ANGIOTENSIN II TYPE 2 (AT2R) AGONIST C21 PREVENTS THE POST-INJURY SHIFT TO A PRO-INFLAMMATORY STATE IN MICROGLIA: THERAPEUTIC IMPLICATIONS FOR THE TREATMENT OF POST-STROKE COGNITIVE IMPAIRMENT

by

WALEED ALTHOMALI, PHARM.D.

(Under the Direction of Susan C. Fagan)

ABSTRACT

Post-stroke cognitive impairment (PSCI) occurs in up to 48% of patients, is an important contributor to long-term disability. Hypertension is a major risk factor for PSCI. Currently, PSCI has no approved treatments. After stroke, the pro-inflammatory microglia activation plays an important role in developing PSCI. Previously, we used C21, a direct AT2R agonist, we discovered that C21 reduces the development of PSCI. However, the exact mechanism of the C21 is undetermined. In this study, we hypothesize that AT2R stimulation by C21 decreases the activated microglial phenotype (M1) and elevates M2 microglial phenotype, causing an improvement in PSCI. We tested our hypothesis has by measuring the level of the IL-1β and IL-4 in brain after stroke. Next, we investigated the direct effect of C21 treatment on microglia polarization *in-vitro*. We

found that C21 treatment prevented the increase of IL-1β in brain tissue and prevented microglia polarization toward a M1-like phenotype.

INDEX WORDS: Stroke; PSCI; Microglia; M1:M2; C21

ANGIOTENSIN II TYPE 2 (AT2R) AGONIST C21 PREVENTS POST-INJURY SHIFT TO A PRO-INFLAMMATORY STATE IN MICROGLIA: THERAPEUTIC IMPLICATIONS FOR THE TREATMENT OF POST-STROKE COGNITIVE IMPAIRMENT

by

WALEED ALTHOMALI, PHARM.D.

PharmD, Taif University, Saudi Arabia

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MMASTER OF SCIENCE

ATHENS, GEORGIA

2019

ANGIOTENSIN II TYPE 2 (AT2R) AGONIST C21 PREVENTS THE POST-INJURY SHIFT TO A PRO-INFLAMMATORY STATE IN MICROGLIA: THERAPEUTIC IMPLICATIONS FOR THE TREATMENT OF POST-STROKE COGNITIVE IMPAIRMENT

by

WALEED ALTHOMALI, PHARM.D.

Major Professor: Susan Fagan

Committee: Somanath Shenoy

S. Priya Narayanan

Electronic Version Approved:

Suzanne Barbour

Dean of the Graduate School

The University of Georgia

August 2019

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Stroke

1.1. Stroke incidence and mortality

Globally, stroke is the second leading cause of death and one of the major causes of long-term disability (Benjamin et al., 2017). In the United States, stroke is the fifth leading cause of death (George et al., 2017). Stroke mortality rates differ based on multiple factors, for example, age, sex, comorbidity, and ethnicity (Thrift et al., 2017).

1.2. Stroke types, risk factors, and complications

Strokes are divided into two major types, ischemic and hemorrhagic. The first type occurs in about 80% of stroke cases. Ischemic strokes are classified based on the etiology, the main subtypes are cardioembolic and atherosclerotic. Hemorrhagic strokes are mainly intraparenchymal or subarachnoid (Adams et al., 1993; Benjamin et al., 2017; Boehme, Esenwa, & Elkind, 2017).

Stroke risk factors are either non-modifiable or modifiable. Non-modifiable risks include age, sex, ethnicity, and genetics. Modifiable risk factors involve some chronic diseases, for example, hypertension, diabetes mellitus, and hyperlipidemia. Behavioral risk factors like smoking and physical activity are also considered modifiable risk factors (Boehme et al., 2017; Guzik & Bushnell, 2017).

Serious medical complications result from stroke, in addition to the immediate

neurologic deficits appearing within days to a few weeks after a stroke. Some complications like cardiac abnormalities, pneumonia, bed sores, venous thrombosis, and falls occur with the first days and weeks after the stroke. Other complications result in long-term disability such as post stroke cognitive impairment (PSCI) (Huang, Yang, & Jia, 2015).

2. *Post-Stroke Cognitive Impairment*

Vascular cognitive impairment (VCI) includes all types of cognitive impairment caused by cerebrovascular diseases such as stroke (O'Brien et al., 2003). Vascular cognitive impairment is the second most common cause of dementia (Erkinjuntti, 2002). Based on multiple diagnostic criteria, 20% to 80% of stroke patients develop PSCI. The severity of PSCI depends on the severity of stroke, stroke recurrence, and the functional outcome (Huang et al., 2015). Most stroke patients develop PSCI within three months of the injury (Yu et al., 2013). After a stroke, the risk for cognitive impairment is higher and is exacerbated by hypertension (HTN), diabetes (DM), and advanced age (He et al., 2018; Qu et al., 2015). The pathophysiology of VCI involves many complex events, start with the disruption of blood flow followed by excitoxicity, oxidative stress, and blood- brain barrier (BBB) dysfunction. This will lead to neuronal disturbance and activates the inflammation system (Kalaria, Akinyemi, & Ihara, 2016). Inflammation following a stroke plays a crucial role in causing PSCI.

3. *Inflammation Process After Stroke*

Inflammatory responses after a stroke are complicated in both human and experimental models. After brain injury, the lack of blood flow leads to energy depletion and cell death. After reperfusion, reactive oxygen species (ROS) will be generated. Reactive

oxygen species can then stimulate cells to produce inflammatory cytokines and chemokines in addition to increasing expression of adhesion molecules and recruiting peripheral leukocytes. After this process, cells could release more cytotoxic agents, for example, matrix metalloproteinases (MMPs) or nitric oxide (NO) (Iadecola & Anrather, 2011). These processes result in disruption of the BBB and extracellular matrix and cause edema. BBB disruption and edema lead to the activation of microglia (Patel, Ritzel, McCullough, & Liu, 2013).

4. *Microglia and the its role on the inflammation process*

Microglia, the resident immune cells in the brain, act as tissue macrophages, and serve as scavenger cells in case of ischemia and neurodegeneration. Microglia activation usually occurs after a brain injury. In the active state, there are two main types of microglia, M1microglia phenotype and M2 microglia phenotype. M1 is a pro-inflammatory phenotype linked with an elevation in pro-inflammatory cytokines such as, IFNγ, Il-1β, TNFα, and Il-6. The M1 phenotype could lead to increase cell damage, compared to M2 microglia (Hu et al., 2012) . M2 microglia are responsible for the production of anti-inflammatory cytokines, including, IL-10, IL-4, and TGF-β. This phenotype is responsible for the reduction of inflammation (Lively & Schlichter, 2018). Secondary neurodegeneration, a progressive loss of the function or the structure of the neurons at the injury site, leads to cognitive impairment. The presence of activated microglia has been implicated in the development of secondary neurodegeneration (Zhang, Zhang, Xing, Liang, & Zeng, 2012).

5. *PSCI treatment*

Currently, there are no FDA approved treatments for PSCI. However, secondary prevention strategies could be used to prevent PSCI and dementia. Hypertension is a potent trigger for stroke and PSCI(Iadecola & Anrather, 2011).

6. *The brain renin-angiotensin system (RAS)*

The renin-angiotensin system (RAS) is a complex system that plays a major role in water and electrolyte balance and blood pressure. The brain has a local RAS system. Angiotensin II (Ang II) is the most active hormone secreted by the RAS. Ang II is a hormone produced from angiotensin I (Ang I) under the effect of the angiotensin converting enzyme (ACE) (Mentz et al., 2013; Sparks, Crowley, Gurley, Mirotsou, & Coffman, 2014). There are many types of Ang II receptors, but the most important two are the angiotensin II type 1 receptor (AT1Rs) and angiotensin II type 2 receptors (AT2Rs). These two receptors play a crucial role in microglia activation after brain injury. AT1R is responsible for the harmful effects that occur from Ang II, enhancing the inflammation process and contributing to the activation of M1 microglia. AT2R provides the protective effect of the RAS system by promoting polarization toward the M2 microglia phenotype (Labandeira-Garcia et al., 2017). AT2R stimulation could be a vital therapeutic target to prevent PSCI. Many studies support using angiotensin receptor blockers (ARBs), widely used antihypertensive medications that selectively block AT1Rs, which allows for greater activation of AT2Rs, in improving stroke outcome, and reducing cognitive impairment (Villapol & Saavedra, 2015). Recent evidence suggests that Compound 21 (C21), an experimental drug and a selective AT_2R agonist, has shown a beneficial effect on PSCI and enhances the activation of microglia (Bennion et al.,

2017; McCarthy et al., 2014). In our lab, we investigated the beneficial effects of C21 and found that C21 potentially improves the behavioral pattern in spontaneously hypertensive rat (SHR) experimental models even with a delayed administration of the drug after stroke (Ahmed et al., 2018). Moreover, the drug showed a neuroprotection with one dose at reperfusion (Alhusban et al., 2015). Additionally, we found that C21 is neuroprotective on female rats(Eldahshan et al., 2019)

PROBLEM STATEMENT AND SPECIFIC AIMS

From our lab, after we used Compound C21 in multiple studies we found that C21 reduced the infarct size, improved the stroke outcomes, and prevented PSCI. Until now, the mechanism by which angiotensin II type 2 (AT2) receptor agonists minimize PSCI is not clear. **Central hypothesis:** Stimulation of AT2R by C21 reduces the activated inflammatory microglial phenotype (M1) and increases the anti-inflammatory microglial phenotype (M2), resulting in an improvement in PSCI.

To address our hypothesis, we will pursue two aims:

Aim 1: We propose to determine the ratio of pro-inflammatory and anti-inflammatory cytokines in brain samples of ovariectomized female spontaneously hypertensive rats (SHRs) after treatment with C21 after stroke. We have evidence that this treatment prevents PSCI in these animals. This hypothesis will be tested by performing the enzyme-linked immunosorbent assay (ELISA) to measure IL-4 and IL-1 beta.

Aim 2: We propose to determine the extent to which treatment with the angiotensin II receptor (AT2R) Agonist, C21, alters M1:M2 ratio of microglia *(in-vitro)*.

We planned to test this hypothesis by performing the following experiments:

2.1 Determine the effect of C21 on microglia polarization in mouse cells (C8-B4 line) by flow cytometry-based analysis of polarization markers.

2.2 Analyze the ratio of mature:pro, which is mainly secreted by M2 microglia, with western blot to inspect the possible underlying mechanism.

References:

Adams, H. P., Jr., Bendixen, B. H., Kappelle, L. J., Biller, J., Love, B. B., Gordon, D. L., & Marsh, E. E., 3rd. (1993). Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke, 24*(1), 35-41.

Ahmed, H. A., Ishrat, T., Pillai, B., Fouda, A. Y., Sayed, M. A., Eldahshan, W., . . . Fagan, S. C. (2018). RAS modulation prevents progressive cognitive impairment after experimental stroke: a randomized, blinded preclinical trial. *J Neuroinflammation, 15*(1), 229. doi:10.1186/s12974-018-1262-x

Benjamin, E. J., Blaha, M. J., Chiuve, S. E., Cushman, M., Das, S. R., Deo, R., . . . Stroke Statistics, S. (2017). Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation, 135*(10), e146-e603. doi:10.1161/CIR.0000000000000485

Bennion, D. M., Isenberg, J. D., Harmel, A. T., DeMars, K., Dang, A. N., Jones, C. H., . . . Sumners, C. (2017). Post-stroke angiotensin II type 2 receptor activation provides long-term neuroprotection in aged rats. *PLoS One, 12*(7), e0180738. doi:10.1371/journal.pone.0180738

Boehme, A. K., Esenwa, C., & Elkind, M. S. (2017). Stroke Risk Factors, Genetics, and Prevention. *Circ Res, 120*(3), 472-495. doi:10.1161/CIRCRESAHA.116.308398

Erkinjuntti, T. (2002). Diagnosis and management of vascular cognitive impairment and dementia. *J Neural Transm Suppl*(63), 91-109.

George, M. G., Fischer, L., Koroshetz, W., Bushnell, C., Frankel, M., Foltz, J., & Thorpe, P. G. (2017). CDC Grand Rounds: Public Health Strategies to Prevent and Treat Strokes. *MMWR Morb Mortal Wkly Rep, 66*(18), 479-481. doi:10.15585/mmwr.mm6618a5

Guzik, A., & Bushnell, C. (2017). Stroke Epidemiology and Risk Factor Management. *Continuum (Minneap Minn), 23*(1, Cerebrovascular Disease), 15-39. doi:10.1212/CON.0000000000000416

He, M., Wang, J., Liu, N., Xiao, X., Geng, S., Meng, P., . . . Wang, Y. (2018). Effects of Blood Pressure in the Early Phase of Ischemic Stroke and Stroke Subtype on Poststroke Cognitive Impairment. *Stroke, 49*(7), 1610-1617. doi:10.1161/STROKEAHA.118.020827

Hu, X., Li, P., Guo, Y., Wang, H., Leak, R. K., Chen, S., . . . Chen, J. (2012). Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. *Stroke, 43*(11), 3063-3070. doi:10.1161/STROKEAHA.112.659656

Huang, Y., Yang, S., & Jia, J. (2015). Factors related to long-term post-stroke cognitive impairment in young adult ischemic stroke. *Med Sci Monit, 21*, 654-660. doi:10.12659/MSM.892554

Iadecola, C., & Anrather, J. (2011). The immunology of stroke: from mechanisms to translation. *Nat Med, 17*(7), 796-808. doi:10.1038/nm.2399

Kalaria, R. N., Akinyemi, R., & Ihara, M. (2016). Stroke injury, cognitive impairment and vascular dementia. *Biochim Biophys Acta, 1862*(5), 915-925. doi:10.1016/j.bbadis.2016.01.015

Labandeira-Garcia, J. L., Rodriguez-Perez, A. I., Garrido-Gil, P., Rodriguez-Pallares, J., Lanciego, J. L., & Guerra, M. J. (2017). Brain Renin-Angiotensin System and Microglial Polarization: Implications for Aging and Neurodegeneration. *Front Aging Neurosci, 9*, 129. doi:10.3389/fnagi.2017.00129

Lively, S., & Schlichter, L. C. (2018). Microglia Responses to Pro-inflammatory Stimuli (LPS, IFNgamma+TNFalpha) and Reprogramming by Resolving Cytokines (IL-4, IL-10). *Front Cell Neurosci, 12*, 215. doi:10.3389/fncel.2018.00215

McCarthy, C. A., Vinh, A., Miller, A. A., Hallberg, A., Alterman, M., Callaway, J. K., & Widdop, R. E. (2014). Direct angiotensin AT2 receptor stimulation using a novel AT2 receptor agonist, compound 21, evokes neuroprotection in conscious hypertensive rats. *PLoS One, 9*(4), e95762. doi:10.1371/journal.pone.0095762

Mentz, R. J., Bakris, G. L., Waeber, B., McMurray, J. J., Gheorghiade, M., Ruilope, L. M., . . . Pitt, B. (2013). The past, present and future of renin-angiotensin aldosterone system inhibition. *Int J Cardiol, 167*(5), 1677-1687. doi:10.1016/j.ijcard.2012.10.007

O'Brien, J. T., Erkinjuntti, T., Reisberg, B., Roman, G., Sawada, T., Pantoni, L., . . . DeKosky, S. T. (2003). Vascular cognitive impairment. *Lancet Neurol, 2*(2), 89-98.

Patel, A. R., Ritzel, R., McCullough, L. D., & Liu, F. (2013). Microglia and ischemic stroke: a double-edged sword. *Int J Physiol Pathophysiol Pharmacol, 5*(2), 73-90.

Qu, Y., Zhuo, L., Li, N., Hu, Y., Chen, W., Zhou, Y., . . . Zhan, S. (2015). Prevalence of post-stroke cognitive impairment in china: a community-based, cross-sectional study. *PLoS One, 10*(4), e0122864. doi:10.1371/journal.pone.0122864

Sparks, M. A., Crowley, S. D., Gurley, S. B., Mirotsou, M., & Coffman, T. M. (2014). Classical Renin-Angiotensin system in kidney physiology. *Compr Physiol, 4*(3), 1201-1228. doi:10.1002/cphy.c130040

Thrift, A. G., Thayabaranathan, T., Howard, G., Howard, V. J., Rothwell, P. M., Feigin, V. L., . . . Cadilhac, D. A. (2017). Global stroke statistics. *Int J Stroke, 12*(1), 13-32. doi:10.1177/1747493016676285

Villapol, S., & Saavedra, J. M. (2015). Neuroprotective effects of angiotensin receptor blockers. *Am J Hypertens, 28*(3), 289-299. doi:10.1093/ajh/hpu197

Yu, K. H., Cho, S. J., Oh, M. S., Jung, S., Lee, J. H., Shin, J. H., . . . Korean-Vascular Cognitive Impairment Harmonization Standards Study, G. (2013). Cognitive impairment evaluated with Vascular Cognitive Impairment Harmonization Standards in a multicenter prospective stroke cohort in Korea. *Stroke, 44*(3), 786-788. doi:10.1161/STROKEAHA.112.668343

Zhang, J., Zhang, Y., Xing, S., Liang, Z., & Zeng, J. (2012). Secondary neurodegeneration in remote regions after focal cerebral infarction: a new target for stroke management? *Stroke, 43*(6), 1700-1705. doi:10.1161/STROKEAHA.111.632448

CHAPTER 2

The direct effect of Angiotensin II Type 2 (AT2R) Agonist C21 on Microglia

Waleed Althomali[1], Ladonya Jakson[1], Wael Eldahshan[1], Bindu Pillai[1], and Susan C. Fagan[1,2].

[1]Program in Clinical and Experimental Therapeutics, Charlie Norwood VA Medical Center and University of Georgia College of Pharmacy, Augusta, GA, Departments of [2]Neurology, Augusta University, Augusta, GA.

Correspondence:

Susan C Fagan, Pharm.D., FAHA

University of Georgia, College of Pharmacy

HM Bldg.;1120 15th St.

Augusta, GA 30912

sfagan@uga.edu

Voice: (706) 721 0130

Fax: (706) 721 3994

(To be submitted to Stroke)

Abstract

Post-stroke cognitive impairment (PSCI) occurs in up to 48% of patients, is an important contributor to long-term disability. Hypertension is a major risk factor for PSCI. Currently, PSCI has no approved treatments. After stroke, the pro-inflammatory microglia activation plays an important role in developing PSCI. Previously, we used C21, a direct AT2R agonist, we discovered that C21 reduces the development of PSCI. However, the exact mechanism of the C21 is undetermined. In this study, we hypothesize that AT2R stimulation by C21 decreases the activated microglial phenotype (M1) and elevates M2 microglial phenotype, causing an improvement in PSCI. We tested our hypothesis has by measuring the level of the IL-1β and IL-4 in brain after stroke. Next, we investigated the direct effect of C21 treatment on microglia polarization *in-vitro*. We found that C21 treatment prevented the increase of IL-1β in brain tissue and prevented microglia polarization toward a M1-like phenotype.

Keywords: Stroke; PSCI; Microglia; M1:M2; C21

Introduction

In the United States, Stroke is the 5th leading cause of death in the US and most of stroke cases are ischemic (Murphy, Kochanek, Xu, & Arias, 2015). Post stroke cognitive impairment (PSCI) is one of most noticeable complications of stroke and occurs in up to 48% of stroke victims (Sun, Tan, & Yu, 2014). PSCI can occur instantly after stroke or it may take a long time to develop. The progression of PSCI correlates with numerous vascular changes (Kalaria, 2012).

PSCI can be exacerbated with several risk factors. While some risk factors such as hypertension, diabetes mellitus, and hyperlipidemia are modifiable, some risk factors are not, for instance, age and race. Many studies showed that managing modifiable risk factors will reduce the incidence of recurrent stroke and PSCI (Huang et al., 2015; Mohd Zulkifly, Ghazali, Che Din, Singh, & Subramaniam, 2016) Unfortunately, there is no approved treatment for PSCI. Many experimental drugs are under the investigation to treat PSCI. Targeting inflammation after stroke is one of the possible future treatment strategies.

Recent studies revealed that after stroke, the inflammatory response plays an important role in repair. In the restoration process, numerous cytokines and chemokines will be produced because of the reactive oxygen species (ROS) activation and that will disturb the blood brain barrier. This leads to activate microglia, the resident immune cell in the brain (Iadecola & Anrather, 2011; Patel et al., 2013). During the activation of the microglia, the M1 phenotype will produce various kind of pro-inflammatory cytokines which include, IFNγ, Il-1β, TNFα. Moreover, the M2 phenotype, is associated with

presence of the anti-inflammatory cytokines, for example, IL-10 and IL-4(Hu et al., 2012; Lively & Schlichter, 2018).

Renin-angiotensin system (RAS) modulators can prevent the development of PSCI in animal models. Angiotensin II (Ang II) plays a major role in microglia activation after stroke by its effect on two main receptors, angiotensin II type 1 receptor (AT1Rs), and Angiotensin II type 2 receptors (AT2Rs). AT1Rs promote the shift toward M1 microglia phenotype and a pro pro-inflammatory state. AT2Rs promote the polarization to M2 microglia, the anti-inflammatory state (Jackson, Eldahshan, Fagan, & Ergul, 2018).

In our lab, we discovered that AT2R stimulation can improve stroke outcome by, minimizing the infarct volume, and reducing behavioral deficits. In order to activate the receptors, we used Compound 21 (C21), an AT2R agonist (Ahmed et al., 2019; Ahmed et al., 2018; Fouda, Pillai, Dhandapani, Ergul, & Fagan, 2017).

In the current study, we aimed to understand the possible mechanism of action of C21 and the underlying signaling pathway. First, we measured the level of IL-4 and IL-1β in brain tissue. Samples were collected from animals involved in our long-term study on female ovariectomized spontaneously hypertensive rats (SHRs), treated with either C21 or saline. To address this goal, we used ELISA assay. Second, we investigated the direct effect of C21 pre-treatment on a C8-B4 microglia cell line.

Materials and Methods

Brain samples collection

Ethical Approval:

The animal protocol was approved by the Institutional Animal Care and Use Committee of the Charlie Norwood VA Medical Center, Augusta, Georgia.

Sample collection:

Brain samples were collected from our long-term study on Young female ovariectomized spontaneously hypertensive rats (SHRs) who were subjected to 1h of MCAO, 6 weeks after ovariectomy. Animals were randomized to be treated with either C21 (0.03 mg/kg/day) or saline IP for the first 5 days then followed by C21 in drinking water or water alone, until the end of the study (6 weeks after stroke). In the study, animals were randomized into four groups as follow: Sham/Saline, Sham/C21, Stroke/Saline, Stroke/C21.

Compound 21 (C21):

C21 was a gift from Vicore Pharma (Mölndal, Sweden) and was freshly prepared for each experiment from 1mg/ml stock in 0.9% saline to a final concentration of 0.03mg/ml for intraperitoneal injection or to a 0.12mg/ml for oral gavage. The dose adjustment for oral administration was based on the drug bioavailability, as described in our previous studies (Ahmed et al., 2018; Alhusban et al., 2015).

Pro-inflammatory cytokines (IL-1β) and Anti-inflammatory (IL-4) levels evaluation

The concentrations of Il-4 and Il-1b were detected using an ELISA kit from RayBiotech Inc. (Peachtree Corners, GA). Diluted samples and the standard were added to the plates and kept at 4 °C overnight. Then, the plates were washed 4 times before adding 100 µL of

biotin antibody anti-rat IL-4 and anti-rat IL-1β and then incubated for 1 hour at room temperature. After another 4 washes, we added 100 µL of Streptavidin solution and the we incubated the plates for 45 min at room temperature. Finally, another four washes were done and then we added 100 µL of TMB (3,3′,5,5′-tetramethylbenzidine) reagent and incubated for 30 min. Finally, we added 50 µL stop solution (0.2 M sulfuric acid) and the readings were measured at 450 nm.

Cell culture:

The direct effect of C21 on microglia polarization was determined in mouse cells (C8-B4 line). We kept the cells at 37◦C in 5% CO2 in Dulbecco's Modification Eagle's Medium (DMEM) with 4.5 g/l glucose, L- glutamine & sodium pyruvate, accompanied with 10% FBS and 1% penicillin/streptomycin. We applied flow cytometry-based analysis of polarization markers (F4/80 TNFa, CD206, and IL-10). We treated cells with LPS (100 ng/ml) and IFNy (20 ng/ml) to prompt the activation M1-like polarization. Cells were pre-treated with C21 (100 nM) 6 hours prior to LPS/IFNy exposure to evaluate whether C21 prevents M1-like polarization. The AT2R blocker, PD 123319 (0.1 µM), was used to determine if the C21 effects were mediated through AT2R agonism. IL-4 (20 ng/ml) was used as a positive control for M2-like polarization. BDNF (which is predominately secreted by M2 microglia) was analyzed with western blot to determine a possible mechanism.

Results

C21 treatment decreased stroke-induced elevation in IL-1β in brain tissue of ovariectomized SHRs.

In female ovariectomized SHRs, 6 weeks after stroke, brain tissue lysate showed a significant elevation in IL-1β level in Saline-treated stroked group compared to the Saline treated-sham group. Interestingly, C21 treatment prevented the stroke related increase in the level of IL-1β (Fig.1).

IL-4 level was decreased under the effect of C21 treatment

With the same brain tissue, IL- 4 was significantly reduced between the treatment groups $P < 0.05$ (Fig. 2).

C21 Blunted the LPS/IFNy Induced Polarization Toward a M1 Phenotype

To evaluate the effect of C21 treatment on microglia, C8B4 mouse cell line were used *in-vitro*. We used F4/80 and TNFα+ to indicate the M1 microglia phenotype. The same cells were gated for CD206+ and IL-10 to indicate the M2 phenotype. Then, we compared TNFα+ and IL-10+ to obtain the M1 and M2 ratio (Fig. 3). LPS/IFNy cell treatment shifted the microglia cells to the pro-inflammatory state (M1) and reduced the percentage of M2 phenotype (Fig 4A-B). 6-hour pre-treatment with C21was able to prevent the polarization toward a M1-like phenotype induced by LPS/IFNɣ (Fig 4A-B).

C21 Enhanced Mature BDNF Expression

Mature BDNF is mainly secreted by M2 microglia. To demonstrate a further confirmation that C21 treatment shifted microglia to the M2 phenotype, we applied western blot to measure the ratio of mature: pro – BDNF and we found that C21 exposure increased the ratio (Fig 5 A-B).

Discussion and conclusion

The main findings of the present study are the following: 1) after stroke, treatment with the AT2R agonist, C21, influences the inflammatory cytokines levels in young female ovariectomized spontaneously hypertensive rats (SHRs) brain lysate. C21 was able to keep the level of the pro-inflammatory cytokine IL-1 beta in the C21 stroke group as the same as in the sham group; 2) the administration of C21 on C8-B4 microglia cell line *in-vitro,* reduced the microglia M1:M2 ratio even after microglia were exposed to LPS/IFNy to induce more polarization toward M1 phenotype. Also, we evaluated the ratio of mature: pro – BDNF and we confirmed that C21 shifted the microglia toward the anti-inflammatory type.

In the current study, we showed that stroke increased the expression of Interleukin-1β (IL-1β), and this was prevented with C21. As is known, IL-1β is a major player of IL-1 family, and a powerful pro-inflammatory cytokine that plays a major role in the defense mechanism after injury (Dinarello, 1996). Within the CNS system, IL-1 is mostly formed by microglial cells (Giulian, Baker, Shih, & Lachman, 1986). This cytokine represents the microglia M1 phenotype. We found that the cytokine level in the sham group was low. That comes along with what we found in the literature that in a healthy state, IL-1β expression is low (Hewett, Jackman, & Claycomb, 2012). In brain injury, the IL-1 family was linked with the increase of the lesion volume and the neurological deficit (Jones et al., 2005). In our study, we found that the increase in IL-1β expression within the brain after stroke, was prevented with C21. It is possible that C21 prevented the shift of microglia towards M1 type which can be detected by the level of IL-1β and other pro-

inflammatory cytokines. This effect ultimately reduces the infarct volume, improves the behavioral outcomes, and prevents PSCI.

Interleukin-4 (IL-4) is a fundamental cytokine that assists in the brain recovery after brain injury by controlling microglia function. The absence of IL-4 worsened PSCI, elevated the expression of M1 microglia cytokines, and reduced the presence of M2 biomarkers. However, IL-4 treatment improved the long-term effect (Liu et al., 2016; Yang et al., 2016). In our study, we detected the IL-4 in brain samples as described in our methods, and we found that there was a reduction in the level of IL-4 with C21 treatment. Previous research showed that IL-1β expression is controlled by IL-10 and IL-4(Pousset, Cremona, Dantzer, Kelley, & Parnet, 1999), suggesting that the reduction in IL-4 seen in the C21 treated animals may be a feedback mechanism, responding to the reduced IL-1β. We also used IL-4 treatment in our microglia cell culture as a positive control, and the percentage of M1 and M2 was almost the same, with no significant difference from control. It is likely that the IL-4 beneficial effect happened due the cytokines ability to control microglia cell number(Soria et al., 2011).

In the *in-vitro* experiments, we produced a huge number of microglial cells, and to differentiate them, we used F4/80 as a marker for M1 phenotype and CD206 as a marker for M2 phenotype. After LPS/INFy treatment, M1 was activated more than M2 but when we pre-treated the cells with C21, the number of M1 microglia were reduced and the M2 population enlarged. Those results are consistent with what Valero-Esquitino and his group identified when they worked with same type of microglia cell line (Valero-Esquitino et al., 2015). We confirmed that C21 polarized the microglia toward the M2

phenotype by running western blot and demonstrating an increase in the expression of mature BDNF.

In summary, C21 reduces chronic inflammation after stroke in hypertensive animals, by a mechanism that involves the polarization of microglia away from the M1 phenotype. The predominance of the M2 phenotype results in an increased expression of mature BDNF, resulting in improved cognition at 6 weeks after stroke.

Figure 1: C21 maintained the level the IL-1β in stroke group

The level of the pro-inflammatory cytokine IL-1β in protein extracted form ovariectomized spontaneously hypertensive rats (SHRs) which exposed to 1 hr MCAO the treated with C21 were determined by enzyme-linked immunosorbent assay (ELISA). N=5 sham/saline group, N=3 sham/C21 group, N=5 stroke/saline group, N=3 stroke/ C21 group. * $P<0.05$

figure 1

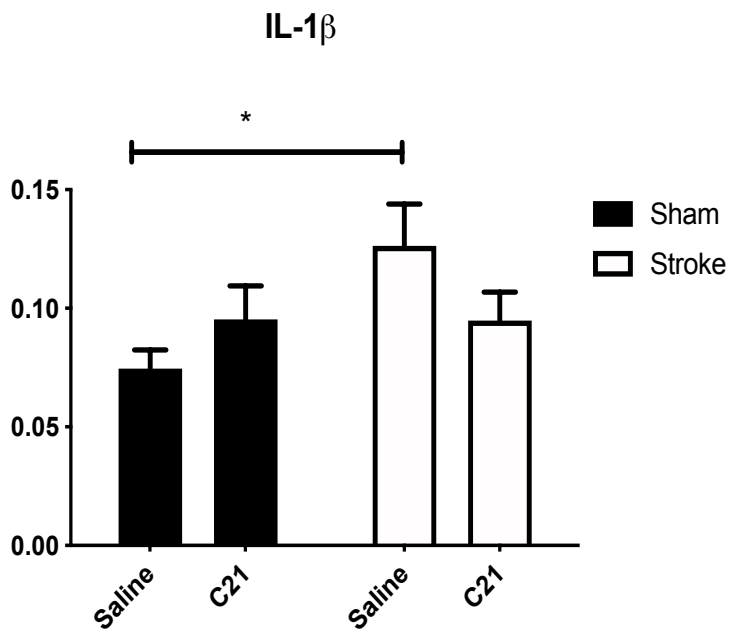


Figure 2: C21 attenuated the level of IL-4 cytokine.

The level of the anti-inflammatory cytokine IL-4 in protein isolated form ovariectomized spontaneously hypertensive rats (SHRs) which exposed to 1 hr MCAO the treated with C21 were determined by enzyme-linked immunosorbent assay (ELISA). N=5 sham/saline group, N=3 sham/C21 group, N=5 stroke/saline group, N=3 stroke/ C21 group. * $P<0.05$

figure 2

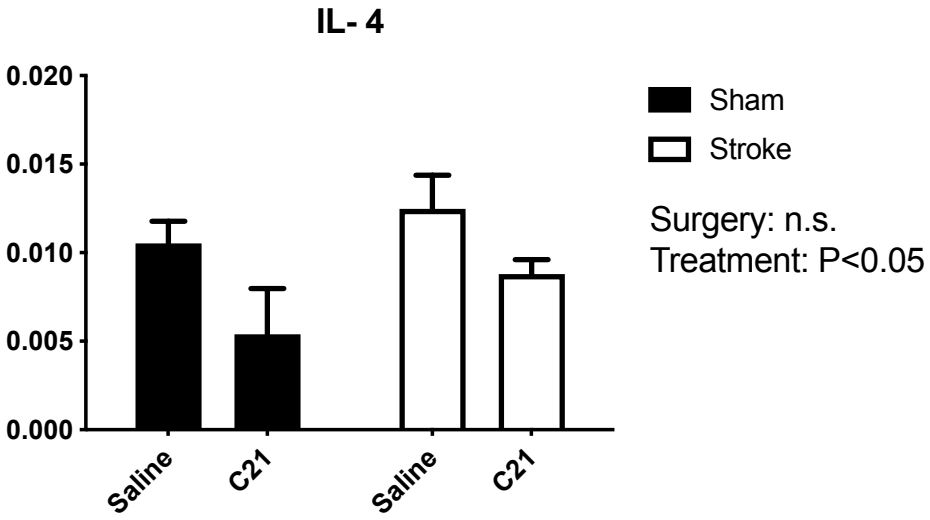

IL- 4
0.020
0.015
0.010
0.005
0.000
Saline
C21
Saline
C21
Sham
Stroke
Surgery: n.s.
Treatment: P<0.05

Figure 3: Flow cytometric representing graph

figure 3

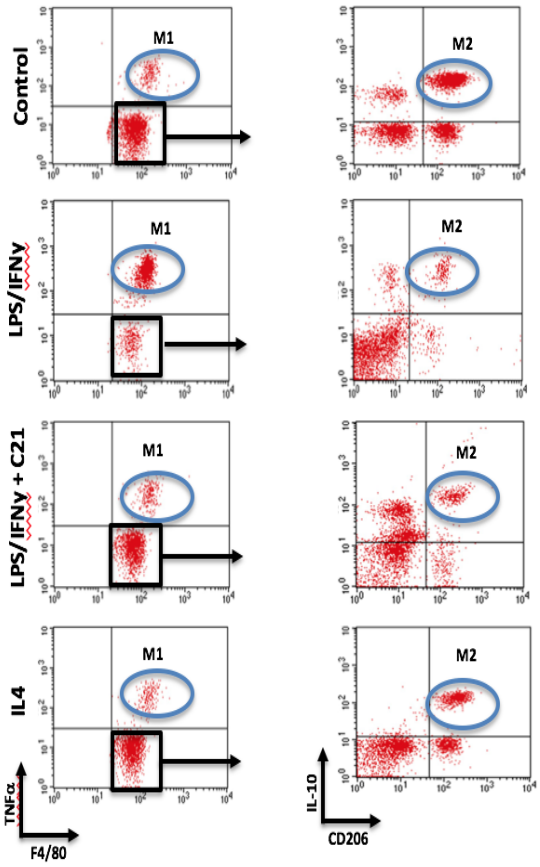
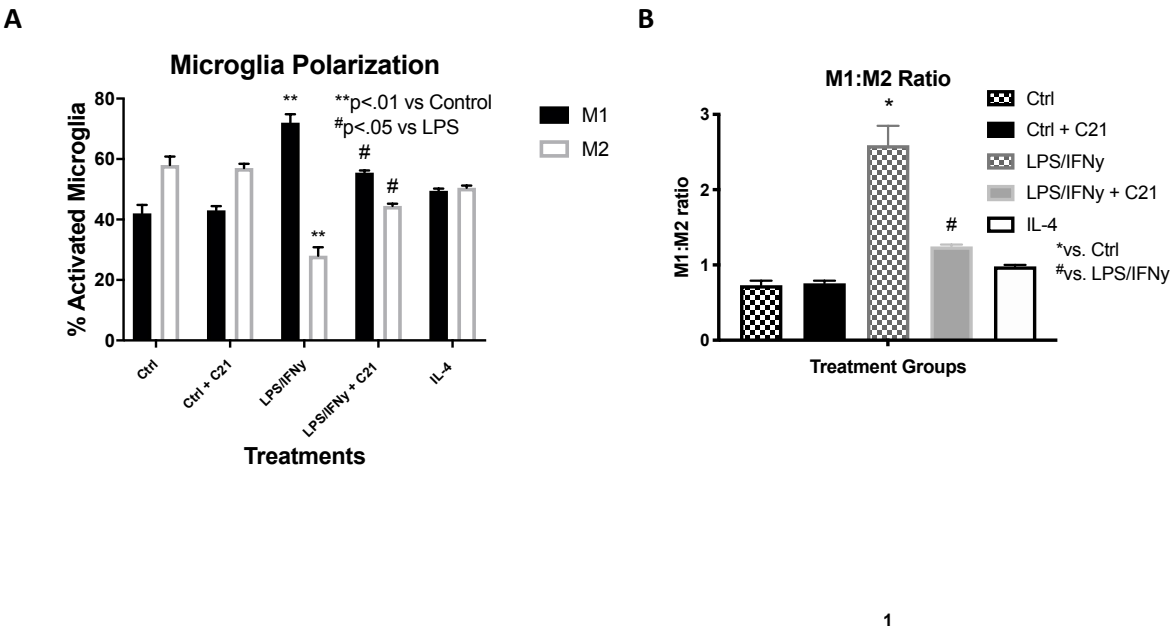


Figure 4: C8-B4 cell line treated with C21

A) LPS/IFNy treatment shifted cells towards M1 phenotype the microglia toward a M1 phenotype and C21 retuned the effect of LPS/IFNy. ** *compared to control ($p<0.01$), # compared to LPS/IFNy* (*$p<0.05$).*

B) C21 reduced M1:M2 ratio in C8-B4 cells polarized toward a M1 phenotype with the under the effect of LPS/IFNy. ** *compared to control ($p<0.01$), # compared to LPS/IFNy* (*$p<0.05$).*

figure 4


A
Microglia Polarization
**p<.01 vs Control
#p<.05 vs LPS
% Activated Microglia
0
20
40
60
80
**
**
#
#
M1
M2
Ctrl
Ctrl + C21
LPS/IFNy
LPS/IFNy + C21
IL-4
Treatments
B
M1:M2 Ratio
*
#
M1:M2 ratio
0
1
2
3
Ctrl
Ctrl + C21
LPS/IFNy
LPS/IFNy + C21
IL-4
*vs. Ctrl
#vs. LPS/IFNy
Treatment Groups

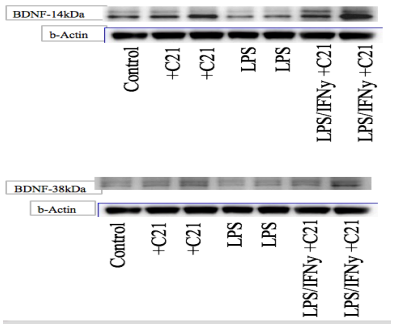
1

Figure 5: C21 Enhanced Mature BDNF Expression

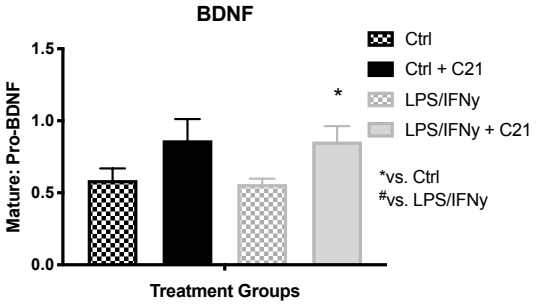
representative western blot. B: C21 Increased the mature:pro BDNF ratio.

Figure 5

A


BDNF-14kDa
b-Actin
Control
+C21
+C21
LPS
LPS
LPS/IFNy +C21
LPS/IFNy +C21
BDNF-38kDa
b-Actin
Control
+C21
+C21
LPS
LPS
LPS/IFNy +C21
LPS/IFNy +C21

B


BDNF
Mature: Pro-BDNF
1.5
1.0
0.5
0.0
Treatment Groups
Ctrl
Ctrl + C21
LPS/IFNy
LPS/IFNy + C21
*
*vs. Ctrl
#vs. LPS/IFNy

References

Adams, H. P., Jr., Bendixen, B. H., Kappelle, L. J., Biller, J., Love, B. B., Gordon, D. L., & Marsh, E. E., 3rd. (1993). Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke, 24*(1), 35-41.

Ahmed, H. A., Ishrat, T., Pillai, B., Bunting, K. M., Vazdarjanova, A., Waller, J. L., . . . Fagan, S. C. (2019). Angiotensin receptor (AT2R) agonist C21 prevents cognitive decline after permanent stroke in aged animals-A randomized double- blind pre-clinical study. *Behav Brain Res, 359*, 560-569. doi:10.1016/j.bbr.2018.10.010

Ahmed, H. A., Ishrat, T., Pillai, B., Fouda, A. Y., Sayed, M. A., Eldahshan, W., . . . Fagan, S. C. (2018). RAS modulation prevents progressive cognitive impairment after experimental stroke: a randomized, blinded preclinical trial. *J Neuroinflammation, 15*(1), 229. doi:10.1186/s12974-018-1262-x

Alhusban, A., Fouda, A. Y., Bindu, P., Ishrat, T., Soliman, S., & Fagan, S. C. (2015). Compound 21 is pro-angiogenic in the brain and results in sustained recovery after ischemic stroke. *J Hypertens, 33*(1), 170-180. doi:10.1097/HJH.0000000000000364

Benjamin, E. J., Blaha, M. J., Chiuve, S. E., Cushman, M., Das, S. R., Deo, R., . . . Stroke Statistics, S. (2017). Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation, 135*(10), e146-e603. doi:10.1161/CIR.0000000000000485

Bennion, D. M., Isenberg, J. D., Harmel, A. T., DeMars, K., Dang, A. N., Jones, C. H., . . . Sumners, C. (2017). Post-stroke angiotensin II type 2 receptor activation provides long-term neuroprotection in aged rats. *PLoS One, 12*(7), e0180738. doi:10.1371/journal.pone.0180738

Boehme, A. K., Esenwa, C., & Elkind, M. S. (2017). Stroke Risk Factors, Genetics, and Prevention. *Circ Res, 120*(3), 472-495. doi:10.1161/CIRCRESAHA.116.308398

Dinarello, C. A. (1996). Biologic basis for interleukin-1 in disease. *Blood, 87*(6), 2095-2147.

Eldahshan, W., Ishrat, T., Pillai, B., Sayed, M. A., Alwhaibi, A., Fouda, A. Y., . . . Fagan, S. C. (2019). Angiotensin II type 2 receptor stimulation with compound 21 improves neurological function after stroke in female rats: a pilot study. *Am J Physiol Heart Circ Physiol, 316*(5), H1192-H1201. doi:10.1152/ajpheart.00446.2018

Erkinjuntti, T. (2002). Diagnosis and management of vascular cognitive impairment and dementia. *J Neural Transm Suppl*(63), 91-109.

Fouda, A. Y., Pillai, B., Dhandapani, K. M., Ergul, A., & Fagan, S. C. (2017). Role of interleukin-10 in the neuroprotective effect of the Angiotensin Type 2 Receptor agonist, compound 21, after ischemia/reperfusion injury. *Eur J Pharmacol, 799*, 128-134. doi:10.1016/j.ejphar.2017.02.016

George, M. G., Fischer, L., Koroshetz, W., Bushnell, C., Frankel, M., Foltz, J., & Thorpe, P. G. (2017). CDC Grand Rounds: Public Health Strategies to Prevent and Treat Strokes. *MMWR Morb Mortal Wkly Rep, 66*(18), 479-481. doi:10.15585/mmwr.mm6618a5

Giulian, D., Baker, T. J., Shih, L. C., & Lachman, L. B. (1986). Interleukin 1 of the central nervous system is produced by ameboid microglia. *J Exp Med, 164*(2), 594-604. doi:10.1084/jem.164.2.594

Guzik, A., & Bushnell, C. (2017). Stroke Epidemiology and Risk Factor Management. *Continuum (Minneap Minn), 23*(1, Cerebrovascular Disease), 15-39. doi:10.1212/CON.0000000000000416

He, M., Wang, J., Liu, N., Xiao, X., Geng, S., Meng, P., . . . Wang, Y. (2018). Effects of Blood Pressure in the Early Phase of Ischemic Stroke and Stroke Subtype on Poststroke Cognitive Impairment. *Stroke, 49*(7), 1610-1617. doi:10.1161/STROKEAHA.118.020827

Hewett, S. J., Jackman, N. A., & Claycomb, R. J. (2012). Interleukin-1beta in Central Nervous System Injury and Repair. *Eur J Neurodegener Dis, 1*(2), 195-211.

Hu, X., Li, P., Guo, Y., Wang, H., Leak, R. K., Chen, S., . . . Chen, J. (2012). Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. *Stroke, 43*(11), 3063-3070. doi:10.1161/STROKEAHA.112.659656

Huang, Y., Yang, S., & Jia, J. (2015). Factors related to long-term post-stroke cognitive impairment in young adult ischemic stroke. *Med Sci Monit, 21*, 654-660. doi:10.12659/MSM.892554

Iadecola, C., & Anrather, J. (2011). The immunology of stroke: from mechanisms to translation. *Nat Med, 17*(7), 796-808. doi:10.1038/nm.2399

Jackson, L., Eldahshan, W., Fagan, S. C., & Ergul, A. (2018). Within the Brain: The Renin Angiotensin System. *Int J Mol Sci, 19*(3). doi:10.3390/ijms19030876

Jones, N. C., Prior, M. J., Burden-Teh, E., Marsden, C. A., Morris, P. G., & Murphy, S. (2005). Antagonism of the interleukin-1 receptor following traumatic brain injury in the mouse reduces the number of nitric oxide synthase-2-positive cells and improves anatomical and functional outcomes. *Eur J Neurosci, 22*(1), 72-78. doi:10.1111/j.1460-9568.2005.04221.x

Kalaria, R. N. (2012). Cerebrovascular disease and mechanisms of cognitive impairment: evidence from clinicopathological studies in humans. *Stroke, 43*(9), 2526-2534. doi:10.1161/STROKEAHA.112.655803

Kalaria, R. N., Akinyemi, R., & Ihara, M. (2016). Stroke injury, cognitive impairment and vascular dementia. *Biochim Biophys Acta, 1862*(5), 915-925. doi:10.1016/j.bbadis.2016.01.015

Labandeira-Garcia, J. L., Rodriguez-Perez, A. I., Garrido-Gil, P., Rodriguez-Pallares, J., Lanciego, J. L., & Guerra, M. J. (2017). Brain Renin-Angiotensin System and Microglial Polarization: Implications for Aging and Neurodegeneration. *Front Aging Neurosci, 9*, 129. doi:10.3389/fnagi.2017.00129

Liu, X., Liu, J., Zhao, S., Zhang, H., Cai, W., Cai, M., . . . Hu, X. (2016). Interleukin-4 Is Essential for Microglia/Macrophage M2 Polarization and Long-Term Recovery After Cerebral Ischemia. *Stroke, 47*(2), 498-504. doi:10.1161/STROKEAHA.115.012079

Lively, S., & Schlichter, L. C. (2018). Microglia Responses to Pro-inflammatory Stimuli (LPS, IFNgamma+TNFalpha) and Reprogramming by Resolving Cytokines (IL-4, IL-10). *Front Cell Neurosci, 12*, 215. doi:10.3389/fncel.2018.00215

McCarthy, C. A., Vinh, A., Miller, A. A., Hallberg, A., Alterman, M., Callaway, J. K., & Widdop, R. E. (2014). Direct angiotensin AT2 receptor stimulation using a novel AT2 receptor agonist, compound 21, evokes neuroprotection in conscious hypertensive rats. *PLoS One, 9*(4), e95762. doi:10.1371/journal.pone.0095762

Mentz, R. J., Bakris, G. L., Waeber, B., McMurray, J. J., Gheorghiade, M., Ruilope, L. M., . . . Pitt, B. (2013). The past, present and future of renin-angiotensin aldosterone system inhibition. *Int J Cardiol, 167*(5), 1677-1687. doi:10.1016/j.ijcard.2012.10.007

Mohd Zulkifly, M. F., Ghazali, S. E., Che Din, N., Singh, D. K., & Subramaniam, P. (2016). A Review of Risk Factors for Cognitive Impairment in Stroke Survivors. *ScientificWorldJournal, 2016*, 3456943. doi:10.1155/2016/3456943

Murphy, S. L., Kochanek, K. D., Xu, J., & Arias, E. (2015). Mortality in the United States, 2014. *NCHS Data Brief*(229), 1-8.

O'Brien, J. T., Erkinjuntti, T., Reisberg, B., Roman, G., Sawada, T., Pantoni, L., . . . DeKosky, S. T. (2003). Vascular cognitive impairment. *Lancet Neurol, 2*(2), 89-98.

Patel, A. R., Ritzel, R., McCullough, L. D., & Liu, F. (2013). Microglia and ischemic stroke: a double-edged sword. *Int J Physiol Pathophysiol Pharmacol, 5*(2), 73-90.

Pousset, F., Cremona, S., Dantzer, R., Kelley, K., & Parnet, P. (1999). Interleukin-4 and interleukin-10 regulate IL1-beta induced mouse primary astrocyte activation: a comparative study. *Glia, 26*(1), 12-21.

Qu, Y., Zhuo, L., Li, N., Hu, Y., Chen, W., Zhou, Y., . . . Zhan, S. (2015). Prevalence of post-stroke cognitive impairment in china: a community-based, cross-sectional study. *PLoS One, 10*(4), e0122864. doi:10.1371/journal.pone.0122864

Soria, J. A., Arroyo, D. S., Gaviglio, E. A., Rodriguez-Galan, M. C., Wang, J. M., & Iribarren, P. (2011). Interleukin 4 induces the apoptosis of mouse microglial cells by a caspase-dependent mechanism. *Neurobiol Dis, 43*(3), 616-624. doi:10.1016/j.nbd.2011.05.010

Sparks, M. A., Crowley, S. D., Gurley, S. B., Mirotsou, M., & Coffman, T. M. (2014). Classical Renin-Angiotensin system in kidney physiology. *Compr Physiol, 4*(3), 1201-1228. doi:10.1002/cphy.c130040

Sun, J. H., Tan, L., & Yu, J. T. (2014). Post-stroke cognitive impairment: epidemiology, mechanisms and management. *Ann Transl Med, 2*(8), 80. doi:10.3978/j.issn.2305-5839.2014.08.05

Thrift, A. G., Thayabaranathan, T., Howard, G., Howard, V. J., Rothwell, P. M., Feigin, V. L., . . . Cadilhac, D. A. (2017). Global stroke statistics. *Int J Stroke, 12*(1), 13-32. doi:10.1177/1747493016676285

Valero-Esquitino, V., Lucht, K., Namsolleck, P., Monnet-Tschudi, F., Stubbe, T., Lucht, F., . . . Steckelings, U. M. (2015). Direct angiotensin type 2 receptor (AT2R) stimulation attenuates T-cell and microglia activation and prevents demyelination in experimental autoimmune encephalomyelitis in mice. *Clin Sci (Lond), 128*(2), 95-109. doi:10.1042/CS20130601

Villapol, S., & Saavedra, J. M. (2015). Neuroprotective effects of angiotensin receptor blockers. *Am J Hypertens, 28*(3), 289-299. doi:10.1093/ajh/hpu197

Yang, J., Ding, S., Huang, W., Hu, J., Huang, S., Zhang, Y., & Zhuge, Q. (2016). Interleukin-4 Ameliorates the Functional Recovery of Intracerebral Hemorrhage Through the Alternative Activation of Microglia/Macrophage. *Front Neurosci, 10*, 61. doi:10.3389/fnins.2016.00061

Yu, K. H., Cho, S. J., Oh, M. S., Jung, S., Lee, J. H., Shin, J. H., . . . Korean-Vascular Cognitive Impairment Harmonization Standards Study, G. (2013). Cognitive impairment evaluated with Vascular Cognitive Impairment Harmonization Standards in a multicenter prospective stroke cohort in Korea. *Stroke, 44*(3), 786-788. doi:10.1161/STROKEAHA.112.668343

Zhang, J., Zhang, Y., Xing, S., Liang, Z., & Zeng, J. (2012). Secondary neurodegeneration in remote regions after focal cerebral infarction: a new target for stroke management? *Stroke, 43*(6), 1700-1705. doi:10.1161/STROKEAHA.111.632448