell within the brain and have several functions including, scavenging neurotransmitters released during synaptic activity, releasing neurotrophic factors and aiding in the formation of the BBB[171]. Failure of these supportive functions will result in neuronal death as neurons are highly dependent on the homeostatic functions of astrocytes[172]. Studies have found astrocytes to express neurotransmitter receptors and respond to neuronal activity via increased intracellular Ca^{2+} which may lead to changes to vascular diameter[173]. Given that the neurovascular unit is composed of astrocytes and cerebral microvessels among other glial cells, astrocytes may have a supportive role in mediating neurovascular coupling. Other extracellular signalling molecules such as glutamate and adenosine have been implicated to inhibit transmission between astrocytes and neurons[174]. The inhibition of synaptic transmissions due to increased glutamate and ATP during an ischaemic event may have detrimental effects on the local control of the microcirculation thereby depriving brain tissue of O_2 and nutrients.

In response to brain injury such as stroke, astrocytes undertake morphological and functional transformations where they become reactive glial cells. Reactive astrocytes migrate to and proliferate at the site of injury forming a barrier around the infarct known as a glial scar. The glial scar restores the physical and chemical integrity of the central nervous system (CNS) as it isolates the infarct core to prevent further spread of cellular damage; however, it prevents neurogenesis thereby inhibiting neurological and functional recovery[175, 176]. Findings from Mukda *et al* demonstrated that endogenous Pinin, a novel protein suggested to be involved in cell adhesion, preserved viable astrocytes surrounding the infarct following ischaemic stroke, which played a pivotal role in the protective process of cells via maintenance of mitochondrial anti-apoptotic and energy metabolic functions[177]. Other studies have also found beneficial effects of astrocytes during stroke, such as compensatory mechanisms for maintaining the BBB, release of antioxidant substances and neurogenesis[178, 179]. Thus, astrocytes are a viable target for potential therapies as they not only promote neuroprotection after stroke but are involved in neuronal apoptosis and inhibit synaptic transmissions[180, 181].

1.2.3 Cognitive Impairment as a Result of Stroke

Each region of the cerebral cortex is responsible for a specific function such as motor, sensory, visual or auditory. Nuclei residing within the frontal and medial temporal lobes synapse with subcortical structures, such as the hippocampus and amygdala, to integrate information for the encoding and consolidation of memory. The formation of memory is not assigned to a single structure of the brain, rather they work in conjunction with related brain structures[182]. Following stroke, damage to cortical or subcortical regions of the brain have been associated with cognitive impairment related to the affected structures[183, 184]. Interestingly, motor impairment typically recovers after stroke, whereas cognition may remain impaired or even progressively worsen[185, 186]. These observations may suggest that separate mechanisms are involved in the repair and maintenance of motor and cognitive functioning, and that further investigations are required to elucidate these underlying mechanisms contributing to the development of cognitive decline after stroke. Post-stroke cognitive impairment affects approximately one-third of individuals recovering from stroke, and these individuals are highly susceptible to the development of dementia within the first year[187]. Significant correlations between motor and cognitive dysfunction after stroke have been observed, and persistence of motor impairment past three-months post-stroke may pose a risk for the development of cognitive impairment[188]. Moreover, the presence of impaired motor and cognitive functions have been associated with a higher risk of stroke[189]. This could be due to a pre-existing condition such as an underlying vascular disease that is a risk factor for stroke, and cognitive and motor impairment. Evidence of white matter lesions within the brain, cerebral microbleeds and changes to autoregulatory dynamics after stroke are suggested to contribute to the development of cognitive impairment[190-193].

1.3 Hypertension, Stroke and Cognitive Impairment

Hypertension is present in approximately 64% of stroke victims and both pathologies are known risk factors for the development of cognitive impairment[194]. Despite attempts of using anti-hypertensives, statins, cholinesterase inhibitors and anti-platelets for treating cognitive impairment[195-198], there is yet to be an effective intervention for improving cognition post-stroke[199]. Furthermore, very few studies investigate post-stroke cognitive impairment (PSCI) in the context of hypertension and are often focused on the prevention of disease[199-201]. The brain undergoes adaptations as a result of hypertension and stroke, in which the immune system is responsible for the repair process of brain parenchyma. Although BBB disruption and reduced CBF are characteristics of hypertension and stroke, the combination of hypertension and stroke may further damage these mechanisms and accelerate the progression of cognitive impairment into dementia[140, 202, 203]. By furthering our understanding of how PSCI is affected by hypertension and how the brain adapts under both conditions, it may bring us closer to unravelling the mechanisms which lead to cognitive impairment and subsequent decline.

The underlying mechanisms for cognitive impairment are not fully understood, as ischaemic damage can only explain a portion of cognitive impairment[204]. Other mechanisms involved in vascular diseases may include amyloid pathology (present even in prodromal dementia); neurofibrillary tangle pathology (critical for the transition of cognitive impairment to AD); neuronal loss in areas associated with memory consolidation; the composition of white matter in the parahippocampal gyrus, which also implicated in memory formation; synaptic degeneration within the hippocampus, prefrontal and temporal lobes of the brain; cholinotrophic innervation from basal forebrain to hippocampal structures; neurotrophic dysfunction; oxidative stress and inflammation in the dysregulation of the hippocampus and neocortex; and concomitant pathogenic factors which contribute to the complexity of cognitive impairment[152, 205-214]. Hypertension induced cerebral vascular dysfunction may contribute to the aforementioned mechanisms of cognitive impairment. Regions of the brain most affected by hypertension are the hippocampus, prefrontal cortex, temporal cortex and the inferior parietal lobule[215], locations where the BBB is most susceptible to damage.

The BBB is at the frontline in protecting the brain from foreign substances, inflammatory cells and oxidative stress. As BBB disruption may occur in both stroke and hypertension[42, 99, 216], changes to the cerebral microenvironment may also result (Figure

5). Current experimental approaches for cognitive impairment include targeting oxidative damage, neuroinhibitory factors and inflammation, which aim to improve brain plasticity and functional outcomes[217]. However, it is uncertain whether treatment of these disease mechanisms would reduce cognitive impairment[113, 218]. DiNapoli *et al* found that the disruption of the BBB was proportional to infarct size and neuronal injury[219]. The implications of this study are that BBB integrity may be vital for the reduction of neuronal damage and preservation of cognition in stroke pathology.

In neurobehavioural studies, both the hippocampus and the PFC have been studied extensively due to link that both regions have on spatial navigation, learning and memory. Interactions between the two structures have been measured by detecting fluctuations in neuronal activity in a short period of time one after another, indicating neuronal synchrony[220]. Neuronal oscillations in the PFC have been demonstrated to be modulated by the theta oscillations of the hippocampus[221], this hippocampal-prefrontal interaction increased neuronal synchrony in animals during tasks involving memory and learning[222, 223]. Thus, the connection between the hippocampus and the PFC demonstrates that cognition does not solely derive from a singular region of the brain, but rather in tandem with other regions.

In clinical practice, various neurological tests are used to assess mild cognitive impairment (MCI), which refers to the deficit of one or more cognitive domains but has yet to develop into dementia[224]. Worsened neurological impairment in hypertension and stroke are often categorised as disability or deterioration and thus not differentiated between their MCI counterparts[225]. Published data have shown that stroke patients with high systolic blood pressure or diastolic blood pressure have a 1.5- to 5-fold increased risk of death, disability and detrioration[225], suggesting that high blood pressure contributes to the risk of post-stroke outcomes. Furthermore, reducing blood pressure may reduce the risk of a fatal stroke[226]. Observational studies have reported that the progression of neurological impairment post-stroke is related to either high[195] or low systolic blood pressure[227]. Inconsistencies in diagnostic criteria, ambiguity in categorising neurological deficits and the methodology in determining hypertensive status may contribute to these discrepancies. These conflicting reports highlight the need for additional studies to clarify this question.

Hypertension has been associated with an increased incidence of recurrent stroke[228-230]. Despite hypertension worsening stroke outcomes, additional consideration is needed when managing blood pressure following ischaemic stroke. Treatment with anti-hypertensives during the acute phases of stroke may worsen long-term outcomes[231]. As chronically hypertensive individuals have altered cerebral autoregulation, aggressive treatment to lower blood pressure in ischaemic stroke has been shown to exacerbate infarct development and cause neurological deficits[232, 233]. It is presently unclear how and to what extent hypertension promotes post-stroke cognitive outcomes. By furthering our understanding of hypertension, stroke and cognitive impairment under conditions where they co-exist, as opposed to independently, it may be possible to develop more effective interventions to either prevent, delay or even reverse the onset of dementia.

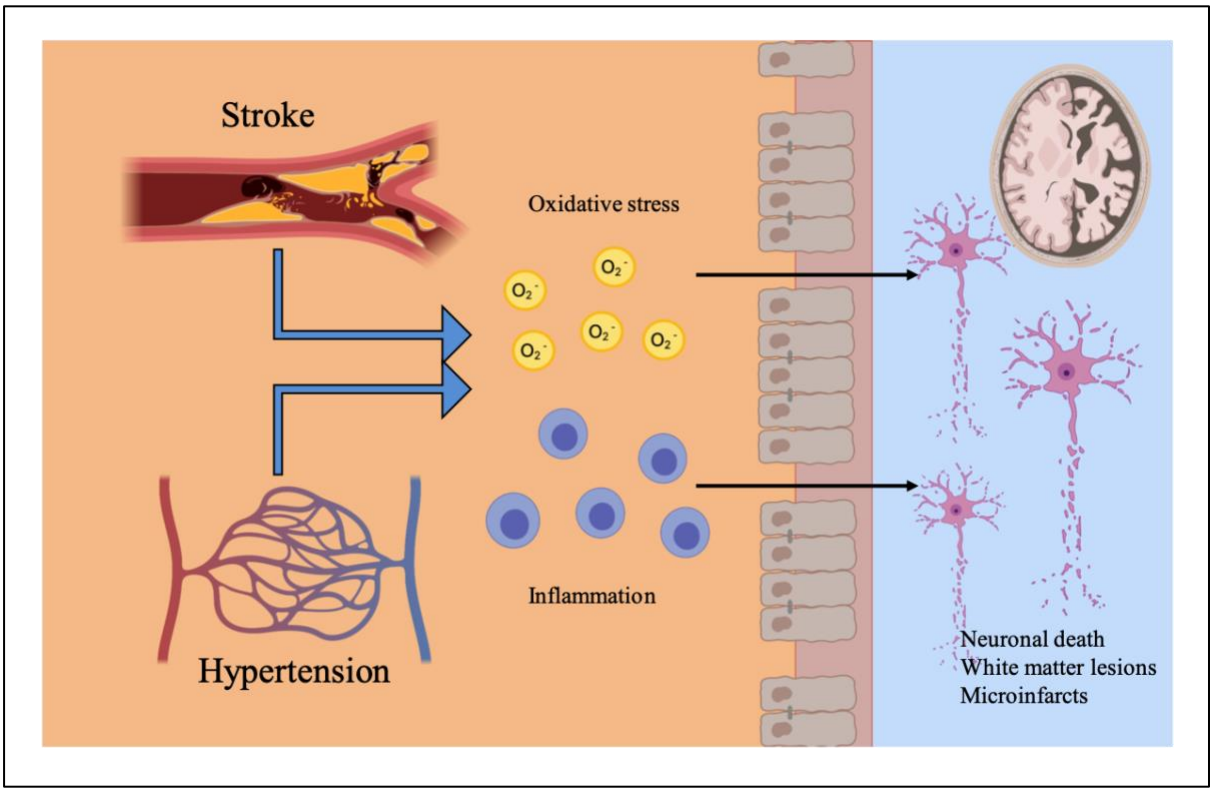


Figure 5. Schematic of Hypertension and Stroke Leading to Neuronal Damage. Both pathologies contribute to the dysfunction of the blood-brain barrier's semipermeable membrane allowing for the transfer of oxidative stress and extravasation of inflammation into the brain. This results in neuronal damage, white matter lesions and microinfarcts contributing to the development of cognitive impairment.

1.4 Conclusion, Hypothesis and Aims

While it is well established that hypertension increases the incidence of stroke, whether these two conditions augment the development and severity of subsequent cognitive impairment is unclear. We hypothesised that either stroke or hypertension alone will result in cognitive impairment. Additionally, hypertension will exacerbate post-stroke cognitive impairment. To test this hypothesis, we sought to address the following aims:

1. Determine the effect of hypertension on post-stroke cognitive outcomes;
2. Assess the impact of concomitant hypertension and stroke on brain injury.

Materials and Methods

2.1 Experimental Animals

Male C57BL/6J mice (3-5 months old) were purchased from Animal Resource Centre (Canning Vale, WA) and individually housed in GM500 cages (Tecniplast, Buguggiate, Italy) in a controlled 12-hour light/dark cycle (7:00 am to 7:00 pm) with access to food and water *ad libitum*. A total of 117 male (29.4 ± 3.7 g) mice were studied. To avoid bias, the investigator was blinded to treatment groups during all procedures and analyses. Mice were excluded from analysis if they were culled prematurely due to an adverse event (n=11). Of these eleven animals, autopsy revealed a ruptured abdominal aortic aneurysm (n=10), or there had been a multifocal haemorrhage and malacia of the spinal cord (n=1). The experiment was divided into two protocols (14 d and 28 d; Figure 6). The 28 d protocol was introduced after the completion of all 14 d protocol cohorts. At the end of the experiment, mice were euthanised via CO_2 asphyxiation followed by decapitation. All procedures and protocols were approved by the La Trobe University Animal Ethics Committee (AEC 16-79). Procedures were performed in accordance with the National Health and Medical Research Council guidelines and with the Australian code for care and use of animals for scientific purposes.

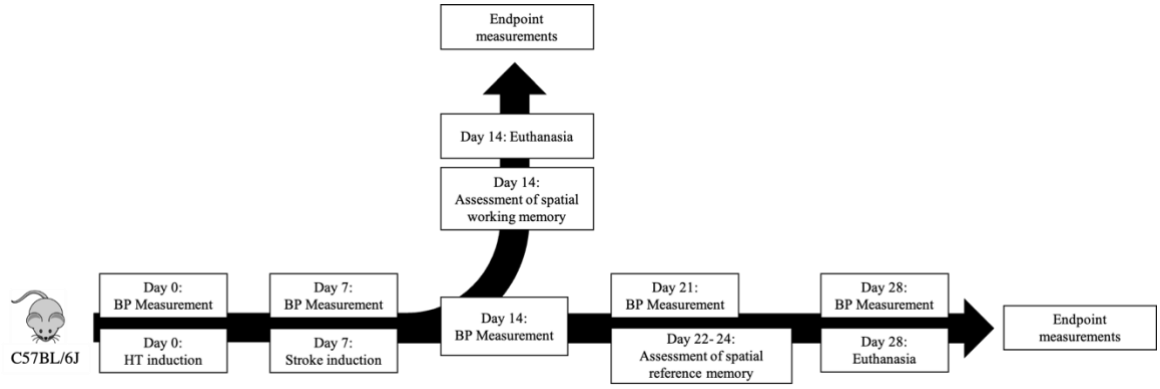


Figure 6. Experimental Design.

C57BL/6J mice were assigned to either the 14 d or 28 d protocol (n=65 and 52, respectively). The induction of hypertension for the 14 d protocol occurred at the beginning of the experiment, and at 7 d post-hypertension induction stroke/sham surgery was performed. Neurobehavioural testing was performed 7 d post-stroke followed by euthanasia for the collection of tissues for endpoint measurements. Hypertension induction and stroke/sham surgery for the 28 d protocol were at the same timepoints as the 14 d protocol, neurobehavioural testing was performed 15-17 d post-stroke, mice were then euthanised four days afterwards for the collection of tissues for endpoint analysis. Blood pressure (BP) was measured weekly. The spontaneous alternation test and the object location test (OLT) were used in the assessment of spatial working memory (day 14). The Barnes maze was used in the assessment of spatial reference memory (day 22-24). HT, hypertension.

2.2 Blood Pressure Measurement

Systolic blood pressure (SBP) was measured using a MC4000 multi-channel BP analysis system (Hatteras Instruments, Cary, USA). Acclimatisation to the tail-cuff plethysmography system was completed one day prior to the induction of hypertension. SBP was measured prior to surgeries; weekly measurements were then taken at the same time of day (7:00- 9:00 am) until endpoint. MC4000 multichannel system blood pressure analysis software was used to analyse SBP readings. Maximum pressure was set to 210 mmHg and platform temperature was set to 40.6 ˚C. Mice were transferred from their cages to the platform and gently restrained with magnetic covers. The tail was threaded through the balloon cuff, then taped down to immobilise the mice. The first set of 10 measurements were discarded and average SBP was obtained over 30-40 cycles.

2.3 Measurement of Cerebral Blood Flow

Mice that underwent the 14 day protocol had their CBF measured as follows (n=8-9). Under anaesthesia, a 3 cm midline incision was made on the scalp. Mice were then transferred to the laser speckle contrasting imaging (LSCI) setup with a heated nose cone delivering isoflurane and oxygen (1.5 % isoflurane, 0.2 L/min O_2). A droplet of Systane Ultra lubricating eye drops (Alcon Laboratories, Frenchs Forest, Australia) was used to lubricate the cranial surface to minimise image glare whilst recording images. Weighted hooks were used to retract the skin to reveal the skull. PIMsoft software (Version 1.5, Pimsoft Inc., Turin, Italy) was used with a Pericam PSI (Perimed AB, Stockholm, Sweden) attached with a LP645 optical filter (Midwest Optical Systems, Inc., Palatine, USA) to acquire 30 images over one minute. A selection tool was used to identify an area of interest 20 ± 1mm^3. Cerebral blood flow images of each mouse were captured on days 0, 7 and 14. Data were calculated within the PIMsoft software.

2.4 Infusion of Saline or Angiotensin II

Mice assigned to the 14 d protocol received 0.7 mg/kg/day of angiotensin II. This dose has been routinely used by several groups and reliably increases systolic blood pressure above 140 mmHg within a short period[234, 235]. Mice assigned to the 28 d protocol received 0.28 mg/kg/day of angiotensin II. This is also a commonly used dose of angiotensin II and typically results in the gradual elevation of systolic blood pressure[236-238]. This dose of angiotensin II has been suggested to be representative of low circulating angiotensin II found in humans[239]. Furthermore, a longer duration is required for the full development of hypertension. Thus, a lower dosage of angiotensin II would be more suitable for the 28 day infusion period.

2.4.1 Preparation of Osmotic Minipumps

An ALZET osmotic pump, Model 2002 (DURECT Corporation, Cupertino, USA) with a reservoir volume of 200 µl was filled with either 0.9 % saline or angiotensin II (0.7 mg/kg/day for 14 days, n=28; or 0.28 mg/kg/day for 28 days, n=27; Auspep, Melbourne, Australia). Prior to the day of surgery, filled osmotic minipumps were primed by submersion in 0.9 % saline at 37 ˚C overnight.

2.4.2 Osmotic Minipump Surgery

Mice were randomly allocated to receive either 0.9% saline or angiotensin II on the day of surgery. Mice were placed in a PVC induction chamber (25 cm x 15 cm x 12 cm) prefilled with isoflurane and oxygen (5 % isoflurane, 1.0 L/min O_2). Once anaesthetised, they were transferred to a heated nosecone which maintained isoflurane delivery (2-3 % isoflurane, 0.2- 0.5 L/min O_2). Body temperature was maintained at an approximate range of 37 ± 1.5 ˚C using heat mats. Eye lubricant (Poly Visc Lubricating Eye Ointment, Frenchs Forest, Australia) was applied to prevent desiccation of the eyes. Once depth of anaesthesia was stable (checked via pedal reflex pinch) surgical procedures were conducted.

Under anaesthesia, the dorsal area between the ears was shaved (approximately 2-3 cm diameter) using electric clippers. The surgical site was prepared by application of an undiluted 4% (w/v) chlorhexidine-s scrub thrice followed by an additional application of a cleanser 0.5% (w/v) of chlorhexidine-c in 70% (v/v) ethanol once. Carprofen (5 mg/kg subcutaneous [s.c.]; general analgesic) was administered contralateral to the area where the osmotic minipump would be implanted. Bupivacaine (2.5 mg/kg s.c; local anaesthetic) was injected in the region where an incision was to be made. A 2 cm incision, perpendicular to the spine, was made at the upper thoracic region using a sterile 15 mm scalpel blade. A pocket was created under the skin on the right flank. The osmotic minipumps was implanted with the flow regulator entering the skin pocket first. Wound clips were used to close and secure the incision site. A 18000-50 Hot Bead Sterilizer FST 350 (Fine Science Tools Inc., CA, US) was used at 270 ˚C to sterilised surgical tools between animal surgeries. The entire procedure was completed in approximately 20 minutes. While still unconscious, the mouse was gently placed in a recovery cage, and standard monitoring procedures were performed immediately afterwards (see section 2.6). Carprofen (5 mg/kg s.c.) was given on the contralateral side of the implanted osmotic minipump daily for two days following surgery.

2.5 Photothrombotic Stroke Surgery

The photothrombotic model of stroke is capable of targeting the PFC without affecting the motor cortex. By only targeting the PFC, which is responsible for spatial working memory, the motor cortex would be preserved to prevent any interferences with the neurobehavioural tests. Additionally, this model allows us to assess the hippocampus, which works in tandem with the PFC, and determine whether the distant injury site would affect this region of the brain. Whereas the middle cerebral artery (MCAO) model of stroke, although a more commonly used, is highly invasive and has a high mortality rate[240] compared to the photothrombotic model of stroke[241]. Clarkson *et al* conducted studies with the photothrombotic model to reliably test functional and behavioural outcomes in stroke, further validating this model of stroke[183, 190, 242, 243].

Mice were randomly assigned to receive either sham or stroke surgery. Mice were anaesthetised prior to surgery as described above for minipump implantation. Under anaesthesia, the scalp of the mouse was shaved, this was followed by sterilisation of the surgical site, local and general anaesthetic administration described as above. A 3 cm midline incision was made on the scalp using a sterile 15 mm scalpel blade to expose the skull. A sterile cotton bud was used to remove any connective tissue and blood. Once the skull became dry the anatomical landmark bregma was identified. The mouse was then placed on a stereotaxic frame connected to a Digital Display Console (Model 940, David Kopf Instruments, CA, USA). Nose and ear bars were adjusted to secure the head. A rectal probe was used to monitor and maintain core body temperature at approximately 37 ± 1.5˚C using a heat mat (Harvard Apparatus, Cambridge, UK.). Isoflurane was maintained (2-3 % isoflurane, 0.4-0.6 L/min) whilst the mouse was attached to this apparatus. A 20X objective lens connected to an LED light source (KL 1600 LED, Schott, Mainz, Germany) was lowered and aligned with bregma. Once positioned, the objective lens was moved 1.2 mm anterior, directly above the pre-frontal cortex (Figure 7).

Mice were injected intraperitoneally with 2 mg/ml Rose Bengal (Sigma-Aldrich Co., Castle Hill, Australia). Rose Bengal was allowed to circulate for 5 min. Following this, the isoflurane settings were reduced (1-1.5% isoflurane, 0.2 L/min O_2) and the light source was switched on with a maximum luminous intensity of 680 lm for 18 min. Once completed, each mouse was removed from the stereotaxic instrument and the exposed skull was closed by bringing the skin together and sealing with Vetbond tissue adhesive

(3M Animal Care Products, Sydney, Australia). Mice that had sham surgery underwent the same procedures with the exception that they were not exposed to any focal light to the skull. While still unconscious, the mouse was gently placed in a recovery cage, and standard monitoring procedures were performed immediately post-surgery (see section 2.6).

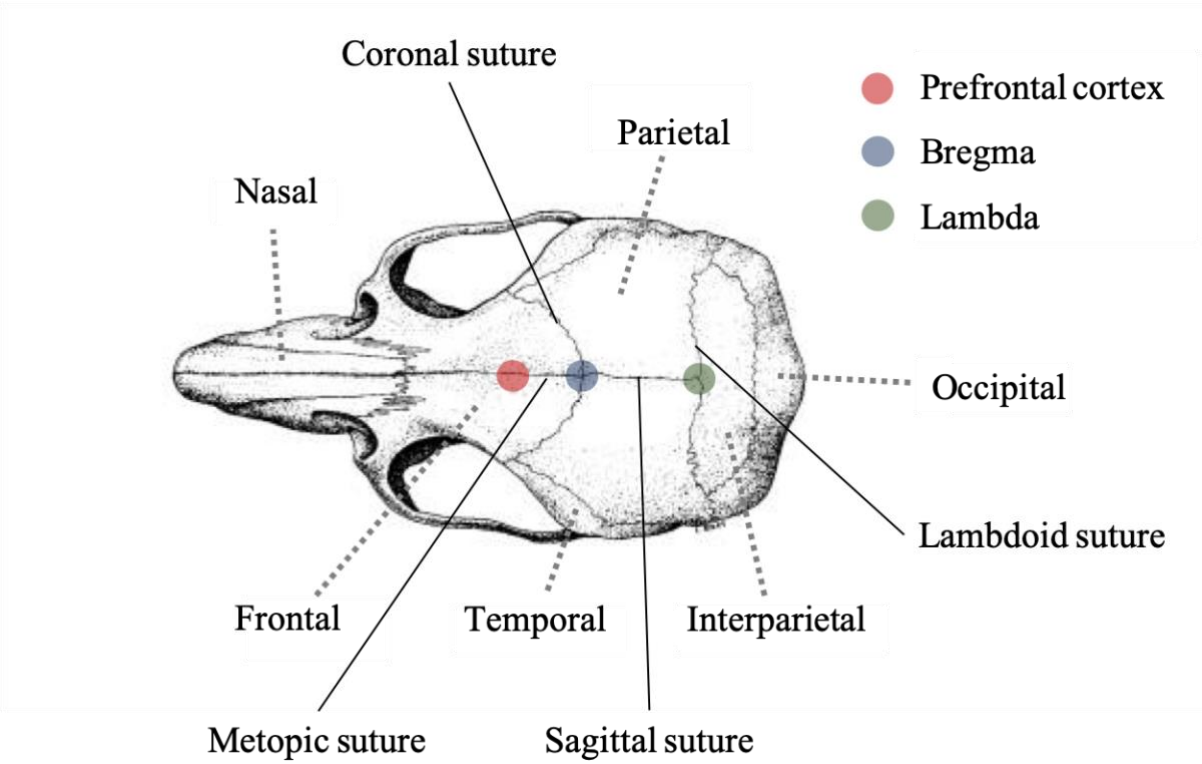


Figure 7. Schematic Diagram of the Mouse Cranial Bones, Sutures and Landmarks. The prefrontal cortex is located on the metopic suture and 1.2 mm anterior to the landmark bregma. This region of the brain is implicated in spatial working memory. Image adapted from the University of South Carolina[244].

2.6 Standard Monitoring Procedures

Following surgery, each mouse was placed in recovery cages under heat mats and were monitored every 15 min until they had regained consciousness and then continued to be monitored every hour for 3 h post-surgery. Following surgical procedures, the animals were monitored and weighed twice daily for the first five days following surgery and then every two-three days (refer to appendix i, ii & iii).

2.7 Behavioural Tasks

The behavioural tasks used in this study assess spatial memory and learning, which tests the functional capability of the PFC and hippocampus. The use of the of the spontaneous alternation test (Y maze) and the object location task (OLT) was used to provide data at a singular time point to assess spatial working memory. Both the Y maze and OLT was performed by the 14 d protocol mice. The Barnes maze provides data at multiple time points to assess spatial reference memory and was performed by the 28 d protocol. The decision to introduction of the Barnes maze was influenced by results of behavioural tasks completed by the 14 d protocol.

2.7.1 Spontaneous Alternation Test

The spontaneous alternation test (Y maze) is a commonly used tool for assessing spatial working memory[245]. The maze (31 x 10 x 6 cm) was comprised of three arms, each at 120˚ from one another. Distant cues were placed at the end of each arm (five-pointed star, seven-pointed star, and no cue) to allow for recognition of the area when previously explored. As mice are inquisitive by nature and typically prefer to investigate new environments, mice prefer to enter the arm that had not previously been entered (Figure 8). The maze was layered with sawdust and the mouse was placed at the centre of the maze and allotted 5 min to freely explore the three arms. Mice were recorded with a Logitech C615 HD Webcam (Logitech, Newark, USA). In between each test, the maze was wiped with 80 % ethanol and fresh sawdust was added. Video footage was assessed manually at half speed using a VLC media player (version 2.2.4, VideoLAN). An alternation was defined as the entry of all three arms without entering the previous arm. Percentage of total spontaneous alternations was calculated by the following formula:

$$Total\ alternation\ \% = \left(\frac{number\ of\ alternations}{total\ number\ of\ possible\ alternations}\right) \times 100$$

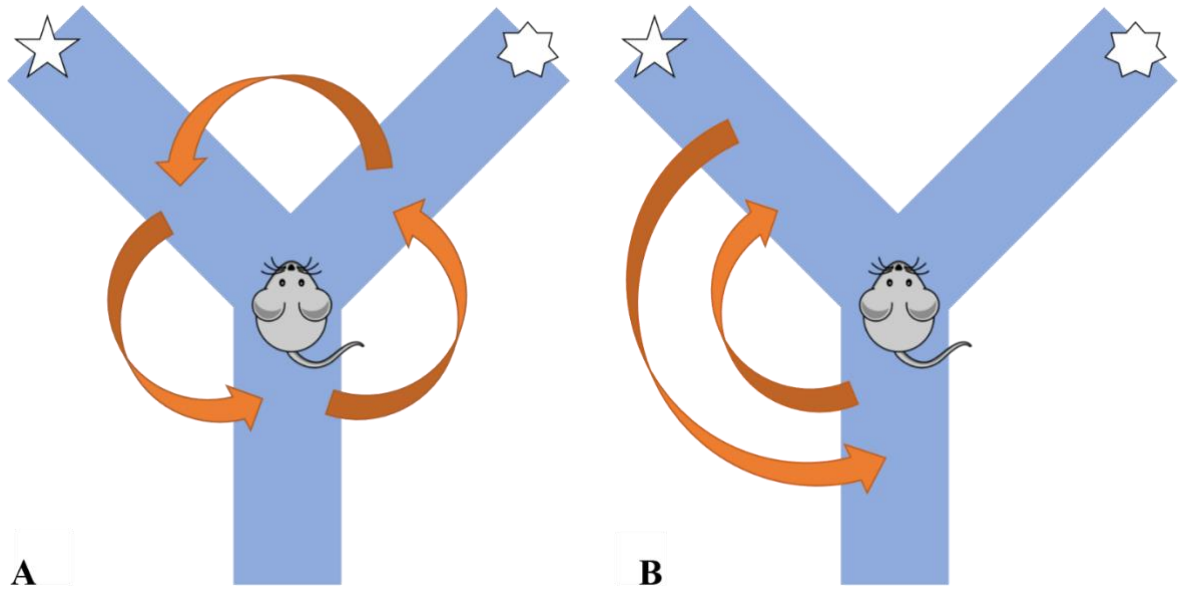


Figure 8. Schematic of Spontaneous Alternation in a Y Maze.

(A) Mouse entering all three arms in any order completes an alternation, **(B)** a mouse entering between two arms repeatedly does not complete an alternation.

2.7.2 Object Location Task

The object location task (OLT) is a behavioural test for assessing spatial working memory[246]. Each mouse underwent a habituation phase, placed in an open field box (41 x 41 x 30 cm) layered with sawdust for 10 min per day for two days prior to testing. In the training phase, the mouse was placed at the centre of the box with two identical objects and allotted 10 min to freely explore and familiarise with the objects. Once completed, the mouse was removed and placed in the home cage for 1 h. In the retention phase, one of the objects, randomly decided by coin-toss, was relocated (Figure 9), and the mouse was placed at the centre of the box with one of the objects now relocated. The mouse was allowed 5 min to explore the arena with the relocated object in this phase. The box was wiped down with 80 % ethanol between each test. A Basler ace GigE Camera (model acA1300-60gmNIR, Ahrensburg, Germany) with infrared heat detection was used to track and record the nose-point and centre-point movements. Within the Ethovision XT software (version 10.0.0828, Noldus Information Technology, Leesburg, USA) detection settings were set to have detection zones around the objects but not in the centre, to avoid calculating the time spent mounting the objects and to more accurately calculate interaction time. The software automatically tracked the nose-point of the mouse to calculate time spent interacting with each object.

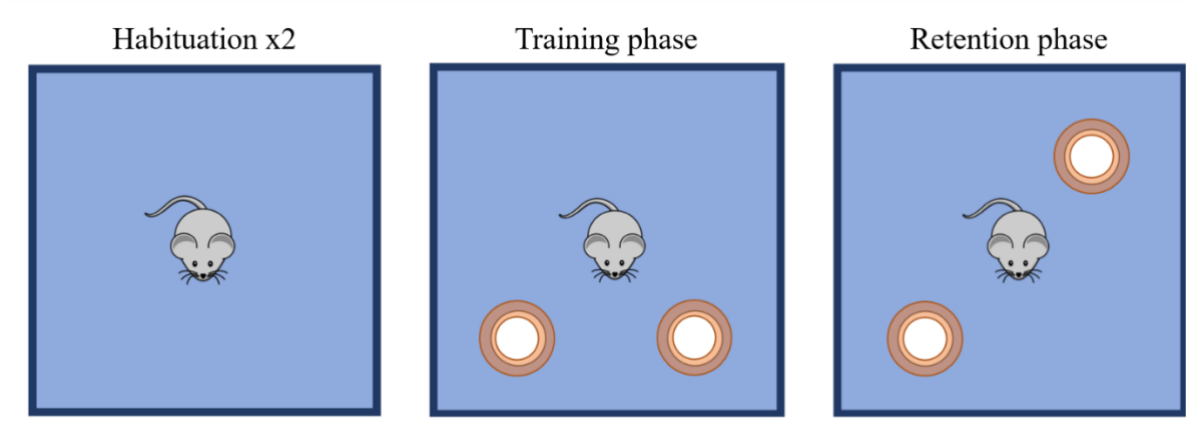


Figure 9. Schematic Diagram of the Object Location Task.

In an open field arena, mice underwent a habituation phase for two days. On the following day, the training phase commences, 10 min were given to familiarise with two identical objects. The retention phase commences an hour later; 5 min was given to interact with the objects but with one relocated. Detection zones were set around the objects (highlighted in orange).

2.7.3 Barnes Maze

The Barnes maze is used to assess spatial reference memory and consists of a circular platform (1 m diameter) with 20 identical circles surrounding the parameter. A removable escape box is attached under one of the circles creating an escape hole. Various objects and images are placed around the maze to assist mice in recognising their location and orientation. Two bright lights (Arlec 45W LED Work with Tripod, Aspley, Australia) are placed in the testing room and a loud buzzer (Dell Latitude 5400 laptop speaker, Round Rock, USA) is used as an aversive stimulus to deter mice from remaining on the platform and to instead locate the escape hole[247, 248]. The buzzer was set to 1 kHz at 85-90 dB using tone generating software (NCH Tone Generator v3.26 NCH Software, Canberra, Australia) and a sound level meter iOS application Decibel X: dB Noise Meter (SkyPaw Co. Ltd, Hanoi, Vietnam) was used for sound calibration. The escape hole for each mouse was assigned via a random number generator with each number corresponding with a circle on the platform. The escape hole was in the same position for each trial and day for each respective mouse. The mouse was placed in a dark room adjacent to the testing room for 20 to 30 m intervals between trials, 3 trials were performed per mouse each day. Mice performed this behavioural test individually; from a distance the escape hole was visually identical to the surrounding circles at the edges of the maze, requiring the mouse to use distant cues to reorientate themselves (Figure 10). No habituation phase was used. During the acquisition phase mice were removed from their cages and gently placed at the centre of the maze and immediately covered by a plastic container. The buzzer and lights were then switched on, container removed, and recording commenced. Mice were given 180 s to locate and enter the escape hole. If the escape hole had not been entered within the 180 s, the mouse was gently placed in the escape box. Once inside, the escape hole was covered for 60 s and the buzzer was switched off. The mouse was returned to their cage until their next trial. The platform and escape box were cleaned with 70% ethanol between each trial to remove any olfactory stimuli that may influence the behaviour of the next mouse. To measure spatial memory, learning and locomotive activity, the following parameters were used: (1) primary latency (time taken to reach the escape hole), (2) escape latency (time taken to enter the escape hole across), (3) distance travelled (path length of mice). A webcam (Logitech HD webcam C615, Newark, USA) was suspended above the maze and used to record footage for analysis via Ethovision XT software. The software automatically calculates the aforementioned parameters and performance was averaged across the three trials.

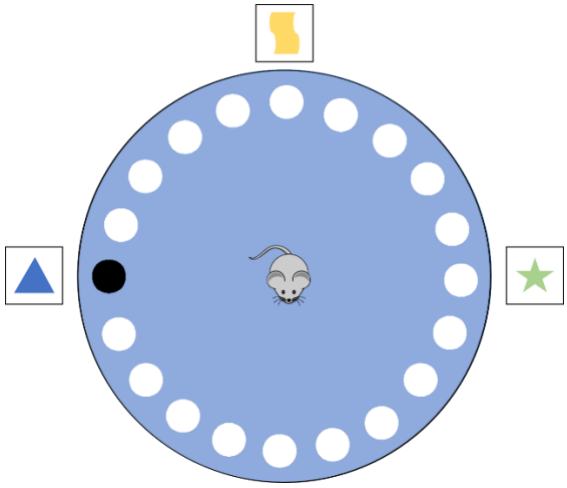


Figure 10. Schematic Diagram of the Barnes Maze.

Layout of behavioural testing room. Distant cues located around the maze, with a dark circle indicating the escape hole. Each mouse was placed in the maze and allowed to explore for 180 s. Visual tracing of its nose-point and body was analysed via Ethovision XT to assess pathlength and escape hole interaction.

2.8 Post-Mortem Analysis

2.8.1 Tissue Harvesting

Mice were euthanased via CO_2 asphyxiation at 3 L/min in their housed cages. Immediately afterwards, blood was cleared from all tissues via transcardial perfusion with 0.01 M PBS with clexane to prevent coagulation. Following this, each mouse was decapitated for the removal of the brain, frozen over liquid nitrogen and wrapped in Parafilm M Sealing Film (Pechiney Plastic Packaging, Chicago, USA) with aluminium foil.

2.8.2 Haemorrhage Grading

Upon collection of the brain, visible haemorrhages were graded depending on severity. A severity score of 0 was given when there was no evidence of haemorrhage, a score of 1 when there was evidence of haemorrhage within the infarct region, and a score of 2 when evidence of haemorrhage was observed outside of the infarct region.

2.8.3 Thionin Staining and Calculation of Infarct Volume

Series 1 Frosted Microscope slides (TRAJAN, Melbourne, Australia) were heated at 200 ˚C for 2 h and coated with poly-L-lysine solution 0.1 % dH_2O. 30 µm coronal sections, 210 µm apart, with 10 µm sections in between of the prefrontal cortex and hippocampus were cut using a Leica CM1850 cryostat (Leica Microsystems Pty Ltd., Wetzlar, Germany) at -18 ˚C. The 30 µm sections were submerged in 0.1 % thionin (2 min), rinsed in dH_2O twice, submerged in 70 % ethanol (2 min), then in 100 % ethanol (2 min), dipped in xylene thrice and coverslipped (22 x 50 mm, Menzel Gläser, Melbourne, Australia) with DPX mounting media (Sigma-Aldrich, St. Louis, USA). Thionin-stained sections were imaged using a Tucsen camera connected to a Leica WILD M3Z microscope (Leica, Wetzlar, Germany) with TCapture software (version 5.1, Tucsen Photonics Co., Ltd, Fuzhou, China), and infarct volume was estimated by tracing around the infarct region of each section using the polygon tool using ImageJ software (version 1.8.0_112, NIH). Total infarct volume was calculated using the formula:

$$Total\ infarct\ volume = \sum [infarct\ area\ \times\ d]$$

where, *d,* is distance between each section. Total infarct volume is expressed as mm^3.

2.9 Immunohistochemistry

2.9.1 Immunofluorescence Staining and Imaging of IgG and GFAP

Eight 10 µm coronal sections for each brain were air-dried (5 min), fixed with 100 % acetone at 4 ˚C (10 min) and washed thrice with 0.01 M PBS (5 min). A wax pen was used to create a barrier around the sections to prevent solutions from spilling. Sections used to assess glial fibrillary acidic protein (GFAP) were incubated with an anti-GFAP primary antibody and anti-rabbit secondary antibody whereas sections used to assess immunoglobulin type G (IgG) were only incubated with an anti-mouse secondary antibody. 0.2 % Triton-X 100 was used to permeabilise cell membranes. Each section was incubated in 50 µl of goat serum (1:10) in antibody diluent (0.2 % Triton-X 100 (1:50) and 5 % BSA/ 0.01 M PBS) to reduce non-specific antibody binding. For binding of the primary antibody, each section was covered by GFAP (goat anti-rabbit GFAP antibody (ab116010), 1:500) (Abcam, Cambridge, UK) in the antibody diluent overnight at 4 ˚C. The following day, sections were washed thrice with 0.01 M PBS (5 min). For binding of the appropriate secondary antibody, sections were incubated in either goat anti-mouse for IgG (goat anti-mouse IgG (ab150118), Alexa 555, 1:200) (Abcam, Cambridge, UK) or goat anti-rabbit for GFAP (Alexa 594, 1:200) in antibody diluent (2 h). Sections were then washed thrice in 0.01 M PBS (5 min). Incubation and washing of secondary antibodies was performed in a dark room. Sections were mounted with VECTASHIELD mounting medium containing the nuclear stain DAPI (Vector Laboratories, Inc. Burlingame, USA) and coverslipped. Edges were sealed with fingernail polish and sections were stored at 4 ˚C until imaging. Images were captured with an Olympus DP73 Camera (Olympus Corporation, Tokyo, Japan) connected to an Olympus BX53 Microscope (Olympus Corporation, Tokyo, Japan) at 100x and 200x magnification running CellSens Standard Software (version 1.17, Olympus Corporation). Exposure settings, ISO and black balance were kept consistent across all images. A DAPI filter was used for imaging cell nuclei and CY4 filter was used for imaging GFAP and IgG. GFAP-stained images were captured at 4 different locations surrounding the infarct or equivalent locations in sham. IgG-stained images were captured at bilateral regions of the infarct or equivalent locations in sham. Regions of interest were chosen to encapsulate the site of injury for the prefrontal cortex (PFC) or representative of each structure in the hippocampus.

2.9.2 Analysis of IgG Accumulation and Astrocyte Activation

Images acquired were analysed with ImageJ software. IgG accumulation was calculated as mean pixel intensity determined by size, density and brightness within the infarct area. For the analysis of GFAP, images were converted into 8-bit binary and thresholded for the segmentation of pixels. Following this, a skeleton analysis script on FIJI, an ImageJ extension, described by Morrison and Filosa[249] was used to convert the image into a skeletal figure. Each skeleton represents a single astrocyte with its branches. The total number of branches and branch lengths were averaged across all images to estimate astrocyte activation.

2.10 Statistical Methods

All data are presented as mean ± SEM. Statistical analyses were performed using unpaired t-test, two-way ANOVA with Tukey's multiple comparisons test or a Kruskal-Wallis test, as appropriate. Alpha was set at 0.05. All data analysis was performed using GraphPad Prism software (Version 7.0, GraphPad Software Inc.; San Diego, USA). Power analysis was run by G*Power software (version 3.1, University of Hamburg, Hamburg, Germany) which determined that 10 mice/group was required for statistical significance for behavioural testing[250]. However, to further ensure sufficient statistical power, n=15 was planned for behavioural testing, and all other experiments comprised n=6-10.

Results

3.1 Blood Pressure Analysis

Systolic blood pressure (SBP) is presented in Table 1 (14 d protocol) and Table 2 (28 d protocol). Baseline SBP was similar in all groups at day 0 ($P > 0.05$) and significantly elevated in the HT group compared with NT group on days 7, 14, 21 and 28 ($P < 0.05$) in both protocols. The infusion of angiotensin II at 0.7 mg/kg/day increased mean SBP to greater than 149 ± 4 mmHg at day 7 and it remained elevated until day 14 (table 1). The infusion of angiotensin II at 0.28 mg/kg/day increased SBP to 139 ± 2 mmHg at day 7 and it remained elevated until day 28. Stroke had no effect on SBP ($P > 0.05$).

Table 1. Systolic Blood Pressure of 14 d Protocol Mice

Groups	Day 0 (mmHg)	Day 7 (mmHg)	Day 14 (mmHg)
NT + sham (n=10)	116 ± 1	123 ± 4	123 ± 4
NT + stroke (n=10)	119 ± 1	118 ± 2	120 ± 1
HT + sham (n=11)	119 ± 1	151 ± 3 *	151 ± 4 *
HT + stroke (n=12)	119 ± 1	149 ± 4 *	151 ± 4 *

Mice had their systolic blood pressure (mmHg) measured on days 0, 7 and 14 via tail-cuff plethysmography. Mice assigned to the normotensive group (NT) were infused with saline 0.9% whilst the hypertensive group (HT) were infused with angiotensin II at 0.7 mg/kg/day. Data are presented as mean ± SEM. n=10-12/group. * indicates $P < 0.05$ HT vs NT, two-way ANOVA with Tukey's multiple comparisons test.

Table 2. Systolic Blood Pressure of 28 d Protocol Mice

Groups	Day 0 (mmHg)	Day 7 (mmHg)	Day 14 (mmHg)	Day 21 (mmHg)	Day 28 (mmHg)
NT + sham (n=12)	120 ± 1	125 ± 1	119 ± 6	116 ± 3	117 ± 2
NT + stroke (n=9)	117 ± 3	122 ± 2	122 ± 2	126 ± 1	120 ± 1
HT + sham (n=12)	121 ± 1	139 ± 2 *	156 ± 7 *	151 ± 3 *	156 ± 2 *
HT + stroke (n=13)	119 ± 1	141 ± 2 *	156 ± 3 *	145 ± 2 *	153 ± 2 *

Mice had their systolic blood pressure (mmHg) measured on days 0, 7, 14, 21 and 28 via tail-cuff plethysmography. Mice assigned to the normotensive group (NT) were infused with saline 0.9% whilst the hypertensive group (HT) were infused with angiotensin II at 0.28 mg/kg/day. Data are presented as mean ± SEM. n=9-13/group. * indicates $P < 0.05$ HT vs NT, two-way ANOVA with Tukey's multiple comparisons test.

3.2 Behavioural Tests

3.2.1 Relocated Object Recognition

Mice assigned to the 14 d protocol had spatial working memory assessed via the object location task 7 d post-stroke surgery. NT + sham mice spent a mean of 57 % of the time interacting with the relocated object compared to 43 % with the original object (Figure 11B; $P < 0.05$). The NT + stroke, HT + sham and HT + stroke groups spent significantly less time interacting with the relocated object compared with NT + sham (Figure 11C; $P < 0.05$) however, no statistical difference was found between each of these treatment groups (Figure 11C; $P > 0.05$).

3.2.2 Y Maze Spontaneous Alternation Performance

Analysis revealed a lower percentage of alternations in hypertensive compared with normotensive mice, although there was no statistical differences between treatment groups (Figure 12).

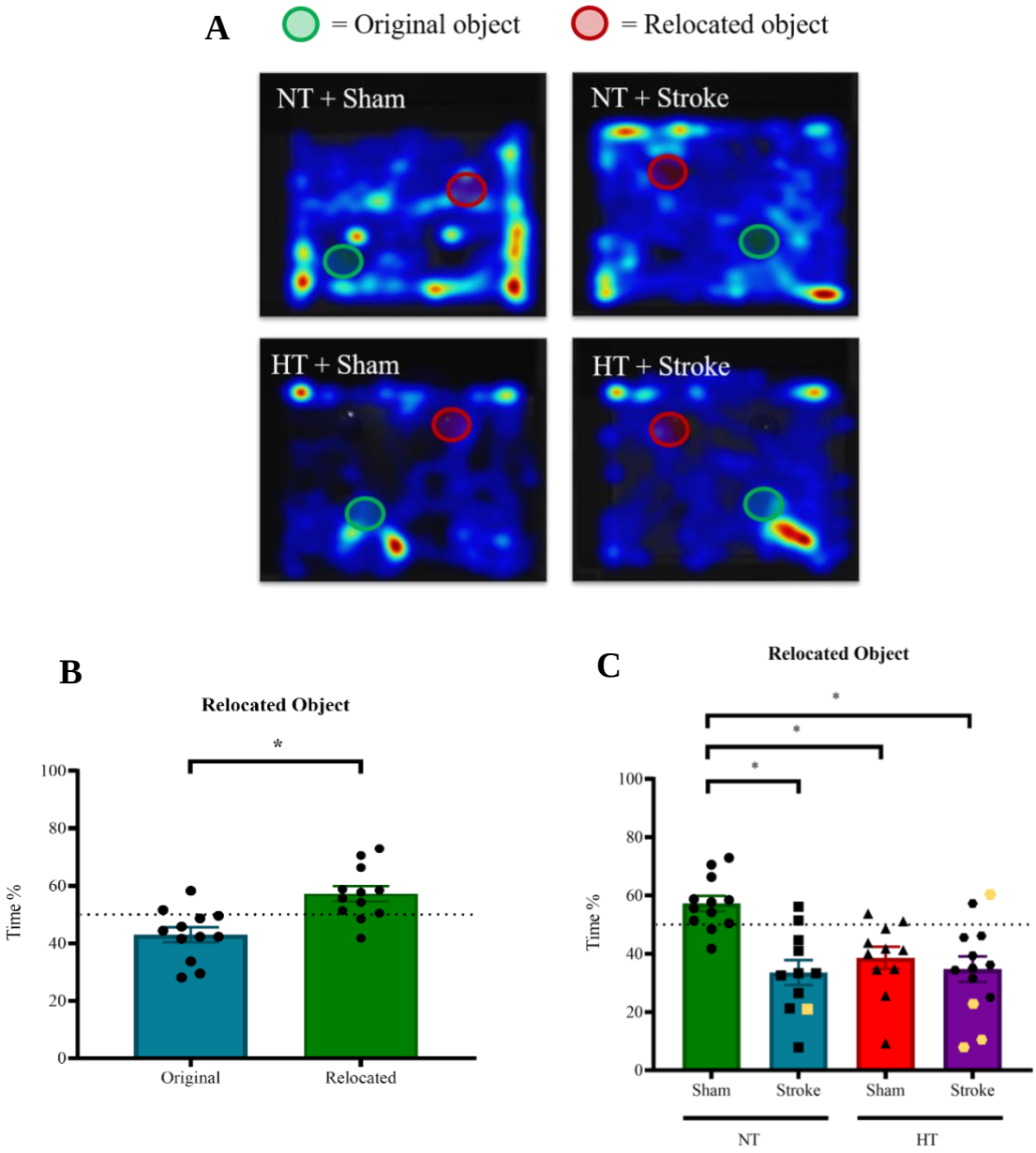


Figure 11. Assessment of Spatial Working memory with the Object Location Task. The OLT was performed in mice undergoing the 14 d protocol. **(A)** Representative heat maps of mouse activity when performing the OLT. Percentage of time spent interacting with **(B)** the original or relocated object in the NT + sham group and replotted in **(C)** as % of time spent interacting with the relocated object in all groups, yellow data points represent mice with evidence of hemorrhaging. Data are presented as mean ± SEM, n=11-14/group. * indicates $P < 0.05$, **(B)** unpaired t-test and **(C)** two-way ANOVA with Tukey's mulitple comparisons test.

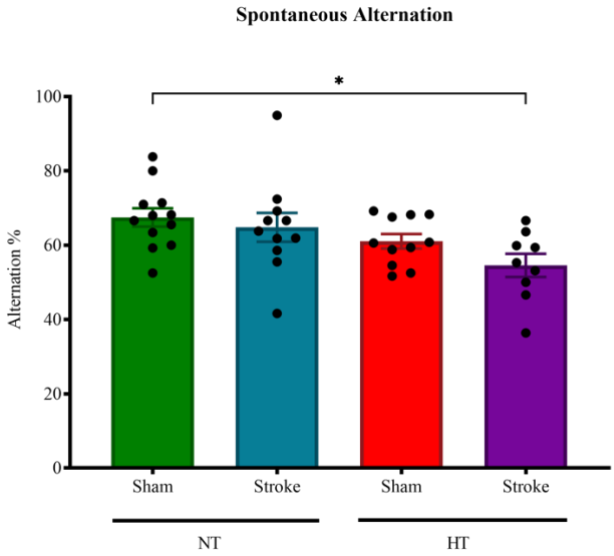


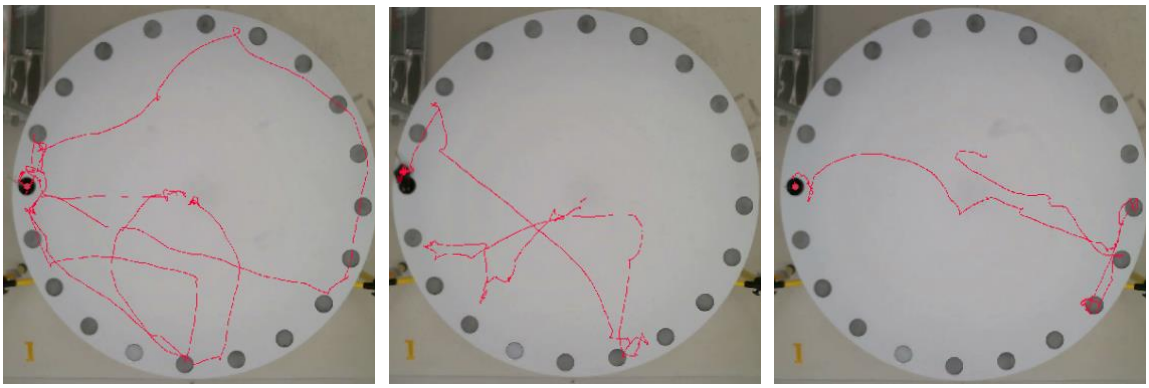
Figure 12. Assessment of Spatial Working Memory via the Y Maze Spontaneous Alternation Test.

The spontaneous alternation test was performed in mice undergoing the 14 d protocol. Data are presented as mean ± SEM, n=9-12/group. Two-way ANOVA with Tukey's multiple comparisons test. * indicates $P < 0.05$, **(A)** unpaired t-test and **(B)** two-way ANOVA with Tukey's mulitple comparisons test.

3.2.3 Barnes Maze Optimisation

The Barnes maze was utilised to assess spatial reference memory and learning across 3 days. Prior to the commencement of the study, a series of trials were completed with untreated C57Bl/6 mice (n=6) to optimise the Barnes maze protocol (Figure 13A-D). Primary latency in mice at day 1 was 98 ± 16 s, day 2 was 81 ± 23 s and day 3 was 47 ± 24 s. Visual tracing of mouse activity across three days is shown in Figure 13A. As seen in Figure 13B the time taken to locate the escape hole at day 2 was comparable to that on day 1, however on day 3 the time was significantly reduced compared to day 1 ($P < 0.05$). Escape latency was comparable across all time points (Figure 13C; $P > 0.05$). Distance travelled on day 2 was significantly less than on day 1 (day 1, 813 ± 114 cm vs day 2, 502 ± 75 cm; $P < 0.05$), however there was no statistical difference between day 3 (545± 97 cm) and day 1 ($P > 0.05$).

A Day 1 Day 2 Day 3



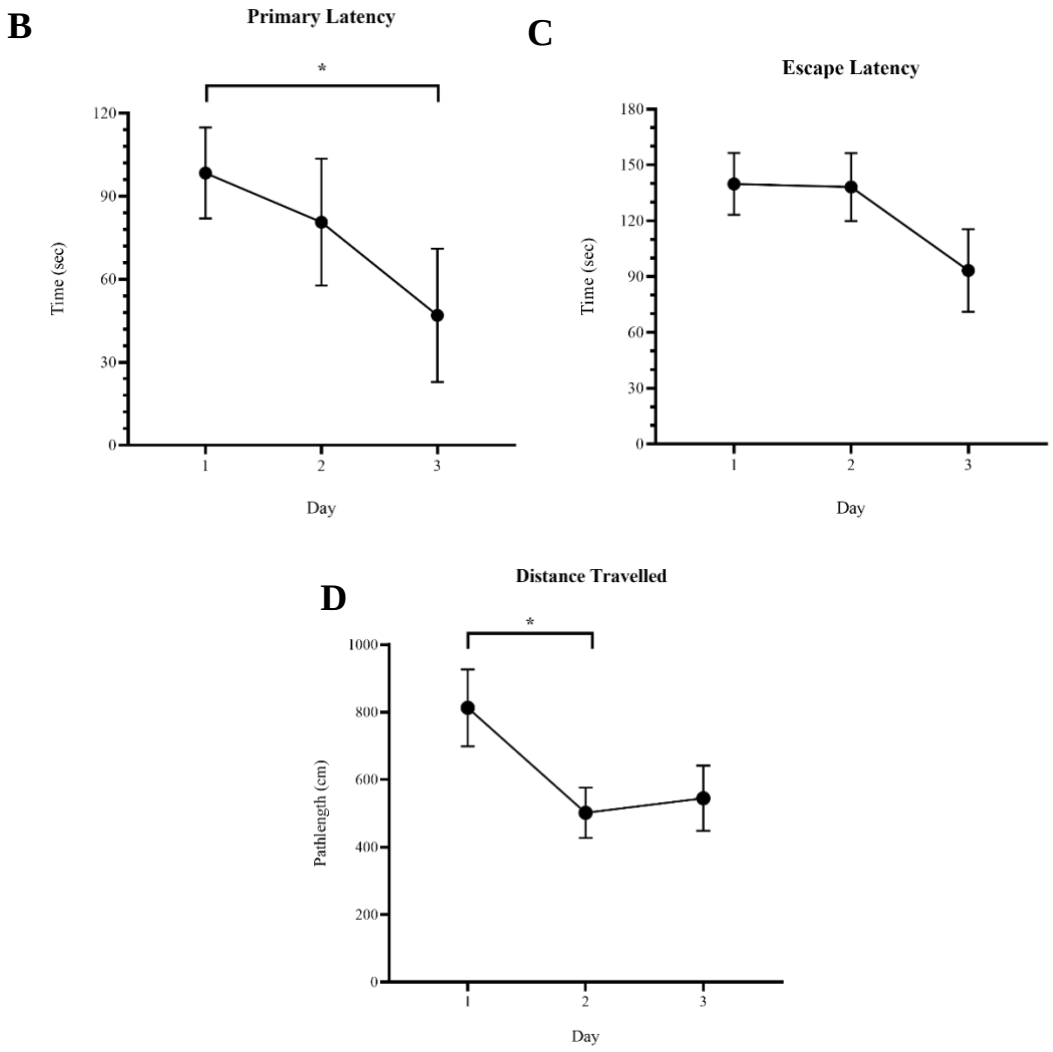
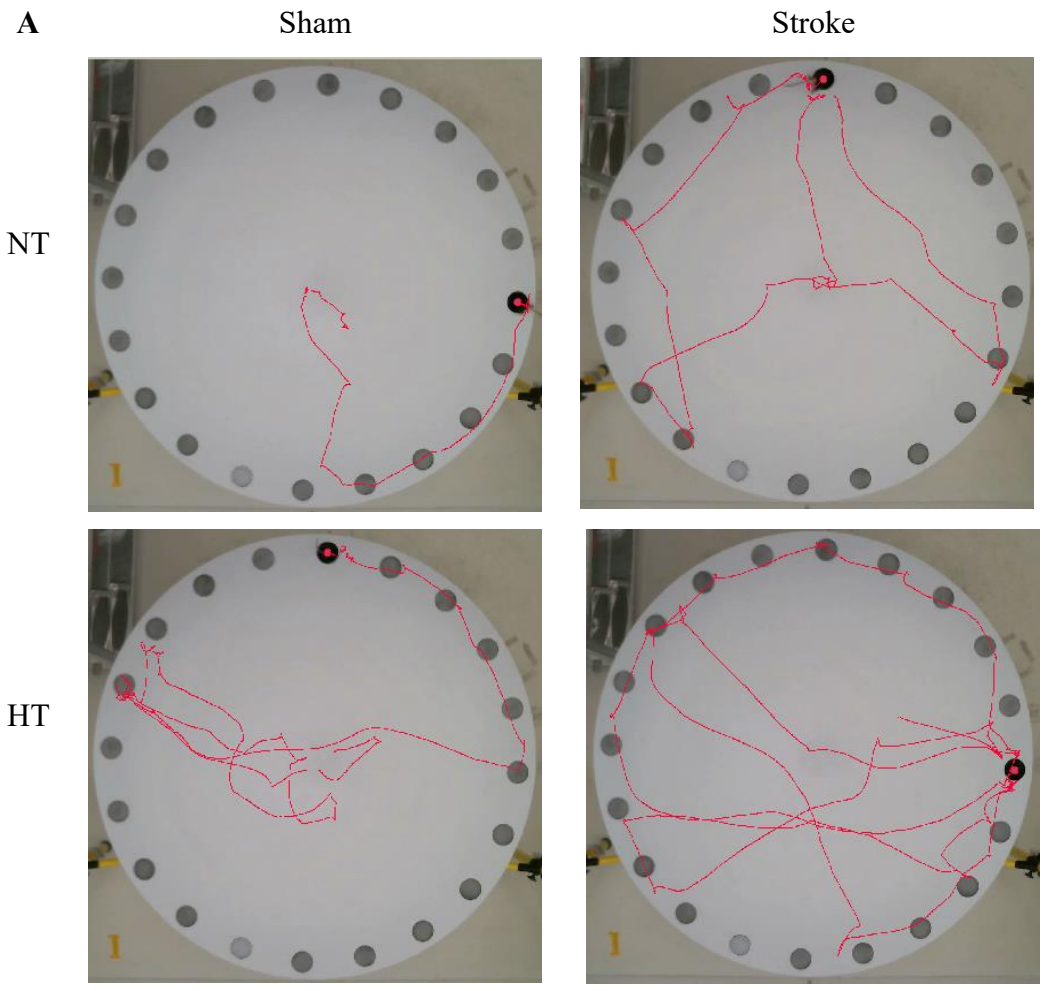


Figure 13. Optimisation of the Barnes Maze.

(A) Visual tracing of mice performing the Barnes maze on days 1-3. The average time taken, across three trials, in seconds to **(B)** locate and **(C)** enter the escape hole. **(D)** Visual tracing of mice movement was calculated (cm). Data is presented as mean ± SEM, n=6. * indicates $P < 0.05$, RM one-way ANOVA with Tukey's multiple comparisons test.

3.2.4 Barnes Maze Testing

Mice assigned to the 28 d protocol performed the Barnes maze. Escape latency at day 1 was similar across all groups. At day 2 the NT + stroke and HT + stroke had an increased latency to enter the escape hole when compared to the NT + sham group ($P < 0.05$). Further, the HT + stroke group took significantly longer to enter the escape hole when compared to the HT + sham and NT + sham at day 3 (HT + stroke 167 ± 6.0 s vs. HT + sham 123 ± 13.8 s vs. NT + stroke 140 ± 10.1 s vs. NT + sham 102 ± 16.7 s; $P < 0.05$) (Figure 14A). The change in performance between day 3 and 1 of latency to escape revealed that the HT + stroke (2 ± 3 %) group was reduced when compared to NT + sham (33 ± 11 %; $P < 0.05$). Primary latency analysis revealed time to be the main effect however, no difference was seen between each of the treatment groups (Figure 14D). Distance travelled also showed time to be the main effect with no differences between each of these treatment groups (Figure 14E).



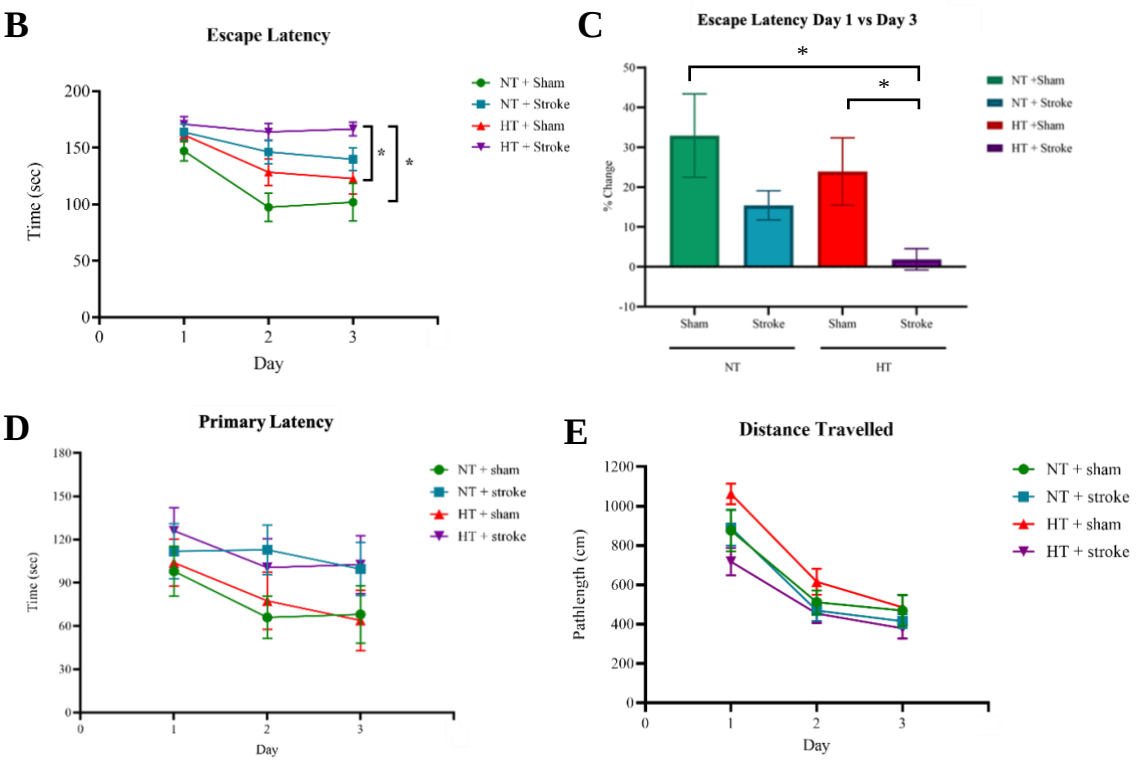


Figure 14. Assessment of Spatial Reference Memory with the Barnes maze.
The Barnes maze was performed in mice undergoing the 28 d protocol. **(A)** Visual tracing of all mice groups performing the Barnes maze on day 1. **(B)** Escape latency, **(C)** the difference in escape latency (%) at day 1 vs day 3, **(D)** primary latency, and **(E)** average distance travelled (cm). Data are presented as mean ± SEM, n=12-13/ group. * indicates $P < 0.05$, two-way ANOVA with Tukey's mulitple comparison test.

3.3 Assessment of Cerebral Blood Flow

Representative speckle traces are shown, including areas in which CBF was measured (black line). Cortical CBF, as measured by LSCI, was not altered on the dorsal surface of the brain in either NT or HT groups (14 d protocol; Figure 15). Stroke reduced CBF (P <0.05) however, the magnitude of this change was comparable in NT and HT groups (Figure 15B). No difference in CBF was observed between NT + stroke and HT + stroke groups at day 14 when compared to baseline (Figure 15C; $P > 0.05$).

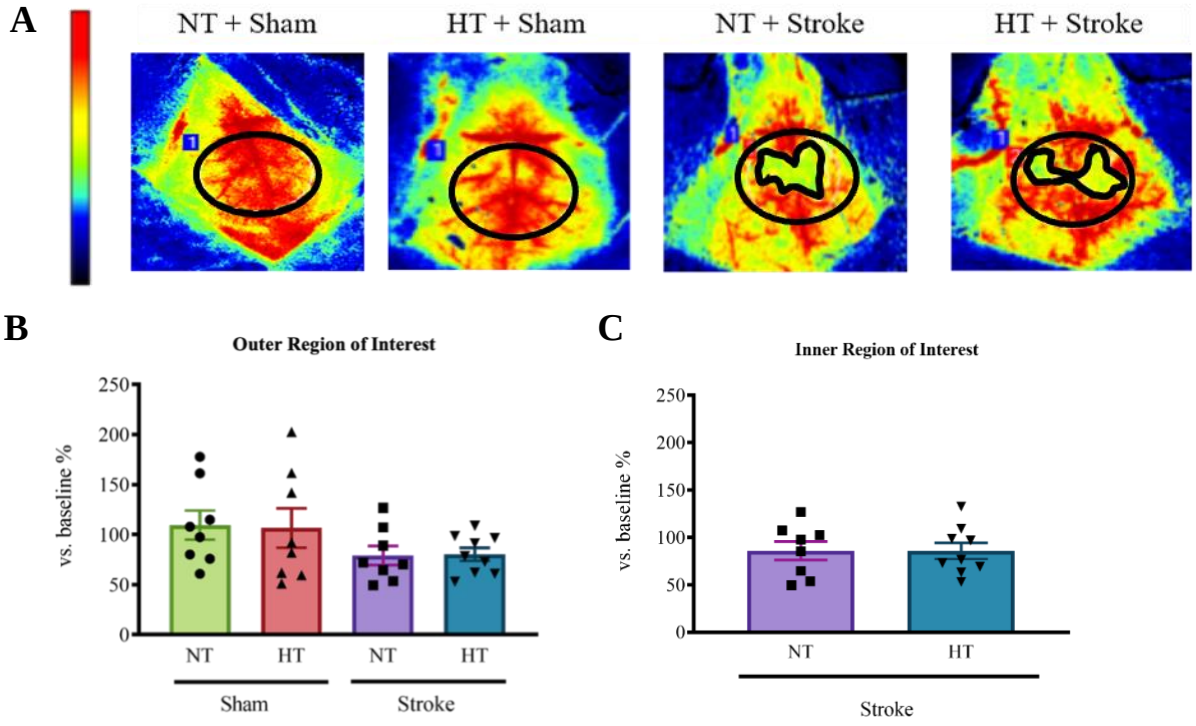
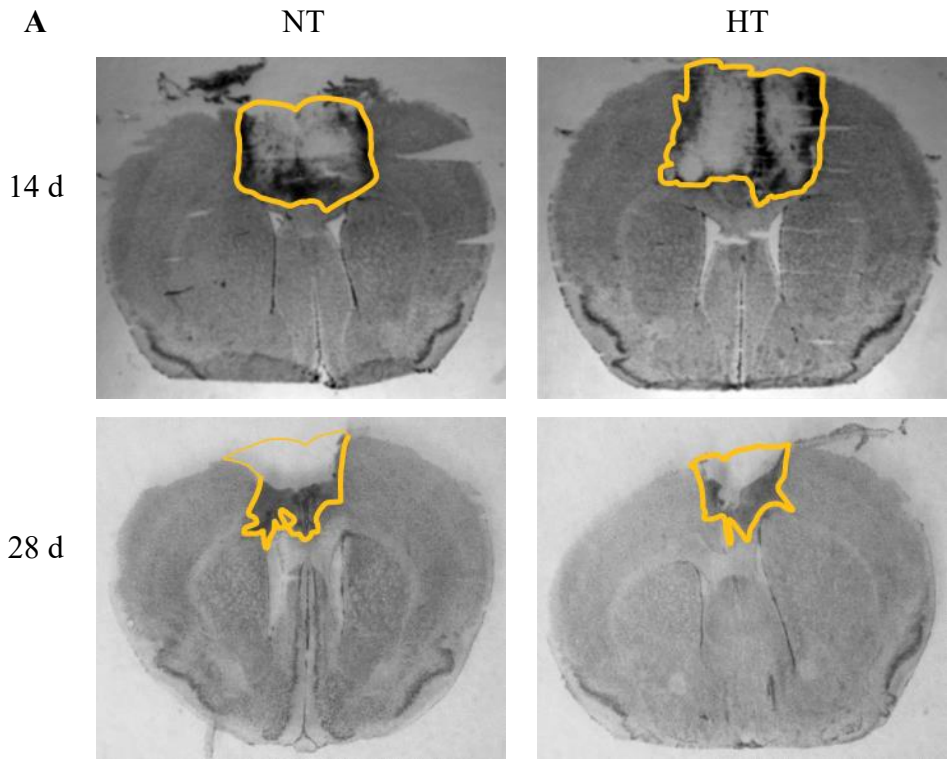


Figure 15. Measurement of Cerebral Blood Flow

(A) Representative images of CBF at day 14. **(B)** The relative change in CBF (vs baseline measurements) in all groups. **(C)** Changes in CBF of the inner region of interest in stroke group (vs baseline measurements). Representative images of CBF at day 14 are shown above. Data are presented as mean ± SEM, n=8-9/group, * indicates $P < 0.05$, two-way ANOVA with Tukey's mulitple comparisons test.

3.4 Infarct Volume Analysis

There was no statistical difference between NT + stroke group and HT + stroke group in either 14 d or 28 d protocols (day 14, 4.2 ± 3.6 mm^3 vs 6.0 ± 3.2 mm^3; day 28, 2.3 ± 1.1 mm^3 vs 2.1 ± 1.3 mm^3; $P > 0.05$) (Figure 16B).



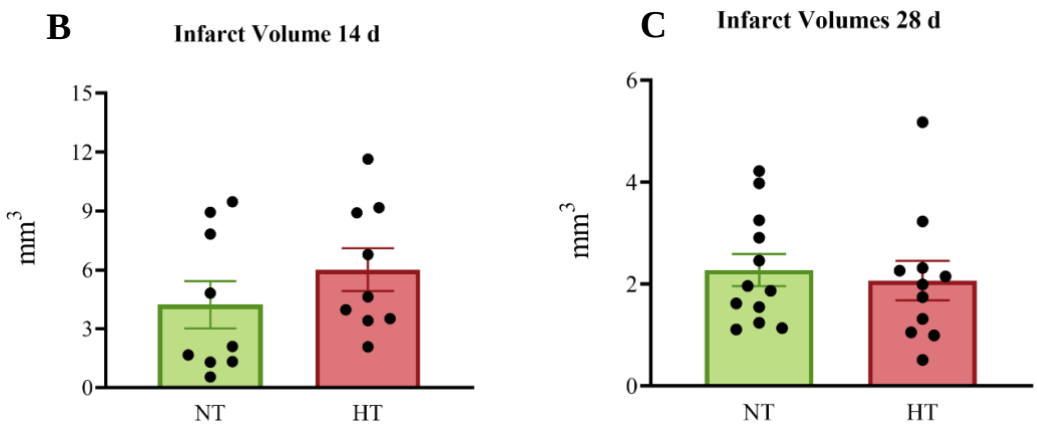


Figure 16. Infarct Volume.

(A) Representative images of infarct volume for NT + stroke and HT + stroke mice. **(B)** Infarct volumes for the 14 d protocol, NT + stroke vs HT + stroke. **(C)** Infarct volumes for the 28 d protocol, NT + stroke vs HT + stroke. Data are presented as mean ± SEM, n=9-12/group. * indicates $P < 0.05$, unpaired t-test.

3.5 Haemorrhage Assessment

The observation of haemorrhages only resided in mice of the 14 d protocol. The incidence of haemorrhage was assessed in isolated brains. Of 10 mice, the NT + stroke group had a single incidence of distal (to the infarct) haemorrhage, whilst of the ten in the HT + stroke group, there were five (4 distal and 1 local; P <0.05) (Figure 17).

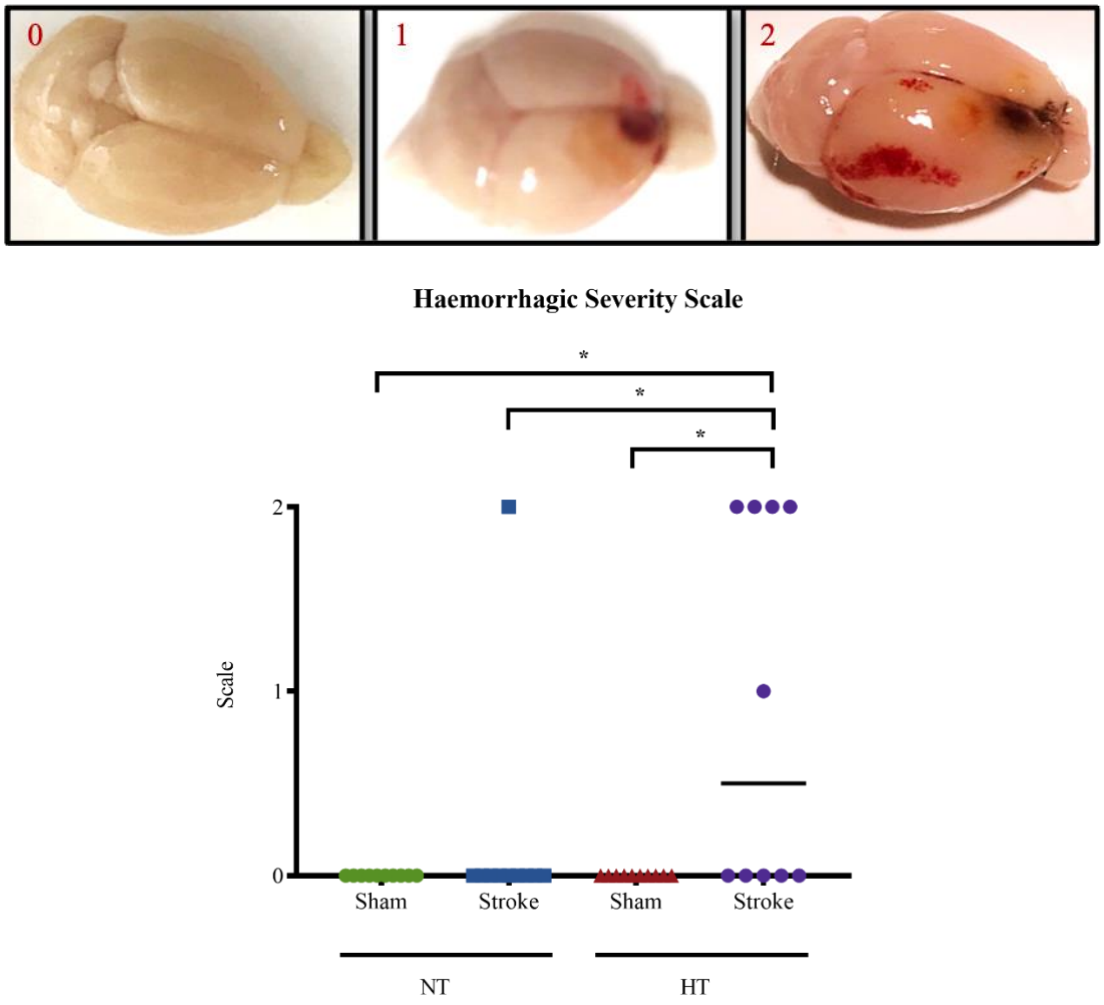
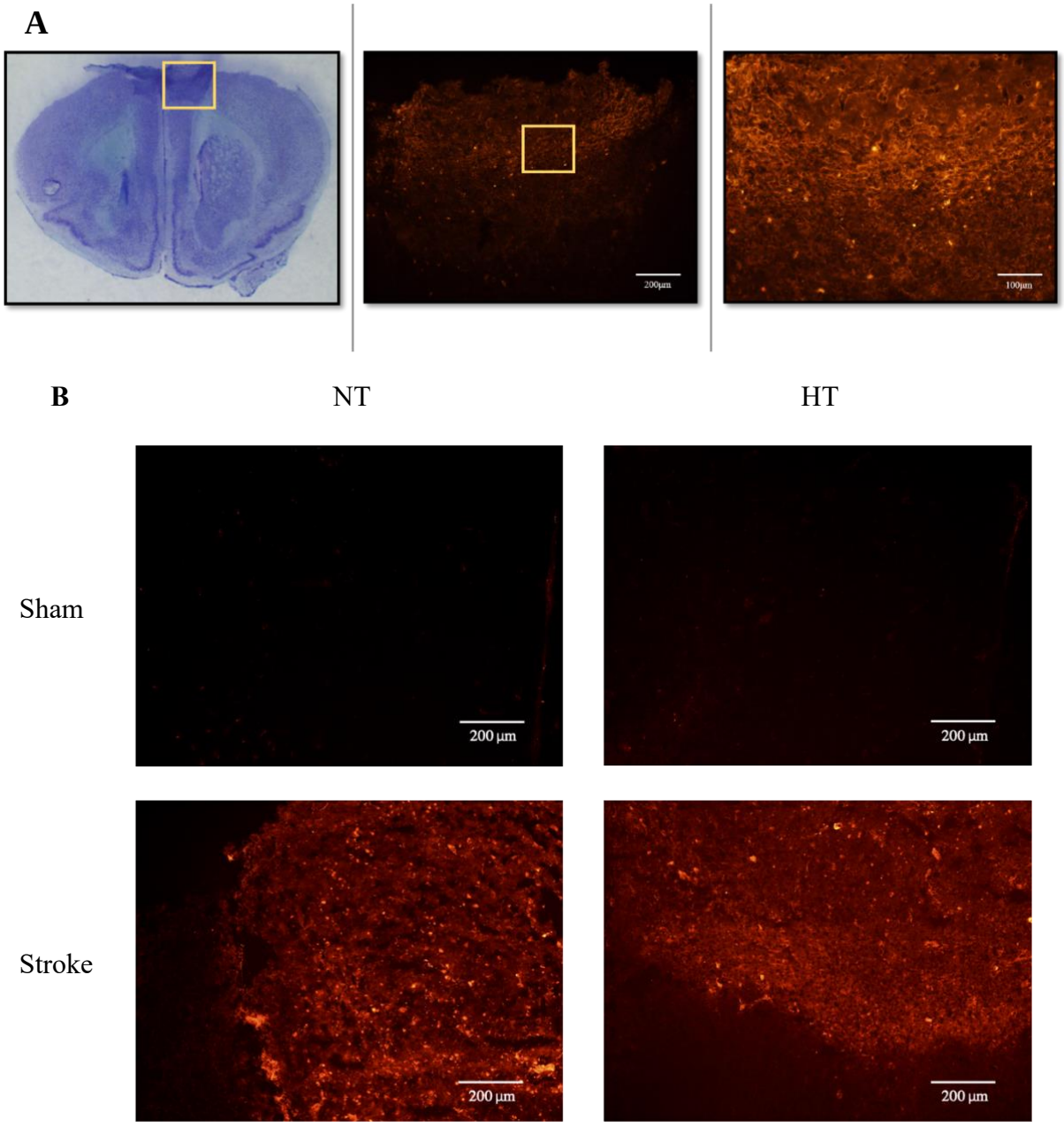


Figure 17. Haemorrhagic Severity Scale.

Representative images of haemorrhage severity shown above; occurrence of haemorrhages across all groups. Median value is indicated, n=10-12/group. * indicates P < 0.05, Kruskal-Wallis test.

3.6 In vivo IgG Immunofluorescence Staining

Representative photomicrographs of IgG within the prefrontal cortex and hippocampus (Figure 18A-C & 19A-C). Mean pixel intensity (MPI) was used to estimate the level of IgG immunofluorescence. Analysis revealed stroke to exert the main effect on IgG immunoreactivity within the prefrontal cortex in both 14 d and 28 d protocols. However, there was no statistical difference between any groups at either time points. No difference was observed in the hippocampus at 14 d. However, in mice from the 28 d protocol, analysis revealed that the HT + sham (1.8 ± 0.4 MPI) had increased IgG immunoreactivity within the hippocampus when compared to the NT + sham (0.7 ± 0.1 MPI) ($P < 0.05$).



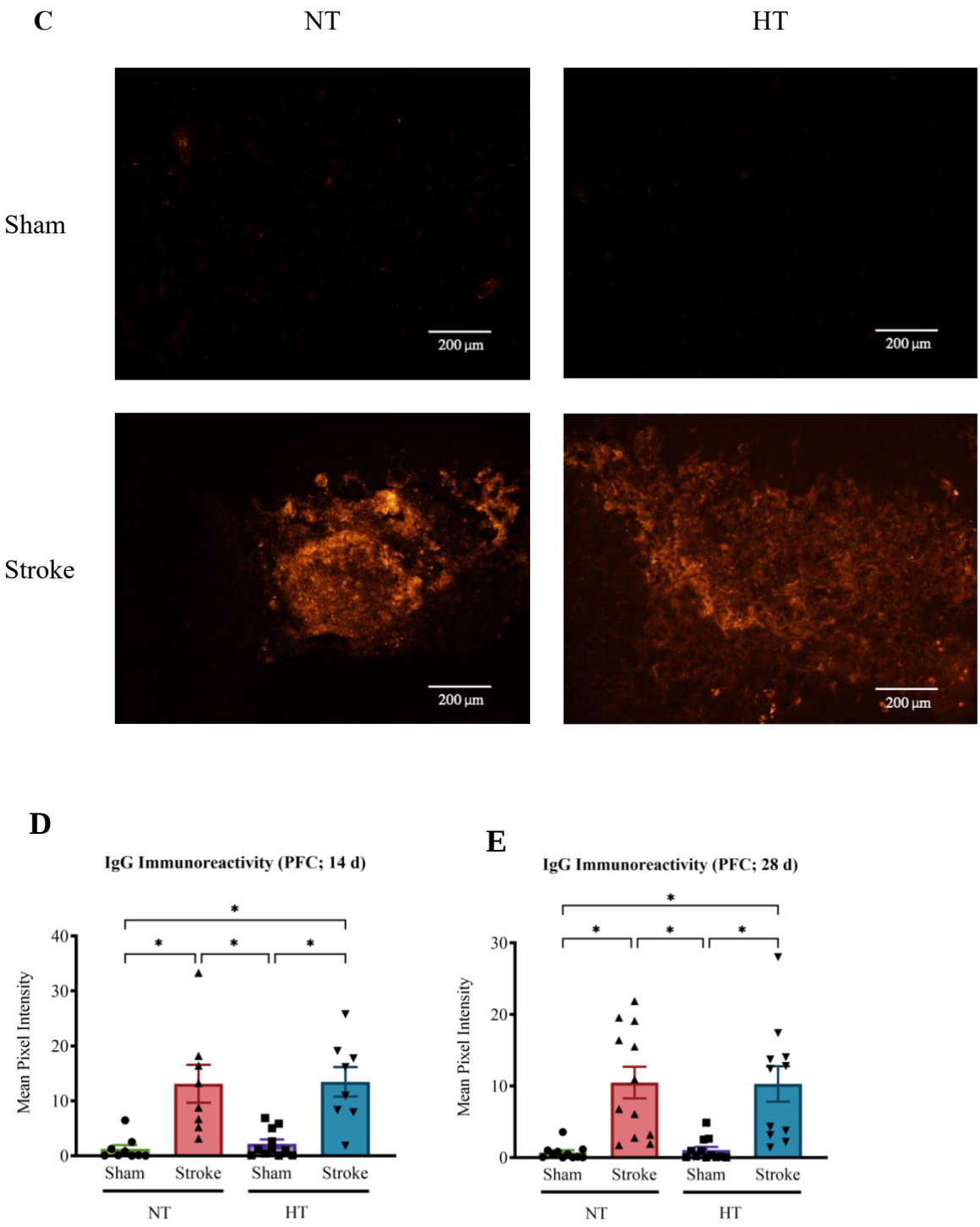
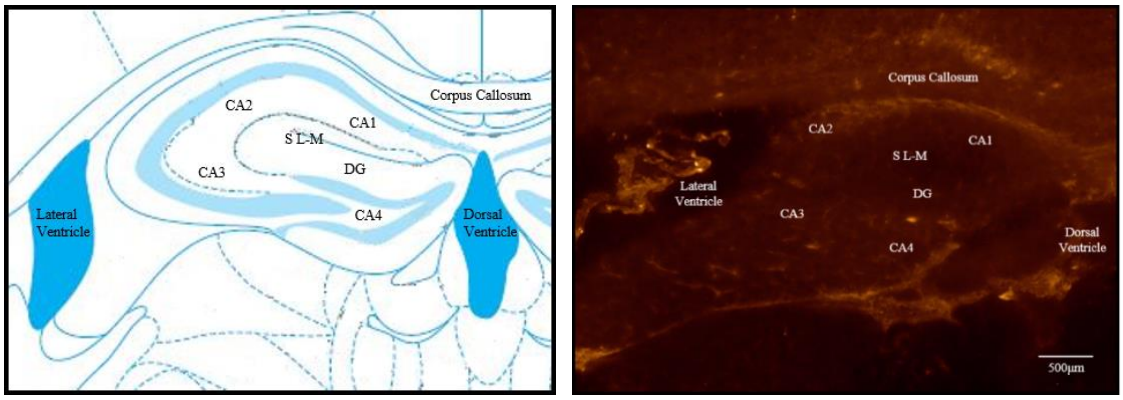


Figure 18. IgG Intensity of the Prefrontal Cortex.

(**A**) Representative photomicrographs of IgG in the prefrontal cortex, images taken were dependent on the location of stroke and equivalent locations were then imaged in shams. **(B)** 14 d and **(C)** 28 d IgG immunoreactivity within the prefrontal cortex (PFC). **(D)** 14 d and **(E)** 28 d mean pixel intensity of the PFC across all groups. Data are presented as mean ± SEM, n=8-12/group. * indicates $P < 0.05$, analysis by two-way ANOVA with Tukey's multiple comparisons test.

A

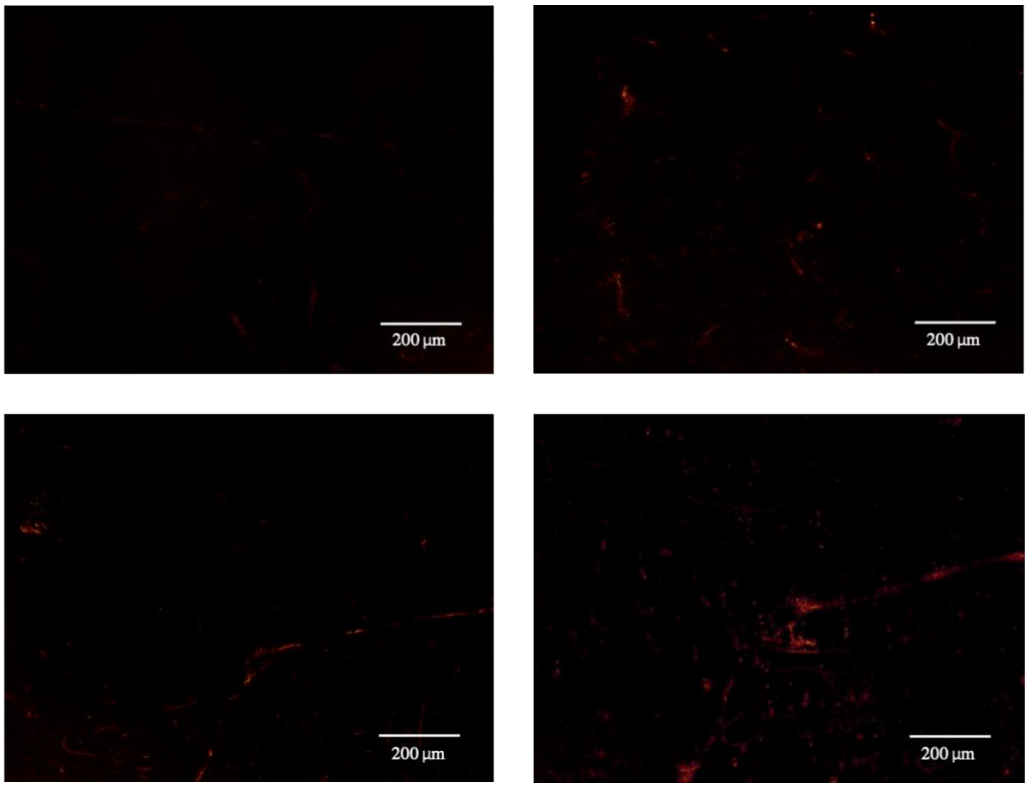


B

NT HT

Sham

Stroke



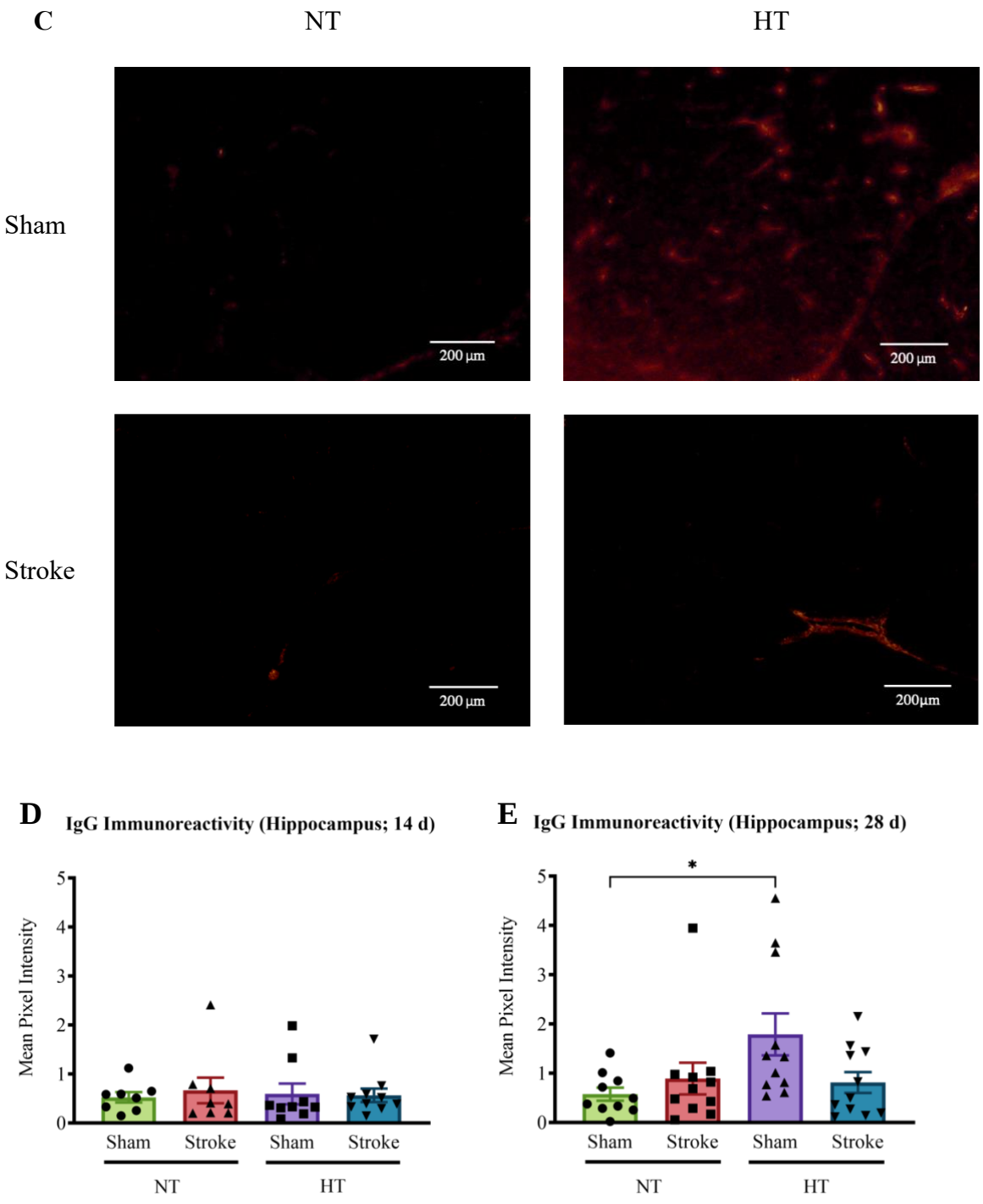


Figure 19. IgG Intensity of the Hippocampus.

(A) Schematic representation of mouse hippocampus with cornu ammonis (CA1-4), dentate gyrus (DG) and stratum lacunosummoleculare (S L-M) labelled approximately. Adapted from Paxinos, G & Franklin, KB. (2007). The Mouse Brain in Stereotaxic Coordinates Third Edition[251]. **(B)** 14 d and **(C)** 28 d IgG immunoreactivity within the hippocampus. **(D)** 14 d and **(E)** 28 d mean pixel intensity across all groups. Data are presented as mean ± SEM, n=8-12/group. * indicates $P < 0.05$, analysis by two-way ANOVA with Tukey's multiple comparisons test.

3.7 Astrocyte Activation

GFAP immunoreactivity was increased in the NT + stroke group and HT + stroke group (14 d protocol; Figure 20).

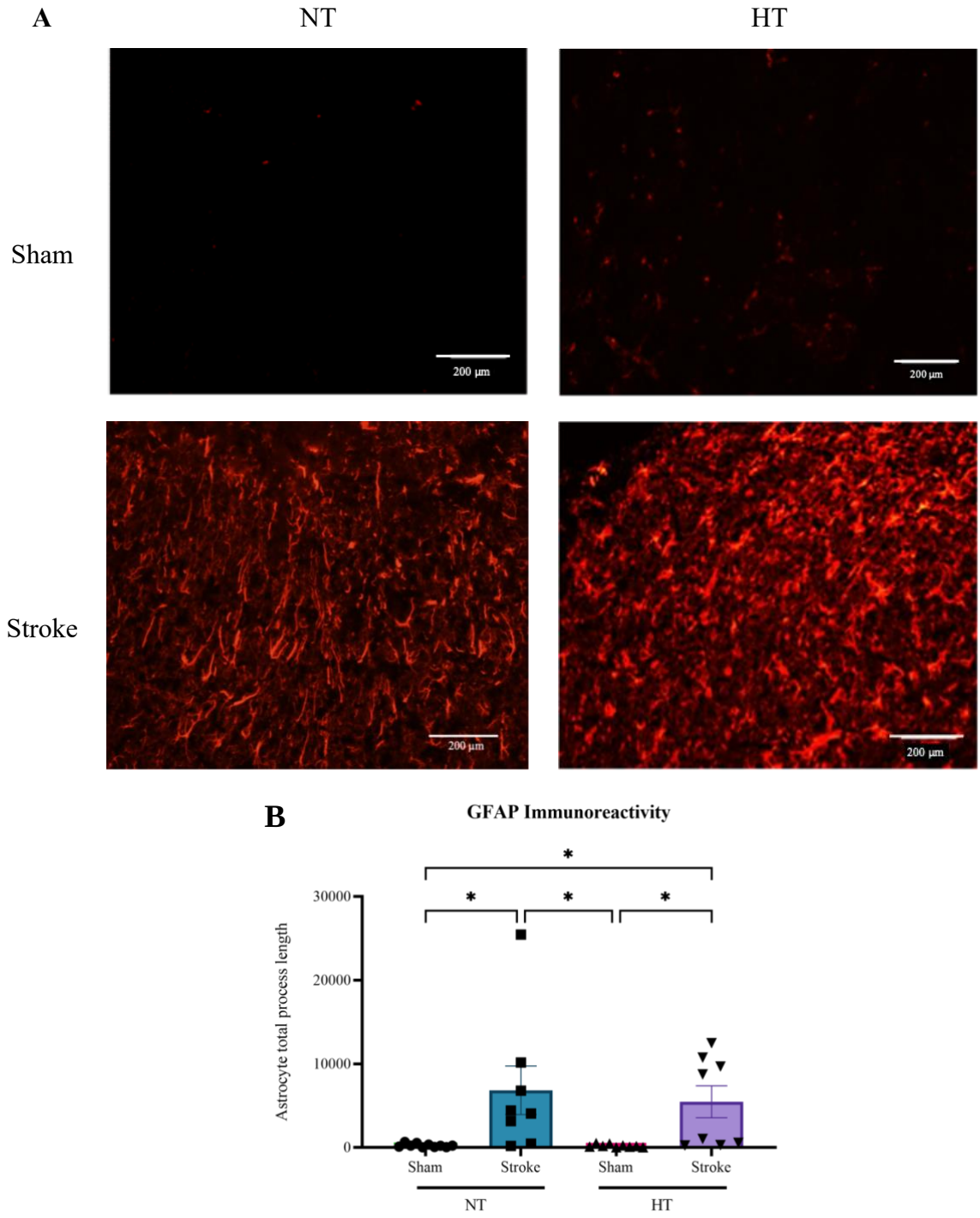


Figure 20. GFAP Intensity in the Prefrontal Cortex.

(A) Representative photomicrographs of GFAP immunoreactivity in the prefrontal cortex. Images taken at x40 magnification. **(B)** Mean pixel intensity across all groups in the 14 d protocol**.** Data are presented as mean ± SEM, n=8-9, * indicates $P < 0.05$, two-way ANOVA with Tukey's multiple comparisons test.

Discussion

In the present study, we hypothesised that either hypertension or stroke alone would result in cognitive impairment and the combination of hypertension and stroke would further increase cognitive impairment. The main findings of this study were: 1) hypertension or stroke impaired spatial working memory and the combination of hypertension and stroke exacerbated learning impairment; 2) hypertension in the presence of stroke resulted in a greater incidence of spontaneous haemorrhage than either hypertension or stroke alone; and 3) hypertension resulted in increased BBB permeability in the hippocampus. Overall, these findings support our hypothesis. Thus, hypertension exacerbates cognitive impairment in stroke leading to worsened cognitive decline in mice and this phenomenon would be expected to result in a reduced quality of life in humans.

4.1 Effect of Angiotensin II on Blood Pressure

Systemic infusion of angiotensin II increases SBP in mice, and this is understood to occur as a result of vasoconstriction, sympathetic outflow, aldosterone production and increased Na^+ and water reabsorption in the kidneys[239, 252]. Vasoconstriction is mediated by the AT1R to which angiotensin II binds and is widely distributed throughout all arteries and arterioles in the body. Upon activation, the AT1R in vascular smooth muscle cells signals the $Ca2^+$/inositol triphosphate pathway (IP3) to release Ca^{2+} from the sarcoplasmic reticulum, and Ca^{2+} then binds to calmodulin and activates myosin light chain (MLC) leading to vasoconstriction[253]. Activation of AT1R can stimulate various pathways such as the NADPH oxidase and Rho Kinase (ROCK) which target lipid rafts promoting atherogenesis, endothelial cell injury and inflammation[254, 255]. Activation of ROCK has been implicated in the pathogenesis of hypertension[256, 257]. ROCK may directly phosphorylate MLC[258] or increase $Ca2^+$ sensitivity of vascular smooth muscle cells, resulting in sustained periods of vascular smooth muscle contractions, thus increasing blood pressure[259-262].

Acute increases of blood pressure do not necessarily result in chronic hypertension. Other suggested mechanisms such as angiotensin II-induced inflammation are likely to play a role[263]. As the pro-inflammatory properties of angiotensin II have been shown to be central to the pathogenesis of hypertension in humans, the angiotensin II infusion model in mice is probably relevant for understanding mechanisms of clinical hypertension[239]. In the present study, and consistent with previous studies, the infusion of angiotensin II increased SBP in both 0.7 mg/kg/day and 0.28 mg/kg/day dosages[264, 265]. The reduction in dosage from 0.7 mg/kg/day to 0.28 mg/kg/day did not change the time taken for mice to

become hypertensive as in each case SBP was approximately 140 mmHg by day 7. This lower dose administered over a longer period allows for a slightly slower, steady increase in blood pressure and is suggested to be a more representative model of chronic essential hypertension in humans[266, 267]. In this study, ischaemic stroke did not have any effect on SBP in normotensive or angiotensin II-infused mice.

4.2 Assessing Cognitive Impairment During Disease

There is consensus that hypertension and stroke can contribute to the development of cognitive impairment[31, 43, 130, 268-271]. The first aim of this study was to determine the effects of hypertension on post-stroke cognitive outcomes. The spontaneous alternation test (Y maze) is commonly used to assess spatial working memory[31, 272]. As expected, our control mice performed the spontaneous alternation test with a 60-70 % alternation rate which is comparable to previous studies[273, 274]. Reduced spontaneous alternation was observed in hypertensive mice; however, no effect of stroke was seen and there was no difference between normotensive and hypertensive groups at 7 d post-stroke. These findings aligned with previous studies that tested spatial working memory in mice following prefrontal stroke and found no impairment after one week[183, 275, 276]. One explanation for the reduction of spontaneous alternations in hypertensive mice could be due to the nature of how hypertension affects the brain. The globalised effects of hypertension may affect both the hippocampus and the prefrontal cortex, whereas in stroke mice only the prefrontal cortex was targeted. Additionally, the time point of when the neurobehavioural tests were performed should also be taken into consideration. Doyle *et al* conducted a study where a reduction in spontaneous alternation was not seen until 7 weeks post-stroke[277]. Suggesting that 7 d post-stroke may not be a sufficient amount of time to detect an effect.

The object location task (OLT) is an effective behavioural test for the assessment of spatial working memory[246]. The inclusion of the learning phase and a retention phase (separated by 1 h) allowed us to test short-term memory. Our findings revealed that the average time spent on the relocated object in our hypertensive mice that received stroke surgery was 23 % less than the control mice. All treatment groups spent less than half of the time on the relocated object as opposed to control mice at 7 days post-stroke surgery. This suggests that all of our treated groups had a reduced capability to recall the spatial placement of the objects, indicating impaired spatial working memory. In contrast to our study, Zhou *et al* only found delayed memory impairment at four weeks post-stroke but not at one week[183]. The location and duration of the stroke, and behavioural tests used

were similar to our study. One potential explanation could be that Zhou *et al* had mice performing four different behavioural tests across four days consecutively; in the study of Zhou *et al* the OLT was performed after the novel object recognition task which may have acted as a training phase to assist mice in remembering the location of the original object. Hillman *et al* also provided supporting evidence of prefrontal stroke producing the delayed onset of spatial memory impairment, where mice performing the OLT had decreased performance at 2 and 4 weeks post stroke; the Y maze performance was reduced 4 weeks post stroke when compared to shams[243]. Additional studies have also reported conflicting outcomes for long-term OLT performance post-stroke[183, 278]. For example, Wang *et al* found memory deficits at one and two weeks post-stroke, however at three and four weeks memory deficits were no longer apparent[278]. Pan *et al* reported memory impairment at just two days post-stroke and further impairment four weeks later[279]. Both studies used a middle cerebral artery occlusion (MCAO) model of stroke, a more severe form of stroke, and both used the novel object recognition task, a variation of the OLT, to assess short-term memory. Discrepancies in long-term memory in the MCAO model may be due to the methodology of stroke induction. This related to the fact that one study injected cholesterol crystals to occlude the artery momentarily[278], whereas the other used a nylon filament to occlude the artery for 90 min[279] resulting to a difference in stroke severity.

The inclusion of the Barnes maze in this study provided greater insight into how memory and learning are affected by hypertension and stroke. Unlike the OLT which produced only qualitative data at a single time-point (i.e. impaired or not impaired), the data from the Barnes maze were quantitative and included several points to assess spatial learning (i.e. degree of impairment and rate of learning)[280, 281]. During the optimisation for the Barnes maze, mice progressively travelled less and spent less time locating their respective escape holes. The primary latency for mice on day 3 was significantly quicker than on day 1. Thus, the 3-day protocol for assessing spatial reference memory was chosen.

The Barnes maze allows for the measurement of spatial learning, short and long-term memory, and behavioural flexibility. Our findings indicate that the hypertensive stroke mice displayed impaired learning across all three days and took longer to enter the escape hole. There are many variations of Barnes maze that can be used to investigate a specific aspect of cognition. Previous studies have extended the time between tests to assess long-term memory, implemented a reversal task to assess behavioural flexibility and calculated primary errors for error recognition[282-284]. Our data were consistent with findings by Cuartero *et al*, where mice subjected to ischaemic injury (MCAO; as opposed to sham surgery) travelled the same distance in the same time to locate the escape hole when performing the Barnes maze[282]. In contrast to the study by Faraco *et al*, in which there was spatial memory impairment at 30 d after the induction of hypertension, we found no impairment at 28 d in our hypertensive mice[285]. This discrepancy may be explained by the different dose of angiotensin II used, as Faraco *et al* used 0.86 mg/kg/day vs our 0.28 mg/kg/day dosage. Thus, higher doses of angiotensin II may contribute to the greater impairment of spatial memory.

4.3 Effect of Hypertension and Stroke on Cerebral Blood Flow

Hypertension has been associated with cerebral hypoperfusion[286-288]. As expected, we observed a 20 % reduction in CBF within the prefrontal cortex in mice subjected to ischaemic stroke. We did not observe any differences in CBF between hypertensive and normotensive mice. The discrepancies between previous reports of reduced CBF in hypertension and our study may be explained by the duration of which mice were exposed to hypertension. For example, Wiesmann *et al* studied the effects of hypertension on CBF in mice over two months[289], compared to our mice in the 14 day protocol. Hypertension has several deleterious effects on the cerebral vasculature such as alterations to autoregulation, endothelial dysfunction, inward remodelling, hypertrophy and rarefaction (a loss of arterioles and capillaries)[91, 103, 290-294]. A combination of these effects is likely to contribute to a reduction in CBF. As there is no energy storage within the brain, it is paramount for there to be a constant supply of O_2 and nutrients. The cerebral microvasculature is closely linked with neurons and glial cells to support normal processes of the brain, so that any disruption to cerebral perfusion will compromise O_2 and nutrient exchange, and thus adversely affecting brain function.

Increased levels of circulating angiotensin II have been associated with increased oxidative stress, inflammation, BBB damage, endothelial damage and thus reduced CBF[46, 99, 295-298]. Gomolak *et al* demonstrated that angiotensin II induces endothelial dysfunction in a dose- and time-dependent manner, where 0.4 mg/kg/day was associated with progressively worsened endothelial dysfunction. Additionally, angiotensin II doses of up to 0.2 mg/kg/day were associated with minimal dysfunction at any given time-point[298]. This suggests that for this study a longer duration may have been required for our 0.7 mg/kg/d dose in order to observe endothelial dysfunction and subsequent consequences leading to reduced CBF.

4.4 Effect of Hypertension in Ischaemic Stroke

During an ischaemic stroke, the occlusion of a cerebral blood vessel leads to the irreversible loss of neurons within the infarct core. In this study we found no differences in infarct volume between normotensive and hypertensive mice following stroke in either 14 d or 28 d protocols, suggesting that hypertension does not contribute to the size of the infarct in this model of stroke. Additionally, we observed variability in infarct sizes in our mice. Similar findings by Zhou *et al* showed that mice subjected to 18 min of photothrombosis had variability in infarct size, whereas mice subjected to 22 min had more consistent infarct sizes[183]. These finding suggest that a longer exposure of light during photothrombosis would produce more consistent infarct sizes compared to our 18 min protocol. Previous studies have shown that longer durations of photothrombosis resulted in larger infarct size[299, 300], thus the 18 min protocol was chosen to produce smaller infarcts, retain adequate function for neurobehavioural testing and observe any additive effect of hypertension and stroke as it would likely be more apparent.

Both normotensive and hypertensive mice subjected to stroke in the 14 day protocol had similar infarct sizes. However, performance during the OLT in the assessment of spatial working memory suggests that the size of the infarct may contribute to the cognitive deficits found in the early time course of stroke. This concept is supported by results from a previous study where smaller infarcts were associated with less behavioural impairment[279].

Surrounding the infarct core, the ischaemic penumbra is a compromised but recoverable area of brain tissue, and the level of ongoing of O_2 and nutrient delivery to this region will impact the potential for recovery[301, 302]. A study by Shin *et al* found that early pharmacological induction of mild hypertension post-stroke improved CBF and O_2 delivery to the ischaemic penumbra via collateral vessels[303]. Despite these findings,

hypertension during stroke is known to be associated with worsened outcomes, haemorrhagic transformations, increased oedema and increased risk of recurrent stroke[225, 304-306]. Thus, mild hypertension may be beneficial as in the study by *Shin et al*, however the risks of such an intervention probably far outweigh the benefits, as demonstrated by the results of this study.

Interestingly, we found that hypertension markedly increased the incidence of brain haemorrhages in mice subjected to stroke in our 14 d protocol. In addition to ischaemic stroke, hypertension is also known to be a risk factor for haemorrhagic strokes[268]. The occurrence of haemorrhage after an ischaemic event is known as a haemorrhagic transformation. The recanalization of occluded arteries can result in worse outcomes and has been associated with early BBB disruption, recanalization may be prevented if treatment is within 3.8 hours following an ischaemic stroke[307, 308]. Evidences of haemorrhage outside of the infarct area were striking and may be related to events following the stroke, such as global inflammation and oxidative stress, which can contribute to the development of endothelial dysfunction and subsequent vessel rupture[309]. Haemorrhagic transformations have also been reported in other animal studies, attributing the cause to hypertension rather than the embolization or thrombotic induction of stroke[305, 310-312]. The occurrences in this study were mainly confined to the 14 d protocol and not observed in the 28 d protocol. This may be due to a greater level of circulating angiotensin II in the 14 d protocol with the 0.7 mg/kg/day dose of angiotensin II potentially increasing binding to the AT1R and its downstream signalling pathways, weakening the endothelium lining the cerebral blood vessels making them more susceptible to haemorrhage. Another explanation for the absence of haemorrhage in the 28 d protocol could be that earlier haemorrhages were cleared via the cerebrospinal fluid (CSF), leaving no evidence of haemorrhage at the time of euthanasia.

4.5 Blood-Brain Barrier Damage During Disease

The BBB provides protection of the brain from infiltration of circulating pathogens, foreign substances, inflammatory cells and plasma proteins including antibodies such as immunoglobulin G (IgG)[42, 313]. IgG is the most abundant antibody class found in the plasma and is normally excluded from the CNS by the BBB. We observed IgG extravasation in the prefrontal cortex in all mice subjected to stroke, indicating a damaged and leaky BBB. No differences were observed between control and hypertension-alone groups. Although IgG immunoreactivity within the hippocampus remained relatively low in the 14 d mice, there was a greater IgG immunoreactivity in the hypertension-alone mice compared to controls at 28 d. The data suggest that the duration of hypertension was a key determinant of BBB damage within the hippocampus. However, no difference in IgG deposition in the hippocampus was observed between the combined hypertension + stroke group, stroke-alone or control group in the 28 d protocol. This is consistent with the concept in which prolonged hypertension leads to BBB dysfunction, and as a result hippocampal neurodegeneration may occur which compromises learning and memory[314]. Future studies will have to investigate the mechanisms behind the increased deposition of IgG within the brain in hypertension-alone mice compared to the combined hypertension + stroke mice.

Immunoreactivity was predominantly localised around endothelial structures supplying the hippocampus, whereas in the prefrontal cortex IgG was seen within the infarct area. Given that the extent of BBB damage after stroke was more severe than in hypertensive mice, this explains the widespread deposition of IgG within the prefrontal cortex This was consistent with findings by Doyle *et al* where large depositions of IgG were found in mice following stroke[315], suggesting that B cells, which form and release IgG, are implicated in the development cognitive dysfunction. In the study of Doyle *et al*, T cell response alone was insufficient in eliciting cognitive dysfunction[315]. Kuang *et al* observed IgG within the brain parenchyma in regions associated with transient changes to BBB permeability due to hypertension[313]. In contrast, Naessens *et al* studied spontaneously hypertensive rats (SHR) to assess IgG in undiluted CSF and did not detect IgG, suggesting no BBB leakage[316]. There are a few explanations for this discrepancy. For example, the SHR at the age of 10-months would have developed inward remodelling of cerebral arteries and an adapted autoregulatory response to prevent damage to the BBB. Another explanation could be due to the sensitivity of CSF sampling methodology as detection limitations may influence the results and conclusions.

The BBB is the structure separating the peripheral circulation from the CNS. Regions impacted by hypertension or stroke have been shown to have degraded tight junction proteins, contributing to BBB damage and neurovascular unit dysfunction[42, 317]. Specialised tight junction proteins expressed by the endothelial cells such as claudins, occludins and zona occludins are vital components that form the BBB. Claudins function to mediate paracellular ion diffusion across the cerebral blood vessels, and when dysfunctional it has been associated with neurological deficits[318]. Occludins induce the formation of tight junctions in both endothelial and epithelial cells and regulate the integrity and permeability of the BBB[319]. Lastly, zona occludins (ZO) not only provide the structural basis of tight junction formation but also participates in cell growth and proliferation[320]. Support structures such as perivascular astrocytes have been implicated in tight junction formation; astrocytes may also produce angiotensinogen which is cleaved into angiotensin II, and when bound to its AT1R in endothelial cells it is proportional to occludin expression[321]. Moreover, AT1R activation restricts ion passage through occludins and its oligomerisation with lipid rafts required for tight junction formation and BBB function[322]. Certain areas of the brain such as the thalamus, basal ganglia, frontoparietal and occipital regions have been shown to be more susceptible to reduced CBF in hypertension[124, 323]. Thus, functional and cognitive impairment associated with these areas may arise from the disruption of structures involved in BBB formation. The BBB has been shown to not be as susceptible to damage in acute hypertension as it is during chronic hypertension and has protective mechanisms such as sympathetic tone to facilitate changes in CBF[324, 325]. However, in chronic hypertension the BBB is not able to sustain neurogenic tone, thus resulting in vascular alterations such as vascular hypertrophy[324, 326].

Overall, our findings indicate that there was a greater disruption of the BBB after stroke which was unaffected by hypertensive status. Hypertension alone may have greater long-term implications for BBB disruption that may result in a greater influx of large molecules and aberrant agents that can potentially cause further damage to the brain.

4.6 Morphometry of Reactive Astrocytes in the Prefrontal Cortex

Glial fibrillary acid protein (GFAP) is an astrocyte marker used to determine glial activation associated with glial scar formation[327]. Observations of GFAP immunoreactivity show elongated processes, indicative of astrocyte activation, surrounding the peri-infarct area. The glial scar has been suggested to act as a barrier to slow the expansion of the infarct core, which may be the initiation site for neurorepair but may also minimise the amount of salvageable tissue[176]. Recently, it has been shown that there are subtypes of reactive astrocytes, namely pro-inflammatory A1 astrocytes and anti-inflammatory A2 astrocytes[328]. Liddelow *et al* showed an abundance of A1 astrocytes in neurodegenerative diseases and a role for them in promoting neuronal death[109]. In contrast, A2 astrocytes are thought to be able to promote the survivability of neurons, support the formation of synapses and contribute to neural plasticity[329]. Astrocytes are crucial for the recovery and function of neurons after stroke[330], as they influence glutamate uptake which is important in the survival process during ischaemia[331]. However, hypertension can inhibit glutamate transporter function of astrocytes[332]. Thus, hypertension may influence the reactive astrocytic subtypes found in stroke. Additional studies are needed to determine the relative abundance of A1 versus A2 astrocytes in ischaemic stroke and/or hypertension. In this study, mice subjected to stroke had increased GFAP immunoreactivity and hypertensive status did not influence this. Our findings may suggest that astrocytes are activated in response to stroke and not hypertension early in this time course.

4.7 Cognition, Neural Plasticity and the Induction of Hippocampal Long-Term Potentiation

Learning, memory and neural plasticity are involved in the remodelling process of neurons and glial cells in response to environmental changes. Under physiological conditions, there is a continuous and dynamic alteration to brain parenchyma that is coordinated and regulated by immune mechanisms[333]. By contrast, in injury or disease, there is an imbalance of pro-inflammatory and anti-inflammatory mediators which disrupts neurophysiological processes which is detrimental to neural plasticity and neurogenesis resulting in memory deficits[334]. Neural plasticity after stroke is the concept in which adjacent brain structures can functionally adapt to the initial injury-induced impairment in the following days and weeks[335, 336]. During the remodelling process, neurons undergo dynamic selective degeneration which leads to morphological and functional adaptations in the brain facilitated by the immune system and initiated by neuronal activity[337].

T cells and B cells have been implicated in memory consolidation and their deficiency may impair hippocampal spatial learning[338-340]. Additionally, $CD4^+$ T cells have been reported to be neuroprotective and have a supportive role in cognition[341]. Cytokines have also been implicated in memory formation. For example, IL-1 is important for the consolidation of memory in the hippocampus[342], whereas IL-6 and TNF-α are reported to be detrimental to memory processes and synaptic plasticity which contributes to cognitive decline[343, 344]. However, IL-6 and TNF-α have also been reported to be beneficial during recovery when they may exert a protective role[345, 346]. Microglial activation induces downstream inflammatory mediators such as IL-1β, TNF-α, IL-6 and COX-2 and this plays a physiological role in modulation of brain plasticity[344]. Microglia also play a critical role in neuronal death and clearance of apoptotic neurons and trigger the activation of astrocytes. Astrocytes may also respond to IL-1 and secrete TNF-α and IL-6, phagocytose cellular processes and debris[347]. Astrocytes also regulate secretions of gliotransmitters to modulate neuronal excitability and synaptic strength, and astrocytic GFAP has been implicated in long term potentiation (LTP) regulation[348]. Thus, alterations to astrocyte function leads to learning, memory and LTP inhibition which has been demonstrated to affect neurobehavioural plasticity[349, 350]. Suppression of inflammatory mediators is shown to impair neuronal plasticity[338-340], suggesting that a balance between anti-inflammatory and pro-inflammatory mediators is required for the proper learning and memory function.

Long term potentiation (LTP) is believed to be involved in the hippocampus and other cortical structures, and has been implicated in the consolidation of short- and long-term memory where synaptic plasticity lay the foundations for this mechanism. Spatial working memory is the ability to integrate diverse forms of information involving spatial properties in a short period of time[351], and reference memory relates to the long-term consolidation and recall of information. The prefrontal cortex has been shown to control higher cognitive functions including spatial memory and has close links with the limbic system and its neural structures such as the hippocampus[352]. The medial temporal lobe has also been shown to be involved in spatial memory[351, 353, 354]. In contrast to localised strokes, hypertension has a global effect on cerebrovascular functions and inhibit LTP induction which could explain the impairment in spatial working memory of our hypertensive mice performing the OLT.

The connection between the hippocampus and the prefrontal cortex is especially important for the encoding of spatial memory and learning, and the disruption of this region of the brain may lead to memory encoding, retention and recall deficits[355]. Within regions of the hippocampus, a relay circuit known as the trisynaptic circuit, involves the entorhinal cortex (EC), the granule cells of the dentate gyrus (DG), pyramidal cells of the cornu ammonis 3 (CA3) and cornu ammonis 1 (CA1)[356]. Communication between the neocortex and the hippocampus is initiated by the EC and transmits information to DG, CA3 and CA1 which are subfields of the hippocampus proper. There have been indications that CA1 processes spatial and nonspatial information and acts as novelty detector[357-359]. Forward feeding information from the EC to the DG and CA3 is suggested to be involved in the consolidation of short-term memory into long-term[359, 360]. An implication of disrupting the trisynaptic circuit of the hippocampus is the inhibition of LTP, thus impairing memory storage and learning capabilities[361].

A previous study showed angiotensin II to completely inhibit hippocampal LTP induction[362]. Further, ACE inhibition has been associated with reduced rates of cognitive decline in dementia patients, consistent with the possibility that angiotensin II contributes to the development of cognitive impairment[363]. Chen *et al* showed that transient global cerebral ischaemia (tGCI) followed by treatment with melatonin improved cognitive impairment via the restoration of myelin, thereby increasing glutaminergic transmission within the ischaemic regions of the brain[364]. Although we did not investigate neuronal death in the hippocampus in this study, Chen *et al* saw reduced numbers of neurons in the stratum pyramidale located in CA1, which disrupt LTP induction and contributed to spatial learning impairment seen in his study.

4.8 Future directions

While hypertension is the greatest risk factor for stroke incidence, it is not entirely clear why this is the case and what the potential mechanisms that underly worsened outcomes. Thus, future studies should determine the subtype of astrocytes within the peri-infarct, investigate the inflammatory profile in regions associated with memory and learning, assess neuronal function within the trisynaptic circuit of the hippocampus and elucidate the function of IgG in these pathologies. Further histological and immunological investigations within the hippocampus may provide greater insight as to how hypertension and stroke, alone and in combination, may affect cognitive outcomes.

4.9 Implications of the Current Study

In this study, we show that the combination of hypertension and stroke worsened learning abilities, whereas either condition alone resulted in either mild impairment or normal behaviour. Clinically, this suggests a need to aggressively treat hypertension in stroke patients. This may result in slowing the progression of cognitive impairment to dementia. Stroke is typically considered as a disease of the elderly (> 65 years)[365]. However, reports suggest that younger individuals are also susceptible[366]. Although mortality is lower in younger people, stroke still has long-term impacts on the quality of life, family, society and the economy[367]. The repercussions of stroke in younger victims cannot be overlooked as they face a lifetime of recovery and loss of valuable years, during which they may develop hypertension which further impacts their quality of life. Therefore, treating hypertension in stroke patients is critically important in preventing worsened cognitive outcomes. However, it is currently unclear whether this strategy is also applicable to older individuals. Our study was performed in young animals and it remains to be determined whether similar protective effects are present in older animals. Clinically, lowering blood pressure has been shown to be detrimental or even fatal in older patients greater than 80 years old[368]. This may suggest that there is an age where anti-hypertensive therapy in stroke patients changes from being protective to detrimental. Thus, alternative strategies may be required for these older patients. From our data, the vulnerabilities of the BBB in hypertension and stroke suggest that the BBB may be an important pharmacological target. By preserving BBB integrity in ischaemic stroke, further damage to brain parenchyma may be prevented and occurrences of haemorrhagic transformations may reduce.

Conclusions

The goal of the current study was to determine the effects of hypertension and stroke on cognitive outcomes, and secondly to assess the impact of the combination of hypertension and stroke on cognitive impairment and brain injury. This study has shown that hypertension and stroke alone can impair spatial working memory. Additionally, learning impairment was augmented by the combination of hypertension and stroke. Surprisingly, the combination of hypertension and stroke resulted in an increase in spontaneous haemorrhage, indicating substantially worsened brain injury, consistent with hypertension contributing to the development of spontaneous post-stroke haemorrhages. The present study highlights the importance of hypertension and its contributions to the development of cognitive impairment and brain pathology, especially in the setting of ischaemic stroke. Therefore, if transferable to humans, the present data suggest that therapeutic approaches to effectively treat hypertension and reduce the risk of stroke are likely to be beneficial for limiting cognitive impairment, a reduced quality of life and the development of vascular dementia.

Appendices

Appendix i. Minipump Post-Operative Monitoring Sheets

ANIMAL MONITORING SHEET – Post-surgery

AEC Project No._____ Investigator _____
Animal ID No._____ Species /Strain/ _____
Animal Details (sex, age, etc.)
_____
Surgery type (circle one) - sham ovariectomy minimpump

- Normal clinical signs are recorded as "N"
- Abnormalities are recorded as "A" and severity is scored in brackets eg Breathing: A (3)
- Comments concerning abnormalities are recorded in the comments section of the table

CLINICAL OBSERVATION (N or A)	DATE/TIME						
UNDISTURBED							
Coat							
Activity/Mobility							
Breathing							
Movement/gait							
Eating							
Drinking							
Alert/Sleeping							
ON HANDLING							
Alert							
Body condition							
Bodyweight (g)							
Body temperature (°C)							
Dehydration							
Eyes							
Faeces							
Nose							
Breathing							
Urine							
Vocalisation							
Incision site							
CARPROFEN GIVEN (5 mg/kg s.c.)							
COMMENTS							
INITIALS:							

_____ _____
Signature of Chief Investigator **Date**

CLINICAL SIGNS SEVERITY SCORE

SIGNS	0	1	2	3
Activity/Mobility	normal	isolated, abnormal posture	huddled/inactive OR overactive	moribund OR fitting
Alertness/Sleeping	normal	dull or depressed	little response to handling	unconscious
Body condition	normal	thin	loss of body fat, failure to grow	loss of muscle mass
Body weight	normal weight and growth rate	reduced growth rate	acute weight loss 10% OR chronic weight loss 15%	acute weight loss >10% OR chronic weight loss >15% OR failure to grow
Breathing	normal	rapid, shallow	rapid, abdominal breathing	laboured, irregular, skin blue
Coat	normal	ruffled fur	unkempt; wounds, hair thinning	bleeding or infected wounds, or severe hairloss or self mutilation
Dehydration	none	skin less elastic	skin tenting	skin tenting & eyes sunken
Drinking	normal	increased OR decreased intake over 24 hrs	increased OR decreased intake over 48 hours	constantly drinking OR not drinking over 24 hours
Eating	normal	increased OR decreased intake over 24 hours	increased OR decreased intake over 48 hours	obese OR inappetence over 48 hours
Eyes	normal	wetness or dullness	discharge	eyelids matted
Faeces	normal	Faeces moist	loose, soiled perineum OR abnormally dry +/- mucus	running out on handling OR no faeces for 48 hrs OR frank blood on faeces
Movement/ gait	normal	slight incoordination OR abnormal gait	hunched, incoordinated OR walking on tiptoe OR reluctance to move	staggering OR limb dragging OR paralysis
Nose	normal	wetness	discharge	coagulated
Urine	normal		abnormal color/volume	no urine 24 hrs OR incontinent, soiled perineum
Vocalisation	normal	squeaks when palpated	struggles and squeaks loudly when handled/palpated	abnormal vocalisation
Incision site	Normal	Redness/irritated/ open		Infected or bleeding

EUTHANASIA/HUMANE EXPERIMENTAL ENDPOINT CRITERIA**

CLINICAL SIGN	ACTION
Score of 0	**Monitor daily for the first 3 days and then every 2-3 days**
Any score of 1	**Monitor twice daily**
Once mouse reaches score of 2 or more	**Euthanise**

** as approved by the AEC, relevant to each specific situation

SCIENTIFIC MEASURES (ie, data or tissues to be collected as part of the experimental use)
(eg animals that are killed should be weighed and have their bodies placed in labelled bags and refrigerated)

Appendix ii: Photothrombotic Stroke Post-Operative Monitoring Sheets

ANIMAL MONITORING SHEET FOR EXPERIMENTAL STROKE IN MICE

AEC Project No.		**Investigator**	
Animal ID No.		**Species/Strain**	
Animal Sex/Age		**Surgery type**	
Surgery date/time		**Cull date/time**	

Date																
Time post-surgery	1h	2h	3h													
	CLINICAL SCORING (0, 1 or 2)															
Clinical Sign - *see scoring chart*																
A. Activity (circling)																
B. Activity (rolling)																
C. Appearance																
D. Body weight score																
E. Body temperature																
F. Breathing																
G. Wound																
H. Feces/urine																
I. Vocalisation																
Any 2 = Euthanase (√):																
Any 1 = Monitor twice daily (√):																
Score of 0 = Monitor daily for the first 5 days post-surgery and then every 2-3 days (√):																
Comments on clinical signs:																
Bodyweight (g)																
Saline given s.c. daily (√)																

SCORING KEY FOR FURTHER ACTION

TOTAL CLINICAL SCORE	ACTION
Score of 0	Monitor daily for the first 7 days and then every 2-3 days
Any score of 1	Monitor twice daily
Any score of 2	Immediately euthanase

SPECIFIC REQUIREMENTS FOR POST-STROKE MONITORING

- Hourly monitoring of all mice for first 3 hours after surgery
- If signs are present within 2 hours of scheduled experimental endpoint, animal will be monitored every 30 minutes
- Provide crushed/crumbed food to the box
- Provide wet mashed food or gel in a dish in the box
- Weigh mice daily
- Monitoring sheet to be kept in the vicinity of the mouse
- After MCAO surgery, keep mice on heat pad until the end of experiment
- Give 1 mL subcutaneous saline once daily AFTER monitoring and scoring

Signature of Chief Investigator **Date**

CLINICAL SIGNS SEVERITY SCORING CHART FOR EXPERIMENTAL STROKE IN MICE

CLINICAL SIGNS	0	1	2
A. Activity (circling)	Normal healthy movement	Not moving spontaneously but moves when lifted by tail OR circling within 2 hours prior to scheduled experimental endpoint	Not moving when lifted by tail OR circling at more than 2 hours prior to scheduled experimental endpoint
B. Activity (rolling)	Normal healthy movement		Rolling at any time OR no righting reflex
C. Appearance	Normal healthy	Hunched	Shaking/Unconsious
D. Body weight	Normal weight	Acute weight loss <10%	Acute weight loss >10% OR Chronic weight loss >15%
E. Body temperature	Feels warm		Feels cold
F. Breathing	Normal	Slow OR Irregular	Gasping OR Laboured
G. Wound	Normal	Redness/irritated OR open	Bleeding OR Infected wounds
H. Feces/urine	Evidence in box	No feces or urine at 9 am on day after surgery	No feces or urine by 9am 2 days after surgery
I. Vocalisation	None	Quiet/low level OR nattering	Vocalisation

SPECIFIC REQUIREMENTS FOR POST-STROKE MONITORING

- Hourly monitoring of all mice for first 3 hours after surgery
- If signs are present within 2 hours of scheduled experimental endpoint, animal will be monitored every 30 minutes
- Provide crushed/crumbed food to the box
- Provide wet mashed food or gel in a dish in the box
- Weigh mice daily
- Monitoring sheet to be kept in the vicinity of the mouse
- After MCAO surgery, keep mice on heat pad until the end of experiment
- Give 1 mL subcutaneous saline once daily

CRITERIA FOR CLOSE MONITORING OR IMMEDIATE EUTHANASIA

CLINICAL SIGN	ACTION
Score of 0	Monitor daily for the first 7 days and then every 2-3 days
Any score of 1	Monitor twice daily
Any score of 2	Immediately euthanase

Appendix iii. Post-Anaesthesia Monitoring Sheet

Post-anaesthesia recovery monitoring sheet

Animal ID:		AEC Approval:				Investigator:	
Procedure type:		Time procedure ended:				Date:	
Time and Initials	Demeanour depressed/ reduced activity levels	Posture hunched	Piloerection present	Increased respiratory effort present	Eyes closing or closed	Total Score	Intervention/other observations

Approved variation to standard post anaesthesia protocol? If yes, give details:

_____

Score 1 in each column signs are present, enter the sum of these scores in "total" column. If no abnormalities are detectable and subject is completely normal then "0" should be noted in the total score column. If you need to consult with LARTF staff, speak to the person in your area or phone LARTF reception on ext. 2732. Out of Hours assistance from LARTF staff can be obtained by phoning ext. 3986.

If you need to consult with LARTF staff, speak to the person in your area or phone LARTF reception on ext. 2732. **Out of Hours assistance can be obtained by phoning ext. 3986.**

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