MECHANISMS OF COGNITIVE IMPAIRMENT DEVELOPMENT IN AGED HYPERTENSIVE RATS: FOCUS ON MACROPHAGES

by

ABDULKARIM ALSHAMMARI

(Under the Direction of SUSAN C. FAGAN and DUO ZHANG)

ABSTRACT

Stroke survivors have an increased risk of developing long-term disability and dementia. Hypertension and aging are major contributing factors to vascular dementia, stroke, and the development of PSCI. Most animal models fail to capture the complex interplay between these pathophysiologic processes. The purpose of this dissertation is to investigate the impact of aging and hypertension on cognitive function, prior to, and following stroke. We utilized aged hypertensive rats to study the trajectory of vascular cognitive impairment, and to investigate the impact of delayed and chronic stimulation of Angiotensin II type 2 receptor on stroke outcomes. Sixty SHRs were housed (in pairs) for 18 months with cognitive assessments every six months and post-surgery. Multiple MRI scans were performed at baseline and throughout the study. At day 3 after stroke, rats were randomized to receive either an angiotensin receptor agonist, Compound 21, or plain drinking water and followed up for 8 weeks. Additionally, we examined the ability of C21 to mediate anti-inflammatory and neurotrophic effects in mouse microglial cell line, C8-B4, and RAW 264.7 macrophages.

INDEX WORDS: Stroke, Hypertension, Vascular cognitive impairment, Neuroinflammation, AT2R, Compound 21

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DEDICATION

This dissertation is dedicated to the loving memory of my father, who passed away before the completion of this work.

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LIST OF ABBERVIATIONS

ATR2: Angiotensin II Type 2 Receptor; ***BBB:*** Blood–Brain Barrier; ***BDNF:*** Brain-Derived Neurotrophic Factor; ***BMDM:*** Bone-Marrow-Derived Macrophage; ***cAMP:*** Cyclic Adenosine Monophosphate; ***CAMS:*** Cell adhesion molecules; ***cDNA:*** Complementary DNA; ***Cox2:*** Cyclooxygenase-2; ***DAMPS:*** Damage-Associated Molecular Patterns; ***DI:*** Discrimination Index; ***dMCAO:*** Distal Middle Cerebral Artery Occlusion; ***DMSO:*** Dimethyl Sulfoxide; ***DWI:*** Diffusion-Weighted Imaging; ***EMCAO:*** Embolic Middle Cerebral Artery Occlusion; ***ENOS:*** Endothelial Nitric Oxide Synthase; ***FLAIR:*** Fluid-Attenuated Inversion Recovery ; ***GFAP:*** Glial Fibrillary Acidic Protein; ***GDNF:*** Glial Cell-Derived Neurotrophic Factor; ***GK RATS***: Goto-Kakizaki Rats; ***HBECS:*** Primary Human Bronchial Epithelial Cells; ***HFD:*** High Fat Diet; ***HIF1α:*** Hypoxia-Inducible Factor 1-Alpha; ***I.P.:*** Intraperitoneal; ***I.V.:*** Intravenous; ***IACUC:*** Institutional Animal Care And Use Committee; ***IBA1:*** Ionized Calcium-Binding Adapter Molecule 1; ***ICV:*** Intracerebroventricular; ***iNOS:*** Inducible Nitric Oxide Synthase; ***K/O:*** Knockout; ***LPS:*** Lipopolysaccharide; ***MAP***: Mean Arterial Pressure; ***MCAO***: Middle Cerebral Artery Occlusion; ***MDM***: Monocyte-Derived Macrophages; ***MMP***: Matrix Metalloproteinase; ***MMP***-2: Matrix Metalloproteinase 2; ***MMP***-3: Matrix Metalloproteinase 3; ***MMP***-9: Matrix Metalloproteinase 9; ***MNSS***: Modified Neurological Severity Scores; ***MPGES***: Microsomal Prostaglandin E Synthase-1; ***MRI***: Magnetic Resonance Imaging; ***MTT***: Mean Transit Time; ***MWM***: Morris Water Maze; ***NO***: Nitric Oxide; ***NOR***: Novel Object Recognition Test; ***OD***: Optical Density; ***OGD***: Oxygen

Glucose Deprivation; ***OVX***: Ovariectomized; ***PAT***: Passive Avoidance Task; ***PGE2***: Prostaglandin E2 ; ***PI***: Preference Index; ***pMCAO***: Permanent Middle Cerebral Artery Occlusion; ***PSCI***: Poststroke Cognitive Impairment; ***RAS***: Renin Angiotensin System; ***ROS***: Reactive Oxygen Species; ***shRNA***: Short Hairpin RNA; ***SHRs***: Spontaneously Hypertensive Rats; ***SIRPA***: Signal Regulatory Protein α; ***STZ***: Streptozotocin; ***T2WI***: T2 Weighted Image; ***tMCAO***: Transient Middle Cerebral Artery Occlusion; ***tPA***: Tissue Plasminogen Activator; ***Trkb***: Tropomyosin Receptor Kinase B; ***TTC***: Triphenyl-tetrazolium chloride; ***UCCAO***: Unilateral Common Carotid Artery Occlusion; ***VCI***: Vascular Cognitive Impairment; ***VEGF***: Vascular Endothelial Growth Factor; ***VEGF A:*** Vascular Endothelial Growth Factor A; ***VEGF B:*** Vascular Endothelial Growth Factor B.

CHAPTER 1

Literature Review

1. Stroke and Post-stroke/ vascular cognitive impairment

1.1. *Incidence, epidemiology, and mortality:*

Stroke is the fifth leading cause of death in the United States, indicated in ≈1 of every 19 deaths.[1] The high incidence of stroke, coupled with improvements in stroke care, has led to a recent decrease in stroke mortality and an increase in stroke-induced disability.[2, 3] Indeed, stroke was among the top 18 diseases contributing to years lived with disability in 2010.[4] Stroke survivors have an increased risk of developing a long-term disability, including sensorimotor and cognitive deficits.[5]

Cognitive impairment frequently affects stroke survivors and is commonly referred to as post-stroke cognitive impairment (PSCI) under the umbrella pathology of vascular cognitive impairment. Generally, PSCI is defined as cognitive impairment persisting three to six months after stroke, but strict diagnostic criteria are lacking. PSCI causes extensive reductions in quality of life and increases the burden of care, with many patients progressing to dementia.[6] Globally, based on the disparities in races, countries, and diagnostic criteria, the prevalence of post-stroke cognitive impairment ranges between 20% and 80% of stroke victims.[5] A separate analysis of the original Framingham cohort has shown that 19.3 % of stroke patients in the United States developed dementia ten years after stroke.[7] Furthermore, stroke doubles the risk of dementia and up to one-third of stroke survivors subsequently develop dementia.[8] Stroke causes an abrupt and accelerated long-

term cognitive decline. Unlike sensorimotor deficits after stroke, which peak shortly after stroke incidents and gradually resolve thereafter, the development of post-stroke cognitive impairment follows a progressive pattern.[9] Patients experience acute decreases in global perception, cognition, learning capability, and verbal memory, as well as accelerated and persistent cognitive decline over the years after stroke.[9] Along with stroke, the presence of other pathophysiological processes, such as advanced age, hypertension, and diabetes, are likely to worsen the post-stroke recovery and accelerate the progression of post-stroke cognitive impairment. [10-12]

1.2. Advanced Age and Hypertension as Important Contributors to Stroke and VCI/PSCI:

Hypertension has emerged as one of the most important contributors to stroke and vascular dementia.[13, 14] Both clinical studies, as well as animal models, revealed an increased risk of cognitive impairment associated with hypertension, independently and by increasing the risk of stroke.[15, 16] In fact, high blood pressure has been linked to accelerated cognitive decline in individuals, and a higher risk of dementia.[17] A prospective cohort of 796 patients with acute ischemic stroke showed that abnormal blood pressure, either elevated or lowered, is associated with a higher risk of PSCI three months after stroke.[18] Furthermore, the evidence supports the notion that mid-life hypertension is strongly linked to late-life vascular dementia, cognitive decline, and Alzheimer's disease.[19, 20] Additionally, the use of antihypertensive treatment was associated with reducing the risk of dementia, with greater protective effects associated with younger age and a longer duration of treatment.[19, 21]

An experimental model of chemically induced hypertension investigated the association between mid-life hypertension and the development of late-life Alzheimer's disease. Chronic hypertension substantially accelerated the development of cognitive decline, vascular inflammation, BBB leakage, as well as hippocampal neurodegeneration.[22] Several mechanisms have been studied to investigate the linkage between hypertension and cognitive impairment. Gorelick et al. elaborated on some of these potential mechanisms in their review.[23] It could be due to either functional, such as vascular endothelial dysfunction, impaired hyperemic response, and a reduction in cerebral amyloid clearance, or structural dysfunction, like the existence of white matter disease or an elevated number of neuritic plaques.[23] Functional hyperemia is crucial to match the cerebral blood flow delivery that is required to maintain brain homeostasis and clear metabolic waste.[23-25] However, support exists for the involvement of a chronic reduction in cerebral blood flow, which in turn leads to white matter injury, lacunar infarct, brain atrophy, as well as memory impairment in rodents.[25, 26] Additionally, another mechanism in which elevated blood pressure induces cognitive impairment is cerebral microvascular endothelial dysfunction. Both vascular cognitive impairment dementia and Alzheimer's disease, share a decrease in endothelial function and barrier integrity as a common feature.[27, 28] Thus, impairment of the cerebral vasculature, particularly endothelial dysfunction, could trigger the development of vascular cognitive impairment.[27, 28] Olivia de Montgolfier et el.[28] demonstrated that elevated systolic blood pressure was associated with brain inflammation as well as memory deficits, and subsequently may contribute to vascular cognitive impairment, dementia, and Alzheimer's disease via its effect on cerebral microvascular endothelial function.[28]

In addition to hypertension, advanced age is a major non-modifiable risk factor that contributes to stroke and further accelerates the development of post-stroke/vascular cognitive impairment.[29-31] Post-stroke cognitive impairment develops progressively after stroke and is substantially associated with age.[29] Abdel Douiri et el.[29], in a population-based study, looked at the development of post-stroke cognitive impairment between 1995 – 2010 (4212 patients were included) and observed that PSCI worsens at a rate of 2% each year following a stroke. The trajectory of cognitive decline after stroke is proportionally increased with age and cardioembolic stroke.[31]

Moreover, another clinical study demonstrated that aging, hypertension, and diabetes were significantly associated with initial MRI findings after stroke (as a percentage of initial DWI/MTT mismatch volume), suggesting that advanced age increases the conversion of ischemic tissues into infarction.[32] Aging also affects synaptic plasticity, as well as causes structural changes in the brain. For instance, advanced age was found to dysregulate astrocyte and microvascular endothelial function, leading to a toxic cellular environment and neuronal loss. Astrocyte dysfunction associated with aging was associated with a loss of ability to differentiate neural progenitor cells into neurons.[33, 34] Lastly, aging is associated with a sustained proinflammatory response that is characterized by excessive production of proinflammatory mediators and less phagocytic activity, worsening the neuroinflammatory response and exacerbating the ischemic injury after stroke.[35-37] Experimental models revealed a significant association between aging and declined hippocampal-related functions.[38-42] In rodents, aging seems to be associated with impaired cognitive function, particularly hippocampal-dependent tasks.[38-42] Hippocampal damage is known to be associated with impaired spatial learning and memory.[43] Aged rodents

exhibited poor performance in tests assessing spatial working memory, such as the Morris water maze, object recognition memory test, and radial maze. [38-42, 44] However, most preclinical studies failed to include advanced age and/or other comorbidities such as diabetes and hypertension. Recently, McCann et el.[45] examined the presence of advanced age or other comorbidities in the experimental stroke literature, as these factors are likely to contribute to the lack of translation and failure of preclinical studies. Indeed, only 11.4% of studies considered aging or other comorbidities, with hypertension being the most studied factor.[45] It was suggested that aging and/or other comorbidities have been avoided due to high mortality, increased cost, as well as the time required to conduct aging experiments.[35]

Collectively, hypertension and aging are leading risk factors for stroke and cognitive decline. Yet, experimental models of stroke have failed to investigate the complex interaction between these two synergistically detrimental pathophysiologic processes, contributing to the lack of translation of promising treatments to clinical use.

1.3. *Available treatments for VCI/ PSCI*

Currently, there are no specific treatments for VCI/ PSCI that have been approved by the FDA. Nonetheless, hypertension is the most common modifiable risk factor for stroke as well as for vascular dementia.[46] Thus, in VCI guidelines, treatment of hypertension is classified as the highest evidence-based grade and is recommended for all those at risk for vascular cognitive impairment (Class I; Level of Evidence A).[19]

2. Inflammatory Response After Stroke:

2.1. General Overview:

Over the past two decades, our knowledge of stroke pathophysiology has dramatically expanded, specifically to include the role of the immune system combined with a more advanced understanding of the neurovascular unit. The injury after ischemic stroke is mediated through a dynamic interaction between all the components of the neurovascular unit, which includes neuronal, glial, and vascular cells.[47] Following the ischemic injury, the activation of glial cells leads to the release of cytokines, chemokines, and infiltration of peripheral leukocytes. Eventually, this furthers the inflammatory response and exacerbates the injury.[48]

Neuroinflammation is well known to be involved in secondary injury following ischemic stroke and results in long-term consequences, including PSCI. Following ischemic injury, damaged or dying neurons will release endogenous molecules known as Damage-associated molecular patterns (DAMPs).[49] DAMPs release triggers the activation of glial cells, astrocytes, and microglia, leading to changes in their morphology and function. Once activated, glial cells secrete various pro-inflammatory cytokines and chemokines. These cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α), exacerbate neuroinflammation. Furthermore, the release of pro-inflammatory cytokines will compromise the blood-brain barrier and lead to the infiltration of peripheral leukocytes. This infiltration leads to further damage in the subacute phase and contributes to secondary damage.[50, 51]

2.2. Roles of Innate immune cells after stroke:

Microglia, the resident innate immune cells, are key players in the inflammatory response in the central nervous system after stroke.[52] Typically, in the brain, the primary role of resident microglia is to survey and maintain homeostasis.[53] In response to ischemic injury, microglia activate continually throughout the course of inflammatory response. Shortly after stroke, microglia are expected to adopt the anti-inflammatory or M2 phenotype. This healthier and anti-inflammatory phenotype results in enhanced phagocytosis, fewer inflammatory mediators, and improved neuronal survival. However, the M2 state is transient and the pro-inflammatory or M1 phenotype begins to dominate the injured area. The M1 microglia are characterized by less phagocytosis and higher production of pro-inflammatory cytokines.[54] The secretion of these pro-inflammatory cytokines initiates the inflammatory response, disrupts the blood-brain barrier, degrades the extracellular matrix, and triggers the infiltration of peripheral leukocytes into the central nervous system.[55] Altogether, these cellular processes will exacerbate the inflammatory response and worsen the injury.

Nevertheless, during the delayed phase of inflammation after stroke, microglia switch back to the M2 anti-inflammatory phenotype and facilitate functional recovery. The anti-inflammatory microglia have favorable effects on promoting blood-brain barrier repair, neurogenesis, and angiogenesis. These neuroprotective and anti-inflammatory effects are mediated via the release of different anti-inflammatory cytokines, such as IL-10, transforming growth factor (TGF)-β, IL-4, and IL-13.[55] The M1 and M2 phenotypic switching has been criticized as being an oversimplification of an exceedingly complex process. [56-59] However, modulation of the microglial inflammatory response after stroke

toward an anti-inflammatory phenotype has emerged as one of the potential targets to reduce the long-term complications of stroke.

2.3. Roles of Monocyte-derived macrophages after stroke:

As described earlier, after stroke, microglia are expected to adopt the anti-inflammatory phenotype, transiently, and thereafter a pro-inflammatory phenotype begins to dominate the injured area leading to the infiltration of peripheral leukocytes into the central nervous system.[55]

The recruitment of peripheral immune cells, including monocyte-derived macrophages, plays a pivotal role in the repair process after CNS injury.[60, 61] Monocyte-derived macrophages are classified into two major sub-population, the proinflammatory ($Ly6C^{high}$) and anti-inflammatory ($Ly6C^{low}$) monocytes.[62] Originally, CCR2/ $Ly6C^{high}$ monocytes were thought to mediate detrimental effects after stroke[56], but recent reports revealed that CCR2/$Ly6C^{hi}$ monocytes promote protective effects after stroke, likely through enhancing the anti-inflammatory effect.[57, 58, 63] Peripheral immune cells infiltrate the injured brain when activated microglia and astrocyte-released inflammatory mediators disrupt the integrity of the BBB and activate endothelial cells.[55, 64, 65] Subsequently, activated endothelial cells induce a rapid secretion of selectins, to facilitate the initial attachment and rolling of leukocytes, and interact with leukocyte integrins through cellular adhesion molecules (CAMs) expressed on endothelial cells, to ensure firm leukocyte adhesion.[66, 67] After stroke, monocytes significantly infiltrate the ipsilateral hemisphere within the first 24 hours, peaking at 3 days.[58, 68] Then, the number of recruited monocytes starts to rapidly decrease throughout the inflammatory response until it reaches the control level at day 14.[58] The inflammatory response mediated through monocyte-derived macrophages is

characterized by dynamic changes in the expression of anti- and pro-inflammatory mediators.[57, 58] As early as day 1 after stroke, the percentage of pro-inflammatory monocytes ($Ly6C^{high}$) was higher in the ischemic brain and it reached its peak at day 3.[58] But then, gradually decreased to around 18% of the monocyte population at day 21.[58] Quite the opposite, the anti-inflammatory monocyte ($Ly6C^{low}$) increased at day 7 and remained elevated throughout 21 days after stroke.[58] Yet, the deletion of $CCR2^{+}$ monocytes resulted in a delayed inflammatory rebound at 15 days after stroke and was associated with worse behavioral outcomes and neurological impairment, indicating a critical role of both subsets of infiltrating monocytes in the recovery after stroke and suggesting that a certain degree of acute inflammatory response might be necessary to promote post-stroke recovery.[57-59] On the whole, these findings reveal a crucial role of monocyte-derived macrophages to promote functional recovery after stroke, mediating an early pro-inflammatory response on day 3 and enhancing an anti-inflammatory response afterward.

2.4. Direct Macrophages to Microglia communication:

As mentioned, neuroinflammation is well known to be involved in secondary injury following ischemic stroke and results in long-term consequences. The post-stroke neuroinflammatory response is mainly mediated through the activation of innate immune cells, microglia, and infiltration of circulating immune cells, including monocytes.[55] Microglia and monocyte-derived macrophages share the same inflammatory response pattern and function. Both mediate an early pro-inflammatory response and release inflammatory cytokines and chemokines to cause further damage and exacerbate the ischemic injury during the acute phase of stroke. Likewise, both cells modulate their phenotypic activation to shift the polarization status to facilitate an anti-inflammatory

response and promote recovery after stroke.[52, 55, 57, 58, 60] Several molecular pathways are involved in modulating the polarization status of microglia/macrophages to either phenotype. For instance, AMP-activated protein kinase (AMPK) is believed to counter-regulate the inflammatory response in macrophages and shift its polarization status toward an anti-inflammatory phenotype.[69] Moreover, downregulation of the NOTCH signaling pathway mediates a pro-inflammatory response and increases pro-inflammatory markers, such as TNF-α, IL-1β, and IL-6, while the activation of the NOTCH signaling pathway decreases the transcription of pro-inflammatory mediators.[70] Several drugs, such as metformin and simvastatin, mediate their action through these molecular pathways. These drugs have been associated with a shift in the polarization of microglia/macrophages toward an anti-inflammatory phenotype and eventually improved functional recovery after stroke.[71-73]

In addition to NOTCH and AMPK signaling pathways, the prostaglandin E receptor is believed to be involved in modulating microglial activation, likely through the production of Camp.[74] Despite the similarities in the function and behavior of microglia and macrophages in the brain, few studies have looked at the direct interaction between them. A recent study revealed that macrophages directly downregulate the expression of pro-inflammatory genes in both mouse and human microglia. Treating BMDMs with LPS and coculturing with microglial cells suppressed the expression of inflammatory genes, including IL-1β, TNF-α, and IL-10.[75] Gene analysis of LPS-activated microglia in the presence of macrophages revealed that around 1076 genes were significantly differentially regulated, mostly those related to the NF-κB signaling pathway and apoptotic cell death. Interestingly, LPS-activated macrophages showed a greater prostaglandin E2 production

in the presence of microglia, suggesting that the prostaglandin E2 signaling pathway might be involved in macrophage-microglia communication. [75] The involvement of the prostaglandin E2 signaling pathway in mediating macrophage-to-microglia communication was also confirmed using a selective EP2 antagonist, PF-04418948, and mPGES-depleted macrophages ($mPGES^{-/-}$).[75] The absence of mPGES, a regulator of PGE2 production and release, in macrophages affected its ability to suppress microglial phagocytosis.[75] These data suggest a critical role of prostaglandin E2 produced by monocyte-derived macrophages in modulating microglia function via prostaglandin E2 receptor.[75]

2.5. Monocyte Phagocytic activity:

Monocytes and macrophages perform various functions throughout stroke recovery. As early as 72 hours after ischemia, recruited monocytes display the most phagocytic activity of any brain phagocytes, with significantly more phagocytosis than microglia, supporting a role in necrotic debris clearance. The phagocytic activity of recruited monocytes is likely to be mediated through the CD47-signal-regulatory protein alpha (SIRPα) pathway.[76] SIRPα is a cell surface receptor-like signaling protein that is expressed by myeloid leukocytes, including monocytes. The interaction between SIRPα, on the surface of macrophages, with CD47 on the encountered cells promotes phagocytosis.[77] Additionally, a recent study revealed that the phagocytic activity of bone marrow-derived macrophages (BMDMs) was enhanced in the presence of microglia. On the contrary, co-culturing microglia with BMDMs decreased its phagocytic activity.[75] Data from an experimental model of spinal cord injury showed that the infiltration of monocyte-derived macrophages to the site of injury significantly reduced the phagocytic activity of microglia, suggesting

a direct communication between microglia and macrophages that affects their phagocytic activity. Also, ex-vivo analysis showed that the phagocytic activity of microglia was significantly increased in *mPGES* $^{-/-}$and CCR2-depleted mice, signifying that the absence of macrophages or the absence of PGE2, directly regulated the phagocytic activity of microglia.[75] Altogether, both in-vivo and in-vitro studies revealed that the production of PGE2 from macrophages mediates an inhibitory effect on microglia phagocytosis, likely through the EP2 receptor.

2.6. The Impact of Advanced Age on the Inflammatory Response:

Aging affects all the components of neurovascular units including microglia, a key player in the neuroinflammatory response. These effects are characterized by increased oxidative stress and sustained proinflammatory response, a phenomenon frequently referred to as "Inflamm-aging".[78] Aging transforms microglia into a "primed" phenotype, a phenotype that is characterized by an increased susceptibility to secondary inflammatory stimuli and an uncontrollable and sustained inflammatory response, which eventually exacerbates the neuroinflammatory response. [79, 80] The idea of microglia priming and its responsiveness to systemic inflammation opened the venue for targeting neurodegenerative diseases by treating systemic diseases.[80] Moreover, a clinical study looked at the expression level of 39 genes, using a large-scale microarray dataset, and it revealed that proinflammatory genes like NFKB1, TRAF6, TLR4, IL1R1, TSPO, and GFAP, were upregulated, while the expression of neurotrophic genes (BDNF, NGF, PDGFA, SYN, and DBN1) was downregulated in aged individuals.[81] These data suggest that aging is linked to increased proinflammatory response and synaptic loss. [81] In a mouse model of systemic inflammation induced via LPS administration, aged mice demonstrated an increased

expression of IL-1β, TNF-α and IL-12 in the brain, suggesting that innate immune cells switched to be "primed" rather than tolerant to systemic infection.[82] Additionally, during stroke recovery in aged mice, aging was associated with an overall failure to upregulate genes associated with cell-cell interaction, like CXCL10, as well as proangiogenic genes, leading to a declined neovascularization.[83] Collectively, Niraula et el.[79] stated that aging triggers a phenotypic switch in microglia leading to a "primed" phenotype that is more likely to display an exacerbated and sustained neuroinflammatory response when exposed to immune stimuli.

3. Renin angiotensin system (RAS) in the brain:

3.1. Overview: RAS as a therapeutic strategy after stroke

The brain renin-angiotensin system (RAS) is believed to be involved in the pathogenesis of stroke and other CNS diseases. Preclinical data showed a direct correlation between angiotensin II (Ang II), the active neuropeptide in the renin-angiotensin system (RAS), and the severity of ischemic injury after stroke.[84] However, angiotensin II (Ang II) binds to different receptor subtypes, mainly angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R). Angiotensin II type 1 receptor (AT1R) is known to be responsible for the pathological actions of angiotensin II (Ang II), while angiotensin II type 2 receptor (AT2R) opposes the action of AT1R.[85] Hyper-activation of AT1R has been shown to worsen cognitive function, induce a proinflammatory response, and increase the production of reactive oxygen species in experimental models. These detrimental effects were associated with increased demyelination as well as impaired neuronal communication after CNS injury. On the other hand, stimulation of AT2R improves cognitive function, enhances remyelination of oligodendrocytes, and is associated with antioxidant and anti-

inflammatory effects. An experimental model of stroke demonstrated that direct stimulation of AT2R, via ICV administration of CGP 42112 (a peptide angiotensin II type 2 receptor agonist), was associated with reduction in infarct volume, enhanced motor function, improved neuronal survival, and increased microglial activation in the ischemic core.[86] Angiotensin receptor blockers (ARBs) have been studied regarding their ability to improve functional outcomes after stroke and attenuate vascular cognitive impairment. Candesartan is one of the largely investigated ARBs after experimental stroke and it has been shown to improve post-stroke neurobehavioral outcomes. Our group, among others, has demonstrated that a single dose of candesartan at reperfusion was sufficient to improve neurobehavior outcomes and reduce neurovascular damage, edema, and infarct size after stroke. These effects were associated with enhanced vascular protection and up-regulation of VEGF A and VEGF B in both ipsilateral and contralateral hemispheres. These beneficial effects, perhaps, were mediated via indirect stimulation of the AT2 receptor and elevated BDNF levels. The blockade of AT1R leads to an increase in the amount of unbound Ang II, which in turn augments its affinity to AT2R.[87, 88] Studies from our research group supporting the role of ARBs, particularly candesartan, on post-stroke recovery are summarized in Table 1.

3.2. Compound 21: A Promising Therapeutic Tool for Delayed, and chronic intervention after stroke

Studies from our laboratory team were the first to demonstrate that compound 21 (C21), the first selective non-peptide angiotensin II type 2 receptor agonist, provides a neurovascular protective effect and enhances sustained functional improvement at 7 days after stroke. After a 3-hour middle cerebral artery occlusion (MCAO), a single dose of C21

was able to reduce the infarct size and enhance functional and behavioral outcomes without affecting blood pressure.[89] Also, C21 treatment downregulated the expression of AT1R in the ipsilateral hemisphere, while augmenting the expression of AT2R in the non-stroked hemisphere. Other pro-survival, neurotrophic, and anti-inflammatory effects were observed as well. A comprehensive overview of these studies is summarized in table 1.

The translation of pre-clinical stroke research into clinically relevant outcomes requires developing comorbid animal models that are able to mimic the complexity of stroke in humans. Unfortunately, almost 90% of stroke research excluded crucial risk factors that are likely to worsen the outcome after stroke.[90] To further contribute to this effort and to ensure the translatability of our early findings, we studied post-stroke functional recovery and the development of PSCI in severely comorbid animal models of stroke, including diabetes, hypertension, and advanced age. We also explored the impact of delayed, and chronic treatment as a potential therapeutic intervention for long-term post-stroke functional recovery.

In the matter of aged hypertensive rats, SHRs were aged until the age of 14 months old, and then underwent permanent unilateral common carotid artery occlusion (UCCAO). 24 hours later, animals were randomized to receive a blinded daily dose of either Candesartan, C21, or vehicle for a total of 8 weeks. During the follow-up, rats were subjected to different neurobehavioral assessments to assess their functional improvement after stroke. Along with post-stroke functional improvements, changes in brain micro- and macrostructure, as well as the accumulation of amyloid were evaluated via MRI and protein expression of Aβ1-42, respectively. UCCAO did not induce a sensorimotor deficit. For this reason, it was not possible to assess the effect of RAS modulation on sensorimotor improvements

after stroke. Novel object recognition test was used to evaluate non-spatial short-term/working memory, and it revealed that both treatments were superior to vehicle in improving non-spatial recognition and working memory. As for aversive associative learning and reference memory, passive avoidance test was performed at baseline and then repeated at week 4 post-UCCAO. However, it was not possible to assess the role of the treatments (RAS modulation) in preserving reference memory due to the inability of these animals to enter the shock arm during the acquisition trial at week 4. On the other hand, The Morris Water Maze (MWM) test demonstrated that RAS modulation was associated with improved outcomes (a reduction in latency to reach the target platform at week 4 and spending more time in the target zone) as compared to vehicle-treated animals. To assess cognitive flexibility, animals' ability to update the initial information (platform location) and learn a new location was evaluated at week 4. Interestingly, rats in RAS modulators groups had a better performance as was evident by lower latencies to the new target. As for brain micro- and macrostructure, T2-weighted brain MRI analysis of aged SHRs at 4 weeks after UCCO showed global brain atrophy, smaller hippocampal volumes, and significant ventricular enlargement. These effects were significantly improved in the Candesartan-treated group but not C21. Also, FLAIR sequence analysis demonstrated white matter and hippocampal hyperintensities in both hemispheres, the effect of which was significantly abolished in candesartan-treated animals. The beneficial effects of RAS modulation in cognitive function were associated with less cortical accumulation of Amyloid beta in the C21-treated group, but not Candesartan.

In young hypertensive rats (~4 months old SHRs), we sought to investigate the impact of chronic AT2R stimulation, either via C21 or Candesartan, on long-term cognitive function

after stroke. Adult SHRs were exposed to either a 60-min tMCAO or SHAM surgery, and then 2 hours after reperfusion were randomized to receive either C21 (0.03 mg/kg/day) or vehicle for 7 days via i.p. administration. After 7 days, rats were either switched to low-dose candesartan (0,3 mg/kg/day) or continued their initial treatments (C21 or vehicle) for a total of 30 days. A battery of neurobehavioral analyses was performed to assess their cognitive function throughout the study, as well as some other molecular analyses. Compound 21 did not affect blood pressure before and after stroke. As for motor deficits, RAS modulation had no effects, where all animals spontaneously recovered to their baseline level at 28 days post-stroke. RAS modulation was associated with intact and superior cognitive performance throughout the follow-up period. In the NOR test, chronic RAS modulation was significantly associated with better performance from day 14 onward. Interestingly, this was profoundly true even when treatment was initiated 7 days after stroke. As for reference memory, animals in RAS modulation groups demonstrated longer latencies in the retention trial, indicating better performance and intact reference memory. On the molecular level, those who were treated with C21 during the first 7 days had a profoundly less hippocampal accumulation of amyloid beta at 30 days post-injury. To further illustrate this effect, the in-vitro analysis revealed that RAS modulation with either Candesartan or C21 prevented Aβ1–42-mediated cytotoxicity in HBEC cells. Moreover, long-term treatment with C21/Candesartan markedly reduced TUNEL-positive cells, indicating less apoptotic cell death, and prevented stroke-induced chronic reactive microgliosis. Collectively, these two studies demonstrated that chronic treatment with RAS modulators effectively slowed the progression of long-term PSCI, even when treatments were initiated well beyond the neuroprotective window of intervention. Suggesting that

delayed, long-term interventions after stroke could be a novel therapeutic target for the later development of PSCI.

As for aged normotensive animals (~14 months old Wistars), we aimed to determine the long-term effect of chronic AT2R stimulation, via C21, on the development of post-stroke cognitive impairment. Stroke was induced via permanent focal ischemia, due to tandem distal middle cerebral artery occlusion. 24 hours post-injury, rats were randomized to receive either C21 or vehicle and then followed up for a total of 30 days. Treatment was blinded and incorporated into their drinking water and was adjusted based on body weight and daily intake. Post-stroke cognitive function was evaluated using different assessment tools, including NOR, PAT, and MWM. Motor function was evaluated at 24 hours and 4 weeks post-injury, and there was no superiority in C21 treated group, where all animals spontaneously recovered. As for cognitive function, C21-treated animals demonstrated a better performance in NOR and preserved spatial/reference and long-term memory in the MWM and PAT tests, respectively. Furthermore, C21 seemed to be associated with improved cognitive flexibility as it was apparent with a better performance in learning a new platform location in the MWM test. On the molecular level, this cognitive superiority was associated with less cortical accumulation of amyloid beta, and no changes in the expression level of BDNF at post-stroke day 30. Yet, it is possible that the expression of BDNF peaked at an earlier time point and facilitated a reparative effect.

Concerning male diabetic animals, we investigated the impact of delayed, and chronic stimulation of AT2R, via C21, on the development of PSCI in diabetic rats. In this study, diabetes was induced via HFD/low-dose STZ in male Wistar rats. Diabetic and control animals were exposed to either 60-minute tMCAO or SHAM surgery. Daily oral treatment

with either C21 or vehicle was initiated 3 days after stroke, avoiding the neuroprotection window of intervention. Then, rats were followed up for a total of 8 weeks, and a battery of motor and cognitive tests were performed at different time points. Diabetic animals had a much higher mortality rate, peaking around week 4 post-surgery. A 40% mortality within 3 days after surgery and prior to treatment randomization, and an additional 55% post-treatment randomization as compared to 25% and 0% in the control group, respectively. Diabetic rats had a worse neurological score 3 days after stroke, and much slower improvement throughout the study, evidenced by ART and neurological score tests. Similarly, diabetic rats demonstrated exacerbated cognitive deficits and progressively steeper cognitive decline after stroke. In fact, diabetes seemed to double the cognitive decline after stroke as compared to the control group. (Baseline to post-stroke decline: 10.6% in the control group vs 27.5% in the diabetic group) Interestingly, considering 33% as a cut-off point for cognitive decline, diabetics had a 67% decline (diabetic SHAM), which further worsen to a 100% decline after stroke (Diabetic stroke), as compared to 0% and 20% in control SHAM and stroke, respectively. These exacerbated functional outcomes were associated with drastic up-regulation of microglia/macrophages (IBA-1) and astrocyte (GFAP) markers in the diabetic group. Also, there was an increase in the number of microglia/macrophages after stroke in diabetic animals, and further analysis revealed that was mainly due to the increase in macrophages but not microglia. Along with the worsened inflammatory response, diabetes was associated with significantly reduced hippocampal myelination. Considering all these effects in the diabetic model of stroke, we can conclude that a chronic neuroinflammatory response after stroke contributed to the chronic deficit in cognition that was associated with diabetes.

In a subsequent investigation of the impact of delayed, and chronic treatment with C21 on post-stroke recovery, diabetic animals treated with C21 had 0% mortality as compared to 55% in the vehicle-treated group. Within a week after stroke, C21-treated groups exhibited better sensorimotor performance in both control and diabetic animals and continued throughout the study. C21 treatment was associated with preserved brain volume in both diabetic and control animals, while enhanced myelination was only observed in diabetic animals. Diabetics treated with C21 exhibited a profound improvement in cognition (week 1 throughout 8), as was evident by a better performance in the 2-trial Y-maze test. This effect was not observed in the control group where all animals, regardless of their treatment assignment, spontaneously recovered. Setting 30% as a cut-off point for cognitive decline, the development of cognitive impairment was reduced from 100% to 0% in diabetic animals treated with C21, where none of these animals had any cognitive impairment at post-stroke week 8. Regarding neuroinflammatory response, C21 did not affect the percentage of activated microglia, nor the number of GFAP+ and IBA-1+ cells. However, it skewed the activated microglia into an anti-inflammatory phenotype by modulating the M1:M2 ratio. The M1:M2 ratio was downregulated after C21 treatment in the ipsilateral hemisphere of diabetic animals, particularly in the prefrontal cortex and hippocampus regions of the brain. Further in-vitro analysis in BV2 microglial cells confirmed the same findings, seemingly independent of AT2R stimulation. From a translational point of view, this study used a clinically relevant model of stroke, exhibiting a similar pattern of high mortality, and functional and cognitive deficits to that observed in patients. Interestingly, C21 still exerted its beneficial effects on functional and cognitive recovery, as well as

neuroinflammation even when initiated well out of the known neuroprotective window of intervention.

In addition to the aforementioned risk factors, we also addressed gender differences in response to C21 treatment in female diabetic rats and ovariectomized hypertensive females, since females are more likely to have a greater negative impact of stroke and dementia than males. A female diabetic study was designed similar to the one done on males, except that they were followed up for a total of 4 weeks. Treatment was initiated beyond the neuroprotection window, 3 days after stroke, and administered via drinking water. C21 treatment was associated with better survivability and improved sensorimotor function after stroke. C21 positively impacted neuroinflammatory response after stroke, as was evident with less pro-inflammatory microglia and decreased M1:M2 ratio. There was no effect on the number of GFAP+ and Iba1+ cells. Similarly, in-vitro analysis of C8-B4 microglia cells showed that C21 treatment down-regulated the M1:M2 ratio and shifted the polarization toward a less pro-inflammatory phenotype. In contrast to male diabetics, C21 did not impact the progression of PSCI, where animals in both C21- and vehicle-treated groups exhibited similar performance in NOR and 2-trial Y-maze. Overall, this study concludes that C21 demonstrated a superior effect in improving survival and sensorimotor skills after stroke, likely mediated via its positive impact on neuroinflammation. A major drawback of this study is the limited sample size and relatively short post-stroke follow-up.

We then evaluated the impact of AT2R stimulation on the development of post-stroke cognitive impairment after stroke in ovariectomized, hypertensive female rats (Female-OVX SHRs). Rats were exposed to ischemia-reperfusion injury via 60-minute tMCAO,

and then, 24 hours post-injury, rats were randomized to receive either C21 or saline. For the first 5 days treatment was administered via intraperitoneal injection (0.03 mg/kg/day), and then orally (0.12 mg/kg/day) for the rest of the study. During the 6-week follow-up, cognitive function, brain structure, and vasculature were evaluated. As for sensorimotor function, stroke induced acute motor deficits, which were sustained throughout the study. There was no difference between C21- and vehicle-treated groups in this matter. Anxiety-like behavior was assessed via elevated plus maze test, and it revealed that stroked animals were more anxious, with no effect of treatment. On the contrary, C21 was associated with an improved fear/reference memory, as well as spatial memory, where C21-treated rats had a better memory as was evident by a longer time exploring the displaced object. Furthermore, Micro-CT angiography demonstrated that C21 improved brain microvasculature after stroke. There was a significant decrease in vessel separation in the ipsilateral hemisphere of C21-treated rats, suggesting an increased collateral blood flow. Collectively, this study provides additional insight into how C21 mediates its beneficial effects in preventing PSCI, mainly spatial and reference memory, in postmenopausal females. These effects are possibly mediated via mechanisms involving vascular protection and restoration.

Equally important, Fouda et al.[91] evaluated the involvement of contralesional AT2R in recovery after stroke, and the expression of AT2R in type 2 diabetic GK rats and control Wistars after stroke. This aim was achieved via two different experiments. First, to determine the impact of AT1 receptor blockade, via candesartan, on post-stroke recovery, and the expression of AT1R and AT2R in GK type 2 diabetic rats. Second, to evaluate the effect of contralesional deletion of AT2 receptor on stroke recovery after C21 treatment.

For the first experiment, GK diabetic rats were exposed to 3-hour tMCAO surgery and randomized to receive either candesartan or vehicle at reperfusion for a total of 24-hour follow-ups. Unlike control Wistars, treatment with Candesartan did not reduce BP level, nor improve acute stroke outcomes in GK type 2 diabetic rats. Likewise, Candesartan treatment had no superiority in preserving vascular integrity, oxidative stress generation, or apoptosis. This study uniquely pointed out the crucial role of the AT2 receptor in the non-ischemic hemisphere after stroke. As anticipated, Candesartan treatment, an Angiotensin type 1 receptor blocker, decreased the expression of AT1 receptor in the ischemic hemisphere, and up-regulate the expression of AT2 receptor in the contralesional hemisphere of Wistar rats. On the other hand, GK diabetic rats exhibited a reduction in the expression of AT2 receptor after stroke, and treatment with Candesartan failed to increase it. Interestingly, Candesartan treatment did not down-regulate the expression of the AT1 receptor in GK diabetic rats, which could attribute to its failure to achieve favorable outcomes after stroke in diabetic rats. To further explore the role of the contralesional AT2 receptor after stroke, a contralesional AT2R knockdown model was generated via intrastriatal injections of short hairpin RNA (shRNA). Then, rats were exposed to tMCAO and treated with C21 daily starting at reperfusion for a total of 7 days. Their post-stroke functional recovery was evaluated over 10 days. Similar to the previous findings, the Contralesional knockdown of AT2R decreased C21-mediated functional recovery after stroke. Altogether, these two studies suggest a potential role of contralesional AT2R in mediating the recovery after stroke

Aside from our research team, several others have looked at the involvement of AT2R stimulation in recovery after stroke and other CNS diseases and reached a similar

conclusion. Claudia et al.[92] demonstrated that pretreatment with C21 5 days before stroke in conscious hypertensive rats resulted in a dose-dependent reduction in infarct size, and that was associated with enhanced motor recovery, and microglial activation, as well as an increase in the expression of BDNF.[92] C21 mediated effects were consistent even when treatment was initiated 6 hours post-injury, suggesting a wide window of neuroprotection against stroke damage. [92] Moreover, Li-Juan Min and colleagues[93] showed that post-stroke treatment with C21 was associated with a reduction in the ischemic lesion, and an improvement in neurological function, without affecting blood pressure. Along with these effects, C21 increased cerebral blood flow after stroke and modulated the inflammatory response toward a less pro-inflammatory phenotype. Surprisingly, C21 blunted stroke-induced BBB disruption in the ipsilateral hemisphere and improved brain edema. [93] Fei Jing et al.[94] revealed that C21 effects were mediated via direct stimulation of AT2 receptors. Daily i.p. Injections of C21 improved cognitive function in C57BL/6 mice, this effect was ameliorated in AT2-deficient animals. Along with an enhanced cognitive function, C21 treatment was associated with increased cerebral blood flow and enhanced hippocampal field-excitatory postsynaptic potential (f-EPSP) in a dose-dependent manner. Also, C21 preserved cognitive function in an animal model of Alzheimer's, intracerebral injection amyloid-b. [94] Martina Füchtemeier et al.[95] suggested that RAS modulation via C21 may have a potential role in vascular cognitive impairment. In a chronic hypoperfusion model of VCI, daily C21 treatment preserved spatial reference memory.[95] Interestingly, C21 had a negative effect on sham animals, where animals spent less time in the target quadrant.[95] Moreover, C21-treated animals had slightly, yet significant, larger basilar arteries than the control group. This may explain the previously observed improvement in

cerebral blood flow.[95] Hippocampal blood supply network is supplied via the posterior cerebral artery, which branches off the superior cerebellar from the basilar artery.[95] Possibly, larger basilar arteries in the C21-treated group may result in a better blood supply to the hippocampus after stroke. [95] Paolo Gelosa et al.[96] investigated the effect of long-term treatment with C21 on spontaneously hypertensive stroke-prone rats associated pathogenesis.[96] C21 treatment delayed the occurrence of brain abnormalities and enhanced survival without affecting blood pressure.[96] Additionally, C21 treatment is associated with favorable outcomes in preserving renal function and structure, via reducing the infiltration of immune cells, collagen disposition, and plasma renin activity as compared to the control group.[96] C21 favorable outcomes after stroke was further confirmed and validated in many other studies. Lastly, Douglas M. Bennion et al.[97] used a transient middle cerebral occlusion model of stroke to induce stroke in Aged male Sprague-Dawley rats (18-20 months old). The study aimed to look at the long-term neuroprotective effects of C21 after stroke in aged rats and to assess the co-localization of AT2 receptor within the infarct zone.[97] Treatments were administered via i.p. Injections at 90 minutes, 1 day, and 2 days post-tMCAO, and followed for up to 28 days.[97] As anticipated, C21 treatment resulted in sustained long-term protective effects up to 3 weeks after stroke, improved neurological function, and smaller infarct lesions.[97] Surprisingly, C21 treatment increased the expression of AT2 receptor in neurons, but not microglia or astrocytes.[97] This study provides profound evidence that C21-mediated neuroprotective effects were consistent in aged rats, and to great effect, there was long-term sustained neuroprotection as well.

All things considered, growing signs are linking the direct and in-direct stimulation of brain AT2 receptor to functional and cognitive improvement after stroke, and suggesting that

RAS modulation exhibited anti-inflammatory, neuroprotective, and pro-angiogenic effects after experimental models of stroke. RAS modulation-mediated neurobehavioral and functional recovery are believed to be independent of the blood pressure-lowering effect. Our recent studies were focused on validating the impact of C21/Candesartan in severely co-morbid models of stroke, including advanced age, diabetes, and hypertension. Notably, these animals exhibited a much worse functional recovery and high mortality after stroke, unlike young-healthy animals. This pattern appears to be more relevant to the trajectory of stroke in patients. Another key point is that RAS modulators, C21/Candesartan, achieved a sustained long-term effect in preserving cognitive function even when treatment was initiated beyond the neuroprotection window (3-7 days). These findings are very interesting and could translate into a novel long-term therapeutic strategy after stroke.

3.3. Failure of RAS modulators in Clinical Trials: Focus on ARBs

Despite the mounting evidence supporting the beneficial effects of candesartan treatment after stroke in experimental models of stroke, clinical studies demonstrated otherwise. The ACCESS trial[98] aimed to study the safety of candesartan in the early treatment of stroke. It demonstrated that 7 days post-stroke treatment with Candesartan, initiated 24 hours after stroke, significantly improved the cumulative 12-month mortality rate and vascular events.[98] This trial concludes that Candesartan is “a safe therapeutic option” after stroke since there were fewer cardiovascular or cerebrovascular events in the Candesartan arm. Yet, The ACCESS trial did not explore post-stroke cognitive and functional improvement after candesartan treatment. One of the largest studies in this matter is the Scandinavian Candesartan Acute Stroke Trial (SCAST).[99] SCAST was a randomized, placebo-controlled, and double-blinded trial aimed to investigate the effect of lowering blood

pressure using candesartan in patients with acute stroke and elevated blood pressure.[99] The primary outcomes were composite endpoint of vascular death, MI, and death within 6 months, and functional recovery at 6 months assessed via modified Rankin score.[99] Patients were randomized to receive either Candesartan, continued for 7 days, or placebo. Candesartan significantly lowered blood pressure, and there were no differences in the composite vascular end-points during the 6 month follow-up period.[99] Surprisingly, patients in candesartan groups had poor functional improvements at 6 months, suggesting a harmful effect.[99] Another analysis for the same cohort investigated whether the blood pressure-lowering effect of candesartan affects long-term cognition and quality of life.[100] Similar to the previous trial, candesartan did not show any superiority regarding cognitive function and quality of life.[100] Quite the opposite, there were signs of harmful effects in candesartan-treated patients.[100] Furthermore, The China Antihypertensive Trial in Acute Ischemic Stroke (CATIS clinical trial) looked at the effect of immediate blood pressure lowering after ischemic stroke on death and disability at 14 days.[101] Their findings conclude that blood pressure lowering via antihypertensive medications did result in outcomes different from the control group, suggesting that blood pressure reduction after ischemic stroke did not improve or worsen the recovery after stroke.[101] The SCOPE trial was designed to examine the impact of candesartan treatment in reducing long-term cardiovascular events, cognitive decline, and dementia in elderly individuals after stroke. After a mean follow-up of 3.7 years, Candesartan treatment did not differ from placebo regarding cognitive decline or occurrence of dementia. As for cardiovascular/cerebrovascular events, there was a significant reduction in non-fatal stroke and a modest, yet non-significant, reduction in major cardiovascular events. The authors

anticipated that cognitive superiority could not be observed for a couple of reasons. First, the study was conducted over a relatively short period, the mean follow-up was 3.7 years. Second, it has been previously reported that high blood pressure is associated with a long-term risk of dementia (10 – 15 years before its onset), while low blood pressure accounts for a short-term risk once dementia is detected. Therefore, considering the inclusion criteria of this trial (elder individuals with mild/moderate blood pressure), patients with a short-term risk of dementia possibly have not been included in the trial. Third, the differences in blood pressure reduction between all groups were relatively small. Lastly, most patients regardless of their assigned group exhibited a good cognitive performance, which makes it challenging to detect cognitive superiority.

Looking at these findings and considering our preclinical studies using candesartan after stroke, one could argue that candesartan, perhaps, mediates its beneficial effects after stroke aside from its antihypertensive effect. Guan et al. [102-104] demonstrated that a single dose of candesartan sufficiently reduced brain edema and infarct size after stroke, as well as an up-regulation of VEGF and protein kinase B, a pro-survival protein. Additionally, Alhusban et al.[105] revealed that candesartan was associated with enhanced pro-angiogenic effects, and increased expression of BDNF, a key neuroprotective molecule in the brain. These effects were likely mediated via its in-direct effects on angiotensin II type 2 receptor. ARBs block the binding of Angiotensin II to Ang II type 1 receptor, which in turn increases the availability and binding affinity of Ang II to Ang II type 2 receptor. Ahmed et al.[88] illustrated that chronic treatment, up to 30 days treatment/follow-up period, with RAS modulators, including Candesartan, enhanced cognition and prevented the development of post-stroke cognitive impairment in hypertensive animals. Interestingly, these effects were

persistent even with delayed treatment, 7 days after stroke, and seemed to be independent of blood pressure. A similar pattern was observed in diabetic animals when treatment with C21 was initiated beyond the neuroprotection window of 72 hours and continued for 4 weeks in female diabetics and 8 weeks in male diabetics.[106, 107]

Collectively, the evidence supporting the role of RAS modulation is mixed at the point and the scientific community has virtually abandoned this pathway as a viable stroke treatment. However, we believe strongly that more investigation is warranted. First, the focus should shift toward the development of post-stroke cognitive impairment as a target for later intervention after stroke. Second, chronic treatment after stroke, beyond the usual 5-7-day treatments studied in clinical trials, should be pursued to improve long-term outcome. Focusing beyond the acute phase after stroke, which involves promoting long-term anti-inflammatory response and neurovascular restoration, could potentially be an alternative strategy to enhance long-term functional recovery after stroke. Therefore, a well-designed clinical trial addressing these important aspects of RAS modulation should target the later development of long-term PSCI.

3.4. Compound 21 in Lung-related Clinical Trials: Vicroe-Pharma VP01 Program

Although C21 is not currently being studied as a stroke treatment, the safety and efficacy of C21 have been tested in a few clinical trials. Currently, three active trials are looking at the safety and efficacy of C21 in different diseases, mainly lung-related diseases. These trials are funded via Vicore Pharma AB, C21's manufacturer. The first trial aims to test the safety/efficacy of C21 as an add-on to the standard of care. It is a randomized, double-blind, placebo-controlled, parallel-group, multicenter phase-2 trial. 600 patients are expected to be enrolled in the trial, with 300 participants in each arm (1:1 randomization).

C21 will be administered orally as 100 mg twice a day for a total of 14 days. (***ClinicalTrials.gov Identifier: NCT04880642***) The other trial is a multi-center, open-label, single-arm phase 2 trial looking at the safety/efficacy profile, and pharmacokinetics of C21 in patients with idiopathic pulmonary fibrosis. A total of 60 participants are expected to be enrolled in the trial. (***ClinicalTrials.gov Identifier: NCT04533022***) Both studies are expected to be completed by December 2022. Moreover, The ATTRACT study (Angiotensin II Type Two Receptor Agonist COVID-19 Trial)[108] was completed in September 2020. ATTRACT was a randomized, double-blind, placebo-controlled, multi-center, parallel-group phase 2 trial, designed to evaluate the safety/efficacy of C21 in hospitalized patients with COVID-19 and CRP $\geq$ 50-150 mg/L.[108] Treatment was orally administered at a dose of 100 mg twice a day for a total of 7 days.[108] A primary outcome was, changes in the level of CRP from baseline, while the secondary outcome was set to be the changes in body temperature, IL-6, IL-10, TNF, CA125, and ferritin, the number of subjects not in need of oxygen supply/mechanical ventilation, need of mechanical invasive or non-invasive ventilation, and time on oxygen supply.[108] These trials are part of the VP01 program, an experimental program assessing the potential effects of C21 in treating fibrosis, inflammation, and vasculopathy in different diseases.

Table 1. Studies from our research group supporting the role of RAS modulation after stroke:

Study	*Purpose*	*Model*	*Intervention*	*Conclusion*
Fagan et al. 2006 [109]	To determine whether BP lowering with candesartan, initiated at reperfusion, can reduce neurovascular damage, and improve outcomes in a model of hypertension after experimental ischemic stroke	Male Wistar 3 hours MCAO	At reperfusion, vehicle vs candesartan (1 mg/kg) IV tail vein Injection Follow-up: 24 hours	-Candesartan decreased post-stroke elevation in MAP, edema, infarct size, and hemoglobin content. -Candesartan administered after reperfusion in acute ischemic stroke reduces neurovascular damage and improves outcome.
Kozak et al. 2009 [110]	To determine the effects of candesartan on pro-angiogenic factors, as well as 7-days	Male Wistar rats 3 hours tMCAO	At reperfusion, animals received a single dose of candesartan (1mg/kg) or saline via IV tail vein injections.	-A single dose of candesartan lowered BP for 4 days. -A single dose of candesartan improved bederson score, elevated body swing test performance, and paw grasp all the way up to day 7.

neurobehavior al outcomes		Follow-up: Either 24 hours or 7 days	-Beam walk test revealed that animals in the candesartan group only showed superiority at 24 hours, but not day 7. -There was no difference in infarct size, while candesartan-treated animals had a slight reduction in cavitation. -In the ipsilateral hemisphere, the candesartan-treated group had elevated levels of MMP-2 and VEGF, and no difference was observed in MMP-9 activity. -At 24 hours, treatment with candesartan decreased vascular permeability in both hemispheres. While at 7 days, the candesartan group had more laminin-positive cells in the striatum, suggesting an increased vascular density. -This study concludes "Candesartan after reperfusion augments ischemia-induced angiogenic state and provides long-term benefits", which

				might be facilitated via vascular protection and enhancement of early angiogenic effects.
Guan et al. 2011 [102]	To determine if early BP lowering with candesartan, in the presence of an occluded cerebral artery, will reduce injury and improve outcome after experimental stroke.	Male Wistar permanent MCAO	Candesartan vs vehicle 3 hours after occlusion Follow-up: either 24 hours or 7 days	-Candesartan reduced MAP, infarct size, edema, and hemoglobin at 24 hours but not day 7 Candesartan-treated animals performed better in Bederson and paw grasp but not beam walk at 24 hours, with no differences at day 7. -There was an Improved cerebral perfusion at 24 hours. -pMCAO increased the expression of MMP-2 and VEGF, not MMP-9, with no effect of candesartan.
Guan et al. 2011 [103]	To determine the vascular protection of candesartan, minocycline, and atorvastatin after stroke in	Male Wistar, SHRs, and GK rats 3 hours MCAO	At reperfusion, a single dose of Vehicle or (Candesartan), (Enalapril), (Minocycline with tPA) (Hydralazine), or (Atorvastatin) Animals in Atorvastatin and	-Candesartan showed more of vascular protection rather neuroprotection in Wistar rats. -Candesartan was predominantly vascular protective, especially in animals with preexisting vascular damage (diabetes, hypertension).

	three different animal models		Minocycline groups received another dose at 12 hours Follow-up: 24 hours	
Guan et al. 2011 [104]	To determine the role of VEGF isoforms and their receptors in vascular protection after experimental stroke	Male Wistar 3 hours tMCAO, and SHAM Surgery	AT reperfusion, a single dose of candesartan (1mg/kg) was administered IV Tail Vein Injection Follow-up: Either 24 hours of 7 days	-A single dose of Candesartan at reperfusion was associated with up-regulation of VEGFA and VEGF B and their respective receptors. -This was associated with an increase in the expression of Protein Kinase B, a pro-survival protein. -Post-stroke treatment with Candesartan enhanced vascular restoration, as well as pro-survival, which is likely to be associated with functional improvement, yet this was not addressed in this study.
Alhusban et al. 2013 [105]	To determine the role of BDNF in the pro-angiogenic effect of candesartan in	Male Wistar, and SHRs rats	Rats were treated with either vehicle or Candesartan (0.3 mg/kg for SHRs or 1mg/kg for Wistar)	-Candesartan induces the pro-angiogenic state of endothelial cells, which is likely mediated via its effects on the expression of BDNF and its

	the brain under hypertensive conditions	SHAM Surgery	IV Tail Vein Injections Follow-up: 24 hours	ability to stimulate the AT2 receptor.
Ishrat et al. 2013 [111]	To evaluate the effect of combination therapy with candesartan and delayed tPA (6 hours) on reversing tPA-induced brain hemorrhage	Male Wistar rats Embolic eMCAO	**Experiment 1:** To evaluate the effects of combination therapy on the neurological outcome, hemorrhage, and infarct size at 48h post-eMCAO **Experiment 2:** To evaluate the effects of a combination therapy on the MMP activity and protein expression at 24h post-eMCAO Combination therapy: Either early (3H) or delayed (6H) Candesartan alone or in combination with tPA (6H), or vehicle	-Candesartan/tPA combination therapy enhanced neurological outcomes and reduced brain hemorrhage as compared to tPA alone. -tPA alone was associated with elevated MMP3 and this was attenuated in tPA/Candesartan combination therapy. -No differences were noticed in MMP9 activity. Candesartan alone or in combination with tPA attenuated the activation of NF-κB pathway and the expression of TNF-α, while increasing the expression of eNOS after eMCAO.

Soliman et al. 2014 [112]	To investigate the pattern of the ARB pro-angiogenic effects in the ischemic brain and its association with VEGF-A and VEGF-B	Male Wistar rats 90 minutes MCAO OGD: in-vitro model mimicking ischemia induced oxygen-glucose deprivation	At reperfusion, animals were injected with either vehicle or candesartan (1mg/kg) IV Tail Vein Injection Follow-up: 8, 24, 48, 72 hours	-Treatment with Candesartan induced prolonged pro-angiogenic effects at 72 hours after stroke Candesartan is associated with a prolonged up-regulation of VEGF A and VEGF B in both hemispheres. -A more profound up-regulation was found in the contralateral hemisphere and lasted up to 72 hours after stroke. While the ipsilateral up-regulation blunted beyond 24 hours. -Candesartan treatment stabilized the expression HIF1a and preserved angiopoietin 1. -As overall, Candesartan-induced VEGF-A and VEGF-B up-regulation and exerts a paracrine neuroprotective effect.
Ishrat et al. 2014 [113]	To determine the contribution of	Male Wistar rats	At reperfusion, rats were treated with either Candesartan	-Candesartan, FeTTPS, and their combination reduced Nitrotyrosine.

	MMP and nitrative stress to the effects of angiotensin blockade in an experimental model of stroke.	3 Hours tMCAO	alone, in combination with (either FeTTPS or GM6001) or vehicle Candesartan: IV Tail Vein Injection FeTTPS and GM6001: i.p. Injections Follow-up: 24 hours	-Candesartan alone reduced NT compared to DMSO and GM6001 alone, this was blunted in Candesartan/GM6001 combination. -Candesartan alone significantly increased MMP-9 when administered with DMSO. -As overall, the acute neurovascular protective effects of candesartan occur regardless of the significant increases in MMP activity and are likely to be at least partially explained by an acute antioxidant effect. -The increase in MMP activity seen after candesartan administration may be necessary to promote long-term recovery after the acute period.
Ishrat et al. 2015 [114]	To determine whether a low, sub-hypotensive	Male Wistar rats	At reperfusion, animals received candesartan (0.3 mg/kg) or saline	-Repeated treatments with a low, sub-hypotensive dose of candesartan promote functional recovery, reduced

	dose of candesartan improves neuroplasticity and functional recovery after stroke, via enhancing neurotrophic factor expression in rats	90 minutes tMCAO	Treatment is repeated every 24 hours for 7 days 1st dose was administered via IV, and then the sub-sequent doses were administered via i.p. Follow-up: 14 days	infarct size, increased the expression of neuroplasticity mediators (VEGF and BDNF), and preserved neurovasculature after stroke. -Blocking AT1R after stroke promotes stroke recovery when excessive BP lowering is avoided.
Alhusban et al. 2015 [89]	To determine the effects single dose of C21 on early and late outcomes after an experimental model of stroke, and its possible underlying mechanism	Male Wistar rats 3 hours tMCAO (For acute neurovas cular endpoints and molecula r analysis)	At reperfusion, animals received a single dose of C21 (0.03 mg/kg) or saline via i.p. AT2R blocker, PD123319, was used in some experiments (3 mg/kg) Follow-up: For acute neurovascular endpoints and	-A single dose of C21 at reperfusion did not affect blood pressure at 24 hours after 3 hours tMCAO At 24 hours after stroke, C21 was associated with a 40% reduction in infarct volume and improved functional recovery. These effects were abrogated when AT2R co-administered. -C21 treatment was associated with a reduction in ischemia-mediated up-regulation of AT1R in the ipsilateral

	90 min tNCAO (For long-term behavioral outcome and immunostaining)	molecular analysis: 24 hours For long-term behavioral outcome and immunostaining: 7 days	hemisphere while augmenting the expression of AT2R in the contralateral hemisphere. -C21 enhanced prosurvival, neurotrophic, and decreased pro-apoptotic effects. It increased the expression of Akt and the phosphorylation of iNOS in both hemispheres while blunting the expression of cleaved caspase 3 in the ipsilateral hemisphere. -C21 reduced ischemia-induced nitrative stress At 7 days post-stroke, C21 enhanced functional recovery, vascular density, and the expression of IL-10, an ani-inflammatory cytokine. -In-vitro analysis of human brain endothelial cells revealed a dose-dependent effect of C21 in up-regulating the expression of BDNF. -In conclusion, “acute AT2R stimulation with C21 provides neurovascular protection and enhances long-term recovery

				via multiple mechanisms, without affecting the BP".
Fouda et al. 2017 [115]	To determine whether ARB-mediated functional recovery after stroke is dependent on the brain-derived neurotrophic factor (BDNF)	Male Wistar rats BDNF knockdown (ICV injection of lentiviral particles) 90 minutes tMCAO	At reperfusion, animals received a single dose of candesartan (1mg/kg) or saline via IV tail vein injections. Follow-up: 14 days Functional recovery was evaluated on days 1, 4, 7, 10, and 14 after MCAO.	-Bilateral ICV injection of lentivirus expressing shRNA BDNF successfully knockdown almost 70 % of BDNF at 14 days. -Candesartan was associated with an improved functional recovery, enhanced vascular density and pro-angiogenic effects, increased VEGF expression, and improved synaptogenesis up to 14 days after stroke, however, these effects were abolished in the BDNF K/O group. -Interestingly, BDNF K/O did not worsen post-stroke outcomes in saline-treated rats. -As an overall conclusion, AT1 blockade with candesartan after stroke mediates a BDNF-dependent functional improvement and this was associated with both

				angiogenesis and increased synaptic plasticity.
Fouda et al. 2017 [116]	To determine whether C21 mediates its neuroprotective effects after stroke via up-regulating the neuroprotective and anti-inflammatory cytokine interleukin (IL)-10	Male Wistar rats 3 hours tMCAO	At reperfusion, animals received a single dose of C21 (0.03 mg/kg) or saline via i.p. Anti-IL-10 neutralizing antibody was used to block the effect of endogenous IL-10 Follow-up: 24 hours	-C21 was associated with a reduction in infarct volume, and an improvement in bederson score and beam walk, however, IL-10 neutralization blunted C21-mediated neuroprotective effects and partially blocked the behavioral recovery. -In an in-vitro model of stroke, OGD, C21 mediated a neuroprotective effect in primary rat cortical neurons, and that was independent of IL-10.
Ishrat et al. 2018 [117]	To determine the direct role of VEGF-B in mediating candesartan beneficial effects after stroke	Male Wistar rats VEGF-B Knockdown (ICV injection of lentiviral particles)	**Experiment 1:** The effect of candesartan on day 3 post-stroke neurological outcome and infarct size. (90-minutes tMCAO) Follow-up: 3 days **Experiment 2:** The effect of Candesartan	-Candesartan mediates its neuroprotective effects via increasing the expression of VEGF-B and the activation of its receptor phosphor-Flt-1. -There was no compensatory up-regulation of VEGF-A after silencing VEGF-B. -Candesartan enhanced neurobehavioral recovery after stroke and reduced infarct

		90-minutes, or 3 hours tMCAO	on vascular damage 24 hours after stroke. (3 hours tMCAO) Follow-up: 24 hours At reperfusion, animals were randomized to receive a single dose of candesartan (1mg/kg) or saline via IV tail vein injections.	size, which was abrogated in VEGF-B silenced group. -Candesartan-mediated vascular protective effect was independent of VEGF-B and its receptor. -VEGF-B has a crucial role in mediating some of the candesartan neuroprotective effects after stroke.
Ahmed et al. 2018 [118]	To determine the role of RAS modulation, via either Candesartan or Compound 21, on different aspects of cognitive functions and pos-stroke recovery in aged	Aged hypertensive rats (14-months old SHRs) Permanent unilateral common carotid artery	24 hours after surgery, animals were randomized to receive daily doses of C21, candesartan, or vehicle C21 (0.12 mg/kg/day via drinking water) Candesartan (1mg/kg/day via drinking water)	-No motor deficits were observed in any of these groups, from day 1 all the way through the end of the follow-up period. -Vehicle-treated aged hypertensive rats showed a progressive worsening in NOR test from baseline to week 4 and week 8 post-UCCAO. -However, C21-treated animals demonstrated a constant performance

hypertensive rats with chronic cerebral hypoperfusion	occlusion (UCCAO)	Treatment with both RAS modulators continued for a total of 8 weeks Follow-up: 8 weeks	throughout the follow-up period, while Candesartan-treated rats has a transitory reduction at week 4 but completely recovered at week 8 post-UCCSO Animals in RAS modulation groups had a superior non-spatial working memory as it was evaluated via the NOR test. -It was not possible to assess learning and memory using PAT since all animals failed to enter the shock arm during the acquisition trial at week 4 post-UCCAO. -T2-weighted brain MRI analysis of aged SHRs with chronic cerebral hypoperfusion, showed global brain atrophy, smaller hippocampal volumes, and significant ventricular enlargement. --These effects were significantly improved in Candesartan treated group but not C21.

				-FLAIR sequence analysis in aged hypertensive rats with chromic cerebral hypoperfusion demonstrated white matter and hippocampal hyperintensities in both hemispheres, the effect of which was significantly abolished in candesartan-treated animals. -C21, but not candesartan, prevented the cortical accumulation of Amyloid beta.
Ahmed et al. 2018 [88]	To determine the impact of RAS modulation (C21 or Candesartan) on long-term cognitive function after stroke in adult hypertensive rats	Adult hypertensive rats (~4 months old SHRs) 60-min tMCAO or SHAM surgery	At 2 hours after reperfusion, animals received either C21 (0.03 mg/kg/day) or vehicle for 7 days Then, animals were either switched to a low dose of candesartan (0,3 mg/kg/day) or continued their initial treatments (C21 or vehicle)	-C21 treatment in SHRs had no effect on BP before and after stroke. -Animals in all groups had a spontaneous motor recovery at 28 days post-stroke. -In the NOR test, saline-treated animals demonstrated a significant cognitive decline from day 14 to 28, and chronic stimulation of AT2R with either C21 or Candesartan prevented this decline. This effect is still pronounced even

		Treatments were administered via i.p. Follow-up: 30 days	with delayed treatment, initiated at post-stroke day 7. -In PAT, rats in the RAS modulation group exhibited longer latencies in the retention trial vs acquisition trial, suggesting an intact reference memory On the molecular level, those who were treated with C21 during the first 7 days had a profoundly less hippocampal accumulation of amyloid beta at 30 days post-injury. -In-vitro analysis of HBECs cells demonstrated that treatment with either Candesartan or C21 prevented Aβ1–42-mediated cytotoxicity Long-term treatment with C21/Candesartan markedly reduced TUNEL-positive cells, indicating less apoptotic cell death, and prevented stroke-induced chronic reactive microgliosis

Ishrat et al. 2019 [119]	To determine the efficacy of Compound 21 in embolic stroke	Male Wistar rats Embolic stroke (eMCAO)	**<u>Experiment 1:</u>** Dose-response effect of C21 at 48 hours Follow-up: 48 hours Treatment: 0.01 mg/kg at 3H (I.V.) and 0.04 mg/kg daily till day 5 (orally) 0.03 mg/kg at 3H (I.V.) and 0.12 mg/kg daily till day 5 (orally) 0.06 mg/kg at 3H (I.V.) and 0.24 mg/kg daily till day 5 (orally) **<u>Experiment 2:</u>** Dose-response effect of C21 at 7-days Follow-up: 7 days Treatment: 0.01 mg/kg at 3H (I.V.)	-Dose-response analysis demonstrated an improvement in motor function at the lowest dose (0.01 mg/kg). -Further time-window analysis reveals that the same dose resulted in an enhanced improvement when administered at 6H and 24H. -C21 alone, or in combination with tPA did not reduce hemorrhage or infarct size after eMCAO This study suggests that future studies should focus on long-term cognitive improvement of C21 rather than acute neuroprotective effects.

		0.03 mg/kg at 3H (I.V.) 0.06 mg/kg at 3H (I.V.) **<u>Experiment 3:</u>** Therapeutic window of C21 7-days Follow-up: 7 days Treatment: 0.01 mg/kg at 3H (I.V.) and 0.04 mg/kg daily till day 5 (orally) 0.01 mg/kg at 6H (I.V.) and 0.04 mg/kg daily till day 5 (orally) 0.04 mg/kg/day daily up to day 5 (orally) **<u>Experiment 4:</u>** C21 in combination with tPA at 48 hours	

		Follow-up: 48 hours Treatment: 0.01 mg/kg at 3H (I.V.) and 0.04 mg/kg at 24H (orally) tPA 10 mg/kg (I.V.) at 4H Combination of both regimen **Experiment 5:** C21 in combination with tPA at 28 days Follow-up: 28 days Treatment: 0.01 mg/kg at 3H (I.V.) and 0.04 mg/kg/day daily till day 5 (orally) tPA 10 mg/kg (I.V.) at 2H	

			Combination of both regimen	
Ahmed et al. 2019 [120]	To determine the effect of long-term C21 treatment on the development of cognitive impairment after stroke in aged animals	Aged male Wistar rats (~14 months old) Permanent focal ischemia (Tandem distal middle cerebral artery occlusion - dMCAO)	24 hours after surgery, animals were randomized to receive daily doses of C21 (0.12 mg/kg/day in drinking water) or vehicle for a total of 30 days Follow-up: 30 days	-As compared to vehicle-treated animals, daily doses of C21 ameliorated stroke-associated weight loss throughout the study, at 8-, 10- and 30-days post-stroke. -All animals, including the control groups, showed similar spontaneous motor recovery at 28 days post-stroke. -There was a significant worsening effect in the NOR test in vehicle-treated animals, the effect of which was abolished in the C21 group where animals had a constant performance at baseline and week 4, similar to the SHAM animals. -The MWM and PAT tests demonstrated that C21 treated animals had preserved spatial, reference, and long-term

				memory in aged animals after stroke C21-treated animals had a better performance in learning a new platform location, indicating improved cognitive flexibility in aged rats. -C21 prevents the cortical accumulation of amyloid beta and had no apparent effect on the expression of BDNF in the prefrontal cortex 30 days after stroke.
Jackson et al. 2020 [106]	To study the impact of delayed, and chronic stimulation of AT2R, via C21, on the development of PSCI in diabetic rats, and to investigate the underlying mechanisms by which C21	Control: Male Wistar rats Diabetic: Male Wistar rats exposed to a combination of HFD/low dose STZ	Treatment was randomized at post-stroke day 3 to avoid the neuroprotection window of intervention At day 3 post-surgery, rats were randomized to receive either C21 (0.12 mg/kg/day) or vehicle implemented in their drinking water, for a total of 8 weeks	-Diabetes was associated with a much worse mortality rate. -Diabetic animals had exacerbated neurological scores and cognitive deficits, and a much slower rate of improvement throughout the study. -Diabetes seems to double the cognitive decline after stroke as compared to control rats. -Considering 33% as a cut-off point for cognitive decline, rats in the diabetic group had a 67% decline (diabetic

exerts its favorable cognitive outcomes, including neuroinflammatory response	Animals were 12 – 15 weeks old 60 minutes tMCAO or SHAM surgeries Inclusion criteria: assessment of sensorimotor deficit and weight loss at day 3 Above 30 seconds in ART	Follow-up: 8 weeks	SHAM), which further worsen to a 100% decline after stroke (Diabetic stroke). -There was drastic up-regulation of microglia/macrophages (IBA-1) and astrocyte (GFAP) markers in the diabetic group after stroke. Further analysis revealed it was mainly due to the increase in macrophages not microglia Diabetes was associated with significantly less myelination. -Diabetic animals treated with C21 had 0% mortality as compared to 55% in the vehicle-treated group. -Within a week after stroke, C21 treated groups exhibited a better sensorimotor performance in both control and diabetic animals and continued throughout the study. -Delayed C21 administration was associated with preserved brain volume in both diabetic

	test and more than 11% weight reduction on day 3		and control animals, while enhanced myelination was only observed in diabetic animals. -Diabetic rats treated with C21 exhibited a profound improvement in cognition (week 1 throughout 8). This effect was not observed in the control group where all animals, regardless of their treatment assignment, spontaneously recovered The development of cognitive impairment was reduced from 100% to 0% in diabetic animals treated with C21, where none of these animals had any cognitive impairment at post-stroke week 8 C21 skewed the activated microglia into an anti-inflammatory phenotype by modulating the M1:M2 ratio. -The M1:M2 ratio was downregulated after delayed and chronic administration of C21 in the ipsilateral

				hemisphere of diabetic animals. -Further in-vitro analysis in BV2 cells confirmed the same findings, seemingly independent of AT2R stimulation.
Jackson et al. 2021 [107]	This study hypothesizes that "administration of C21 at 3 days post-stroke improves long-term stroke outcomes in diabetic female rats through modulation of microglia/macrophage polarization toward a more anti-inflammatory profile"	Female diabetic Wistar rats (14 weeks old) 60-minute tMCAO or SHAM surgeries	Treatment was randomized at post-stroke day 3 to avoid the neuroprotection window of intervention At day 3 post-surgery, rats were randomized to receive either C21 (0.12 mg/kg/day) or vehicle implemented in their drinking water, for a total of 4 weeks Follow-up: 4 weeks	-C21 significantly improved survival starting from week 1 through week 4, C21-treated group had no mortality after treatment randomization C21 improved fine sensorimotor function after stroke. -C21 modulated neuroinflammatory response after stroke via decreasing the M1:M2 ratio toward an anti-inflammatory phenotype. -Further analysis revealed that C21 treatment decrease both M1-like microglia and macrophages. -C21 did not decrease the number of microglia or astrocyte in the ipsilateral hemisphere.

				-Microglial in-vitro analysis further confirms that C21 decreases the M1:M2 ratio, mainly via decreasing the proinflammatory phenotype. -Interestingly, unlike what we observed in male diabetic rats, C21 had no effect in altering cognitive function after stroke.
Eldahshan et al. 2021 [121]	To determine the impact of AT2R stimulation, via compound 21, on the development of post-stroke cognitive impairment after stroke in ovariectomized (OVX) spontaneously hypertensive rats (SHRs)	Young female SHRs (3 months old) Animals were ovariectomized, mimicking the extended period without estrogen that is likely to worsen	24 hours after stroke, rats were randomized to receive either C21 or saline for 6 weeks C21 treatment was administered via i.p. (0.03 mg/kg/day) for 5 days, and then switched to 0.12 mg/kg/day administered orally for the rest of the study Follow-up: 6 weeks	-Delayed C21 treatment did not improve sensorimotor deficits after stroke Stroke induced anxiety-like behavior in OVX female rats, with no apparent effect of C21 treatment. -C21-treated animals had a preserved aversive reference and spatial memory after stroke C21- and saline-treated groups had a similar infarct size at 6 weeks after stroke There were no white matter hyperintensities after stroke. -Stroke induced a significant increase in ventricular volume in the ipsilateral hemisphere

		cognitive impairment in females		of both C21- and saline-treated animals, along with a decrease in the ipsilateral volume, indicating brain atrophy at 6 weeks after stroke. -microCT angiography revealed a significant increase in vessel separation after stroke, this was abolished after C21 treatment, indicating an increased in the collateral blood flow. -C21 had no effect in altering vascular density or inflammatory response. - This study concludes that "Compound 21 could be beneficial in preventing PSCI in postmenopausal women, specifically in domains related to spatial awareness and memory" likely via improving vascular protection and restoration
Fouda et al. 2022 [91]	To determine the expression of AT2R after	Male Wistar rats	**Experiment 1:** The effect of AT1R blockade on stroke	-A single dose of candesartan reduced BP after stroke in GK diabetic rats, however, unlike

stroke in type 2 diabetic GK rats and control Wistar, and to assess the role of contralesional AT2R in recovery after stroke	GK type 2 diabetic rats	recovery and the expression of AT1R and AT2R in GD type 2 diabetic rats 3 hours tMCAO Follow-up: 24 hours Treatment: At reperfusion, animals received a single dose of candesartan (1mg/kg) or saline via IV tail vein injections. **Experiment 2:** The effect of unilateral AT2R knockdown (contralateral hemisphere) on stroke recovery in GK diabetic rats treated with C21 ShRNA lentiviral particles against AT2R	previously published studies in normotensive and normoglycemic Wistar rats, Candesartan failed to bring it down to pre-stroke BP level Also, candesartan failed to achieve a favorable functional outcome, as well as no superiority at the molecular level. Suggesting that candesartan had no favorable outcomes in post-stroke recovery in GK diabetic rats, possibly due to an impaired sensitivity to angiotensin blockade. -In GK diabetic animals, there was a reduction in the expression of AT2R after stroke, and candesartan failed to promote the expression of AT2R, unlike Wistar rats where the expression of AT2R increased after stroke and further augmented with candesartan treatment. -On the other hand, contralateral deletion of AT2R

		(AT2R knockdown in contralateral hemisphere) 90 minutes tMCAO Follow-up: 10 days Treatment: At reperfusion, animals received a single dose of C21 (0.03 mg/kg) or saline via i.p., and then daily for 7 days	resulted in worsened recovery after stroke and C21 treatment, as it was assessed from day 7 throughout 10. -This study concludes that "Contralesional AT2R may be involved in C21 mediated functional recovery after stroke and suggest that the contralesional hemisphere plays an important role in brain healing after stroke".

Specific Aims:

All these studies highlight the promise of the renin-angiotensin system as a potential therapeutic target for PSCI. However, more evidence is needed to overcome the barriers to clinical translation. We, therefore, chose an animal model with comorbid conditions replicating those seen in humans at risk of vascular dementia. In the current study, we are the first to investigate the development of vascular cognitive impairment (VCI) in 18-month-old spontaneously hypertensive rats (SHRs) prior to and following minor stroke. This study aims to explore the significance of delayed-chronic stimulation of AT2 receptor in preventing the development of long-term PSCI in an aged hypertensive population. Ultimately, this will improve the translatability of our previous findings and open venues for long-term treatment strategies after stroke. Our ***central hypothesis*** is that chronic and delayed stimulation of the AT2 receptor, via C21, improves post-stroke long-term cognitive impairment by a mechanism involving neuroinflammation.

The specific aims of this are as follows:

1. **To determine the impact of chronic stimulation of the AT2 receptor, via delayed administration of C21, on long-term post-stroke recovery in *aged hypertensive rats*.**
    - *1.1.1. To evaluate the ability of aged hypertensive rats to serve as a suitable model to study vascular cognitive impairment.*
    - *1.1.2. To assess the development of post-stroke cognitive and sensorimotor deficits in aged hypertensive rats after a minor stroke.*

1.1.3. To study the impact of angiotensin II type 2 receptor activation on post-stroke functional recovery, changes at the molecular level, as well as brain structure.

For this aim, we will utilize the transient middle cerebral artery occlusion (tMCAO) model to investigate the long-term impact of chronic and delayed treatment with C21 on post-stroke sensorimotor and cognitive function in aged hypertensive rats. To generate a model that is able to capture the complex interaction between aging and hypertension, young spontaneously hypertensive rats (SHRs) will be maintained up until the age of 18 months. At 18 months old, rats will be randomized to undergo either, 30 min tMCAO or SHAM surgeries. Then, at day 3, rats will be randomized to receive either, C21 (0.12mg/kg/d) or vehicle incorporated in their drinking water and adjusted based on their daily consumption and body weight.

2. To investigate the impact of C21 on the polarization status of microglia/macrophages in an in-vitro setting.

2.1. To determine the direct effect of C21 on the polarization status of mouse microglia cells (C8B4):

2.1.1. Pro-inflammatory gene expression analysis by real-time qPCR.

2.1.2. Realtime qPCR analysis of neuroprotective genes.

2.1.3. Analysis of pro-inflammatory cytokines/chemokines by ELISA.

2.1.4. Generation of reactive oxygen species and free radicals.

2.2. To determine the direct effect of C21 on the polarization status of mouse RAW264.7 macrophages:

2.2.1. Pro-inflammatory gene expression analysis by real-time qPCR.

2.2.2. Realtime qPCR analysis of neuroprotective genes.

2.2.3. Analysis of pro-inflammatory cytokines/chemokines by ELISA.

2.2.4. Generation of reactive oxygen species and free radicals.

2.3. To examine the crosstalk between macrophages and microglia; Macrophages to microglia communication: Via assessing the ability of C21-treated macrophages to alter the polarization status of microglia; using conditioned media concentrates.

2.3.1. Pro-inflammatory gene expression analysis by real-time qPCR.

2.3.2. Realtime qPCR analysis of neuroprotective genes.

2.3.3. Analysis of pro-inflammatory cytokines/chemokines by ELISA.

2.3.4. Generation of reactive oxygen species and free radicals.

For this aim, we will use an in-vitro model of the inflammatory response, the LPS-induction model, that allows us to investigate the potential effects of C21 in reversing the inflammatory response in macrophage/microglial cells. C21 effects on the inflammatory response will be tested in a dose-dependent manner in both cell lines. The inflammatory response will be assessed at multiple levels, the expression of pro-inflammatory genes and protein as well as the generation of free radicals, to ensure that different aspects of neuroinflammation are addressed. Compound 21 is thought to be blood-brain barrier impermeable, hence, we will test the potential for the macrophage to microglia communication by examining the effect of C21-treated macrophages on the microglial inflammatory response, suggesting that various stimuli might change the phenotypic activation of macrophages and that subsequently affects the microglial response.

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CHAPTER 2

MINOR STROKE ACCELERATES THE DEVELOPMENT OF VASCULAR COGNITIVE IMPAIRMENT IN AGED HYPERTENSIVE RATS [1]

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Abstract

Hypertension and aging are leading risk factors for stroke and cognitive decline. Most animal models fail to capture the complex interplay between these pathophysiologic processes. In the current study, we examined the development of cognitive impairment in 18-month-old spontaneously hypertensive rats (SHRs) before and following a 30-minute transient middle cerebral artery occlusion (tMCAO) or SHAM surgery. Sixty SHRs were housed (in pairs) for 18 months with cognitive assessments every six months and post-surgery. Multiple MRI scans were performed at baseline and throughout the study. At day 3 after stroke, rats were randomized to receive either an angiotensin receptor agonist, C21, or plain drinking water and followed up for 8 weeks. Results: SHRs demonstrated a progressive cognitive decline and significant abnormalities on MRI prior to stroke, and low peri-operative mortality within 72 hours of tMCAO. Minor stroke resulted in sustained sensorimotor deficits and exacerbated the progressive cognitive decline. There was no evidence of anhedonia at 8 weeks. C21 enhanced sensorimotor recovery and ischemic lesion resolution by MRI at week 8. Stroke induced significant brain swelling at day 3 and brain atrophy at post-stroke week 8. ***Conclusions:*** Minor stroke accelerated the progression of cognitive decline, resulted in sustained sensorimotor deficits, and was associated with brain edema and atrophy in aged hypertensive rats. Angiotensin modulation improved outcomes, even when initiated at 3 days after stroke.

Keywords

Aging, Compound 21, Hypertension, Stroke, Vascular cognitive impairment/dementia (VCID),

Introduction

Stroke is the fifth leading cause of death in the United States, indicated in ≈1 of every 19 deaths.[1] The high incidence of stroke, coupled with improvements in stroke care, has led to a recent decrease in stroke mortality and an increase in stroke-induced disability.[2, 3] Stroke survivors have an increased risk of developing a long-term disability, including sensorimotor and cognitive deficits.[4] PSCI causes extensive reductions in quality of life and increases the burden of care, with many patients progressing to dementia.[5, 6]

Hypertension and aging are major contributing factors to vascular dementia, stroke, and the development of PSCI.[7-9] [10, 11] Mid-life hypertension is strongly associated with late-life vascular dementia, cognitive decline, and Alzheimer's disease.[12-15] Post-stroke cognitive impairment develops progressively after stroke and is proportionally associated with age.[7, 9] Preclinical models demonstrated that chronic hypertension and advanced age substantially accelerated the development of cognitive decline and hippocampal neurodegeneration after stroke.[16] [17-21]

The brain renin-angiotensin system (RAS) is involved in the pathogenesis of stroke.[22-38] Studies have demonstrated that compound 21 (C21), the first selective non-peptide AT2R agonist, provides a neurovascular protective effect and enhances sustained functional improvement after stroke.[23, 34, 37-50] Our recent studies were focused on validating the impact of C21/Candesartan in severely co-morbid models of stroke, including aging, diabetes, and hypertension.[23, 37, 41-43] Notably, these animals exhibited a much worse functional recovery and high mortality after stroke. RAS modulation was an effective strategy to preserve cognitive function, even when initiated 3 days after stroke.[23, 37, 41-43] This is in contrast to most post-stroke clinical interventions where treatment is initiated

acutely after stroke and discontinued thereafter, missing an opportunity of improved long-term outcomes. The delayed intervention approach has been replicated and shown to be effective in delaying the incidence of PSCI.[23, 37, 41-43]

Most preclinical stroke research failed to include aging and hypertension, limiting the translation of preclinical studies.[51] In the current study, we aimed to investigate the development of VCID in 18-month-old spontaneously hypertensive rats (SHRs) prior to and following a minor stroke. We hypothesize that the development of vascular cognitive impairment in hypertensive rats will progressively evolve, and stroke will accelerate the progression of PSCI. This study is also designed to explore the significance of delayed-chronic stimulation of the AT2 receptor in preventing the development of long-term PSCI in an aged hypertensive population.

Material and methods

Ethical approval

All experiments were performed following the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood Veterans Affairs Health Care System, and Augusta University, Augusta, Georgia.

Experimental design

A total of 60 young hypertensive rats (7-week-old SHRs) were obtained from Charles River laboratories. Rats were double housed in a pathogen-free, temperature-controlled environment with free access to food and water, and on a 12:12-hours dark: light cycle. The experimental design and flowchart are shown in Figure 1.1 and Supplementary Figure 1.1. Rats were maintained up until the age of 18 months. During the aging process, body

weight and cognitive function were monitored. 18-month-old rats were subjected to either, 30-min tMCAO or sham surgery. At post-surgery day 3, rats were randomized to blindly receive either an oral dose of C21 (0.12 mg/kg/day; incorporated in drinking water) or plain drinking water. Treatment was initiated on day 3 and continued for a total of 8 weeks. A series of sensorimotor and cognitive assessments, and MRI scans were performed at baseline and throughout the study (Figure 1.1 and Supplementary Figure 1).

Stroke model

Stroke was induced via a transient middle cerebral artery occlusion (tMCAO). 18-month-old SHRs, averaging around 382 g, were subjected to a 30-min tMCAO surgery using silicon-coated nylon suture (Doccol 404356). Thirty-minute tMCAO was chosen to reduce mortality in these vulnerable animals. As previously described[28, 52], animals were anesthetized using 2 – 5 % isoflurane, and then a ventral midline incision was made. After that, the right common carotid artery was exposed, and the external carotid artery was ligated and cut. Afterward, the suture was inserted through a small nick at the external carotid artery into the internal carotid artery until a mild resistance was encountered, which indicates the branching of the anterior and middle cerebral artery. The suture was tied off with a knot once appropriately placed. Considering the short occlusion time, rats were kept under anesthesia for the whole occlusion time. Then, the suture was removed to allow reperfusion, and the external carotid artery was permanently ligated. In sham surgery, the common carotid artery was isolated and manipulated without cutting or inserting a suture.

Occlusion time justification

In our previous studies[52, 53], we successfully established a 60 min tMCAO model with high survivability and sufficient neurological deficits in young SHRs. However, in these

vulnerable animals, we thought opting for a shorter occlusion time would translate into better survivability. It has been reported that occlusion times longer than 22-min consistently resulted in adequate lesion size in young SHRs.[54] A separate cohort of 5 rats was used to validate the use of 30-min occlusion time, suture size, and surgical condition in 18-month-old hypertensive rats.

Dose justification

The dose was adopted from our previous studies.[37, 41-44] Previously, we studied C21 in two different routes of administration, i.p. (0.03 mg/kg/day) or an equivalent oral dose (0.12 mg/kg/day) based on oral bioavailability of 20 - 30% and estimated half-life of 4 hours in rats.[55] These doses were shown to have beneficial effects on cognition in experimental stroke in rats.[37, 41-44] Oral treatment with C21 was initiated on day 3 after stroke to avoid the window of acute neuroprotection.

Blinding and randomization

Treatments were administered in a randomized-blinded manner. Treatment bottles were prepared by an individual not involved in the surgical procedure or behavioral assessments. Rats were numbered upon arrival and maintained the same number throughout the study. All behavioral analyses were performed/analyzed by a blinded investigator.

Power analysis

Power analysis was based on the 8-week recognition index in which aged SHRs were subjected to chronic hypoperfusion and treated with vehicle or C21. It was predicted that 10/group would provide at least 95% power to detect a difference between groups (saline vs. C21) for aged SHRs (0.30±0.15 vs. 0.60±0.15, respectively).

Inclusion criteria

Visible ischemic lesion at day 3 MRI scans was used as a pre-set inclusion criterion to ensure only animals with a degree of ischemic injury are included in the analysis. Animals with severe cognitive decline at 12-month assessment (preference index <50%) were excluded from the analysis; to establish a stable baseline prior to surgery.

Assessment of functional outcome

Body weight. Weight monitoring is an exceedingly helpful tool to assess animals' health and welfare. Body weight was recorded monthly throughout the aging process, daily after surgery, and weekly afterward. All rats were provided with a supplementary Nutra-Gel Complete Nutrition (Bio-Serv®) to support their recovery, and Ringer's lactate solution if necessary.

Blood pressure measurement. A separate cohort of aged SHRs was implanted with a telemetry transmitter to record their arterial pressure throughout 14 days. Arterial pressure was monitored in 10-minute intervals. 7-day-average arterial pressure was used to assess their mean arterial pressure (MAP), as well as their circadian rhythm.

Behavioral assessment. A diverse set of neurobehavioral tests were performed, recorded, and analyzed in a blinded fashion (Supplementary Figure 1). Neurobehavior analyses were designed to evaluate sensorimotor function (modified neuro-severity score (mNSS), beam walk), non-spatial working memory (Novel object recognition test-NOR), spatial memory (Y-maze spontaneous alteration test), associative learning and reference memory (Passive avoidance test-PAT), locomotor activity and anxiety-like behavior (Open field-OF), and depression-related behavior (Sucrose preference test).

Assessment of sensorimotor function. Sensorimotor function was evaluated via mNSS, adopted from Yu et al.[56], and beam walk tests on days 1,7,14, and weeks 5, and 8.

Modified neurological severity score (mNSS) is a 14-point neurological test that is designed to test sensory and motor function, abnormal movement patterns, absence of reflexes, and beam balance. Each domain is assigned a certain score that reflects its neurological status with lower scores indicating better performance. A maximum of 14 points indicates a severe neurological deficit.[56]

Beam walk test was performed to assess body balance and motor coordination after surgery. Briefly, rats were placed on an elevated beam and allowed to move across the beam toward the narrow end for 60 seconds. Latency to cross the beam was used as an endpoint to evaluate motor function. A maximum score of 60 seconds was assigned in case of inability to cross the beam.

Cognitive function assessments

NOR. NOR test was performed, as previously reported[37, 41, 52], to evaluate non-spatial working memory related to both cortical and hippocampal damage.[57-59] NOR relies on the natural tendency of rats to explore a novel object in their environment. Rats were permitted to habituate to the testing environment for a total of 10 min for three consecutive days. In the familiarization trial, rats were allowed to explore two identical objects placed in the testing box for a total of 10 min, and then returned to their cages for a retention period. In the preference trial, rats were exposed to two different objects, one from the previous trial and a novel object, for a total of 5 minutes. The time spent exploring each object was noted and used to calculate the preference index, time spent exploring the novel object divided by the total exploration time. A minimum of 20 seconds total exploration time is required.

PAT. The passive avoidance test is a fear-aggravated behavior test and was performed to assess associative learning and reference memory. It involves two trials: an acquisition trial, when animals are exposed to an aversive stimulus, and a test trial, to check their latency to enter the arm where an aversive stimulus was previously introduced. The test apparatus was divided into two equal chambers with a shock grid floor. The activity was automatically monitored by 16 photobeams. In the acquisition trial, rats were individually placed into the "shock-free" arm, with the door separating the two chambers closed. After a 60-second habituation period, the door leading to the shock compartment was raised, allowing the animal to enter and explore the dark compartment. Once the animal had fully entered the shock arm, the door immediately lowered, and a brief foot shock was delivered through the grid floor for 2.0 seconds. Twenty-four hours later, the test trial was performed in the same manner as the acquisition trial except that no shock was delivered. Animals were allowed to explore the compartments for a total of 300 seconds and their latency to enter the shock arm was noted and used as an indication of memory consolidation. A shorter latency to enter the shock arm indicates a long-term memory deficit, while a longer latency suggests better memory. Those who did not cross over to the dark compartment were assigned a latency of 300 seconds.

Spontaneous alternation test. The Y-maze spontaneous alteration test assesses spatial working memory, and it relies on the natural tendency of rats to explore a novel environment. Rats were introduced to the center of a Y-shaped maze and allowed to explore all three arms freely for a total of 7 minutes. Cognitively intact rats tend to explore the least recently visited arm and alternate their entries between all three arms. An entry occurs when all four limbs are within the arm. ANY-maze software was used to video record and

automatically detect the total number of arm entries, and the number of triads. Alterations are described as successful entries into all three arms in consecutive triplet sets (i.e., ABC, ACB, BAC, BCA, etc.). The percentage of spontaneous alternation is defined as the ratio of actual alternation to possible alternation. The formula is described as follows,

$$\%\ of\ Alternation = \frac{Total\ Alternations}{(Total\ arm\ entries - 2)} * 100$$

Open field test. The open field test was performed to evaluate anxiety and animals' exploratory behavior. Rodents tend to have an aversion toward open areas. Typically, an anxious animal would spend more time in the periphery, while less anxious animals will travel evenly in both areas. Rats were individually placed into the open field activity monitors (43.2 X 43.2 cm, Med Associates St. Albans, VT) and all behavioral events were controlled using Med-Associates software. Horizontal distance traveled (cm) was recorded during a 30-min test session. Total distance traveled, and distance traveled across the peripheral zone and central zone were analyzed as an indicator of locomotor activity and anxiety-like behavior.

Sucrose preference test. In our prior work with young hypertensive animals, we revealed significant anhedonia development in our singly housed animals, leading to us utilizing double housing for the duration of this study. To test whether this tactic was effective, sucrose preference test was performed at week 8 to assess anhedonia and depression-related behavior. Briefly, animals were separated apart from their partners and provided with a 1.5% sucrose solution for habituation. Twenty-four hours later, rats were presented with 2 bottles, their normal drinking water, and 1.5% sucrose solution for 24 hours. The positions of these two bottles were switched halfway to eliminate side bias. Water and sucrose intake were measured and used to calculate sucrose preference over water. Rodents

show a preference toward sweetened solutions or food, while those with anhedonia, a key sign of depression, lose that interest.

Magnetic resonance imaging (MRI)

MRI scans were performed to assess the pathological structural changes in the brain. All rats underwent two types of MRI scans, T2-weighted (T2WI) and fluid-attenuated inversion recovery (FLAIR) scans. All MRI scans were performed at the Augusta University Cancer Center by the Core Imaging Facility for Small Animals (CIFSA). Total brain and hemispheric ventricular volume, and ischemic lesion volume were evaluated using Mango multi-image analysis software. For each brain scan, 35 slices were obtained with a thickness of 0.8 mm for each slice.

Statistical analysis

GraphPad Prism 9 software was used for statistical analyses. All data were presented as mean ± SD. Data generated for two factors (two surgeries and two treatments) were analyzed using 2-way ANOVA. Data generated for three factors (two surgeries, two treatments, and multiple time-point) were analyzed using 3-way ANOVA. Two groups comparison (6- vs 12-months, stroke vs sham, vehicle vs C21) was performed using unpaired t-test for variables with normal distribution, or by pairwise comparisons using Mann-Whitney U tests for variables without normal distribution. Data distribution was evaluated via the Shapiro-Wilk test. Data were considered significant at a Type I error rate of 5%.

Results

Aged hypertensive rats demonstrate preserved overall health, brain pathology, and progressive cognitive decline consistent with VCID

Aged-SHRs maintained good health and showed a continuous weight gain over the course of 18 months (Figure 1.3(a)). Aged hypertensive rats illustrated a high level of survivability throughout the aging process, with a survival rate of 86.7% (52 out of 60 rats) (Figure 1.1). Cognitive status was evaluated at 6- and 12 months via NOR. Rats showed no sign of cognitive impairment at 6 months, with an average preference index of 75.7%. (Figure 1.3(b)) The average preference index dropped to 66% at 12 months of age (Figure 1.3(b)). This significant decrease suggests progressive cognitive decline.

A 50% preference index was used as a cut-off point between cognitively intact and impaired rats. At 6 months, all rats showed a preference index of > 50%. However, at 12 months, 30% of rats had a preference index of ≤ 50%, and more than 20% were excluded due to the lack of exploratory activity (Figure 1.3(b) and Supplementary Figure 1.2(d)).

18-month-old hypertensive rats displayed a high incidence of brain pathology and evidence of significant abnormalities consistent with vascular cognitive impairment. The most prevalent abnormality was enlarged ventricles (72%) followed by hematoma, old ischemic lesion, white matter hyperintensities, and hydrocephalus (Figure 1.3(c)).

Aged hypertensive rats demonstrate a normal circadian rhythm despite severely elevated mean arterial pressure

Aged-SHRs displayed an elevated MAP that ranges between 177 – 183 mm Hg. Despite their elevated arterial pressure, aged hypertensive animals retained a normal circadian rhythm during the dark/light cycle (Figure 1.3(d)).

Aged hypertensive rats are able to survive a minor stroke

There was no intraoperative mortality, but one rat died under anesthesia before undergoing either surgery. Mortality within 72 hours was 14% in the tMCAO group (4 rats out of 27) and 0% in the sham group. We anticipated a high mortality rate after surgery; however, overall survivability was better than expected. In the tMCAO-operated group, the survival rate to week 8 was 100% and 88.9% in C21- and vehicle-treated groups, respectively (Figure 1.2(a)). In the sham-operated-rats, the probability of survival was 75% in the C21 group and 88.9% in the control group (Figure 1.2(a)).

Minor stroke causes an acute and sustained sensorimotor deficit, which was ameliorated with C21 treatment

Animals subjected to tMCAO showed a significant sensorimotor deficit 24 hours after stroke (Figure 1.2(b,d)). Rats in the control group had a prolonged sensorimotor deficit throughout the study (Figure 1.2(c,e)). Delayed C21 treatment was associated with a significant improvement in mNSS (Figure 1.2(c)), but not in the beam walk test (Figure 1.2(e))

Stroke induces brain edema at day 3 and chronic brain atrophy in the ipsilateral hemisphere at week 8

MRI analyses revealed a significant reduction in the brain ventricular volume after stroke in both hemispheres at day 3 (Figure 1.4(a-c). This was prior to treatment being initiated and indicates acute cerebral edema in response to ischemic injury. There was a significant increase in the ipsilateral but not contralateral ventricular volume at week 8, evidence of chronic brain atrophy (Figure 1.4(d-f)).

C21 improves ischemic resolution at the chronic phase after stroke

C21 resulted in a large reduction of more than 70% at post-stroke week 8, as compared to around 44% in the control group (Figure 1.5). The reduction was assessed as a percent reduction relative to lesion volume at post-stroke day 3 (Figure 1.5(a)).

Minor stroke exacerbates the progression of cognitive decline in aged hypertensive rats

Passive avoidance test, performed at week 5, demonstrated a trend for the interaction between surgery and treatment, but no significant difference was detected (2-way ANOVA, *p*-value=0.0642). After excluding those with severe cognitive decline prior to surgery, we observed a significant interaction between surgery and treatment (2-way ANOVA, *p*-value =0.0298) (Figure 1.6(a)). A post-hoc Dunn's multiple comparisons test revealed a significant reduction in the latency to cross the shock arm in the stroke group (*p*-value=0.0437). Indicating that minor stroke exacerbated the cognitive decline at post-surgery week 5. There was no significant difference between treatment and control (*p*-value =0.3158).

Rats exhibited cognitive deficits that could not be captured via NOR and Y-maze. At the pre-surgery analysis, we established a stable baseline by reducing the retention time to 30 minutes instead of 1 hour in NOR. 30% of the rats were excluded from the analysis for not meeting the minimum total exploration time of 20 seconds, and the remainder had ≥ 50% preference index (Supplementary Figure 2(a,d). The lack of exploratory activity progressively worsened after stroke and more rats were excluded in post-stroke week 5 and 8 analyses. Almost 48% and 70% of rats were excluded at post-stroke weeks 5 and 8, respectively (Supplementary Figure 2(b-d)). Similarly, the Y-maze alternation test

revealed a wide range of variability with more rats within or less than 50% alternation at baseline and post-stroke week 4 (Figure 1.6(b-d)).

Alternatively, we analyze NOR data using total exploration time instead of preference index. There was a continuous deterioration in total exploration time as they aged (Figure 1.6(e)). After surgery, the relative reduction was 36% in the sham group and 55% in the stroke group (Figure 1.6(f)). C21-treated animals displayed more interest in exploring their surroundings at week 5 (8% relative reduction vs 23%) and week 8 (35% relative reduction vs 55%) (Figure 1.6(f)). 3way-ANOVA analysis revealed significant effects of time and treatment; *p*-value=0.0002 and 0.0205, respectively. There was no significant difference between stroke and sham (*p*-value=0.1024).

Aged hypertensive rats exhibit significant hyperactivity after stroke, with no indication of anxiety-like behavior

Gross locomotor activity and anxiety-like behavior were evaluated via open field test at week 5. Animals in the tMCAO group significantly covered more distance in the open field compared to the control group. Neither the treatment group nor the interaction between surgery and treatment showed any difference (2-way ANOVA *p*-value=0.0131 for surgery) (Figure 1.7(a-c)). Further analysis demonstrated that all rats traveled comparable distances in the inner, and outer zone, indicating no signs of anxiety at week 5 (Figure 1.7(b-c)).

Aged hypertensive rats do not display depression-related behavior at the chronic phase after stroke

Sucrose preference test was performed at week 8 to assess anhedonia and depression-related behavior. All animals, regardless of their treatment or surgery assignment, showed

an interest to consume more sucrose solution than water, indicative of no depression-like symptoms (Figure 1.7(d)).

Discussion

Animal models of stroke typically failed to include major comorbidities such as advanced age and hypertension, which likely contributed to a consistent lack of translation into clinical care. It has been reported that only 11.4% of preclinical studies of stroke included advanced age and/or other comorbidities.[51] These comorbidities have been overlooked due to uncertain mortality and increased cost.[60] Ignoring these detrimental risk factors led to huge inconsistencies between clinical and preclinical studies. In the current study, to the best of our knowledge, we are the first to report the development of VCID in 18-month-old SHRs prior to and following a 30-minute tMCAO.

Aged hypertensive rats displayed a progressive cognitive decline, brain atrophy, and abnormalities that are consistent with VCID. Rats started to show signs of cognitive decline as early as 12 months, and progressively worsened thereafter. Brain pathological changes were present in most animals. Enlarged ventricular volume was the most prevalent abnormality. Hypertension and/or advanced age are well-known to be contributors to brain atrophy in patients.[61] There is an association between white matter lesions and a regional pattern of gray matter atrophy, which was exacerbated by aging and reduced by blood pressure control.[61] Enlarged ventricular volume and brain atrophy were observed in older individuals, and in those with elevated systolic blood pressure.[62] This agrees with our findings where most aged hypertensive animals exhibited brain atrophy and early cognitive

decline as early as 12 months old, further validating the clinical relevance of our model to study VCID and the development of PSCI.

In this study, we had a relatively low mortality rate after surgery. We previously reported a higher mortality rate after stroke in diabetic animals as well as young hypertensive animals.[42, 52] High mortality rate in aged animals following stroke was observed in several other studies as well.[63-66] In the current study, we reported no intraoperative mortality as well as low overall mortality in the 8-week follow-up. This is likely due to the reduction in occlusion time from 60 min to 30 min. A 30-min tMCAO effectively produced a visible ischemic lesion in day 3 MRI, as well as significant sensorimotor deficit 24h after stroke. Improved survivability in aged hypertensive rats after stroke is an encouraging finding to move preclinical studies toward a more relevant model of stroke.

Minor stroke has resulted in acute and prolonged sensorimotor deficit throughout the study. This uniquely differs from what has been previously reported in young hypertensive and aged normotensive animals, where they recovered to their baseline shortly after stroke.[37, 41] This is consistent with the manifestation of stroke in patients. The long-term sensorimotor deficit was profoundly blunted by the delayed-chronic stimulation of AT2R. MRI analysis revealed an acute ischemic lesion formation, cerebral edema, and long-term brain atrophy in the ipsilateral hemisphere after stroke. Delayed C21 treatment was associated with a 70% reduction in lesion volume at the chronic phase after stroke, and no effect on brain atrophy.

Aged hypertensive rats showed early signs of cognitive decline at 12 months. We anticipated that this cognitive decline progresses over time and deteriorates after stroke. The study was designed to investigate different aspects of cognition over multiple time

points. Long-term cognitive assessments were performed at post-stroke week-5 and beyond. Passive avoidance test demonstrated an impaired long-term reference memory at week 5. This impairment could not translate into measurable deficits in NOR and Y-maze tests due to the test limitation.

A major shortcoming of this study is the inability of our testing strategies to capture the severe cognitive decline in such a comorbid model. There was a distinct cognitive decline, some of which could not be captured using the standard assessment tools. In NOR, rats exhibited a lack of interest in exploring either object after stroke. The lack of interest in exploring objects could be explained, perhaps, by either depression or the lack of ambulation. However, these were ruled out since animals did not exhibit anhedonia in the sucrose preference test, and the open field test demonstrated stroke-induced hyperactivity. The percentage of animals excluded exponentially increased over time. It started at a slower rate, 1.7% and 22.8% at 6- and 12 months, respectively, and then increased steeply to 30.2%, 48%, and 70%, at pre-surgery baseline and post-surgery subsequent analysis. It has been previously suggested to increase the exploration time from 5 min to 10 min and keep scoring beyond 5 min until the total exploration time reaches 20 seconds.[67] However, we did not anticipate this lack of exploration in our animals and decided to keep parameters other than retention time static.

This extremely affected our ability to assess their cognitive decline after stroke and led us to adopt an alternative strategy. We decided to use total exploration time in spite of the time spent exploring the novel object as an indicative of cognitive decline. This strategy appeared to be effective in showing the progressive nature of cognitive decline in aged hypertensive rats over the course of their lifespan. There was a significant effect of

treatment in slowing the deterioration in total exploration time, even in the sham group, however, there was no significant difference between sham and stroke. Similarly, Y-maze test revealed a wide range of inconsistency with more rats within or less than 50% alternation at baseline and week 4. Two possibilities may explain the lack of interest in engaging with objects in NOR. First, the test is not intended to assess cognitive decline over time and probably is not sensitive enough to capture such a decline via repeated testing. Second, NOR and Y-maze were not designed to evaluate cognitive decline in severely comorbid models of stroke.

One of the most noteworthy findings in this study is the ability of C21 to mediate beneficial effects even when treatment was delayed. Chronic treatment with RAS modulators enhanced cognitive function and prevented the development of PSCI in hypertensive animals.[23] These effects were persistent even with delayed treatment, 7 days after stroke, and believed to be independent of blood pressure.[23] A similar pattern was observed in diabetic animals when treatment with C21 was initiated 72 hours after stroke and continued for 4 and 8 weeks.[42, 43] This study further corroborates these findings and points toward the importance of long-term treatment after stroke.

The evidence supporting the role of RAS modulation is mixed at this point and the scientific community has virtually abandoned this pathway as a viable stroke treatment. However, we believe strongly that more investigation is warranted. First, the focus should shift toward the development of post-stroke cognitive impairment as a target for later intervention after stroke. Second, chronic treatment after stroke, beyond the usual 5-7-day treatments studied in clinical trials, should be pursued to improve long-term outcomes. Focusing beyond the acute intervention after stroke could potentially be an alternative

strategy to enhance long-term functional recovery after stroke. A well-designed clinical trial addressing these important aspects of RAS modulation should target the later development of long-term PSCI.

In summary, we demonstrated that aged hypertensive rats developed a progressive cognitive decline, brain abnormalities, and high survivability pre- and post-stroke. Minor stroke resulted in acute-prolonged sensorimotor deficits, accelerated cognitive decline, cerebral edema, and brain atrophy. There was no evidence of post-stroke depression, likely due to double housing and maintaining the same partner throughout the study. Delayed stimulation of AT2R resulted in a sustained improvement in sensorimotor function and a significant reduction in lesion volume at the chronic phase after stroke. Classical cognitive assessment tools failed to capture the cognitive decline in aged hypertensive rats, and proper optimization are needed.

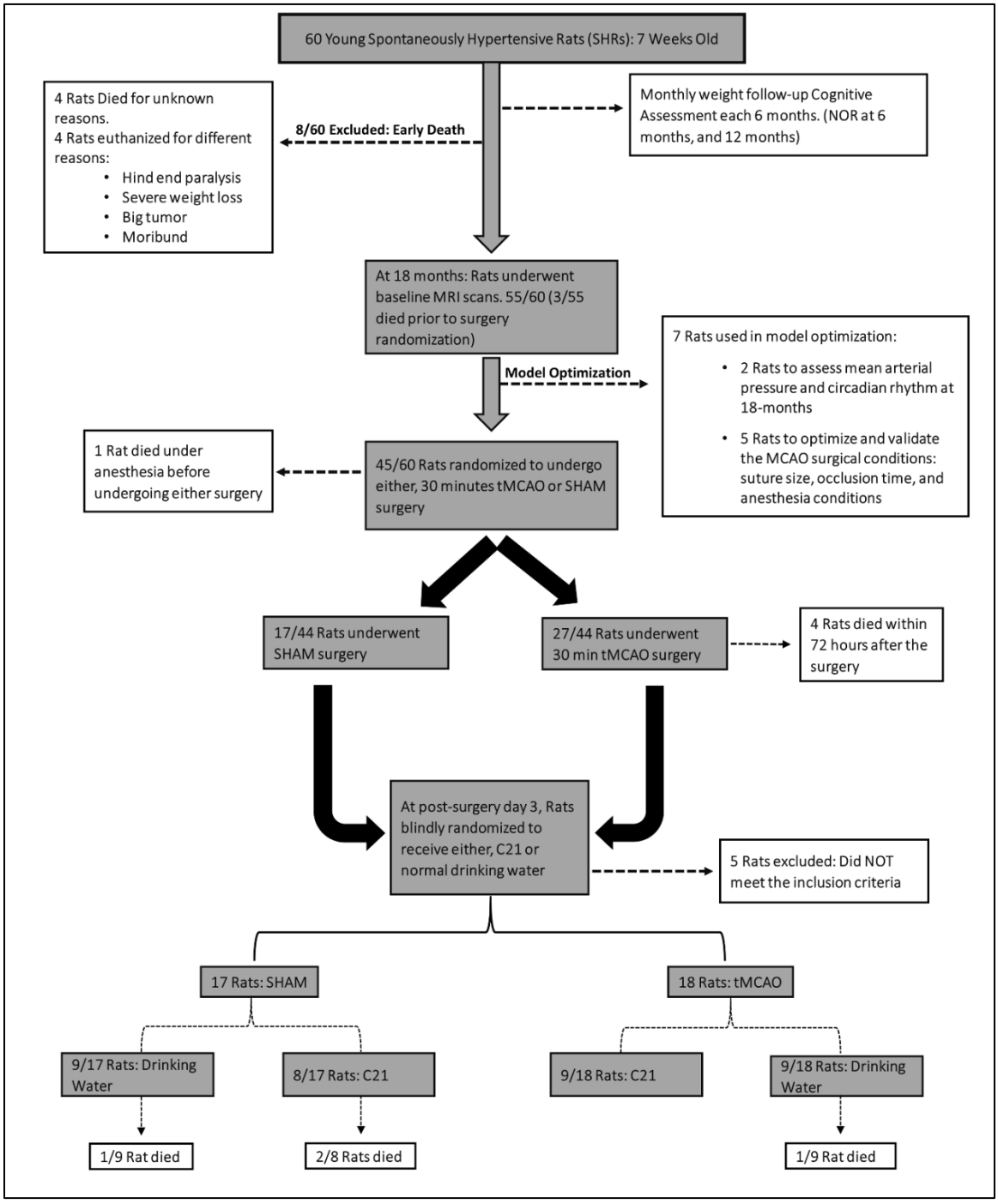


Figure 1.1: Overall study design flow chart.

Figure 1.2. Aged hypertensive rats demonstrated high survivability, and a prolonged sensorimotor deficit after minor stroke, which was ameliorated with C21 treatment. (a) Survival analysis of aged SHRs after a 30-min tMCAO over an 8-week follow-up. Survival proportions were 88% (1/8) in the vehicle-treated sham, 75% (2/6) in the C21-treated sham, 88.9% (1/8) in vehicle-treated tMCAO, and 100% (0/9) in C21-treated stroke; *p*-value= 0.4531. The acute sensorimotor deficit was assessed 24 hours after stroke via mNSS (n=16 in sham group; n=21 in tMCAO group); Mann Whitney test; *p*-value <0.0001 (b), and beam walk test (n=16 in sham group; n=21 in tMCAO group); Unpaired t-test; *p*-value=0.0035 (c). Long-term sensorimotor deficits, over 8 weeks, were observed at post-stroke days 1,7, weeks 2,5, and 8. Minor stroke caused an abrupt and persistent sensorimotor deficit in mNSS; 3way-ANOVA (*p*-value <0.0001 for surgery and time, and 0.0003 for treatment) (d), and beam walk test; 3way-ANOVA (*p*-value <0.0001 for surgery and time, and 0.2045 for treatment) (e).

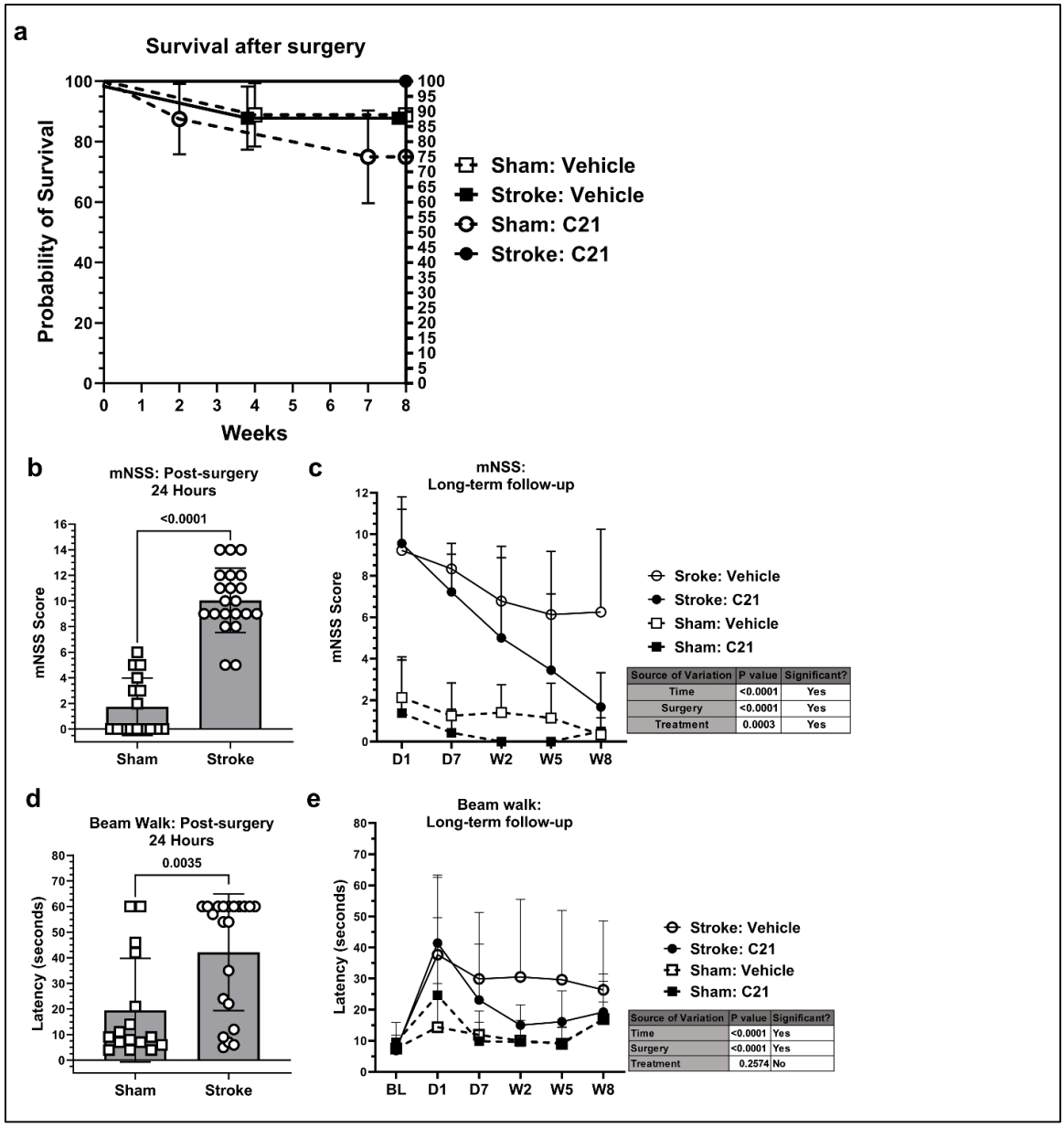

a
Survival after surgery
Probability of Survival
Weeks
Sham: Vehicle
Stroke: Vehicle
Sham: C21
Stroke: C21
b
mNSS: Post-surgery
24 Hours
<0.0001
mNSS Score
Sham
Stroke
c
mNSS:
Long-term follow-up
mNSS Score
D1
D7
W2
W5
W8
Sroke: Vehicle
Stroke: C21
Sham: Vehicle
Sham: C21
Source of Variation | P value | Significant?
Time | <0.0001 | Yes
Surgery | <0.0001 | Yes
Treatment | 0.0003 | Yes
d
Beam Walk: Post-surgery
24 Hours
0.0035
Latency (seconds)
Sham
Stroke
e
Beam walk:
Long-term follow-up
Latency (seconds)
BL
D1
D7
W2
W5
W8
Stroke: Vehicle
Stroke: C21
Sham: Vehicle
Sham: C21
Source of Variation | P value | Significant?
Time | <0.0001 | Yes
Surgery | <0.0001 | Yes
Treatment | 0.2574 | No

Figure 1.3. Aged hypertensive rats exhibited a progressive cognitive decline and brain abnormalities that are consistent with vascular cognitive impairment. (a) Average body weight (g) over an 18-month follow-up. (b) Preference index (%) in 2-object novel object recognition test at 6 months (n=59) versus 12 months (n=44). Unpaired t-test; *p*-value <0.0001. (c) Qualitative analysis of brain pathological changes at 18 months of age, assessed via brain MRI scans (n=55). (d) Representative of two light/dark cycles for 7-day average mean arterial pressure readings. Readings were monitored via a telemetry transmission device (n=2).

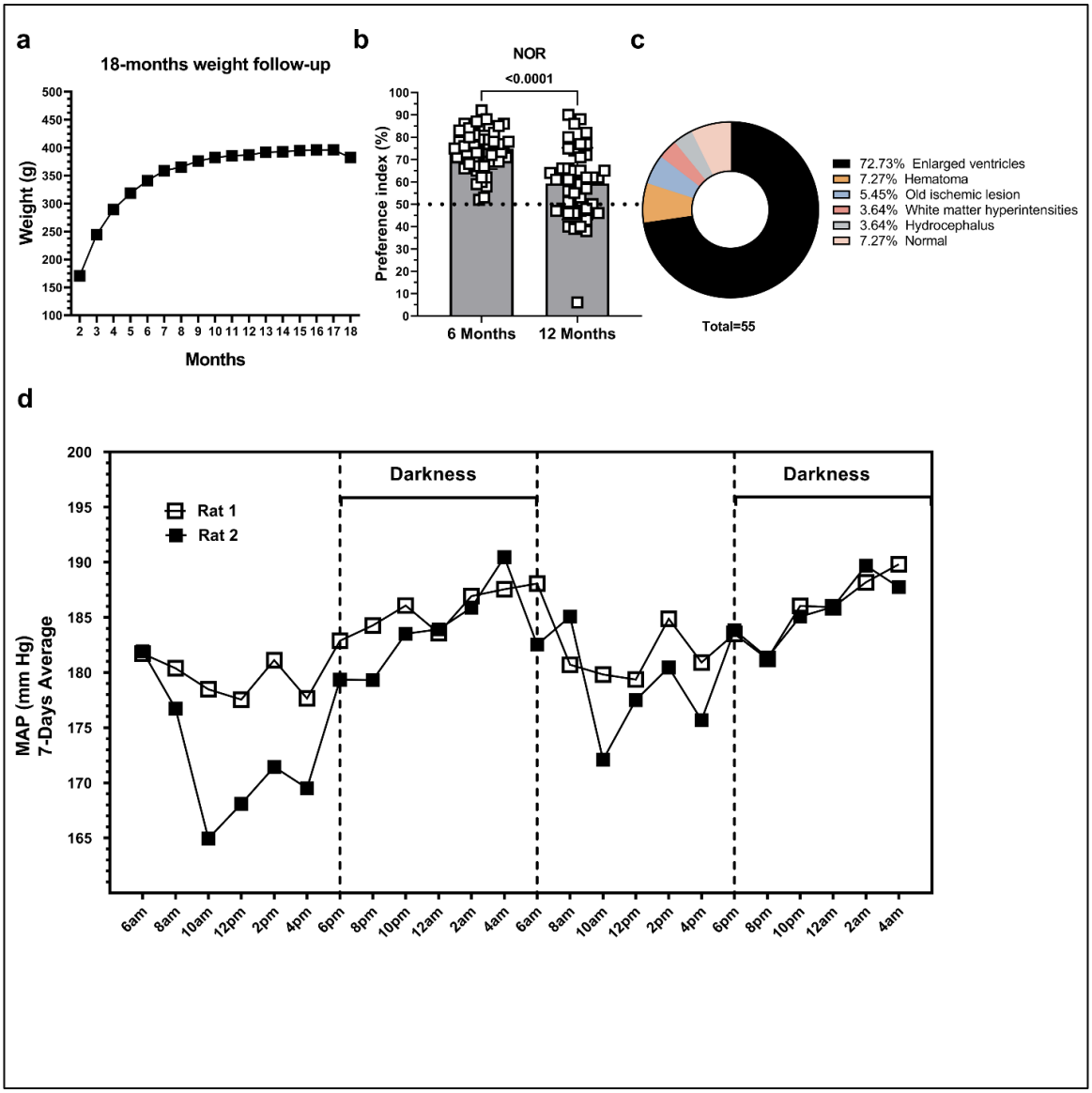

a
18-months weight follow-up
Weight (g)
Months
b
NOR
<0.0001
Preference index (%)
6 Months
12 Months
c
72.73% Enlarged ventricles
7.27% Hematoma
5.45% Old ischemic lesion
3.64% White matter hyperintensities
3.64% Hydrocephalus
7.27% Normal
Total=55
d
Darkness
Darkness
Rat 1
Rat 2
MAP (mm Hg)
7-Days Average

Figure 1.4. Minor stroke resulted in acute cerebral edema on day 3, and chronic brain atrophy in the ipsilateral hemisphere at week 8. Brain MRI scans were performed on day 3 (a-c) and week 8 (d-f) to assess changes in brain ventricular volume. On day 3, minor stroke reduced the ventricular volume in both hemispheres, as was assessed via total ventricular volume; Unpaired t-test *p*-value=0.0022 (a), ventricular volume in the contralesional; Unpaired t-test *p*-value=0.0055 (b), and ipsilateral hemispheres; Unpaired t-test *p*-value=0.0027 (c). (n=8 for Sham, and n=14 for tMCAO). At post-stroke week 8, there was no significant differences in the total ventricular volume; Unpaired t-test *p*-value=0.1685 (d) and contralesional ventricular volume; Unpaired t-test *p*-value=0.4200 (f). A significant increase in the ipsilateral ventricular volume was observed at week 8; Unpaired t-test *p*-value=0.0191 (e). n=14 for Sham, and n=17 for tMCAO. (g) Representative images of brain MRI scans of sham- and tMCAO-operated rats at baseline, day-3, and week-8. Since there was no significant effect in the treatment groups (Supplementary figure 3.), data were pooled based on surgery group, and regardless of treatment.

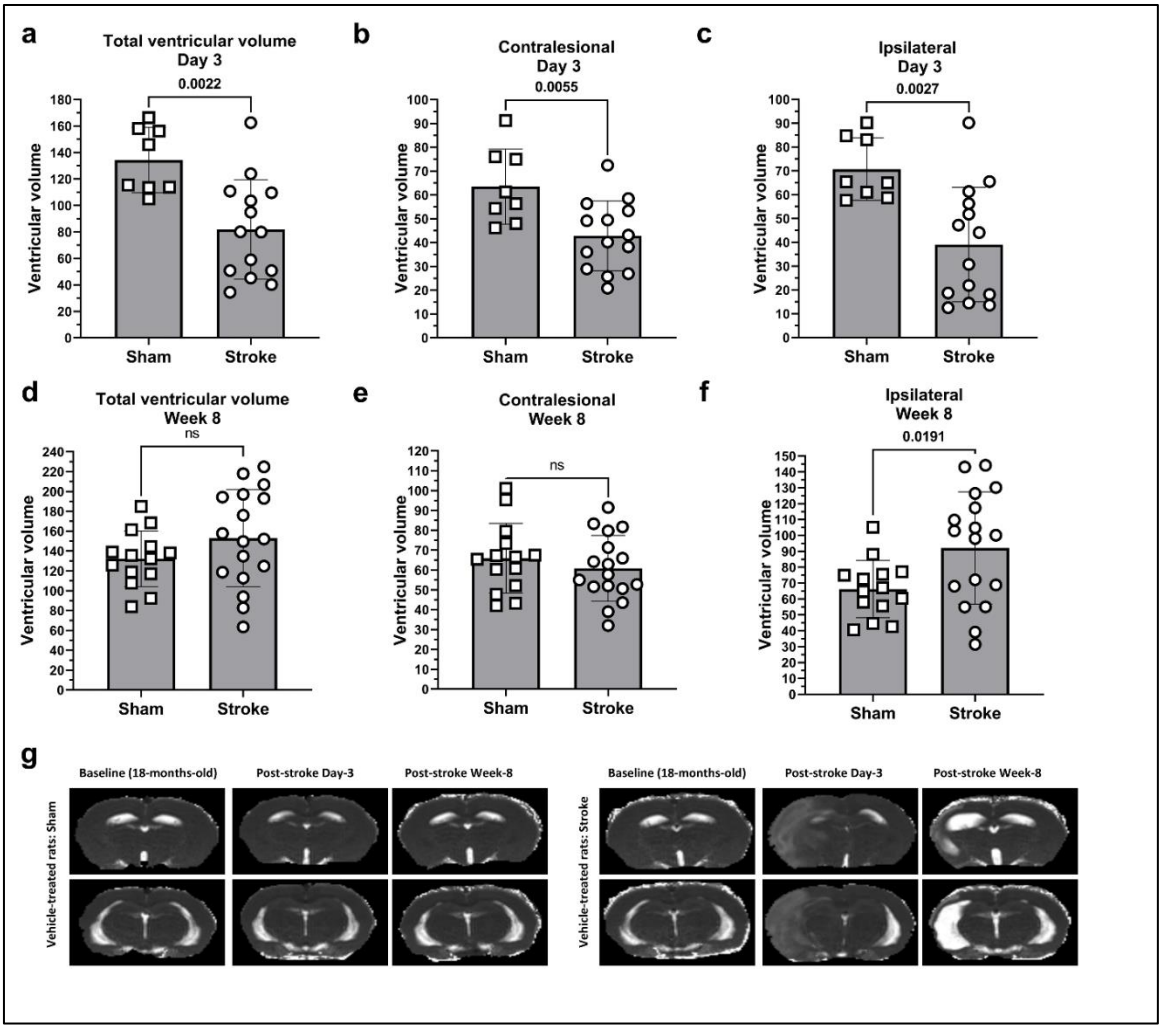

a
Total ventricular volume
Day 3
0.0022
Ventricular volume
Sham
Stroke
b
Contralesional
Day 3
0.0055
Ventricular volume
Sham
Stroke
c
Ipsilateral
Day 3
0.0027
Ventricular volume
Sham
Stroke
d
Total ventricular volume
Week 8
ns
Ventricular volume
Sham
Stroke
e
Contralesional
Week 8
ns
Ventricular volume
Sham
Stroke
f
Ipsilateral
Week 8
0.0191
Ventricular volume
Sham
Stroke
g
Baseline (18-months-old)
Post-stroke Day-3
Post-stroke Week-8
Vehicle-treated rats: Sham
Baseline (18-months-old)
Post-stroke Day-3
Post-stroke Week-8
Vehicle-treated rats: Stroke

Figure 1.5. C21 was associated with a better lesion resolution. Lesion volume was assessed at post-stroke day-3 and week-8. C21 was associated with a significant >70%reduction in lesion size (as compared to 44% in the control group). (a) Percentage reduction in lesion size between C21- and vehicle-treated rats (week 8 vs day 3); Unpaired t-test *p*-value=0.0034. (b) Absolute lesion volume at day-3 and week-8 in both C21- and vehicle-treated animals; 2way-ANOVA *p*-value=0.0050 for treatment, and *p*-value=0.0004 for time. (c) Representative images of brain MRI scan at day 3 and week 8 after stroke; n=5 per group.

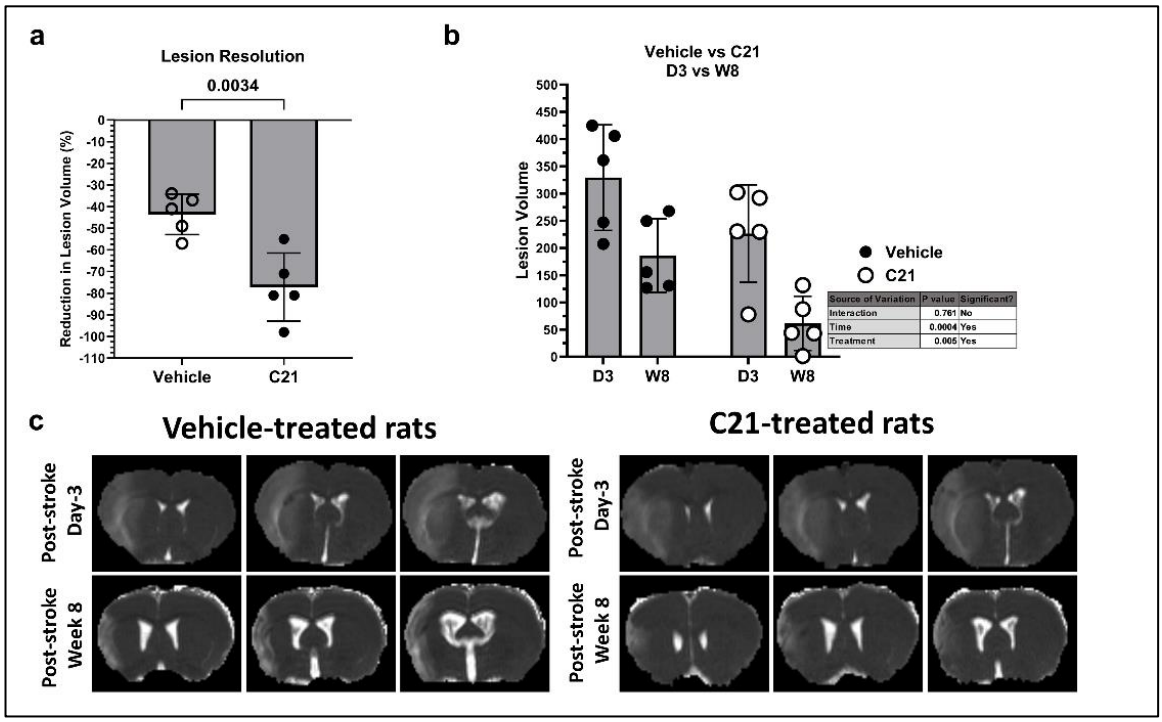

a
Lesion Resolution
0.0034
Reduction in Lesion Volume (%)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
Vehicle
C21
b
Vehicle vs C21
D3 vs W8
Lesion Volume
500
450
400
350
300
250
200
150
100
50
0
D3
W8
D3
W8
Vehicle
C21
Source of Variation | P value | Significant?
Interaction | 0.761 | No
Time | 0.0004 | Yes
Treatment | 0.005 | Yes
c
Vehicle-treated rats
C21-treated rats
Post-stroke Day-3
Post-stroke Week 8
Post-stroke Day-3
Post-stroke Week 8

Figure 1.6. Minor stroke accelerated the progression of post-stroke cognitive impairment in aged hypertensive rats. Post-stroke cognitive decline was assessed at weeks 4 (Y-maze), 5 (NOR, and PAT), and 8 (NOR). (a) A significant interaction between surgery and treatment in favor of the C21-treated group was observed in passive avoidance test (2-way-ANOVA, p=0.0298). A post-hoc pairwise analysis via Dunn's multiple comparisons test revealed a significant decline after minor stroke (p-value=0.0437), with no difference between treatment groups (p-value=0.3158); n= 7 for vehicle-treated sham, n=6 for C21-treated sham, n=8 for vehicle-treated stroke, and n=8 for C21-treated stroke. (b-d) Y-maze data was inconclusive due to the high variability. Most rats had an alternation of <50%, even before the surgery (c) and in the sham group(b,d). Suggesting a severe cognitive decline as a result of advanced age and/or hypertension; n=8 for vehicle-treated sham, n=6 for C21-treated sham, n=8 for vehicle-treated stroke, and n=9 for C21-treated stroke. 2way-ANOVA (p-value=0.7956 for surgery, p-value=0.2069 for treatment, and p-value=0.8011 for time point). NOR was reanalyzed using total exploration time instead of preference index. (e) Progressive reduction in total exploration time over rats lifespan; Ordinary one-way ANOVA followed by Tukey's multiple comparisons test. p-value <0.0001 for 6 months vs 12, 18, 19 (Post-surgery weeks 5), and 20 months (Post-surgery week 8). p-value=0.0152 for 12 vs 18 months. p-value=0.0021 for 12 vs 19 months. p-value <0.0001 for 12 vs 20 months. p-value=0.9443 and 0.0517 for 18 months vs 19 and 20, respectively. (d) Post-stroke deterioration in total exploration time revealed a significant effect of treatment and time, but not surgery; 3way-ANOVA (p-value=0.0205 for treatment, 0.1024 for surgery, and 0.0002 for time). The relative reduction in total exploration time at week 8 was 42% (shame: control), 55% (stroke: control), and 35% (stroke: C21).

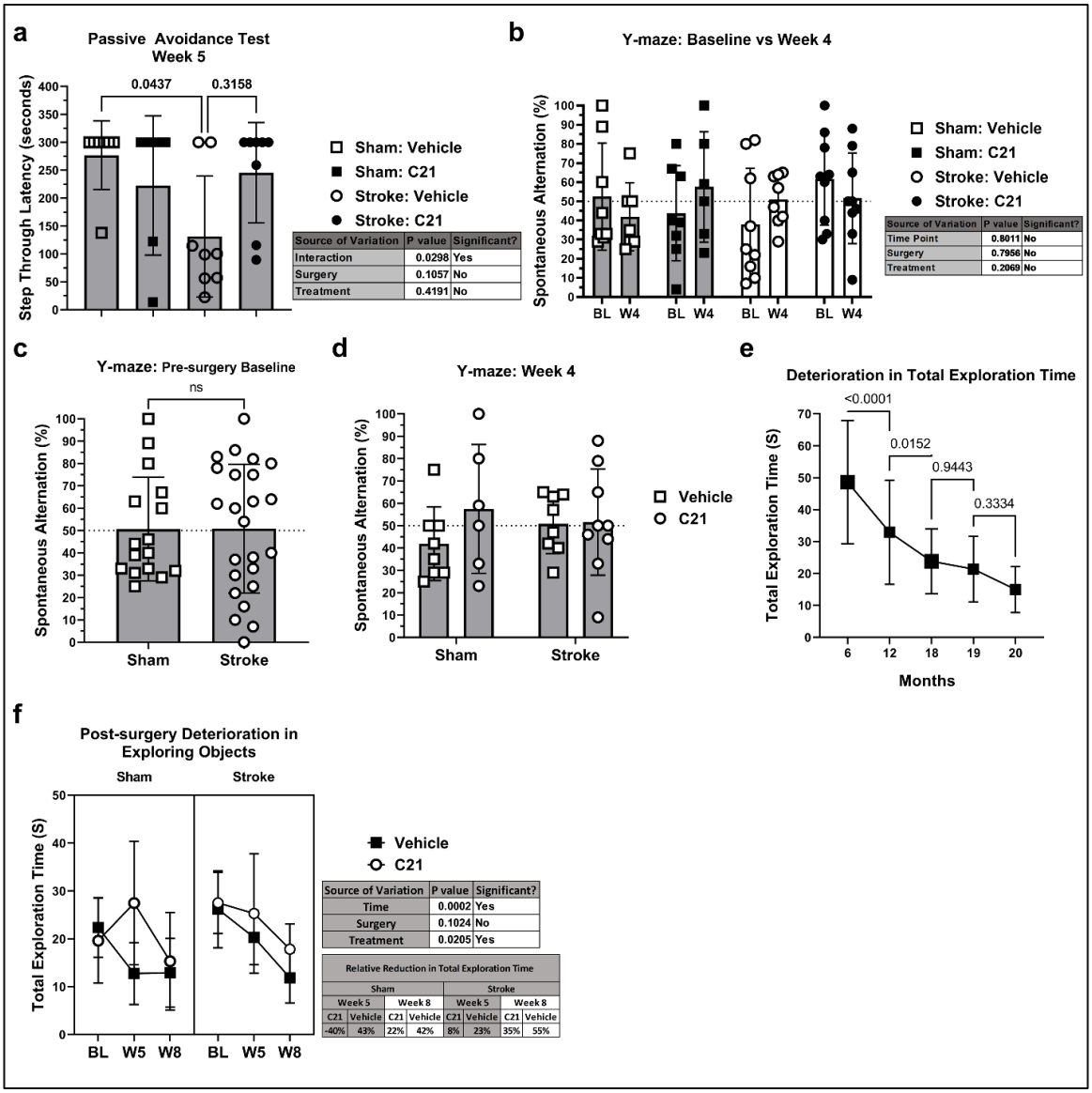
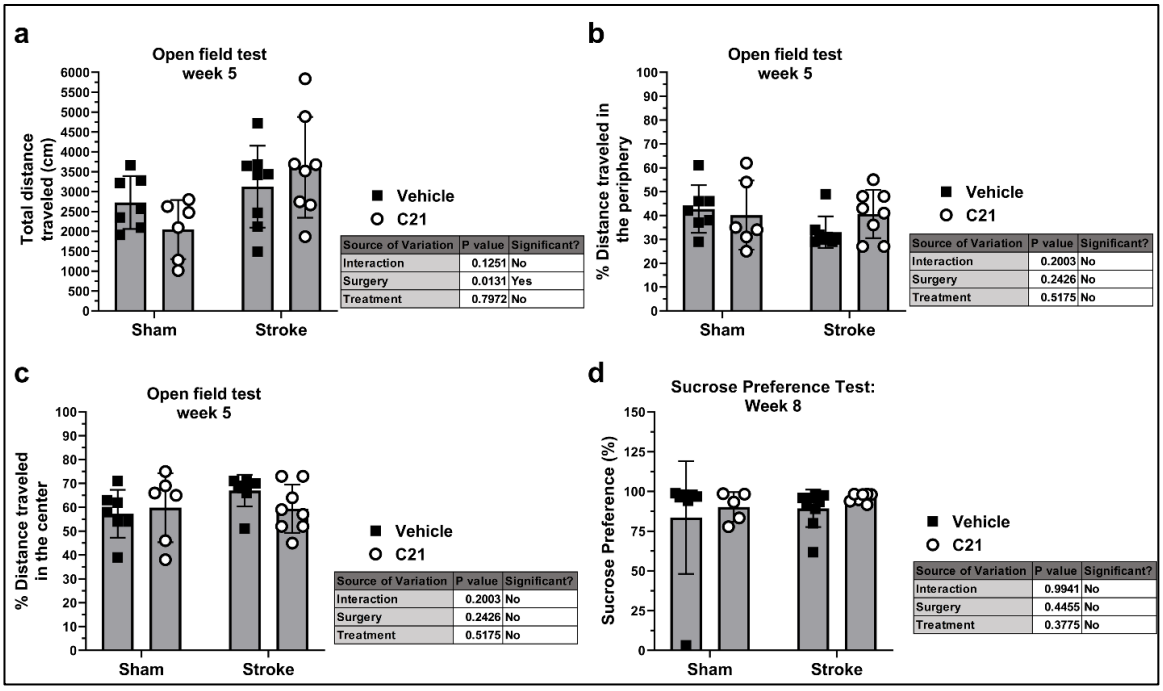
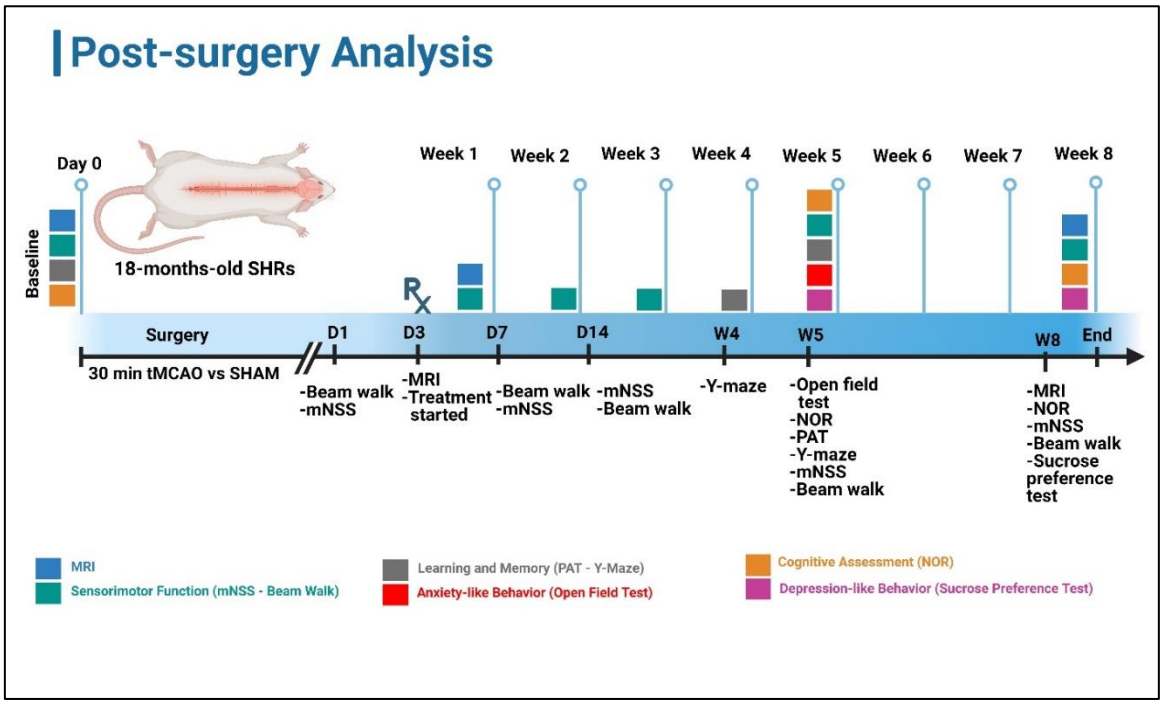

a
Passive Avoidance Test
Week 5
0.0437
0.3158
Step Through Latency (seconds)
Sham: Vehicle
Sham: C21
Stroke: Vehicle
Stroke: C21
Source of Variation | P value | Significant?
Interaction | 0.0298 | Yes
Surgery | 0.1057 | No
Treatment | 0.4191 | No
b
Y-maze: Baseline vs Week 4
Spontaneous Alternation (%)
BL W4 BL W4 BL W4 BL W4
Sham: Vehicle
Sham: C21
Stroke: Vehicle
Stroke: C21
Source of Variation | P value | Significant?
Time Point | 0.8011 | No
Surgery | 0.7956 | No
Treatment | 0.2069 | No
c
Y-maze: Pre-surgery Baseline
ns
Spontaneous Alternation (%)
Sham
Stroke
d
Y-maze: Week 4
Spontaneous Alternation (%)
Sham
Stroke
Vehicle
C21
e
Deterioration in Total Exploration Time
<0.0001
0.0152
0.9443
0.3334
Total Exploration Time (S)
6 12 18 19 20
Months
f
Post-surgery Deterioration in Exploring Objects
Sham
Stroke
Total Exploration Time (S)
BL W5 W8 BL W5 W8
Vehicle
C21
Source of Variation | P value | Significant?
Time | 0.0002 | Yes
Surgery | 0.1024 | No
Treatment | 0.0205 | Yes
Relative Reduction in Total Exploration Time
Sham | Stroke
Week 5 | Week 8 | Week 5 | Week 8
C21 | Vehicle | C21 | Vehicle | C21 | Vehicle | C21 | Vehicle
-40% | 43% | 22% | 42% | 8% | 23% | 35% | 55%

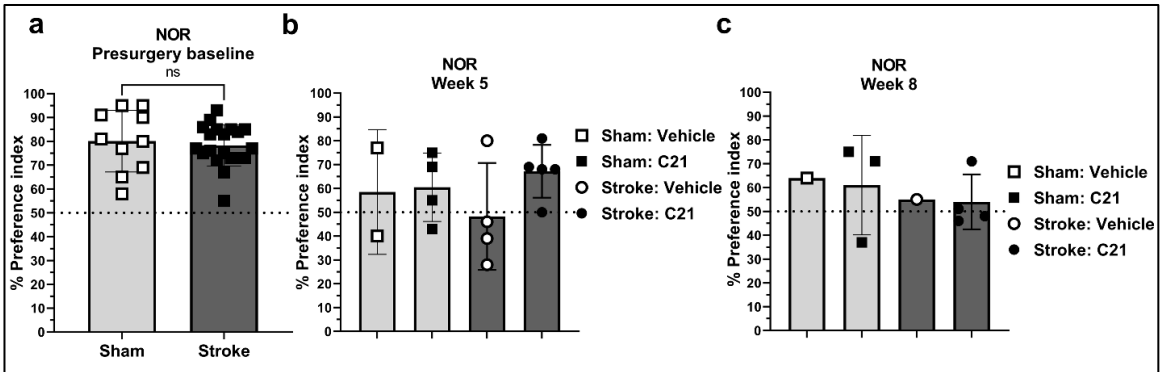
Figure 1.7. Minor stroke induced hyperactivity in aged hypertensive rats, with no signs of anxiety- and depression-like behavior. Open field test was performed at week-5 to assess locomotor activity and anxiety-like behavior. Aged hypertensive rats demonstrated stroke-induced hyperactivity as was assessed via the total distance traveled; 2way-ANOVA (*p*-value=0.0131 for surgery, and 0.7972 for treatment) (a). Minor stroke did not affect the anxiety level in aged hypertensive rats, where all animals traveled comparably in the periphery (b) and the center (c); 2way-ANOVA (*p*-value=0.2426 for surgery, and 0.5175 for treatment). Sucrose preference test was performed at week 8 to assess anhedonia. Anhedonia was evaluated via the percentage of preferring sucrose over plain drinking water. All rats regardless of their group assignment displayed an interest to consume more sucrose than water; 2way-ANOVA (*p*-value=0.4455 for surgery, and 0.3775 for treatment) (d).


a
Open field test
week 5
Total distance traveled (cm)
Sham
Stroke
Vehicle
C21
Source of Variation | P value | Significant?
Interaction | 0.1251 | No
Surgery | 0.0131 | Yes
Treatment | 0.7972 | No
b
Open field test
week 5
% Distance traveled in the periphery
Sham
Stroke
Vehicle
C21
Source of Variation | P value | Significant?
Interaction | 0.2003 | No
Surgery | 0.2426 | No
Treatment | 0.5175 | No
c
Open field test
week 5
% Distance traveled in the center
Sham
Stroke
Vehicle
C21
Source of Variation | P value | Significant?
Interaction | 0.2003 | No
Surgery | 0.2426 | No
Treatment | 0.5175 | No
d
Sucrose Preference Test:
Week 8
Sucrose Preference (%)
Sham
Stroke
Vehicle
C21
Source of Variation | P value | Significant?
Interaction | 0.9941 | No
Surgery | 0.4455 | No
Treatment | 0.3775 | No



Supplementary Figure 1. Schematic depiction of experimental design. *Adapted from "Mouse Experimental Timeline", by BioRender.com (2023). Retrieved from* https://app.biorender.com/biorender-templates

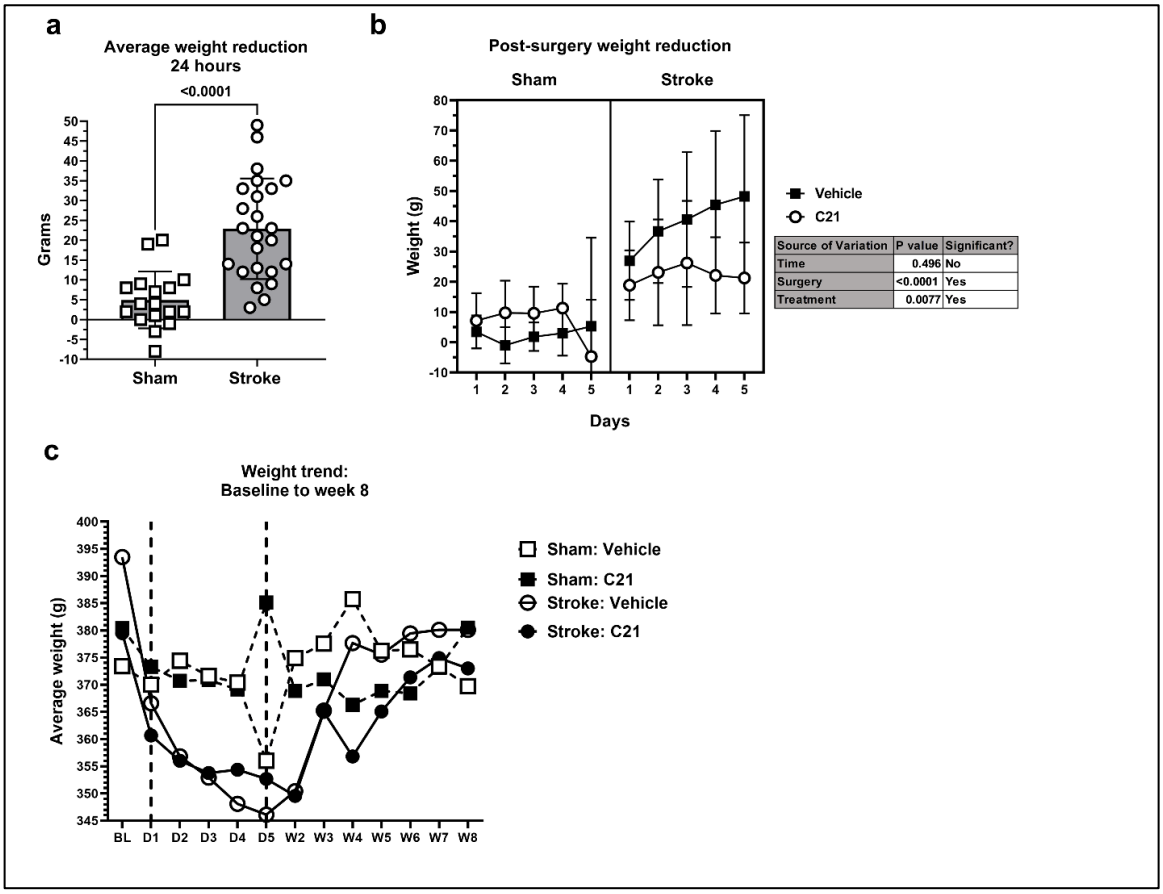
Supplementary Figure 2. Most animals were excluded at post-stroke NOR analysis due to the lack of interest in exploring objects. The retention period was decreased to 30 min instead of an hour at pre-surgery baseline analysis. All animals exhibited a comparable baseline performance at 18 months. Unpaired t-test (*p*-value <0.6535); n=10 in the sham group, and 20 in the tMCAO group (a). It was not possible to assess their performance at weeks 5 and 8, where most of the animals had to be excluded for not meeting the minimum requirement of total exploration time (20 seconds) (b-c). The percentage of animals who had to be excluded exponentially increased as time progressed (d).



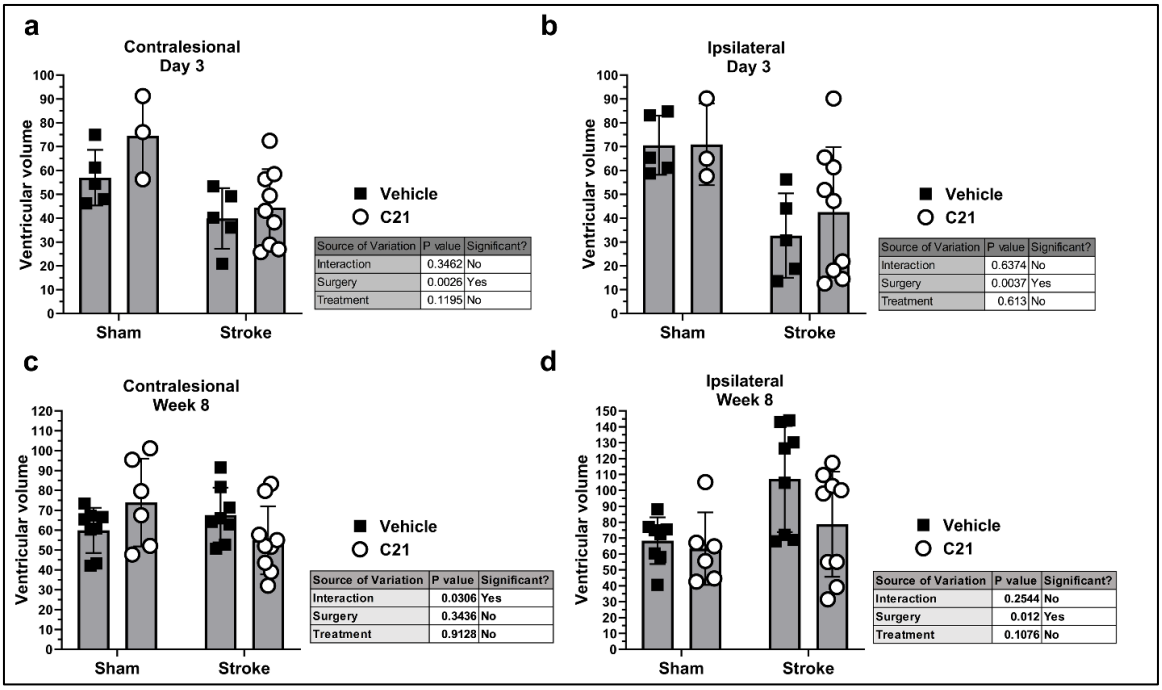
d

Rats excluded from the analysis due to the lack of interest engaging with objects (Total Exploration Time < 20 seconds)						
Surgery	Treatment	6-months	12-months	18-months	Post-stroke W5	Post-stroke W8
Sham	Vehicle	1/60 Rat (1.67%)	13/57 Rats (22.81%)	13/43 Rats (30.23%)	4/6 Rats (66.67%)	6/7 Rats (85.71%)
	C21				2/6 Rats (33.33%)	3/6 Rats (50%)
Stroke	Vehicle				4/8 Rats (50%)	7/8 Rats (87.50%)
	C21				4/9 Rats (44.44.%)	5/9 Rats (55.56%)

Supplementary Figure 3. Minor stroke resulted in a significant weight loss at 24 hours, and C21 slightly helped them maintain their weight. On average, rats in the sham group lost 4.9 grams, while those in the tMCAO group lost 22.9 grams. Unpaired t-test; *p*-value <0.0001 (a). Post-stroke 5-day average body weight was comparable between groups. For the sham group it was 378g and 375g, in vehicle- and C21-treated rats, respectively. Vehicle-treated tMCAO group averaged around 354g, while those treated with C21 averaged around 357g; however, upon treatment initiation, C21-treated group started to maintain/gain weight, and vehicle-treated animals continued to lose weight (d). 3way-ANOVA analysis revealed significant differences between treatments, and surgeries; 3way-ANOVA (*p*-value <0.0001 for surgery, 0.0077 for treatment, and 0.496 for time).


a
Average weight reduction
24 hours
<0.0001
Grams
Sham
Stroke
b
Post-surgery weight reduction
Sham
Stroke
Weight (g)
Days
Vehicle
C21
Source of Variation | P value | Significant?
Time | 0.496 | No
Surgery | <0.0001 | Yes
Treatment | 0.0077 | Yes
c
Weight trend:
Baseline to week 8
Average weight (g)
BL D1 D2 D3 D4 D5 W2 W3 W4 W5 W6 W7 W8
Sham: Vehicle
Sham: C21
Stroke: Vehicle
Stroke: C21

Supplementary Figure 4. C21 had no effect in stroke-induced acute cerebral edema, and chronic brain atrophy. Brain MRI scans were performed on day 3 (a-b) and week-8 (c-d) to assess changes in brain ventricular volume. Minor stroke significantly lowered the ventricular volume in both hemispheres at day 3 MRI scans; 2way-ANOVA (*p*-value=0.0026 for surgery, and 0.1195 for treatment in the contralesional hemisphere) (a) and (*p*-value=0.0037 for surgery, and 0.613 for treatment in the ipsilateral hemisphere) (b). Minor stroke resulted in a significant increase in the ipsilateral ventricular volume at week 8, with no difference between the treatment groups; 2way-ANOVA (*p*-value=0.3436 for surgery, and 0.9128 for treatment in the contralesional hemisphere) (c) and (*p*-value=0.012 for surgery, and 0.1076 for treatment in the ipsilateral hemisphere) (d).



Source of Variation	P value	Significant?
Interaction	0.3462	No
Surgery	0.0026	Yes
Treatment	0.1195	No

Source of Variation	P value	Significant?
Interaction	0.6374	No
Surgery	0.0037	Yes
Treatment	0.613	No

Source of Variation	P value	Significant?
Interaction	0.0306	Yes
Surgery	0.3436	No
Treatment	0.9128	No

Source of Variation	P value	Significant?
Interaction	0.2544	No
Surgery	0.012	Yes
Treatment	0.1076	No

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CHAPTER 3

STIMULATION OF ANGIOTENSIN II TYPE 2 RECEPTOR MODULATES PRO-INFLAMMATORY RESPONSE IN MICROGLIA AND MACROPHAGES: THERAPEUTIC IMPLICATIONS FOR THE TREATMENT OF POST-STROKE COGNITIVE IMPAIRMENT [1]

[1]Abdulkarim Alshammari, Yohan Han, Timothy W. Jones, Bindu Pillai, Duo Zhang, Payaningal R. Somanath, Adviye Ergul, Susan C. Fagan. To be submitted to [**Frontier in Immunology**]

Abstract

Sustained pro-inflammatory microglial activation contributes to the development of post-stroke cognitive impairment (PSCI). Compound 21 (C21), an angiotensin II type 2 receptor agonist, has shown some neurovascular protection after stroke, mainly due to its anti-inflammatory effects on neuroinflammatory response. This study aimed to investigate the direct anti-inflammatory effects of C21 on macrophages, as well as brain innate immune cells. Murine microglial cell line (C8-B4) and RAW 264.7 macrophages were exposed to lipopolysaccharide (LPS) and co-treated with C21. Pro-inflammatory mediators were assessed via RT-qPCR and ELISA. Cellular reactive oxygen species (ROS) were evaluated via CellROXGreen staining, and nitrate production was assessed using Griess assay. Cells were activated with 100 ng/ml LPS and co-treated with different concentrations of C21 for 24 hours. C21 suppressed LPS-induced inflammatory response and the generation of ROS in both cell types in a dose-dependent manner. In microglia, C21 blunted LPS-induced mRNA expression of IL-1β, IL-12b, COX-1, iNOS, and IL-6, and protein expression of IL-1β. As for macrophages, a similar pattern was observed, where C21 suppressed LPS-induced proinflammatory response (IL-1β, TNF-α, CXCL1). C21 also regulated the production of ROS and nitric oxide. These anti-inflammatory effects in microglia and macrophages were associated with increased neuroprotective gene expression, including GDNF and BDNF. Our findings suggest a protective effect of C21 against the inflammatory response, in both macrophages and microglia, via suppressing the release of pro-inflammatory cytokines/chemokines and the generation of ROS while stimulating the production of neurotrophic factors.

Keywords: Stroke, PSCI, Neuroinflammatory response, ROS, Compound 21, Renin-Angiotensin System (RAS).

Introduction

Over the past two decades, our knowledge of stroke pathophysiology has dramatically expanded, specifically to include the role of the immune system combined with a more advanced understanding of the neurovascular unit. Neuroinflammation is known to be a secondary injury following ischemic stroke and results in long-term consequences, such as post-stroke cognitive impairment (PSCI)[1]. The injury after an ischemic stroke is mediated through a dynamic interaction between all the neurovascular unit components, including neuronal, glial, and vascular cells[1].

Microglia, the resident innate immune cells, is a key player in neuroinflammatory response after stroke[2]. Typically, the primary role of resident microglia in the brain is to survey and maintain homeostasis[3]. In response to ischemic injury, microglial activation goes through dynamic functional changes throughout the neuro-inflammatory response. Shortly after stroke, microglia are expected to adopt an anti-inflammatory phenotype, facilitating enhanced phagocytosis, fewer inflammatory mediators, and improved neuronal survival. However, the anti-inflammatory status is transitory; shortly thereafter, pro-inflammatory microglia begin dominating the injured area[4, 5].

The pro-inflammatory microglia are characterized by less phagocytosis and higher production of pro-inflammatory cytokines and ultimately start a cascade of events leading to disrupt the BBB, degrading the extracellular matrix, and triggering the infiltration of peripheral leukocytes into the central nervous system[4, 5]. The recruitment of monocyte-derived macrophages (MDM) to the site of injury has a crucial role in the repair processes[6, 7]. MDMs are classified into two major sub-population, the pro-inflammatory ($Ly6C^{high}$) and anti-inflammatory ($Ly6C^{low}$) monocytes[8]. Classically, MDMs were believed to

mediate detrimental effects after stroke [9]; however, recent studies demonstrated that Ly6C^{hi} monocytes promote protective effects after stroke, likely through enhancing delayed-anti-inflammatory effects[10-12].

The brain renin-angiotensin system (RAS) is believed to be involved in the pathogenesis of stroke. Preclinical data showed a direct correlation between angiotensin II (Ang II), the active neuropeptide in the renin-angiotensin system (RAS), and the severity of ischemic injury after stroke[13]. Several studies have shown that stimulation of the AT2R either indirectly via candesartan or directly by C21 provided acute and long-term neurovascular protection and improved sensorimotor and cognitive outcomes after stroke[14-18]. These effects, in part, were mediated by regulating the neuroinflammatory response[18-20]. In the current study, we sought to further confirm the ability of C21 to mediate anti-inflammatory and neurotrophic effects in mouse microglial cell line, C8-B4, and RAW 264.7 macrophages.

Materials and methods

Cell maintenance, culture, and treatment conditions

C8-B4 mouse microglial cell line and RAW 264.7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were cultured in DMEM media supplemented with 10% heat-inactivated fetal bovine serum and 1% Penicillin-Streptomycin (ATCC, Manassas, VA) at 37 °C in a humidified 5% CO_2 incubator. Cells were seeded at a density of $1 – 1.5 \times 10^6$ in 60 mm dishes for mRNA and protein expression experiments, $10 – 12 \times 10^4$ in 96 well plates for MTS viability assays, 1×10^4 in 6-well plates for trypan blue staining, and 1×10^4 in 60 mm dishes for ROS generation experiments. For all experiments, cells were allowed to adhere for 24 hours and then maintained in a serum-

free condition overnight, before any treatment. C8-B4 and RAW 264.7 cells were treated with lipopolysaccharide (LPS) (100 ng/ml) to induce a pro-inflammatory response and co-treated, either with different doses of C21 or vehicle for a total of 24 hours[21].

RNA isolation, cDNA reverse transcription, and quantitative real-time rt-PCR

RT-qPCR was performed to measure the expression of pro-inflammatory and neurotrophic genes. Briefly, total RNA from cells was extracted using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA). The quantity and quality of isolated RNA were measured and assessed using Nanodrop (NanoDrop Technologies, Wilmington, DE). An equal amount of RNA (500 ng) from each sample was reverse transcribed into complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed by StepOnePlus™ Real-Time PCR System, using SYBR Green Master Mix. The expression of the housekeeping gene, PPIA, was used as an endogenous control. The amplification condition was as follows: initial pre-incubation at 95 °C for 10 min, followed by amplification of the target DNA for 40 cycles (95 °C for 15 seconds followed by 60 °C for 1 min). The primers used in this study and their sequences are listed in Table 1.

ELISA

ELISA kits for pro-inflammatory cytokines, IL-1β (ab197742) and CXCL1 (ab216951), were purchased from Abcam, and all the analyses were performed according to the manufacturer's protocol. Following treatment conditions, cells' supernatants were collected and centrifuged at 2000 g × 10 minutes for complete removal of cells' debris and then used to assess the secreted cytokines/chemokines using ELISA kits.

MTS viability assay

MTS viability assay (Promega, Madison, WI) was used to determine the safety profile of C21. Briefly, 1×10^4 cells were seeded into 96 well plates and allowed to adhere, and then cells were cultured in a serum-free medium overnight. Cells were treated with different concentrations of C21 (1 µM, 10 µM, 20 µM, 50 µM, and 100 µM) for a total of 24 hours and then incubated with MTS reagent for ≈ 3 hours at 37 °C. Absorbance was recorded at a wavelength of 490 nm using a microplate reader (Bio-TEK). Accordingly, the percentage of cell viability was calculated based on the OD readings, using the following equation.

% Cell viability= (OD/ Mean OD of control) * 100

Trypan blue staining: viability analysis

Cell viability was assessed via trypan blue staining. C8-B4 cells were seeded at a density of 1×10^5 in 6-well plates and left to adhere for 24 hours. Afterward, cells went through overnight serum starvation and were then treated with different doses of C21 (1 µM, 10 µM, 20 µM, 50 µM, and 100 µM) or vehicle for a total of 24 hours. At this point, cells were trypsinized using 0.25% Trypsin and resuspended with PBS. Cells suspensions were mixed with 0.4 % trypan blue solution (1:1 dilution) and allowed to stand for ≈ 2 - 3 min at room temperature. A 10 ul of cell suspension/trypan blue mixture was used to count viable/non-viable cells using TC20 automated cell counter (Bio-Rad).

Cellular ROS generation

Cellular production of reactive oxygen species was detected using the CellROX™ Green Reagent (Invitrogen, Waltham, MA, USA). Cells were seeded at a density of 1×10^6 and then treated according to our experimental design. Briefly, cells were induced with LPS (100 ng/ml) and co-treated with either different concentrations of C21 (1 µM, 10 µM, 20

μM, and 50 μM) or vehicle for 24 hours. Then, cells were washed with PBS and incubated with 5 μM CellROXGreen Reagent for 30 minutes at 37 °C. Fluorescence images were obtained using a Zeiss Observer Z1 microscope (Carl Zeiss).

Nitrite and nitrate measurement

The level of nitric oxide was quantified using Griess assay kit (Invitrogen, Waltham, MA, USA). Cell culture supernatant was collected from LPS-induced and C21-treated cells. Cells were centrifuged to eliminate cell debris, mixed with Griess reagent, and let stand for approximately 30 minutes at room temperature. After 30 min, absorbance was recorded at 548 nm using a microplate reader (Bio-TEK). Sodium nitrate was used as a reference to detect the level of nitrate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Multiple groups analysis (three or more) was performed using one-way ANOVA with either Dunnett's multiple comparisons test for variables with normal distribution, or by pairwise comparisons using Mann-Whitney U tests for variables without normal distribution. Data distribution was evaluated via the Shapiro-Wilk test. Data are shown as mean ± SD, and a *P*-value of ≤ 0.05 was considered to be statistically significant.

Results

Compound 21 demonstrates acceptable cytotoxicity at lower concentrations

The safety of Compound 21 in microglia/macrophages was evaluated via MTS viability assay and trypan blue staining. Different concentrations of C21 (1 μM, 10 μM, 20 μM, 50 μM, and 100 μM) were evaluated, and our data demonstrate an acceptable safety profile

up to 20 µM in C8-B4 microglia (***Fig. 2.1A and B,***), and 50 µM in macrophages ***(Fig. 2.1C).***

Compound 21 exhibits a predominant anti-inflammatory response in microglia via decreasing the expression of LPS-induced pro-inflammatory cytokines/chemokines in a dose-dependent manner

C8-B4 microglial cells were co-cultured with LPS to induce the release of pro-inflammatory cytokines/chemokines, mimicking post-stroke inflammatory response, and treated with different concentrations of C21 (1 µM, 10 µM, and 100 µM). The LPS-mediated inflammatory response was evaluated at both the mRNA and protein levels. C21 demonstrated a dose-dependent reduction in the expression of pro-inflammatory cytokines/chemokines at the transcription level, including IL-1β, IL-12b, IL-6 ***(Fig. 2.2A, 2B, 2C)***, and protein level of released IL-1β ***(Fig. 2.2D).***

Compound 21 significantly reduces the expression of pro-inflammatory cytokines/chemokines in macrophages, following a dose-dependent fashion

We examined the direct impact of C21 on the polarization status of RAW 264.7 macrophages. As illustrated in ***Fig. 2.3***, C21 significantly suppressed the expression of pro-inflammatory genes, IL-1β ***(Fig. 2.3A)***, TNF-α ***(Fig. 2.3B)***, and CXCL1 ***(Fig. 2.3C),*** and pro-inflammatory proteins, IL-1β ***(Fig. 2.3D)***, TNF-α and CXCL1 ***(Fig. 2.3E)***. Indicating an anti-inflammatory effect of C21 via suppressing the release of pro-inflammatory cytokines/chemokines in both microglia and macrophages.

Compound 21 effectively regulates the production of reactive oxygen species (ROS), nitric oxide (NO), and the activity of pro-inflammatory enzymes, iNOS, and COX-2, in microglia/macrophages

As demonstrated in ***Fig 2.4*** and ***Fig. 2.5***, C21 showed a profound effect in neutralizing the expression of COX2 and iNOS in C8-B4 microglia ***(Fig. 2.4A, 4B)*** and RAW 264.7 macrophages ***(Fig. 2.5A, 2.5B)***, Also, C21 regulated the generation of free radicals, ROS and NO production in both microglia ***(Fig. 2.4C)*** and macrophages ***(Fig. 2.5C, 2.5D)***. Our observations demonstrate that C21 normalizes the release of proinflammatory mediators and reduces the production of reactive oxygen species and other free radicals.

Compound 21 is associated with increased neuroprotective activity in microglia/macrophages

We sought to investigate whether C21 increases the level of BDNF and other neurotrophic molecules via its direct effect on microglia and macrophages. ***Fig. 2.6*** demonstrates the neuroprotective effects of C21 in both microglia and macrophages, as it was evaluated via the expression of neurotrophic factors, BDNF and GDNF. C21 upregulated GDNF in C8-B4 microglia ***(Fig. 2.6C) and*** increased the expression of GDNF and BDNF in RAW 264.7 macrophages ***(Fig. 2.6A, 2.6B)***. This indicates that C21 plays a neuroprotective role after stroke, which is mediated by the upregulation of neurotrophic factors both centrally and peripherally.

Discussion

After a stroke, microglia follow biphasic activation throughout the neuroinflammatory response[22]. There is a transient activation of the anti-inflammatory phenotype followed by the activation of the M1-like phenotype[22]. However, during the delayed phase of

neuroinflammation, microglia are designed to switch back to the anti-inflammatory phenotype to facilitate long-term functional recovery, BBB repair, neurogenesis, and angiogenesis.[4] Similarly, at the early stage after stroke, the newly recruited macrophages assume the anti-inflammatory phenotype and gradually changed to an M1-like phenotype[22, 23]. Modulating microglia/macrophage activation after stroke toward an M2-like phenotype would be beneficial in modulating the inflammatory response and ultimately facilitate long-term functional recovery.

In the current study, we demonstrated that the stimulation of AT2R effectively modulates the polarization of microglia and macrophages toward a less pro-inflammatory phenotype. Our findings suggest that C21 exhibited an anti-inflammatory effect and reduced the expression of pro-inflammatory mediators in a dose-dependent manner, in both mouse microglial cells and RAW 264.7 macrophages. The expression of proinflammatory chemokines/cytokines was evaluated at both protein and mRNA levels. Furthermore, C21 was able to neutralize the expression level of iNOS and COX-2, as well as the generation of ROS, which has been shown to suppress post-stroke injury and mediate neuroprotective activities[24-27]. The current study provides additional insights into how C21 mediates its beneficial effects after stroke. As evident from our study, C21 has dual anti-inflammatory and neurotrophic effects, affecting both microglia and monocyte-derived macrophages. C21 may stimulate the immune cells peripherally to produce neuroprotective/anti-inflammatory mediators, which in turn start a cascade of neuroprotective effects centrally. This could be facilitated via direct macrophage-microglia crosstalk or through stimulating macrophages to release neuroprotective molecules that can cross BBB and mediate its effects centrally.

Microglia and monocyte-derived macrophages share the same inflammatory response pattern and function[2, 4, 6, 11, 12]. Both mediate an early pro-inflammatory response and release inflammatory cytokines/chemokines to cause further damage and exacerbate the ischemic injury during the acute phase of stroke[2, 4, 6, 11, 12]. Likewise, both cells modulate their phenotypic activation to shift the polarization status to facilitate an anti-inflammatory response and promote delayed recovery after stroke[2, 4, 6, 11, 12]. A recent study revealed that macrophages directly downregulate the expression of pro-inflammatory genes in both mouse and human microglia[28]. LPS-stimulated BMDMs suppressed the expression of inflammatory genes in microglia, including IL-1β, TNFα, and IL-10[28]. Gene analysis of LPS-activated microglia in the presence of macrophages revealed that around 1076 genes were significantly differentially regulated, primarily those related to the NF- κB signaling pathway and apoptotic cell death[28]. With this in mind, our observations might indicate that the anti-inflammatory effects of C21 on macrophages could synergistically modulate the inflammatory status of microglia. We attempted to assess this by examining the impact of C21-treated macrophages on microglial inflammatory response using conditioned media concentrate, however, we could not reliably demonstrate such a concept due to experimental limitations.

The cytotoxic effect of C21 on microglia/macrophages is a major limitation of our study. C21 exhibited a slightly different pattern of toxicity on macrophages and microglia. In microglia, the highest two concentrations (50 and 100 μM) induced a significant reduction in cell viability, while in macrophages only 100 μM did. This made it a bit confusing since some of the neurotrophic and anti-inflammatory effects were only observed at the highest concentration. In microglia, the positive effect of C21 on the expression of GDNF,iNOS,

IL-12B, and IL-6, was only observed at 100 μM. In macrophages, a similar pattern was observed in the expression of TNFa, cxxl1, COX-2, iNOS, GDN, and BDNF. However, C21 still enhanced an anti-inflammatory effect in smaller concentrations, within its cytoprotective range. This is evident in the expression level of Il-1b, Cxcl1, COX-2, nitric oxide production, and the generation of ROS. These concentrations had been used in mouse macrophages and were shown to reduce nitric oxide production in a dose-dependent manner. The authors anticipated this to be unlikely related to the loss of viability, yet cell viability was not tested.

This controversy regarding the efficacy of C21 following different concentrations could be explained by the duration of treatment. Inflammatory cytokines peak at different time points, and the ability of a compound to reverse it differs accordingly. The ability of smaller doses of C21 in modulating the inflammatory response was previously tested in THP-1 cells, following different treatment conditions, 3,6, and 12 hours.[29] In that study, different patterns of reduction were observed at 3, 6, and 12 hours.[29] Also, the trend at the mRNA level did not necessarily translate into protein expression across these time points.[29] For instance, C21 significantly regulated the mRNA expression of IL-6 at 3 hours, but not at 6 and 12 hours. Conversely, at the protein level, C21 significantly reduced the expression of IL-6 at 6 and 12 hours, but not at 3 hours.[29] Therefore, perhaps, the smaller concentration of C21 we used (1 and 10 μM) induced an anti-inflammatory response at an earlier time point, and that could not be captured in our 24-hour experiment.

In conclusion, C21 exhibited anti-inflammatory effects in microglia and macrophages via suppressing the mRNA/protein expression of proinflammatory mediators, as well as

regulating the generation of free radicals. C21-mediated anti-inflammatory effects were associated with an increase in neurotrophic activity.

Figure 2.1. Compound 21 demonstrates acceptable cytotoxicity at lower concentrations. The effect of different concentrations of C21 on cell viability was assessed via MTS viability assay and Trypan Blue staining. C8-B4 microglia and RAW 264.7 macrophages were seeded into 96 well plates and treated with different concentration of C21 (1 µM, 10 µM, 20 µM, 50 µM, and 100 µM) for a total of 24 hours. Then, cells were incubated with MTS reagent for ≈ 3 hours at 37 °C. Absorbance was measured at a wavelength of 490 nm and the percentage of cell survival was calculated accordingly. C8-B4 cells proliferation was assessed using MTS assay **(A)**, and trypan blue staining **(B).** Cell viability was assessed in RAW 264.7 macrophages via MTS viability assay **(C).** Values are the mean ± SD of 3-4 independent experiments analyzed by one-way ANOVA. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

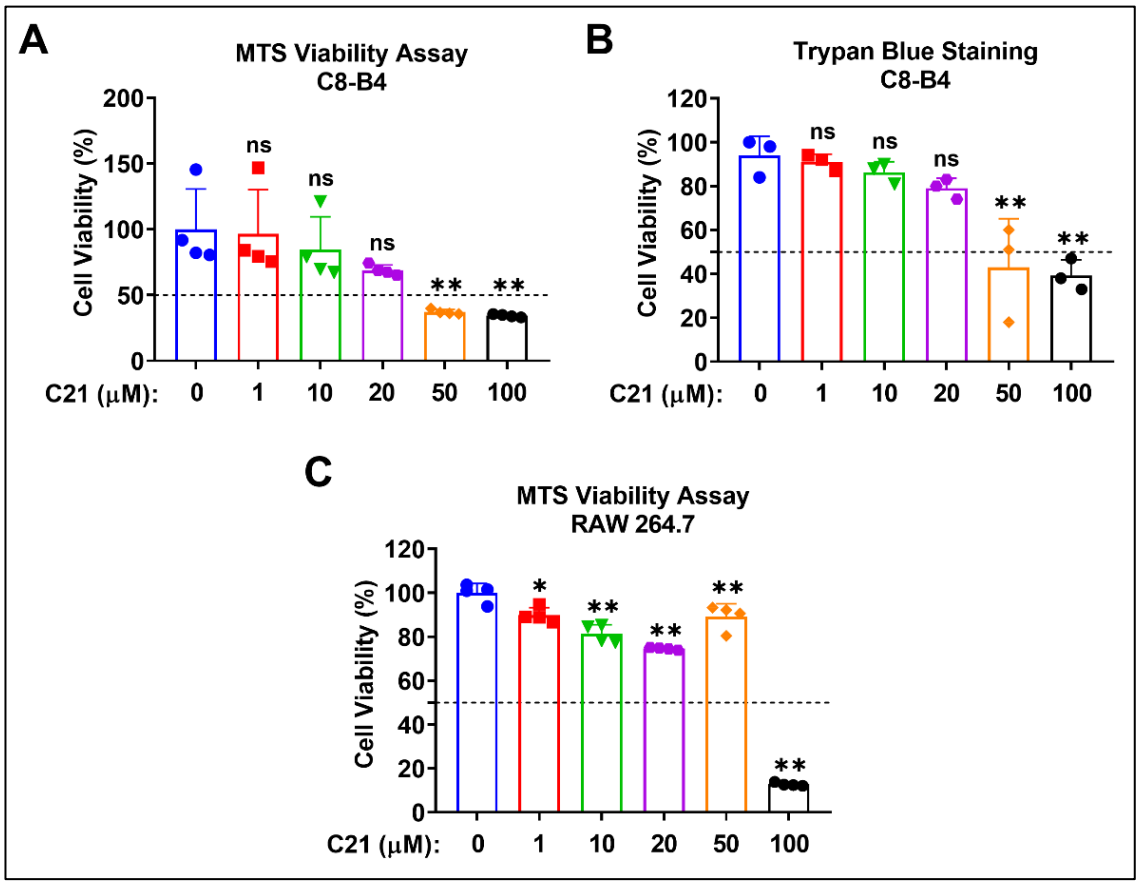
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Figure 2.2. Compound 21 exhibits a profound anti-inflammatory response in Microglia, via decreasing the expression of LPS-induced pro-inflammatory cytokines/chemokines in a dose dependent manner. C8-B4 mouse microglia cells were activated by LPS treatment (100 ng/ml) and co-treated with either different concentration of C21 as indicated or vehicle for 24 hours. 500 ng of total RNA were reverse transcribed into complementary cDNA, and then gene expression was detected using SYBR Green-based RT-qPCR. PPIA was used as an endogenous control. As for protein expression, supernatants of cell culture medium were collected from the same experiment and centrifuged at 2000 g × 10 minutes for complete removal of cell debris and then the secreted cytokines/chemokines were assessed using ELISA kits. **(A-C)** mRNA expression level of proinflammatory cytokines and chemokines, IL-1β **(A),** IL-12b **(B),** and IL-6 **(C). (D)** Protein expression level of proinflammatory cytokine, IL-1β. Values are the mean ± SD of 3 - 5 independent experiments analyzed by one-way ANOVA. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

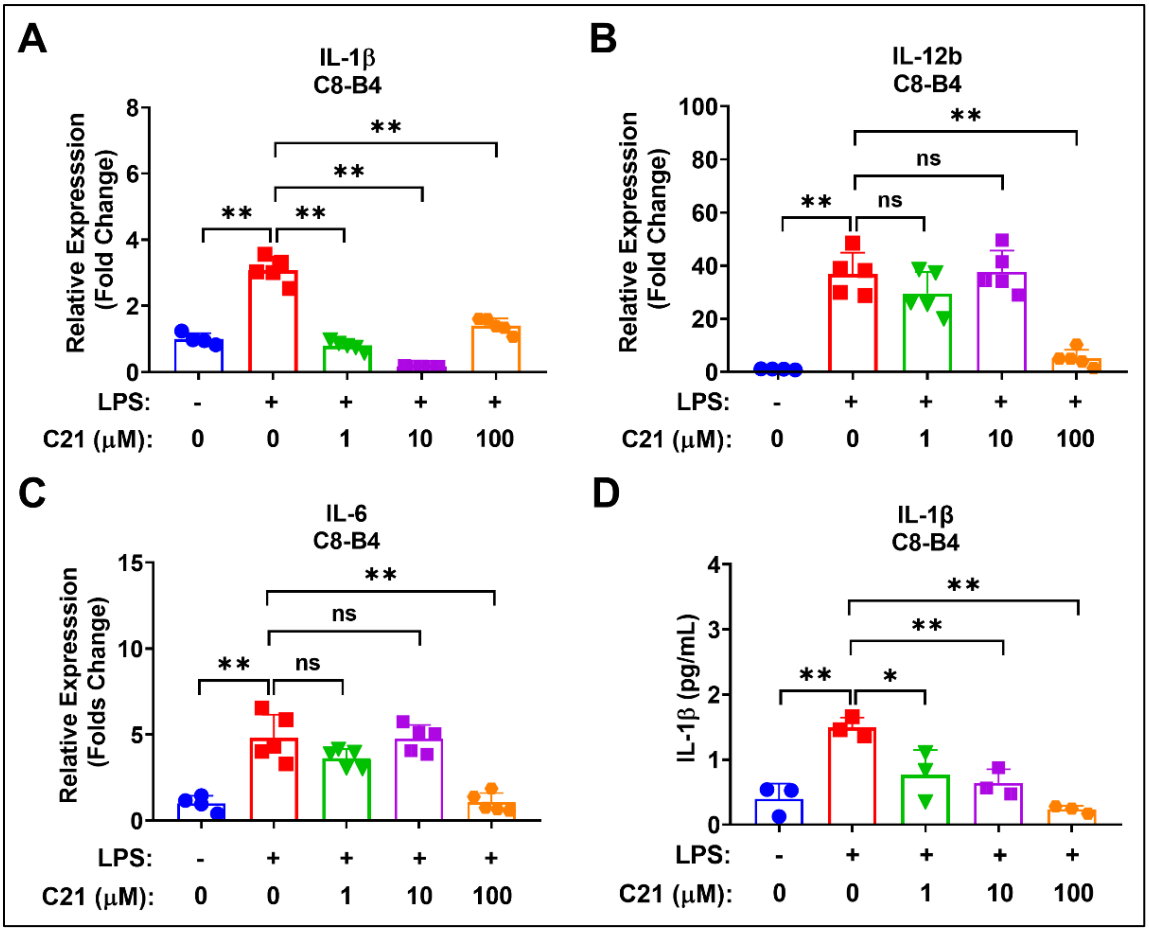
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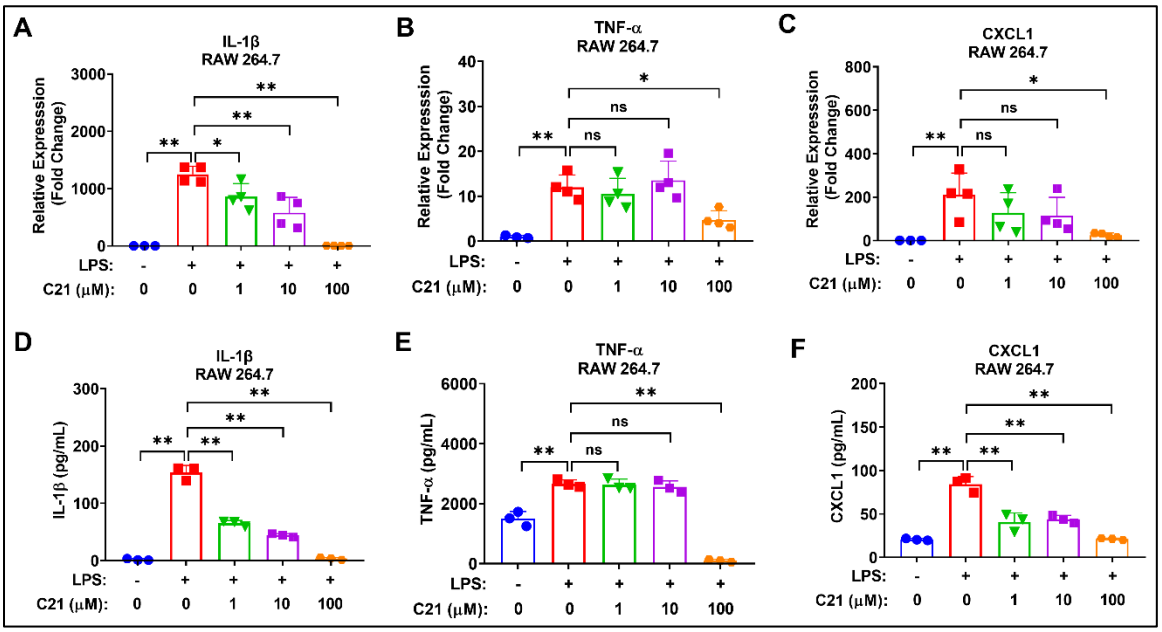
Figure 2.3. Compound 21 effectively regulates the production of ROS, NO, and the activity of pro-inflammatory enzymes, iNOS and COX2 in microglia C8-B4 cells were incubated with LPS (100 ng/ml) and co-cultured with different concentration of C21 as indicated for 24 hours. mRNA expression level of proinflammatory mediators, COX-2 **(A)** and iNOS **(B).** Griess assay kit was used to quantify NO production. Cell culture supernatant was collected and centrifuged to eliminate cell debris, and then mixed with Griess reagent for 30 minutes at room temperature. The 548 nm absorbance was measured. Sodium nitrate was used as a reference. The level of nitrate (μM) was recorded using a microplate reader **(C).** Results are the mean ± SD of 3-5 independent experiments analyzed by one-way ANOVA. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.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LPS: - + + + +
C21 (μM): 0 0 1 10 100

Figure 2.4. Compound 21 significantly ameliorates LPS-induced pro-inflammatory response in Macrophages, following a dose dependent fashion. RAW 264.7 macrophage cells were cultured with LPS (100 ng/ml) and co-treated with different concentration of C21 as indicated or vehicle for 24 hours. 500 ng of total RNA were reverse transcribed into complementary DNA, and then gene expression was detected using SYBR Green-based RT-qPCR. PPIA was used as an endogenous control. Protein expression was evaluated via ELISA. Cell culture supernatants were collected following similar experimental design and centrifuged at 2000 g × 10 minutes for complete removal of cells' debris and then the secreted cytokines/chemokines were assessed. **(A-C)** mRNA expression level of proinflammatory cytokines and chemokines, IL-1β **(A)**, TNF-α **(B)**, and CXCL1 **(C). (D-F)** Protein expression level of proinflammatory cytokine/chemokines, IL-1β **(D),** TNF-α **(E)**, and CXCL1 **(E).** Values are the mean ± SD of 3-4 independent experiments analyzed by one-way ANOVA. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

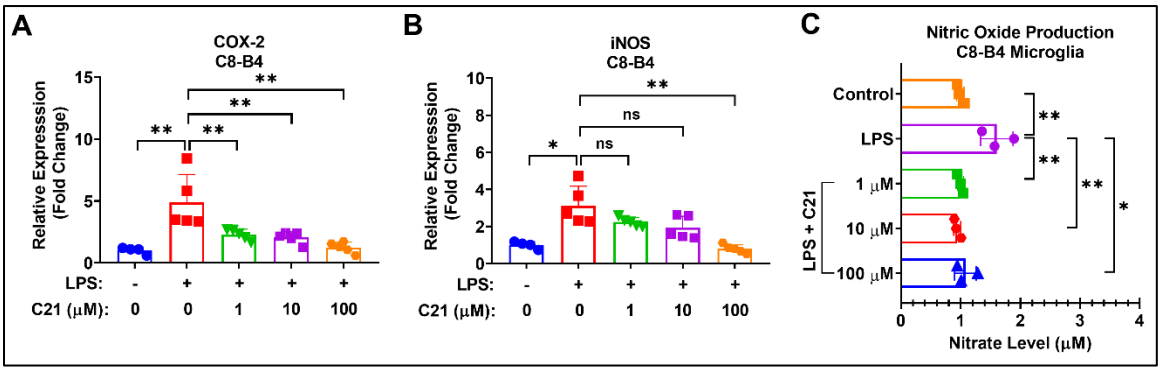

A
COX-2
C8-B4
Relative Expresssion (Fold Change)
15
10
5
0
**
**
**
**
LPS: - + + + +
C21 (μM): 0 0 1 10 100
B
iNOS
C8-B4
Relative Expresssion (Fold Change)
10
8
6
4
2
0
**
ns
*
ns
LPS: - + + + +
C21 (μM): 0 0 1 10 100
C
Nitric Oxide Production
C8-B4 Microglia
Control
LPS
1 μM
10 μM
100 μM
LPS + C21
**
**
**
*
0 1 2 3 4
Nitrate Level (μM)

Figure 2.5. Compound 21 regulates the generation of ROS, NO, and the activity of pro-inflammatory enzymes, iNOS and COX2 in Macrophages. RAW 264.7 macrophage cells were incubated with LPS (100ng/ml) and/or different concentration of C21 as indicated for 24 hours. 500ng of total RNA were reverse transcribed into complementary DNA, and then gene expression was detected using SYBR Green-based RT-qPCR. PPIA was used as an endogenous control. mRNA expression level of proinflammatory mediators, COX-2 **(A)** and iNOS **(B).** As ROS production, Cells were cultured with LPS (100 ng/ml) and co-treated with either different concentrations of C21 or vehicle for 24 hours. Then, cells were washed with PBS and treated with 5 µM CellROXGreen Reagent for 30 minutes. **(D)** Fluorescence images were obtained using a Zeiss Observer Z1 microscope (Carl Zeiss). Nitrate level (µM) was evaluated by Griess assay kit **(C).** After 24-hours treatment with LPS (100ng/ml) and/or C21, cells supernatant were centrifuged, and incubated Griess reagent. Absorbance was measured at 548 nm and Sodium nitrate was used as a reference. Values are the mean ± SD of 3-5 independent experiments analyzed by one-way ANOVA. ns $p > 0.05$, ** $p < 0.01$.

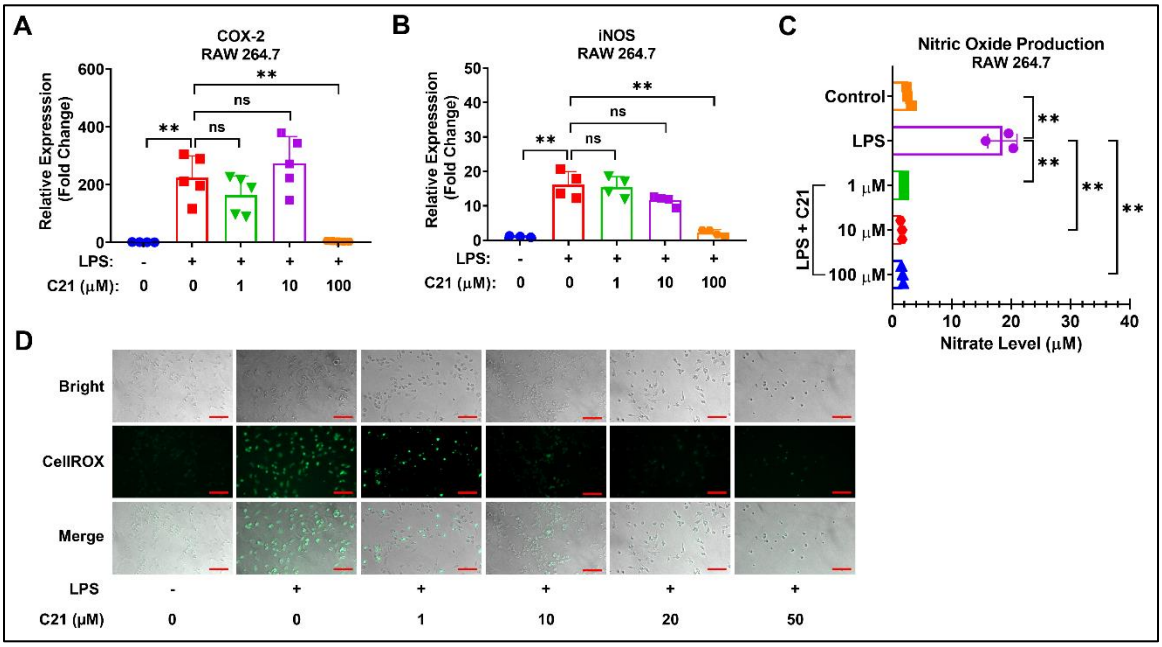
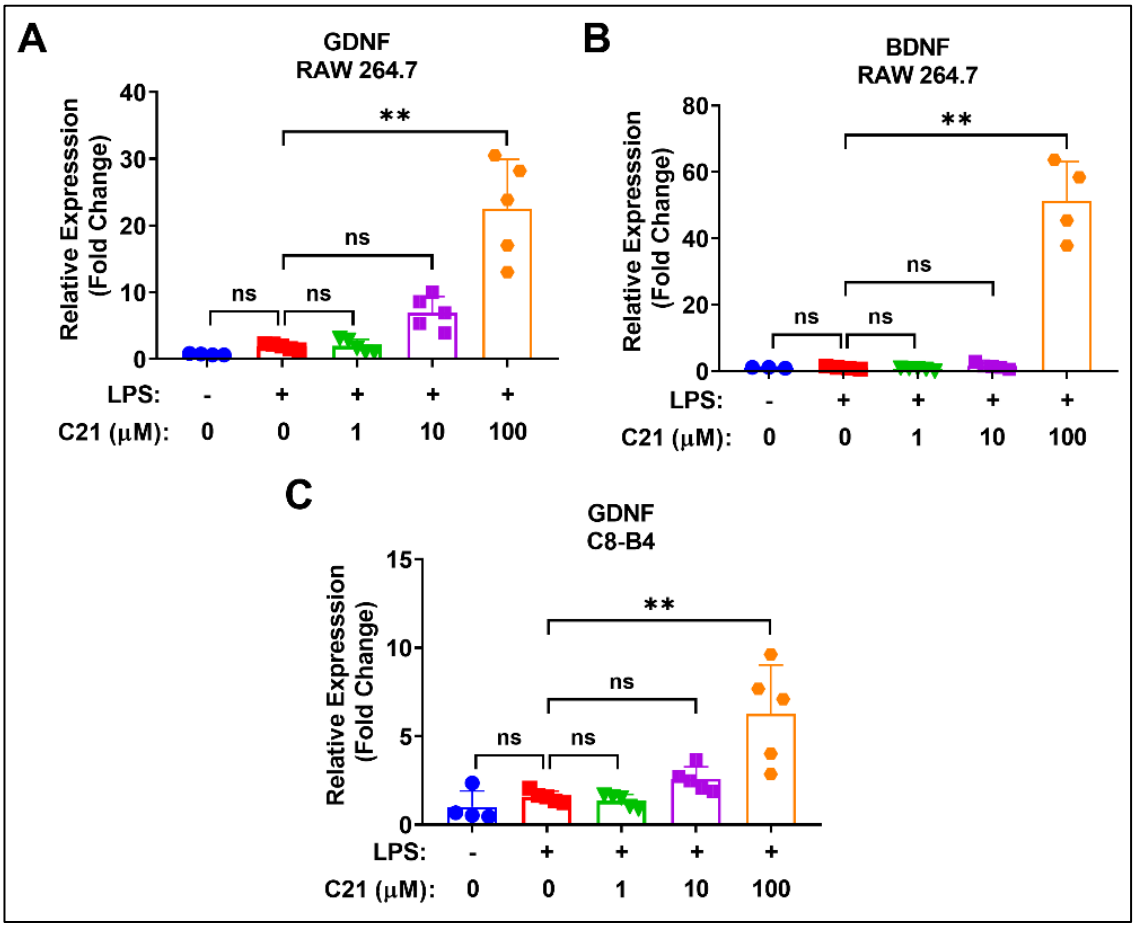

A
COX-2
RAW 264.7
Relative Expresssion (Fold Change)
600
400
200
0
**
ns
**
LPS: - + + + +
C21 (μM): 0 0 1 10 100
B
iNOS
RAW 264.7
Relative Expresssion (Fold Change)
50
40
30
20
10
0
**
ns
**
LPS: - + + + +
C21 (μM): 0 0 1 10 100
C
Nitric Oxide Production
RAW 264.7
Control
LPS
1 μM
10 μM
100 μM
LPS + C21
**
**
**
**
0 10 20 30 40
Nitrate Level (μM)
D
Bright
CellROX
Merge
LPS - + + + + +
C21 (μM) 0 0 1 10 20 50

Figure 2.6. Compound 21 exhibits an increased neurotrophic activity. RAW 264.7 macrophage and C8-B4 microglial cells were incubated with LPS (100 ng/ml) +/- C21 for a total of 24 hours. mRNA expression levels of neurotrophic factors were analyzed using SYBR Green-based RT-qPCR. PPIA was used as an endogenous control. mRNA expression level of neurotrophic factors, GDNF **(A)** and BDNF **(B)**, in RAW 264.7 macrophages **(A** and **B),** and C8-B4 microglia **(C).** Values are the mean ± SD of 5 independent experiments analyzed by one-way ANOVA. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.


A
GDNF
RAW 264.7
Relative Expresssion
(Fold Change)
40
30
20
10
0
**
ns
ns
ns
LPS: - + + + +
C21 (μM): 0 0 1 10 100
B
BDNF
RAW 264.7
Relative Expresssion
(Fold Change)
80
60
40
20
0
**
ns
ns
ns
LPS: - + + + +
C21 (μM): 0 0 1 10 100
C
GDNF
C8-B4
Relative Expresssion
(Fold Change)
15
10
5
0
**
ns
ns
ns
LPS: - + + + +
C21 (μM): 0 0 1 10 100

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CHAPTER 4

INTEGRATED DISCUSSION

The primary objective of this dissertation was to investigate the interplay between hypertension, advanced age, and stroke and their impact on the development of post-stroke/vascular cognitive impairment. These factors are likely to correspond to the complexity of stroke in patients. The study was aiming to look at the impact of aging and hypertension on the development of vascular cognitive impairment before and after stroke. Additionally, we sought to investigate the therapeutic impact of delayed-chronic stimulation of AT2R in preserving cognitive function and delaying the progression of PSCI. Lastly, we aimed to investigate the ability of C21 to regulate the inflammatory response and neutralize the generation of free radicals in both microglia and peripheral macrophages. We hypothesize that the development of vascular cognitive impairment in hypertensive rats will progressively evolve, and stroke will accelerate the progression of PSCI. We believed that the deterioration significance of delayed-chronic stimulation of the AT2 receptor in preventing the development of long-term PSCI, by a mechanism involving neuroinflammation.

Our hypothesis was developed based on encouraging preliminary data from different preclinical stroke models and therapeutic strategies. First, the advancement of new therapeutic strategies targeting stroke has been limited due to the focus on acute neuroprotection after stroke. Second, preclinical stroke research is primarily orientated toward the use of younger, healthier animals. These two factors combined further widen

the gap between clinical and preclinical studies and limit the development of new therapeutics. Studies published by our group and others have demonstrated the progressive nature of post-stroke cognitive impairment and urge the need to develop treatment strategies targeting the later development of PSCI. Our preliminary data strongly indicate a slower rate of recovery and higher mortality in diabetic and hypertensive animals.[1-3] RAS modulation, either via Candesartan or C21, has shown tremendous success to preserve cognitive function after stroke and slow the progression of PSCI, even in animals with comorbidity[1, 2, 4-6]. In these studies, we implemented delayed-chronic intervention as a novel therapeutic strategy, targeting the progression of PSCI and avoiding the acute neuroprotective effects. The impact of delayed intervention with RAS modulators was robust, even when initiated as late as 7 days after stroke[4, 5 1, 2, 6, 7]. Since hypertension and aging are the most common risk factors for stroke and the progression of VCI/PSCI, we needed to investigate whether their complex interplay affects the development of VCI and whether stroke further accelerates cognitive decline.

First, we demonstrated that aged hypertensive rats are a suitable animal model to study vascular cognitive impairment (VCI). Typically, most models of VCI are based on cerebral hypoperfusion, achieved via carotid artery occlusion.[8] Our study was unique in demonstrating a progressive, and spontaneous cognitive decline over time. The complex interplay between aging and hypertension resulted in progressive cognitive decline, brain pathological changes, and elevated arterial pressure. Despite severely elevated MAP, aged SHRs maintained a normal circadian rhythm that peaks and dips over a 24-hours cycle. This mild cognitive decline and MRI abnormalities are closely corresponding to the manifestation of mild cognitive impairment in elderly hypertensive population[9, 10].

Hypertensive elder individuals experience white matter lesions, grey matter atrophy, larger ventricles, and smaller brain volume[9, 10]. These findings, along with high survivability throughout the aging process, further validate the use of aged hypertensive rats to study vascular cognitive impairment.

In the second part of the study, we focused on aged hypertensive rats as a clinically relevant model to study stroke and the potential effect of delayed-chronic stimulation of AT2R in preventing the development of long-term PSCI. A successful stroke is characterized by, acute sensorimotor deficits, weight loss, and the formation of an ischemic lesion. We established a model of minor stroke in aged hypertensive rats, and these parameters were used to validate its applicability. Aged hypertensive rats demonstrated an acute sensorimotor deficit 24 hours after stroke, along with a significant weight loss within 5 days. Additionally, MRI analysis revealed an acute ischemic lesion formation and cerebral edema after stroke. Also, there was long-term brain atrophy in the ipsilateral hemisphere. We anticipated high intra- and perioperative mortality. However, overall short- and long-term survivability was better than expected. We demonstrated that minor stroke has resulted in acute and sustained long-term sensorimotor deficit throughout the study. This uniquely differs from what have been previously reported in young hypertensive and aged normotensive animals, where all animals exhibited a spontaneous recovery.[4, 6] This is important to validate the use of animals with severe comorbidities in stroke research. Most stroke patients experience a long-term disability and reduced mobility. Also, chronic sensorimotor impairment, in elderly stroke patients, is associated with smaller ipsilateral hippocampal volume[11].

Minor stroke further accelerates the progression of cognitive impairment. Long-term cognitive assessments were performed at week-5 and beyond. There was a distinct cognitive decline, some of which could not be captured using the standard assessment tools. Passive avoidance test demonstrated an impaired long-term reference memory at week 5. This impairment could not translate into a measurable deficit in NOR test due to the test limitation. Most animals did not spend enough time exploring their environment at weeks 5 and 8. The percentage of animals excluded exponentially increased over time. It started at a slower rate, 1.7% and 22.8% at 6- and 12 months, respectively, and then increased steeply to 30.2%, 48%, and 70%, at pre-surgery baseline and post-surgery subsequent analysis. This extremely affected our ability to assess their cognitive decline after stroke and led us to adopt an alternative strategy. We decided to use total exploration time in spite of the time spent exploring the novel object. This strategy appeared to be effective in showing cognitive decline after stroke. Aged hypertensive rats demonstrated a progressive decline in total exploration time. This trend corresponds with the trajectory of cognitive decline we observed in the NOR test at an earlier time point, and in young hypertensive rats after stroke. The lack of exploratory activity could be explained by either depression or anxiety; however, these were ruled out. For these reasons, we believe the modification that we made is an acceptable way of assessing cognitive decline, considering the limitation of current tests. Yet, it is crucial to have more studies validating this approach and establishing a standard way of assessing cognitive decline in severely impaired animals.

As for therapeutic intervention, we implemented delayed-chronic treatment with C21, targeting the development of long-term PSCI. Treatment was initiated on day 3 and continued over an eight-week follow-up period. The sole inclusion criteria to be included

in the analysis was a visible ischemic lesion on day three MRI scans. Our data showed a profound effect of delayed AT2R stimulation in reversing the sensorimotor deficit after stroke. This was not anticipated, considering that in our previous studies, all animals recovered after a short period of time. In this study, C21-treated animals completely recovered to the sham level by the end of the study. Additionally, treatment with C21 significantly ameliorated lesion resolution at the chronic phase after stroke. These two findings are of great importance considering the ability of a delayed intervention to reverse a severe long-term sensorimotor impairment and improve ischemic injury. On the other hand, C21 had no superior long-term effect on brain atrophy and cognitive decline. However, when we modified the testing parameters of NOR at weeks 5 and 8 after stroke, animals in the control group exhibited a steeper reduction in exploratory activity as compared to the C21-treated group. The difference between the groups was significant and suggests that delayed AT2R stimulation had a superior effect in preserving cognitive function after stroke.

In the last part of this dissertation, we sought to further confirm the ability of C21 to mediate anti-inflammatory and neurotrophic effects in mouse microglial cell line, C8-B4, and RAW 264.7 macrophages. We demonstrated a dose-dependent reduction in the transcription and translation of pro-inflammatory cytokines/chemokines. Also, there was a significant reduction in cellular ROS and nitrate production. C21 mediated a neurotrophic activity at higher doses in both microglia and macrophages. These effects suggest that C21 mediates its beneficial effect after stroke, in part, via enhancing a dual anti-inflammatory in both microglia and macrophages.

This dissertation has some major limitations. First, we were not able to assess PSCI using the standard testing strategies. This could be explained by several reasons. The spatial memory assessment test is not intended to assess cognitive decline over time and probably is not sensitive enough to capture such a decline. Likely, animals were not interested in engaging with the same repetitive task over time. Another possibility is that the NOR test is not designed to evaluate cognitive decline in severely comorbid models of stroke. The trajectory of cognitive decline as they got older clearly indicated a progressive cognitive decline and made us believe that the test was not sensitive enough to capture PSCI in aged hypertensive animals. These concerns were not anticipated. As a result, we made some necessary modifications that have not been validated. The development of sensitive cognitive tests that are responsive to repetitive testing and able to capture severe cognitive decline is of great importance. The second major limitation is that most of the observed anti-inflammatory effects in the in-vitro analysis were within the cytotoxic range. Although we observed a dose-dependent reduction in some of the cytokines/chemokines, others were only effective at the highest concentration. These discrepancies could be explained by the inability of the isolated in-vitro system to assess the pharmacokinetics of a certain compound. Also, the experiments were designed to assess the inflammatory response at 24 hours post-LPS injury. Perhaps, a smaller concentration was able to induce an anti-inflammatory response earlier than 24 hours. It has been previously reported that immune cells respond to C21 treatment in a time-dependent fashion[12]. An ideal in-vitro experiment would be designed to look at dose- and time-dependent responses to C21 treatment.

Together, this dissertation provides a better understanding of the complex interplay between aging and hypertension, and its impact on the development of vascular cognitive

impairment. We demonstrated that aged hypertensive rats developed a progressive cognitive decline, brain abnormalities, and high survivability pre- and post-stroke. Also, minor stroke resulted in acute-prolonged sensorimotor deficits, accelerated cognitive decline, cerebral edema, and brain atrophy. There was no evidence of post-stroke depression, likely due to double housing and maintaining the same partner throughout the study. Delayed stimulation of AT2R resulted in a sustained improvement in sensorimotor function and a significant reduction in lesion volume at the chronic phase after stroke. The limitation of the current cognitive function tests limited our ability to come up with a clear conclusion regarding the ability of C21 to prevent the progression of PSCI. All in all, some novel aspects of this study are worth pointing out. First, considering all the findings, aged hypertensive rats present as a clinically relevant model to study VCI/PSCI. Stroke research should adopt this model and similar severely comorbid models. Second, targeting the later development of PSCI via delayed-chronic intervention seems to be an effective strategy after stroke. We demonstrated this in previous studies and this study further confirms a similar finding. We are optimistic that more preclinical and clinical studies start to implement a similar therapeutic strategy after stroke. Third, although the clinical data supporting the role of RAS modulation is mixed at this point, this study further supports our previous findings and shows the significance of delayed, long-term intervention with C21 after stroke.

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6. Ahmed HA, Ishrat T, Pillai B, et al. Angiotensin receptor (AT2R) agonist C21 prevents cognitive decline after permanent stroke in aged animals-A randomized double-blind pre-clinical study. *Behav Brain Res* 2019; 359: 560-569. 2018/10/09. DOI: 10.1016/j.bbr.2018.10.010.
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