Published in final edited form as:

Behav Brain Res. 2018 July 02; 346: 29–40. doi:10.1016/j.bbr.2017.12.007.

ROLE OF ANGIOTENSIN SYSTEM MODULATION ON PROGRESSION OF COGNITIVE IMPAIRMENT AND BRAIN MRI CHANGES IN AGED HYPERTENSIVE ANIMALS- A RANDOMIZED DOUBLE- BLIND PRE-CLINICAL STUDY

Heba A. Ahmed^a, Tauheed Ishrat^b, Bindu Pillai^a, Kristopher M Bunting^c, Ashni Patel^a, Almira Vazdarjanova^c, Jennifer L. Waller^d, Ali S. Arbab^g, Advije Ergul^e, and Susan C. Fagan^{a,f}

^aProgram in Clinical and Experimental Therapeutics, Charlie Norwood VA Medical Center and University of Georgia College of Pharmacy, Augusta, GA

^bDepartment of Anatomy and Neurobiology, College of Medicine, Neuroscience Institute, University of Tennessee Health Science Center, Memphis, TN

^cDepartment of Pharmacology and Toxicology, Augusta University, Augusta, GA

^dDepartment of Biostatistics & Epidemiology, Augusta University, Augusta, GA

^eDepartment of Physiology, Augusta University, Augusta, GA

^fDepartment of Neurology, Augusta University, Augusta, GA

^gGeorgia Cancer Center, Augusta University, Augusta, GA

Abstract

Growing evidence suggests that renin angiotensin system (RAS) modulators support cognitive function in various animal models. However, little is known about their long-term effects on the brain structure in aged hypertensive animals with chronic cerebral hypoperfusion as well as which specific domains of cognition are most affected. Therefore, in the current study we examined the effects of Candesartan and Compound 21 (C21) (RAS modulators) on aspects of cognition known to diminish with advanced age and accelerate with hypertension and vascular disease. Outcome measures for sensorimotor and cognitive function were performed using a sequence of tests, all blindly conducted and assessed at baseline and after 4 and 8 weeks of chronic hypoxic hypoperfusion and treatment. Magnetic resonance imaging (MRI) was performed at the end of the 8 week study period followed by animal sacrifice and tissue collection. Both Candesartan and C21 effectively preserved cognitive function and prevented progression of vascular cognitive impairment (VCI) but only candesartan prevented loss of brain volume in aged hypertensive animals. Collectively, our findings demonstrate that delayed administration of RAS modulators

Correspondence: Susan Fagan, Pharm., 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.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

effectively preserve cognitive function and prevent the development/progression of VCI in aged hypertensive animals with chronic cerebral hypoperfusion.

Keywords

vascular cognitive impairment; MRI; hypertension; angiotensin type 1 receptor blockade; renin-angiotensin; AT2 receptor

1. INTRODUCTION

1.1 Vascular Cognitive Impairment

Vascular cognitive impairment (VCI) and dementia are potentially avertable tragedies that rob individuals of their dignity and independence. The two most important risk factors for development and progression of cognitive impairment/dementia, including both VCI and Alzheimer's disease (AD) are advanced age and hypertension [1, 2]. Hypertension is especially important as it increases the risk of dementia both independently and by increasing the risk of stroke. In fact, hypertension alone can predict the development of dementia in nearly 60% of subjects with executive dysfunction [3]. Unlike with AD and other forms of dementia where disturbances in episodic memory may be considered hallmark, the salient feature of VCI is executive dysfunction, which is highly dependent upon the pre-frontal cortex (PFC) [3, 4]. Although long-term memory deficits may be present, it is not a requirement for the diagnosis of VCI. The cognitive deficits, related to PFC regions, ensue long before other symptoms of long-term memory impairment become apparent [3]. Executive dysfunction is therefore an essential component of the comprehensive cognitive/neuropsychological battery for suspected VCI [5].

1.2 Pathophysiology

The structural and functional cerebrovascular alterations, induced by hypertension, are all part of a vicious cycle (Fig. 1). These are the major contributors of the progressive neuropathological abnormalities responsible for VCI [6]. Chronic cerebral hypoperfusion (CCH)/reduced cerebral blood flow (CBF), due to hypertension and vascular insufficiency, result in a cascade of events with hypoxia, increased oxidative stress and inflammation that ultimately end in cognitive dysfunction [7]. The state of hypoxia also facilitates amyloid β ($A\beta$) production, by activating the amyloid precursor protein (APP) cleavage enzyme β -secretase [8]. $A\beta$ sustains microglial activation and neuroinflammation [9]. Moreover, being a potent vasoconstrictor, $A\beta$ further reduces cerebral blood flow (CBF), decreasing its own clearance leading to accumulation, endothelial cell (EC) toxicity and neurodegeneration [8]. The resultant endothelial dysfunction also causes damage and death to pericytes, neurons and glia, which in turn worsen EC atrophy and microvascular rarefaction, owing to their mutual, co-dependent, trophic support of one another [8, 10]. Therefore, despite the diversity of underlying brain pathologies, the neurovascular alterations all have a similar mechanistic basis, with hypoperfusion, oxidative stress and inflammation leading to endothelial and pericyte damage, blood brain barrier (BBB) breakdown, demyelination and disruption of trophic coupling between neurovascular unit (NVU) components.

1.3. Renin-Angiotensin System (RAS) Modulation

Growing evidence suggests that RAS modulators, including candesartan, which acts by selectively blocking the angiotensin type 1 receptor (AT1R) and allowing an unopposed stimulation of the less abundant angiotensin type 2 receptor (AT2R) by unbound Angiotensin II [6, 7] and the direct AT2R agonist C21, support cognitive function in various animal models [7, 31]. However, little is known about their long-term effects on the brain structure in aged hypertensive animals with chronic cerebral hypoperfusion as well as which specific functions are most affected. The purpose of this investigation was to determine the impact of the RAS modulators, candesartan and C21, on the various aspects of cognitive function involved in the development of VCI in aged hypertensive rats with chronic cerebral hypoperfusion as well as to correlate structural changes, seen with neuroimaging, with those findings at the end of the study period.

2. MATERIALS AND METHODS

2.1. Animals

We conducted our studies using aged, spontaneously hypertensive rats (SHRs), thus satisfying both advanced age and hypertension, as major co-morbidity requirements and primary risk factors for VCI. This is also the strain of choice owing to the similarity of its pathophysiology with human essential hypertension. In addition, the evolution of behavioral and neuropathological characteristics as well as microstructural and cognitive changes are similar to those occurring in human neurodegenerative disorders and VCI [13]. The hemodynamic alterations and cardiovascular adaptations also seem to follow a very similar course in SHRs as with human hypertension [13].

2.2. Experimental Design

A total of 28 young adult (4 months old), male SHRs were obtained from Charles River Laboratories and singly housed with free access to food and water, on a 12:12 h light–dark cycle. These animals were kept for 10 months before starting experiments. Once they were approximately 14 months old (weight range 400–440g), these animals were subjected to permanent unilateral common carotid artery occlusion (UCCAO) and randomly assigned to receive daily C21, candesartan, or vehicle 24 hours after surgery (Fig. 2). Drugs were administered in a blinded manner. All experimental procedures and animal protocols were approved by the IACUC of the Charlie Norwood Veterans Affairs Medical Center; Augusta, GA.

2.3. Unilateral Common Carotid Artery Occlusion (UCCAO)

We utilized the permanent UCCAO model to simulate mild, hypoxic hypoperfusion. This model is associated with gradual reductions in CBF and presents a pattern of injury that is apparent in both hemispheres, closely resembling the asymptomatic carotid artery occlusion seen in human atherosclerosis with aging and vascular cognitive impairment/dementia [14]. For this, SHRs were anesthetized using 2–5 % isoflurane inhalation and a ventral mid-line incision was made followed by right common carotid artery separation and ligation of the artery with 4-0 silicon-coated nylon suture.

2.4. Treatment

Oral administration of C21 (VicorePharma, Göteborg, Sweden), Candesartan cilexetil (Astra Zeneca, Cambridge, UK) or vehicle was initiated 24h after surgery and administered daily for a total of 8 weeks. Treatments were incorporated into the drinking water and adjusted, according to their daily intake, to provide them with 0.12 mg/kg/d of C21 an oral dose equivalent to 0.03 mg/kg/day IP [15]. The latter dose was chosen based on our previous publications [11, 12] and shown to have superior cognitive benefits. The dose of candesartan (1 mg/kg/d) was chosen and expected to reduce blood pressure significantly in SHR, according to our published results obtained with blood pressure (BP) telemetry [18, 19]. All treatments were administered by a blinded investigator.

2.5. Outcome Measures

2.5.1. Assessment of Functional Outcome—Since rodents are nocturnal animals and exhibit the highest level of activity during the dark phase, all behavioral tests were conducted during the 12 hour dark cycle to ensure the most reliable results.

2.5.1.1. Body Weight and Water Intake: Weight monitoring gives an independent and unambiguous measure of an animal's overall health and welfare [20]. Water intake was monitored periodically, over the entire course of the study. Animals were weighed before surgery and then daily thereafter until sacrifice.

2.5.1.2 Neurobehavioral Testing: Animals underwent neurobehavioral testing at baseline and again at 4 and 8 weeks after the insult, allowing each individual animal to serve as its own control, hence presenting a more accurate determination of motor and cognitive abilities. The testing sequence, illustrated in Fig. 2, was designed to minimize task interference - tasks with aversive components, i.e. Inhibitory Avoidance (IA) and Morris Water Maze (MWM), used for assessing spatial learning and long-term/reference memory, were conducted after assessing sensory-motor function (Bederson test) and non-aversive short-term/working memory, Novel Object Recognition (NOR) test. All functional outcomes were assessed using validated, reproducible and blinded tests.

2.5.1.2.1. Sensorimotor Testing: To assess sensorimotor function, animals underwent the Bederson test. A score of 0–3 was assigned based on the following parameters: forelimb flexion; diminished resistance to lateral push; and contralateral circling. The animal was given one point for each parameter, with lower scores indicating better performance and a score of zero indicating complete absence of a deficit [16][17] [20, 21]. In addition, swimming abilities were evaluated using the MWM (limb movement, swim speed and ability to climb onto the platform).

2.5.1.2.2. Cognitive Testing

The Novel Object Recognition (NOR) Test: The NOR test was performed to evaluate non-spatial short-term/working memory involving frontal-subcortical circuits [23]. This test, based on the spontaneous tendency of rodents to explore and interact with a novel object more than a familiar one, typically consists of 2 trials separated by a retention period and preceded by a habituation phase. The habituation phase (15 min/d) was conducted on 2

separate days before the start of the test, to allow animals to acclimate to their arena, which consisted of an empty standard size box ($45 \times 45 \times 35\text{cm}$). On the designated test day, animals were first subjected to an acquisition/sample trial, where the animal was introduced into the box containing 2 identical (sample) objects and allowed to explore for 10 min [22, 23, 24]. Following sample object exposure, the animal was returned to its home cage for a 15 min retention period. The second preference/test trial (5 min), which followed the retention period, was conducted in the same manner as the first trial, except that a new/novel object replaced one of the familiar/sample objects. This interval (delay) between sample and test trials was adjusted to 15 min for selective testing of non-spatial short-term/working memory. The discrimination index (DI), which is the difference in exploration time for the familiar and novel objects divided by the total time of exploration, and the recognition index (RI), which is the time spent exploring the novel object relative to the total time of exploration, were taken as indicators of working memory [22, 24].

$$\text{Discrimination index (DI)} = (T_N - T_F) / (T_N + T_F)$$

$$\text{Recognition index (RI)} = T_N / (T_N + T_F)$$

T_F and T_N are the times spent interacting with the familiar and novel object respectively.

The Inhibitory Avoidance (IA) Test: The Inhibitory Avoidance (IA) Test (commonly referred to as Passive Avoidance Test) was used to assess aversive associative learning and related reference memory. During IA, the animal must actively inhibit the desire and withhold the response, to re-enter a dark chamber or explore a novel compartment, after it has experienced an aversive stimulus in that location [26]. For this test, one of the compartments in a Y-maze equipped with a metal floor, is connected to an electric circuit box adjusted to deliver brief, moderate intensity electric shocks (3s duration, 1.5 mA). For the acquisition trial, the shock compartment/arm was blocked and the animal introduced into one of the “safe” arms and allowed 10 mins to explore the 2 open arms. After 10 mins, the door blocking the shock arm was opened allowing the animal to enter. Once the animal had fully entered the shock arm (base of the tail had crossed into the arm), its initial latency was recorded and it received a brief electric shock before being returned to its cage. After a 72 hour retention period, the test trial was conducted. This was performed in a manner similar to that of the acquisition trial except that the foot shock was omitted and all 3 arms were accessible to the animal from the start. The difference, between training and test sessions, in latency for entering into the desired compartment (shock arm) was used as a measure of retention. This latency was recorded for up to 300s, as the index of long-term aversive associative memory consolidation [26, 27, 28]. Acquisition and retention trials were both performed before (pre) and again after (post) surgical procedure. This was done in order to assess the effect of surgery/treatment by comparing the differences in transfer/step-through latency times between acquisition and retention trials at 2 time points (before and 4 weeks after surgery).

The Morris Water Maze (MWM): The MWM test was used to assess spatial learning, long-term memory and cognitive flexibility. All water maze tests were conducted in a large

circular pool of water, 120cm in diameter, 55cm height, filled to a depth of 35 ± 1 cm with water at $25\pm 2^\circ\text{C}$. This was separated into quadrants designated northeast (NE), northwest (NW), southeast (SE) and south-west (SW), based on the 4 equally spaced cardinal points N (North), S (South), E (East), and W (West) around the edge of the pool. One of these quadrants contained a transparent escape platform (10.5 cm diameter), submerged 1.5 cm below the water surface and obscured from view. Visual extramaze cues were mounted to aid spatial navigation.

MWM- Training/Learning sessions: The initial training consisted of a single daily session of 4 trials (120s each) per day for 7 consecutive days. Animals were initially placed on the escape platform located in a fixed position in the NE quadrant for 10s before the start of the first trial. Each trial consisted of releasing the rat into the water from 1 of the 4 starting locations and allowing it to find the platform. If they did not reach the platform within 120s, they were gently guided to it and kept there for 10s, then removed. Trials were spaced 30s apart. The starting location of each daily session was varied. All trials were recorded and video tracked by the computerized tracking system Etho-Vision XT 7 (Noldus, Leesburg, VA, USA). This automated system monitored animals' swim patterns and calculated mean escape latency (s), total distance travelled to target (cm), and velocity to target (cm/s). Data from all training sessions were pooled for each individual animal, evaluated and compared between groups at the different time points [30].

MWM-Spatial Reference Memory Test: Spatial reference memory was assessed with a probe test 24 hours after the last daily session. For this test all procedures were kept the same as during training, except that the platform was removed and rats were allowed to swim for 60 s in an attempt to find it. Performance was evaluated by measuring time spent in the target quadrant/zones, proximity to the target location, and initial latency to the target zone [30]. The target zone was centered on the platform location and was 2.5 times bigger.

MWM- Learning and Cognitive Flexibility/Reversal Training: Cognitive flexibility, which allows for flexible updating of representations in response to changing environmental contingencies, and "new" learning, was assessed by the reversal training/reversal probe test. Such ability is critically dependent on PFC systems and strongly affected by the aging process across species [31]. For reversal training, all parameters were kept the same, except that the platform was moved to a new location, in the SE quadrant. Each rat was given a single daily session of 4 trials (120s each) per day for 2–3 consecutive days, to learn the new location. The reversal probe test was conducted similarly to the initial probe test, for which all procedures are kept the same as during training, except that the platform was removed and rats are allowed to swim for 60s in an attempt to find it. Performance was evaluated by measuring time spent in the target and previous quadrants/zones, proximity to the target locations, and initial latency to the target and previous locations [30].

2.5.2. Magnetic Resonance Imaging (MRI)—To determine the changes in the brain, ventricular volume, and white matter hyper intense areas, animals underwent T2 -weighted and fluid attenuated inversion recovery (FLAIR), 8 weeks after UCCAO. This was performed using a horizontal 7.0 T BioSpec MRI spectrometer (Bruker Instruments, Billerica, MA) equipped with an 8.9-cm micro imaging gradient insert (100 gauss/cm. All

T2-weighted MRI and FLAIR images were obtained at Augusta University by the Core Imaging Facility for Small Animals (CIFSA) [32]. All MRI images were registered DICOM sequences, analyzed using FIJI [33]. Total brain volume as well as hippocampal and ventricular volumes (regions of interest), were determined on binarized sequences obtained by thresholding [33]. For each region of interest, the volumes were calculated by adding the areas measured on each slice and multiplying it by the slice thickness (1 mm in all cases).

2.6. Animal Sacrifice and Tissue Collection

At day 60, animals were anesthetized with IP ketamine/xylazine and transcardially perfused with 300 ml of ice cold 1X PBS. Animals were decapitated and their brains collected. Sections A and B from the brain matrix, which consisted of the prefrontal cortical region were snap frozen and kept for ELISA.

2.7. Protein Expression

Quantitative determination of A β ₁₋₄₂ concentrations in cortical lysates were carried out using a rat specific sandwich amyloid- β ELISA kit (Wako, USA), according to the manufacturer's protocol [34].

2.8 Statistical Analysis

All statistical analyses were performed using SAS 9.4 and statistical significance was assessed using an alpha level of 0.05, unless otherwise noted. Descriptive statistics (means and standard deviations) for behavioral data measures in aged animals within group and at indicated measurement times were determined. Repeated measures ANOVA mixed models were used to examine differences in outcomes, cognitive functional outcomes-NOR, PAT, MWM as well as water intake between the three groups over time. For each outcome, the mixed model contained fixed effects of group, time and the two-factor interaction between group and time. Animal nested within group was considered a random effect. The statistical test of interest was the F-test for the two-factor interaction between group and time. If this interaction was statistically significant it would indicate that the changes in the studied parameter over time are different in the three groups. A Bonferroni adjustment to the overall alpha level for the number of post hoc pair-wise comparisons performed within group between measurement weeks and within weeks between groups was used to control for the number of tests performed. To examine differences in weight over the 8 week/56-day period (from baseline/pre-surgery, day 0) to the day of sacrifice (56 days following surgery) between groups, a quadratic growth curve model was utilized. Day of measurement was considered a continuous variable. The fixed effects in the model included group and random effects included the intercept, linear day term, and the quadratic day² term. A compound symmetric variance co-variance structure provided the best model fit. Differences between groups for specific days (0, 1, 7, 10, 29, and 56) were examined using the estimated least squares means from a repeated measures mixed model and using a Bonferroni adjustment to the overall alpha level for the number of comparisons made between groups. For the MWM reversal training and testing outcomes, as well as MRI and molecular parameters, one-way ANOVA was used to examine differences between the groups. A Tukey-Kramer multiple comparison test was used to examine post hoc pair-wise differences.

3. INTEGRATED RESULTS AND DISCUSSION

3.1. Mild cerebral hypoperfusion does not affect the fluid intake or body weight of aged hypertensive animals

The mild cerebral hypoperfusion induced by UCCAO did not alter the aged SHR's normal activities of daily living. All animals retained their ability to groom and maintain their appearance. Food and water intake was consistent and did not differ across the groups. There was no statistically significant difference in post hoc pair-wise comparisons in mean fluid intake between groups within measurement time or between measurement times, within groups, (Fig. 3A). Animal body weight was also stable throughout the study duration with no statistically significant differences between any of the groups (Fig. 3B).

3.2. Mild cerebral hypoperfusion does not affect the motor function of aged hypertensive animals

None of the groups displayed any visible motor impairment at 24 hours post-UCCAO. This was evidenced by a Bederson score of zero which indicated absence of deficits in the 3 parameters, namely gait, motor coordination and forelimb function. All animals continued to show impeccable motor function including fine motor skills (reflected by the animals' ability to easily peel and eat sunflower seeds), locomotion and gross motor skills (gait, overall movement, motor performance and swimming ability (Fig. 4). These aspects did not differ between animals at baseline, 4 weeks or 8 weeks after UCCAO in any of the treatment groups. This was consistent with previous publications [35].

3.3. RAS modulators resulted in preservation of non-spatial recognition and short-term working memory in aged hypertensive animals with chronic cerebral hypoperfusion: Novel Object Recognition (NOR)

A statistically significant two-factor interaction between group and time was found for both discrimination index (DI) (Fig. 5A) and recognition index (RI) (Fig. 5B), indicating that the changes for each outcome over time in the groups had a different pattern. Specifically, vehicle treated animals showed progressive, significant reductions both in DI and RI from baseline to 4 and 8 weeks post-UCCAO. Their DI at week 4 ($P=0.0004$) and week 8 ($P<0.0001$) were significantly lower than baseline, and week 8 was significantly lower than week 4 ($P=0.0002$). This pattern was similar to that of the RI, with baseline values significantly higher than those of week 4 ($P=0.0006$) and week 8 ($P<0.0001$), and week 4 was significantly higher than week 8 ($P=0.0003$).

The C21 treated animals, on the other hand, stayed relatively constant from baseline to 4 and 8 weeks, showing no statistically significant differences in DI or RI between any of the different time points. Candesartan treated animals had a temporary reduction at 4 weeks ($P=0.0015$) with complete recovery at 8 weeks post UCCAO. There were no statistically significant differences between any of the groups at baseline or at 4 weeks post-UCCAO. However the DI and RI of the vehicle group was significantly lower than both the C21 ($P=0.0023$) and candesartan ($P=0.0002$) groups at week 8 post-UCCAO. A major advantage of this test is that it can be considered a "pure" recognition memory test, which reliably evaluates non-spatial working memory related to the frontal sub-cortical circuits. It does not

involve reference memory components (e.g. explicit rule learning) and is unaffected by lesions of the hippocampus. Rodents with hippocampal damage can perform normally on the NOR test, making it a more selective index of mPFC function [35–37]. Another advantage of the NOR is that this test doesn't involve positive reinforcements (e.g. food, so doesn't require food restriction) or negative reinforcements with strong aversive stimuli (e.g. electric shocks likely to produce stress and may influence performance) thus making it more comparable to the memory tests used in humans. This test, being quick and easy to implement, is widely used for assessing cognitive impairment in pre-clinical research [24, 25, 38–40]. The groups treated with RAS modulators showed superior performance on the NOR test, used to evaluate non-spatial working memory, compared to vehicle treated animals.

3.4. RAS modulators reinforced spatial reference memory and reduced progression of cognitive impairment in aged hypertensive animals with chronic cerebral hypoperfusion: Morris water maze probe test

At baseline (before the surgical procedure), the PAT transfer/step-through latency time significantly increased on the retention trial as compared to the acquisition trial for all the animals. These animals showed similarly high “differences” in the transfer/step-through latency times between acquisition and retention trials at baseline. This not only indicates an intact reference memory, with retention of the aversive event, it also signifies effectual associative learning and recall of the connection between properties of the chamber and the foot shock. However, when this test was conducted again 4 weeks post-insult, none of the animals entered the shock arm on the acquisition trial and were not exposed to the aversive stimulus (electric shock). Consequently, it was not possible for us to proceed with the retention trial. This may have been due to the initial baseline shock intensity which may have been too high for the aged SHRs known to have low tolerance as well as exaggerated responses to acute stressful stimuli [3]. This may have been negatively affected by the harsh experience as was evident by a complete lack of desire to explore the novel arm on the pre-acquisition trial. This was in contrast to their baseline pre-acquisition latency which averaged to 23.4 s. All animals entered the novel arm quickly and took an average <25s). To assess spatial reference memory, the MWM probe test was conducted both at baseline and after 4 weeks of treatment (4 weeks post-UCCAO). Although all animals showed a similar ability to learn the initial platform location (NE) at baseline, some of these animals showed signs of memory decline (mild cognitive impairment) as indicated by poor performance on the probe test. Although most parameters including the total distance traveled and total time (cumulative duration) spent in the target quadrant did not differ significantly, these animals did take slightly longer to reach the actual platform zone (longer latency) at 4 weeks post-UCCAO (Fig. 6A). These animals also spent significantly less time in the platform zone at 4 weeks post-UCCAO (Fig. 6B and C). The probe test was repeated at 4 weeks post-UCCAO and a statistically significant two-factor interaction between group and time existed for several parameters, indicating that the changes in such outcomes over time had a different pattern for the different treatment groups. While the aged vehicle treated rats showed a significant increase (worsening) in latency to reach the target platform zone and spent less time (cumulative duration) in the target zone at 4 weeks post-stroke compared to their initial baseline values, the C21 and candesartan treated animals actually showed the opposite

pattern. These RAS modulator treated animals actually showed reductions (improvements) in latency at 4 weeks compared to their initial baseline values, and also tended to spend more time in that target zone. These findings may signify a possible reinforcement of learning with treatment, resulting in a stronger, more stable long-term memory.

3.5. RAS modulators resulted in improved cognitive flexibility and reduced preservative behavior in aged hypertensive animals with chronic cerebral hypoperfusion

Although all animals displayed similar learning patterns on the initial (NE platform location) training, their ability to update this information at 4 weeks post-stroke and to learn a new location differed significantly between the treatment groups. Animals treated with RAS modulators showed significantly lower (better) latencies to the new target (SE) quadrant and platform zone on the reversal training at 4 weeks post-surgery, compared to those of vehicle treated animals, $P=0.0107$ and $P=0.0257$ for C21 and Candesartan respectively (Fig. 7A and B). These animals also showed significantly lower latencies to the new target zone on the reversal testing (when the platform was removed) compared to those of vehicle treated animals, $P=0.0152$ and $P=0.0056$ for C21 and Candesartan respectively. They also had lower mean distances to this new target zone compared to vehicle treated animals (Fig. 7C). This means their swim pattern was more focused as they were more inclined to swim closer to this target location while attempting to find it. Moreover, these C21 and candesartan treated animals tended to spend more time in the new target (SE) zone (Fig. 7D). What was also interesting was that the vehicle treated animals spent significantly more time in the previous target (NE) quadrant compared to C21 ($P=0.0006$) and Candesartan ($P=0.0022$) treated animals (Fig. 7E). This preservative behavior, seen in the vehicle treated animals, is an important indicator of the decreased cognitive flexibility often associated with VCI. These deficits are attributed to difficulty with the updating of information in response to changing environmental contingencies. This may result from impaired consolidation or retrieval of brand new memories or with difficulty inhibiting an adaptive or previously learned response when it became no longer appropriate [30]. Heat maps were also generated, for the reversal, to illustrate the relative time spent in each of the different locations (Fig. 7F).

3.6. RAS modulators reduced negative changes in the brain macrostructure of aged hypertensive animals with chronic cerebral hypoperfusion: MRI

Representative images (Fig. 8A–C), T2-weighted brain MRI scans of aged SHR with chronic cerebral hypoperfusion, revealed global brain atrophy (Fig. 8D), smaller hippocampal volumes (Fig. 8E) and significant ventricular enlargement (Fig. 8F) in vehicle treated animals compared to the RAS modulator treated animals. These findings are consistent with those of others [3, 32, 34]. Although these changes were reduced in both candesartan and C21 treated animals, only the candesartan group achieved statistical significance when compared to the vehicle treated animals ($P<0.05$).

3.7. RAS modulators tended to reduce negative changes in the brain microstructure of aged hypertensive animals with chronic cerebral hypoperfusion

The FLAIR sequence images displayed hyperintense areas in the white matter and hippocampus in both hemispheres of aged hypertensive animals (Fig. 8G). These were significantly lower in candesartan treated animals ($P<0.05$) (Fig. 8H). This is an interesting

finding. Although such diffuse WMHs are a frequent finding in elderly, hypertensive patients, these have not been consistently seen in animal models. One study found severe white matter lesions in 5 month old stroke prone SHR (SHR-SP) but not in standard SHRs [40]. This was also seen by other groups who used SHRs that were 6 months [33] and 10 month old [41]. These groups failed to detect WMHs despite significantly enlarged, hyperintense lateral ventricles and smaller brain volumes, in addition to significant impairments in non-spatial working memory [33]. Therefore, ours is the first study to detect WMHs in standard SHRs. This may have been partly because we conducted our MRIs on 16 month old SHRs while the maximum age used in the other MRI studies was 10 months. This is also the first and only study to evaluate the effect of RAS modulators (candesartan and C21) on appearance of WMH in aged SHRs. Although we found these WMHs to be significantly lower in candesartan treated animals, they were also reduced to some extent in C21 treated animals compared to vehicle treated controls. While this difference did not reach statistical significance, it may become apparent with future studies using a larger sample size or a higher dose of C21.

3.8. C21 prevents cortical accumulation of A β ₁₋₄₂ in aged hypertensive animals with chronic cerebral hypoperfusion

A β is known to be neurotoxic substance. It damages nerve cells by oxidative stress and activates a variety of inflammatory and pro-apoptotic pathways, all of which play an important role in the pathogenesis of AD [42]. Therefore, an important area of AD research involves the identification of agents capable of inhibiting A β mediated neurotoxicity [42]. Unfortunately, the relationship between A β and dementia is complex. Reports of associations between A β with cross-sectional measures of brain atrophy and dementia are not consistent [43]. Some reports indicate that individuals with A β alone do not show progressive atrophy or dementia [42, 43, 45]. On the other hand, a combination of A β and cerebrovascular disease, including either chronic cerebral hypoperfusion and/or acute ischemia, is known to negatively affect cognition [46–48]. In our study, animals treated daily with C21, starting at 24 h after UCCAO had markedly lower concentrations of amyloid beta (A β ₁₋₄₂) in their prefrontal cortex at 8 weeks post-ischemia than those treated with vehicle (Fig. 9). This finding, although novel, may not be responsible for the cognitive benefits associated with RAS modulators, since animals treated with candesartan also displayed preserved cognitive function, despite sustained elevations in cortical A β ₁₋₄₂. It is possible, however, that candesartan also achieved its benefits by reducing A β ₁₋₄₂ mediated cytotoxicity. In an in vitro cell culture study we conducted on human brain endothelial cells, candesartan effectively prevented A β ₁₋₄₂ mediated cytotoxicity under both normoxic and hypoxic conditions (data not shown). These findings were in line with the those of earlier studies which also demonstrated that ARBs are effective at preventing A β -induced cytotoxicity and vascular damage [47].

4. CONCLUSION- SIGNIFICANCE OF THE PROPOSED RESEARCH AND ITS RELEVANCE TO POPULATION HEALTH

Vascular dementia is, after Alzheimer's disease, the second most common cause of acquired dementia and represents between 25 and 30% of total dementia cases [3]. In VCI, PFC-

supported executive functions (mainly working memory, processing speed and cognitive flexibility) are those primarily affected [23, 30]. This is a pattern of cognitive impairment that is most commonly encountered in those with long-standing hypertension and is also particularly sensitive to decline with age [6, 28]. Currently, there is no established method/therapy for prevention of VCI, apart from general vascular risk factor management, including blood pressure control. Moreover, no cure is available once the cognitive deterioration manifests. To our knowledge, this is the first long-term study to compare the effects of C21 and Candesartan to vehicle on the specific cognitive parameters known to be associated with VCI and further accelerated with aging. This thorough investigation was comprised of a sequence of neurobehavioral tests assessing the various aspects of learning and memory in aged hypertensive animals with chronic cerebral hypoperfusion. This was conducted both at baseline and at different stages during treatment. The present study demonstrated that RAS modulators, candesartan and C21, effectively prevented progression of VCI in aged hypertensive animals and that C21 was not inferior to Candesartan. Treatment was initiated in aged (14 month old) SHR and continued for a total of 8 weeks. The more potent effect of candesartan on brain atrophy and white matter damage, as quantified using MRI, is likely due to the BP lowering effects of candesartan or perhaps a failure to utilize an equivalent dose of C21. Either way, this means that initiating treatment with the RAS modulators even later in life may help preserve cognitive function in older individuals with hypertension and mild cerebral hypoperfusion due to carotid artery stenosis.

References

1. Rouch L, Cestac P, Hanon O, Cool C, Helmer C, Bouhanick B, Chamontin B, Dartigues JF, Vellas B, Andrieu S. Antihypertensive drugs, prevention of cognitive decline and dementia: A systematic review of observational studies, randomized controlled trials and meta-analyses, with discussion of potential mechanisms. *CNS Drugs*. 2015; 29:113–130. [PubMed: 25700645]
2. Liu W, Yamashita T, Kurata T, Kono S, Hishikawa N, Deguchi K, Zhai Y, Abe K. Protective effect of telmisartan on neurovascular unit and inflammasome in stroke-resistant spontaneously hypertensive rats. *Neurological Research*. 2015; 37:491–501. [PubMed: 25591419]
3. López-Gil X, Amat-Roldan I, Tudela R, Castañ A, Prats-Galino A, Planas AM, Farr TD, Soria G. DWI and complex brain network analysis predicts vascular cognitive impairment in spontaneous hypertensive rats undergoing executive function tests. *Frontiers in Aging Neuroscience*. 2014; 6:1–33. [PubMed: 24478697]
4. Gorelick PB, Scuteri A, Black SE, DeCarli C, Greenberg SM, Iadecola C, Launer LJ, Laurent S, Lopez OL, Nyenhuis D, Petersen RC, Schneider JA, Tzourio C, Arnett DK, Bennett DA, Chui HC, Higashida RT, Lindquist R, Nilsson PM, Roman GC, Sellke FW, Seshadri S. Vascular Contributions to Cognitive Impairment and Dementia: A Statement for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke*. 2013; 42:2672–2713.
5. Gorelick PB, Nyenhuis D. Understanding and treating vascular cognitive impairment. *Continuum (Minneapolis Minn)*. 2013; 19:425–37.
6. Iadecola C, Yaffe K, Biller J, Bratzke LC, Faraci FM, Gorelick PB, Gulati M, Kamel H, Knopman DS, Launer LJ, Sackin JS, Seshadri S, Al Hazzouri AZ. Impact of Hypertension on Cognitive Function: A Scientific Statement from the American Heart Association. *Hypertension*. 2016; 68:e67–e94. [PubMed: 27977393]
7. Iwanami J, Mogi M, Tsukuda K, Jing F, Ohshima K, Wang XL, Nakaoka H, Kan-No H, Chisaka T, Bai HY, Min LJ, Horiuchi M. Possible synergistic effect of direct angiotensin II type 2 receptor stimulation by compound 21 with memantine on prevention of cognitive decline in type 2 diabetic mice. *European Journal of Pharmacology*. 2014; 724:9–15. [PubMed: 24361310]

8. Iadecola C. Dangerous Leaks: Blood-Brain Barrier Woes in the Aging Hippocampus. *Neuron*. 2015; 85:231–233. [PubMed: 25611503]
9. Torika N, Asraf K, Danon A, Apte RN, Fleisher-Berkovich S. Telmisartan Modulates Glial Activation: In Vitro and In Vivo Studies. *Plos One*. 2016; 11:1–15.
10. Madri JA. Modeling the neurovascular niche: implications for recovery from CNS injury. *Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society*. 2009; 60:95–104.
11. Gallo-Payet N, Guimond MO, Bilodeau L, Wallinder C, Alterman M, Hallberg A. Angiotensin II, a neuropeptide at the frontier between endocrinology and neuroscience: Is there a link between the angiotensin II type 2 receptor and Alzheimer's disease? *Frontiers in Endocrinology*. 2011; 2:1–10. [PubMed: 22649356]
12. Alhusban A, Kozak A, Ergul A, Fagan SC. AT1 receptor antagonism is proangiogenic in the brain: BDNF a novel mediator. *The Journal of Pharmacology and Experimental Therapeutics*. 2013; 344:348–59. [PubMed: 23211364]
13. Leong XF, Ng CY, Jaarin K. Animal Models in Cardiovascular Research: Hypertension and Atherosclerosis. *BioMed Research International*. 2015; 2015:1–12.
14. Thong-asa W, Tilokskulchai K. Neuronal damage of the dorsal hippocampus induced by long-term right common carotid artery occlusion in rats. *Iranian Journal of Basic Medical Sciences*. 2014; 17:220–226. [PubMed: 24847426]
15. Wan Y, Wallinder C, Plouffe B, Beaudry H, Mahalingam aK, Wu X, Johansson B, Holm M, Botoros M, Karlén A, Pettersson A, Nyberg F, Fändriks L, Gallo-Payet N, Hallberg A, Alterman M. Design, synthesis, and biological evaluation of the first selective nonpeptide AT2 receptor agonist. *J Med Chem*. 2004; 47:5995–6008. [PubMed: 15537354]
16. Ishrat T, Pillai B, Soliman S, Fouda AY, Kozak A, Johnson MH, Ergul A, Fagan SC. Low-Dose Candesartan Enhances Molecular Mediators of Neuroplasticity and Subsequent Functional Recovery After Ischemic Stroke in Rats. *Mol Neurobio*. 2015; 51:1542–1553.
17. Alhusban A, Fouda AY, Pillai B, Ishrat T, Soliman S, Fagan SC. Compound 21 is pro-angiogenic in the brain and results in sustained recovery after ischemic stroke. *J Hypertens*. 2015; 33:170–180. [PubMed: 25304472]
18. Kozak W, Kozak A, Johnson MH, Elewa HF, Fagan SC. Vascular protection with candesartan after experimental acute stroke in hypertensive rats: a dose-response study. *J Pharmacol and Exp Ther*. 2008; 326:773–782. [PubMed: 18559971]
19. Proakis AG. Review and evaluation of pharmacology and toxicology data, Center for drug evaluation and research, Application Number 20-838. *Pharmacology*. 1998:1–47.
20. Freret T, Bouet V. Improvements of the Stroke Model Guidelines – Animal body weight and long-term functional concerns. *Journal of Experimental Stroke and Translational Medicine*. 2009; 2:28–31.
21. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke*. 1986; 17:472–476. [PubMed: 3715945]
22. Guan W, Somanath PR, Kozak A, Goc A, El-Remessy AB, Ergul A, Johnson MH, Alhusban A, Soliman S, Fagan SC. Vascular protection by angiotensin receptor antagonism involves differential VEGF expression in both hemispheres after experimental stroke. *PloS One*. 2011; 6:1–8.
23. Ma J, Xiong JY, Hou WW, Yan HJ, Sun Y, Huang SW, Jin L, Wang Y, Hu WW, Chen Z. Protective Effect of Carnosine on Subcortical Ischemic Vascular Dementia in Mice. *CNS Neuroscience and Therapeutics*. 2012; 18:745–753. [PubMed: 22943141]
24. Wietrzyk M, Meziane H, Sutter A, Ghyselinck N, Chapman PF, Chambon P, Krezel W. Working memory deficits in retinoid X receptor gamma-deficient mice. *Learn Mem*. 2005; 12:318–326. [PubMed: 15897255]
25. Antunes M, Biala G. The novel object recognition memory: Neurobiology, test procedure, and its modifications. *Cognitive Processing*. 2012; 13:93–110. [PubMed: 22160349]
26. Webster SJ, Bachstetter AD, Nelson PT, Schmitt FA, Van Eldik LJ. Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Frontiers in Genetics*. 2014; 5:1–23. [PubMed: 24567736]

27. Radahmadi M, Hojjatallah A, Sharifi M, Nasrin H. The effect of synchronized running activity with chronic stress on passive avoidance learning and body weight in rats. *Asian Journal of Sports Medicine*. 2013; 4:54–62. [PubMed: 23785577]
28. Alonso M, Bekinschtein P, Cammarota M, Vianna MRM, Izquierdo I, Medina JH. Endogenous BDNF is required for long-term memory formation in the rat parietal cortex. *Learning & Memory*. 2005; 12:504–510. [PubMed: 16204202]
29. Palit P, Mukherjee D, Mandal SC. Reconstituted mother tinctures of *Gelsemium sempervirens* L. improve memory and cognitive impairment in mice scopolamine-induced dementia model. *Journal of Ethnopharmacology*. 2015; 159:274–284. [PubMed: 25459447]
30. Vazdarjanova A, Bunting K, Muthusamy N, Bergson C. Calcyon upregulation in adolescence impairs response inhibition and working memory in adulthood. *Molecular Psychiatry*. 2011; 16:672–84. [PubMed: 21403673]
31. Bizon JL, Foster TC, Alexander GE, Glisky EL. Characterizing cognitive aging of working memory and executive function in animal models. *Frontiers in Aging Neuroscience*. 2012; 4:1–14. [PubMed: 22514534]
32. Shankar B, Iskander TfA, Nr V, Br A, Jain M, Mikkelsen T, Am G, Wb C, Shankar A, Borin TF, Iskander A, Varma NRS, Achyut BR, Jain M. Combination of vatalanib and a 20 HETE synthesis inhibitor results in decreased tumor growth in an animal model of human glioma. 2016; 9:1205–1219.
33. Kaiser D, Weise G, Möller K, Scheibe J, Pösel C, Baasch S, Gawlitza M, Lobsien D, Diederich K, Minnerup J, Kranz A, Boltze J, Wagner DC. Spontaneous white matter damage, cognitive decline and neuroinflammation in middle-aged hypertensive rats: an animal model of early-stage cerebral small vessel disease. *Acta Neuropathologica Communications*. 2014; 2:169–183. [PubMed: 25519173]
34. Jing F, Mogi M, Sakata A, Iwanami J, Tsukuda K, Ohshima K, Min LJ, Steckelings UM, Unger T, Dahlof B, Horiuchi M. Direct stimulation of angiotensin II type 2 receptor enhances spatial memory. *J Cereb Blood Flow Metab*. 2012; 32:248–255. [PubMed: 21971355]
35. Jalal FY, Yang Y, Thompson J, Lopez AC, Rosenberg GA. Myelin loss associated with neuroinflammation in hypertensive rats. *Stroke*. 2012; 43:1115–1122. [PubMed: 22363061]
36. Oliveira AMM, Hawk JD, Abel T, Havekes R. Post-training reversible inactivation of the hippocampus enhances novel object recognition memory. *Learning & Memory*. 2010; 215:155–160.
37. Rekik K, Francés B, Valet P, Dray C, Florian C. Cognitive deficit in hippocampal-dependent tasks in Werner syndrome mouse model. *Behavioural Brain Research*. 2017; 323:68–77. [PubMed: 28119126]
38. Leger M, Quiedeville A, Bouet V, Haelewyn B, Boulouard M, Schumann-Bard P, Freret T. Object recognition test in mice. *Nature Protocols*. 2013; 8:2531–2537. [PubMed: 24263092]
39. Moscardo E, Salvetti B, Becchi S, Bertini G, Fabene PF. The Novel Object Recognition Test in Rodents : Which Are the Essential Methodological Aspects ? *Proceedings of Measuring Behavior*. 2012; 2012:476–478.
40. Lin JX, Tomimoto H, Akiguchi I, Wakita H, Shibasaki H, Horie R. White matter lesions and alteration of vascular cell composition in the brain of spontaneously hypertensive rats. *Neuroreport*. 2001; 12:1835–1839. [PubMed: 11435908]
41. Brittain JF, McCabe C, Khatun H, Kaushal N, Bridges LR, Holmes WM, Barrick TR, Graham D, Dominiczak AF, Mhairi Macrae I, Hainsworth AH. An MRI-histological study of white matter in stroke-free SHRSP. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*. 2013; 33:760–3.
42. He Y, Yao Y, Tsirka SE, Cao Y. Cell-culture models of the blood-brain barrier. *Stroke*. 2014; 45:2514–2526. [PubMed: 24938839]
43. Jagust W. Is amyloid-beta harmful to the brain? Insights from human imaging studies. *Brain*. 2016; 139:23–30. [PubMed: 26614753]
44. Herrup K. The case for rejecting the amyloid cascade hypothesis. *Nat Neurosci*. 2015; 18:794–799. [PubMed: 26007212]

45. Choi YJ, Chae S, Kim JH, Barald KF, Park JY, Lee SH. Neurotoxic amyloid beta oligomeric assemblies recreated in microfluidic platform with interstitial level of slow flow. *Scientific Reports*. 2013; 3:1–7.
46. Caughlin S, Hepburn JD, Park DH, Jurcic K, Yeung KKC, Cechetto DF, Whitehead SN. Increased expression of simple ganglioside species GM2 and GM3 detected by MALDI Imaging Mass Spectrometry in a combined rat model of A β toxicity and stroke. *PLoS ONE*. 2015; 10:1–17.
47. Li NC, Lee A, Whitmer Ra, Kivipelto M, Lawler E, Kazis LE, Wolozin B. Use of angiotensin receptor blockers and risk of dementia in a predominantly male population: prospective cohort analysis. *BMJ (Clinical Research Ed)*. 2010; 340:1–10.

HIGHLIGHTS

- RAS modulators preserved cognition in aged SHR with cerebral hypoperfusion
- C21 reduced accumulation of A β ₁₋₄₂ in the prefrontal cortex of aged SHR
- C21 improved cognitive function in aged SHR, as did candesartan
- Candesartan prevented progressive loss of brain volume in aged SHR

Fig. 1.
Mechanism of cognitive dysfunction induced by chronic hypertension and cerebral hypoperfusion/vascular insufficiency

Fig. 2.
Schematic depiction of the experimental design

Fig. 3. Effect of mild cerebral hypoperfusion on the fluid intake and body weight of aged hypertensive animals

The (A) fluid intake and (B) weight trend of aged SHRs, at various time points, before and after UCCAO (n=8–10 animals/group). Symbols and error bars indicate mean and SEM, compound symmetric variance co-variance structure provided the best model fit. Differences between groups over time were examined using the estimated least squares means from a repeated measures mixed model and using a Bonferroni adjustment to the overall alpha level for the number of comparisons made between groups, any differences were nonsignificant.

Fig. 4. Effect of mild cerebral hypoperfusion on the motor function of aged hypertensive animals Swim speeds on the initial and reversal training/test of the Morris Water Maze test were taken as measures of swimming ability, a parameter of motor function ($n = 8-9$ animals/group). Error bars indicate SEM, Repeated measures ANOVA mixed models were used to examine differences in outcomes, for the initial training, between the groups over time. Statistical significance for post hoc comparisons using Bonferroni adjustment to the overall alpha level for the number of post hoc pair-wise comparisons, $*P < 0.0056$, the Bonferroni adjusted alpha. For the reversal training and testing outcomes, a one-way ANOVA was used to examine differences between the groups and a Tukey-Kramer multiple comparison test was used to examine post hoc pair-wise differences $*P < 0.05$. Any differences were nonsignificant.

Fig. 5. Effect of RAS modulator treatment on the non-spatial working memory of aged hypertensive animals with mild cerebral hypoperfusion

Effect of treatment on the (A) Discrimination Index (DI) and (B) Recognition Index (RI) of aged hypertensive animals, at different time points after ischemic insult (n=8–10 animals/group). Symbols and error bars indicate mean and SEM. Repeated measures ANOVA, mixed models, were used to examine differences in outcomes, between the three groups over time with post hoc pair-wise comparisons denoted by * $P < 0.0042$, the Bonferroni adjusted alpha.

Fig. 6. Effect of RAS modulator treatment on spatial reference memory of aged hypertensive animals with mild cerebral hypoperfusion

Spatial reference memory evaluated with the Morris Water Maze probe test in aged SHR. The platform was removed and rats were allowed to swim for 60 s in an attempt to find it. Performance was evaluated by measuring (A) initial latency to the target zone as well as (B) time spent in the target zones. (C) Heat maps illustrating relative time spent in the various locations during the probe tests both before (pre-probe) as well as 4 weeks after (pre-probe) after initial ischemic procedure. The target (NE) quadrant is indicated with black lines (n=8–10 animals/group). Symbols and error bars indicate mean and SEM. Repeated measures ANOVA, mixed models, were used to examine differences in outcomes between the three groups over time with post hoc pair-wise comparisons denoted by * $P < 0.0056$, the Bonferroni adjusted alpha.

Fig. 7. Effect of RAS modulator treatment on cognitive flexibility/new learning in aged hypertensive animals with mild cerebral hypoperfusion

Cognitive flexibility and “new” learning was assessed by the reversal training/test. Performance was evaluated by measuring (A) Latency to target quadrant (B) Latency to target/platform zone (C) Distance to platform zone, which is how close they swim to target location while attempting to find it (D) Duration in target zone (E) Duration in previous quadrant, which indicates preservative behavior in aged hypertensive animals and (F) Heat maps illustrating relative time spent in the various locations during the reversal, the target (SE) quadrants is indicated with black lines (n = 8–10 animals/group). Bars and error bars indicate mean and SEM. A one-way ANOVA was used to examine differences between the groups and a Tukey-Kramer multiple comparison test was used to examine post hoc pairwise differences. Statistical significance for post hoc comparisons using Tukey’s multiple comparison procedure are denoted by *P<0.05.

Fig. 8. Effect of RAS modulator treatment on brain structure in aged hypertensive animals with mild cerebral hypoperfusion using Magnetic Resonance Imaging (MRI)

Representative T2-weighted (T2W) (A) coronal trans-axial MRI slices and (B) axial whole brain images of the different treatment groups compared to young normotensive wistar control (C) Coronal trans-axial FLAIR images of the 3 treatment groups. Relative measures of brain damage/atrophy (D) Total brain volume (E) Hippocampal volume (F) Ventricular volume (H) Representative trans-axial FLAIR images (G) White matter hyperintensities (WMH). The yellow arrows indicate those affecting the periventricular white matter tracks; the red arrows indicate those affecting the lateral septal nucleus in addition to the dorsal and intermediate portions. (n =3–5 animals/group). Error bars indicate SEM. A one-way ANOVA was used to examine differences between the groups and a Tukey-Kramer multiple comparison test was used to examine post hoc pair-wise differences. Statistical significance for post hoc comparisons using Tukey's multiple comparison procedure are denoted by *P < 0.05.

A β 42 pre-frontal cortex

Fig. 9. Effect of RAS modulator treatment on A β ₁₋₄₂ concentrations in the pre-frontal cortex of aged hypertensive animals with mild cerebral hypoperfusion

Quantitative determination of A β ₁₋₄₂ concentrations in cortical lysates of aged hypertensive animals, comparing the different treatment groups at 8 weeks post-UCCAO (n = 8–9 animals/group). Error bars indicate SEM. A one-way ANOVA was used to examine differences between the groups and a Tukey-Kramer multiple comparison test was used to examine post hoc pair-wise differences. Statistical significance for post hoc comparisons using Tukey's multiple comparison procedure are denoted by *P<0.05.