THE EFFECT OF LARGE ARTERY STIFFNESS ON COGNITIVE DECLINE, INFLAMMATION, AND OXIDATIVE STRESS

by

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A THESIS

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Stiffer large elastic arteries are a consequence of advancing age and are associated with cognitive impairment. Recent studies in young mice have shown that even short-term exposure to increased large artery stiffness leads to cerebrovascular dysfunction. However, the direct effects of long-term exposure to increased large artery stiffness are unknown. Therefore, this study looked at the combination of age and large artery stiffness on cognitive function, cerebrovascular function, inflammation, and oxidative stress. Old elastin haploinsufficient mice with large artery stiffness did not have impaired memory and motor coordination compared to both young and old controls. However, old elastin haploinsufficient mice had impaired cerebral artery endothelial function due to lower nitric oxide bioavailability. Old wildtype mice had higher levels of inflammation in the entorhinal cortex, hippocampus, and thalamus than young mice, while elastin haploinsufficient mice trended towards having higher levels of inflammation in the entorhinal cortex than control mice. Finally, old elastin haploinsufficent mice have more endothelial nitric oxide synthase uncoupling in the cerebral artery compared to control mice, indicating a greater likelihood of oxidative stress. These results suggest that while large artery stiffness leads to impaired cerebral artery endothelial function, increased oxidative stress, and increased inflammation, it does not lead to cognitive decline.

Acknowledgements

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Introduction

Aging and Age-Related Diseases

As a result of increased healthcare, nutrition, and standards of living, people are living longer than ever. By the year 2050, one in 5 Americans will be over the age of 65 (Bureau, 2018), and the proportion of the global population above the age of 65 is expected to rise as well. As a consequence of the aging population, the prevalence of age-related diseases such as cardiovascular disease, Alzheimer's disease, and other kinds of dementia is expected to rise significantly, with one paper even estimating that 13.8 million people will have Alzheimer's disease by the year 2050 in the United States alone (Hebert et al., 2013). With these numbers in mind, it is increasingly important to understand the causes of age-related diseases as well as find a viable prevention method or treatment option.

Alzheimer's disease (AD) is a progressive brain disorder whose symptoms include problems with memory, thinking, and behavior, causing significant impairment to daily life. Risk factors for getting AD include age, genetics, diabetes, poor diet, and social isolation. AD is one type of dementia, a general term that includes many specific medical conditions that are characterized by a decline in cognitive abilities. There are currently no treatments that have been proven to be effective at reversing dementia, which is why more research into the causes of dementia is necessary. Many previous approaches to studying AD and other kinds of dementia have looked at the nervous tissue of the brain, but drugs developed from this method have a 99.6% failure rate

(Cummings et al., 2014). Therefore, a new approach is necessary, and recent work has shown that targeting the aging cardiovascular system is a promising route.

The Aging Cardiovascular System

As we age, large elastic arteries of the cardiovascular system, like the aorta and the carotid artery, stiffen. Stiffness is due to fragmentation of the elastin protein, the protein that makes an artery elastic, as well as increased levels of collagen (Henson et al., 2014). Aortic stiffness has been shown to be one of the most important factors leading to cardiovascular diseases (Mitchell et al., 2010). As the stiff large artery cannot expand, accommodate, and dampen the large changes in pressure caused by pumping of the heart compared to the elastic artery, the spikes in pressure are carried up to the smaller arteries in the brain, causing greater pulsatility. The small vessels of the brain are not structured to be exposed to prolonged, increased pulsatility, so therefore large artery stiffness leads to damaged surrounding tissue. Consequences include greater levels of neuroinflammation, cerebral blood flow dysregulation, damage to the endothelium (inner lining) of cerebral arteries, and cognitive decline (Thorin-Trescases et al., 2018). In humans, large artery stiffness has been shown to predict the conversion of mild cognitive impairment to dementia, even independent of other risk factors (Rouch et al., 2018).

Damage to the endothelium is significant because the endothelium is responsible for vasodilation and blood flow regulation (Cines et al., 1998). In an organism, vasodilation of an artery occurs when shear stress of increased blood flow causes the endothelial cells to release vasodilators. These vasodilators then cause relaxation of the smooth muscle cells in the tunica media layer of the artery, which results in

vasodilation. Another signal for vasodilation is acetylcholine (ACh). ACh binds to muscarinic receptors on the endothelium and activates eNOS, endothelial nitric oxide synthase (Elhusseiny et al., 1999). eNOS produces nitric oxide (NO), a vasodilator that relaxes the smooth muscle of the tunica media layer, leading to dilation. While in an organism ACh isn't used as much to cause vasodilation compared to shear stress, it still is a powerful tool for studying *ex vivo* vasodilation. Previous studies have used young mice with elastin haploinsufficiency, a model of large artery stiffness that mimics stiffness seen in old mice. These studies show that large artery stiffness leads to decreased cerebral artery endothelial function by causing decreased vasodilation in response to ACh (Walker et al., 2015). Therefore, the elastin haploinsufficient mouse model can be used to mimic the cardiovascular changes experienced by old mice, and through these mice the molecular effects of large artery stiffness can be elucidated.

Molecular Mechanisms of Cardiovascular Aging

The two main mechanisms of cardiovascular aging are oxidative stress and inflammation. Oxidative stress occurs by the increased production of reactive oxygen species (ROS) byproducts such as superoxide (O_2^-) and the inability of antioxidant pathways to defend against ROS. Superoxide is produced by the electron transport chain in the mitochondria (Ungvari et al., 2007) and by the enzyme NADPH oxidase (Donato et al., 2007). Though superoxide and other ROS have normal immune functions in the cell, increased ROS production due to increased NADPH oxidase activity and decreased antioxidant enzymes with age can cause damage to the arteries (Ungvari et al., 2010).

NO bioavailability also changes with age. Decreased NO is partially due to decreased eNOS expression (Ungvari et al., 2010) but also increased eNOS uncoupling. eNOS uncoupling is caused by the phosphorylation of eNOS at the threonine (Thr) 495 amino acid. This phosphorylated isoform of the enzyme interferes with calmodulin binding, which attenuates eNOS activity and decreases NO bioavailability (Heiss et al., 2014). The phosphorylated eNOS uncoupling also leads to the production of more superoxide than NO, which goes on to cause structural damage in the arteries. Superoxide also further decreases NO bioavailability, as it reacts with NO to form peroxynitrite ($ONOO^-$), another ROS (Harrison, 2012). As NO acts as a vasodilator, decreased bioavailability leads to decreased vasodilation and decreased cerebral artery endothelial function (Walker et al, 2015).

Another mechanism is chronic neuroinflammation. This can result from increased damaging pulsatility in smaller vessels of the brain. Chronic inflammation has been shown to be extremely detrimental to cognitive function (Kinney et al., 2018). Proliferation of microglia, cells of the immune system that contribute to inflammation, is correlated with AD severity (Olmos-Alonso et al., 2016). This inflammation is especially prominent in the hippocampus, entorhinal cortex, and thalamus (Neuroinflammation Working Group et al., 2014).

Oxidative stress and inflammation are not separate, unrelated processes. As NO inhibits inflammatory processes, decreased NO bioavailability due to oxidative stress can lead to increased inflammation (Harrison, 2012). Increased expression of inflammatory factors such as nuclear factor-kβ (NFkβ) in old mouse arteries leads to oxidative stress-mediated endothelial dysfunction, which can be restored by inhibiting

inflammatory processes (Lesniewski et al., 2011). Increased oxidative stress leads to increased inflammation, which in turn leads to increased oxidative stress, and so on.

While oxidative stress, inflammation, and large artery stiffness has been shown to lead to cerebral artery dysfunction, the interaction of age and large artery stiffness has not been studied. Also, the causal role of large artery stiffness on cognitive decline, the precursor to dementia, has not been established. Therefore, the goal of this study is to establish the causal role of large artery stiffness in old mice on inflammation, oxidative stress, and cognitive decline.

Methods

Model Organism: Elastin Haploinsufficient Mice

3 groups of C57BL6 transgenic mice, both male and female, were used for this study. Young elastin wildtype mice (young Eln+/+) and old elastin wildtype mice (Old Eln+/+) were used for the young and old control groups respectively. Old elastin haploinsufficient mice (Old Eln+/-), mice with a heterozygote deletion of exon 1 of the elastin gene, were used for the large artery stiffness model (Walker et al., 2015). Elastin genotypes were verified by polymerase chain reaction (PCR) and gel electrophoresis.

Different sets of mice were used for different experiments. Characteristics of mice used for the novel object recognition test and the accelerating rotarod test are shown in Table I.

	Young Eln+/+	Old Eln+/+	Old Eln+/-
n	9	15	14
Age (months)	6.9±0.0	25.4±0.1*	25.3±0.1*

Table I. Characteristics of animals used for novel object recognition test and accelerating Rotarod test. *$p < 0.05$ vs Young Eln+/+

Characteristics of mice used for cerebral artery endothelial function experiments are shown below in Table 2.

	Young Eln+/+	Old Eln+/+	Old Eln+/-
n	9	8	8
Age (months)	6.9±0.0	25.4±0.1*	25.3±0.1*

Table 2. Characteristics of animals used for cerebral artery endothelial function experiments. *p < 0.05 vs Young Eln+/+

Characteristics of mice used for eNOS/phosphorylated-eNOS immunofluorescence and Iba-1 immunofluorescence are shown in Tables 3 and 4, respectively.

	Old Eln+/+	Old Eln+/-
n	8	8
Age (months)	25.4±0.1	25.3±0.1

Table 3. Characteristics of animals used for eNOS and phosphorylated-eNOS immunofluorescence

	Eln+/+	Eln+/-	Young	Old
n	8	13	19	15
Age (months)	15.7±1.1	15.7±1.1	7.0±0.7	30.8±1.4*

Table 4. Characteristics of animals used for Iba-1 immunofluorescence. *p < 0.05 vs Old

All animals were housed in an animal care facility on a 12-hour/12-hour light-dark cycle at 24°C and had *ad libitum* access to normal chow food and water. All animal procedures conformed to the *Guide to the Care and Use of Laboratory Animals* (8th edition, revised 2011) and were approved by the Institutional Animal Care and Use Committees at the University of Oregon.

Novel Object Recognition Test

The following procedure was adapted from Villasana, et al., 2013, with some modifications made. Mice were habituated to the testing room for 1 hour prior to the beginning of testing each day the experiment was conducted. Mice were temporarily individually housed, and a white noise machine was placed in the room. Mice were individually handled by the experimenter for 60 seconds each day for 3 consecutive days in order to acclimatize mice to testing conditions. On the fourth day, the open field test was conducted by first handling each mouse for 60 seconds and then placing the mouse in a well-lit arena (40 cm × 40 cm). Each mouse had 10 minutes to explore the arena. Generally, mice that are less anxious will spend more time in the center of the arena. In between each trial the arena was cleaned with 5% acetic acid to remove any lingering odors.

On the fifth day, the novel object recognition test was conducted. First, each mouse was handled for 60 seconds and then placed in the arena with 2 identical objects. Each mouse had 10 minutes to investigate the objects, after which the mouse was removed and the arena and objects were cleaned with 5% acetic acid. 4 hours later, each mouse was placed in the arena with two different objects. One of the objects was the same as the objects of the previous trial, and the other object was new to the mouse, the novel object. Figure 1 shows the two types of objects used.

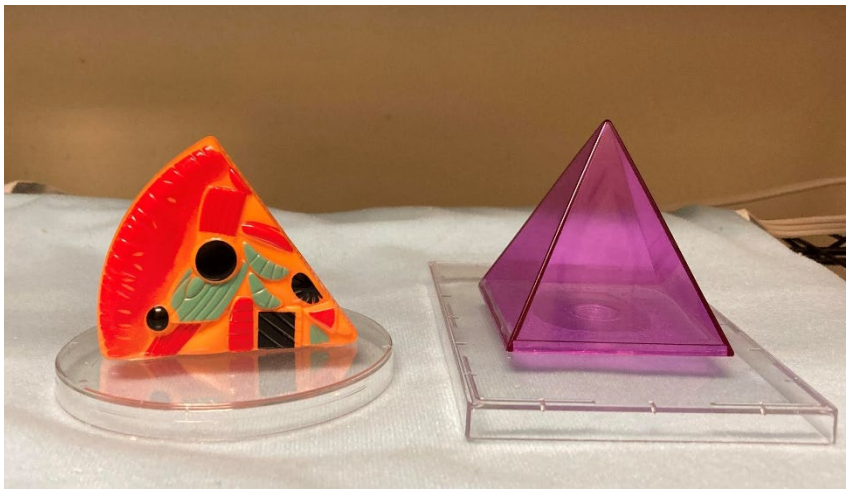

Figure 1

Two kinds of objects were used for the novel object recognition test. Mice either began with two pyramids (right) or two pizzas (left). After 4 hours, mice were placed in the arena with one of each kind of object.

Each mouse had 10 minutes to investigate the objects. Figure 2 shows a summary of the experiment.

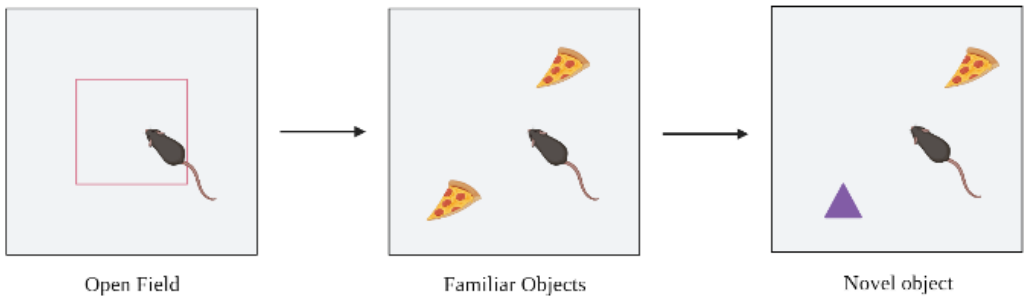

Figure 2

The novel object recognition test protocol begins with the open field test to measure anxiety levels. The next day, the mouse is placed in the arena with 2 similar objects, such as two pizzas. 4 hours later, the mouse is presented to one pizza and one pyramid, one of which is novel and one which is familiar. Image created with biorender.com.

The time spent in the center of the arena was recorded for the open field test, and the time spent investigating the objects was located for the novel object trials. Data was collected using software that can track the nose point of a mouse from a video file

(Ethovision XT, Noldus, Leesburg, VA). Each video file was studied to make sure the nose point tracking system is accurate. The mouse is considered to be investigating an object if its nose point is within 2 cm around the object. The discrimination index was calculated as (time investigating novel object – time investigating familiar object)/total time investigating objects.

Accelerating Rotarod Test

To assess motor coordination, mice were tested using a Rotarod apparatus shown in Figure 3 (47650 Rota-Rod NG, Ugo Basile, Gemonio, Italy).

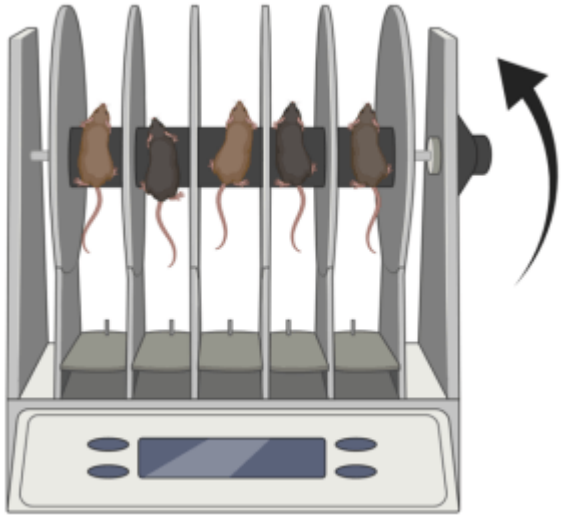

Figure 3

The Rotarod apparatus used to assess motor coordination in young and old mice. Adapted from Xhako et al., 2020

Mice were habituated to the testing room for 1 hour prior to the beginning of testing each day the experiment was conducted (Dayger et al., 2012). Mice were temporarily individually housed, and a white noise machine was placed in the room. On the first day, mice were placed on an elevated rod rotating at 4 rpm in order to

acclimatize them to testing conditions. Mice needed to stay on the rod for 90 seconds, and if they fell off before 90 seconds elapsed, they were placed on the rod again. On the second day, mice were placed on the rod that initially rotated at 4 rpm but increased to 40 rpm over the course of 5 minutes. Mice ran on the rod until they fell off or until 6 minutes elapsed and the time of fall was recorded by the apparatus. Mice received three trials spaced 10 minutes apart.

Cerebral Artery Endothelial Function

Endothelial function was assessed *ex vivo* in isolated, pressurized posterior cerebral arteries, PCAs (Walker et al., 2015). First, animals were euthanized by exsanguination under isoflurane. PCAs were taken from the brain and were cannulated onto glass capillaries inside a myograph chamber (DMT Inc., Hinnerup, Denmark) and tied with nylon (11-0) sutures. The chamber was filled with a physiological solution which contained 145 mM NaCl, 4.7 mM CaCl2, 1.17 mM MgSO4, 1.2 mM NaH2PO4, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM MOPS buffer, and 1 g/100 mL BSA, pH 7.4. The chamber was kept at 37°C and the artery was pressurized to 50 mm Hg. Endothelium dependent dilation was measured by first pre-constricting the artery with phenylephrine (PE) followed by doses of increasing concentration of acetylcholine (ACh). The percent dilation of the artery's luminal (inner) diameter compared to the pre-constricted value was calculated. Arteries were incubated with N-nitro-L-arginine methyl ester (L-NAME), an eNOS inhibitor, for 30 minutes, after which the ACh response was repeated. Finally, endothelium independent dilation was measured by first pre-constricting the artery with PE and then administering doses of

increasing concentration of sodium nitroprusside (SNP). The percent dilation of the artery's luminal (inner) diameter compared to the pre-constricted value was calculated.

Immunofluorescence

eNOS & phosphorylated-eNOS

At the time of cerebral artery endothelial function tests, sections of middle cerebral arteries (MCAs) were frozen in optimal cutting temperature (OCT). Each MCA was sliced into 8 μm sections using a cryostat (Nikon, Minato, Tokyo, Japan) and was adhered to a charged slide (Walker et al., 2015). 3-4 sections of the MCA were placed on each slide, and 8-10 slides were prepared for each animal.

Two slides from each animal were first fixed in acetone for 10 minutes, after which a PAP pen was used to draw hydrophobic barriers around each section that prevent solutions from crossing the barrier. Slides were then incubated in a blocking buffer for 15 minutes (0.1% Triton-X, 1% bovine serum albumin, phosphate-buffered saline). On each slide, 2-3 sections were incubated with a primary antibody at a 1:100 concentration for 2 hours, while the last section served as a no-primary control. One slide per animal was incubated with a primary antibody for eNOS (Invitrogen PA1-037, ThermoFisher Scientific, USA), and the other slide was incubated with a primary antibody for a version of eNOS that is phosphorylated at the Thr495 position (Bioss BS-3731R, ThermoFisher Scientific, USA). After incubation with the primary antibody, slides were incubated with the secondary antibody Alexa Fluor 647 (AF-647) (Life Technologies, Grand Island, NY, USA) for 1 hour. Then, slides were mounted with Prolong Gold with DAPI (Life Technologies) in order to stain cell nuclei in the samples

and were allowed to dry. Slides were imaged using a Leica DM4 Microscope (Leica, Wetzlar, Germany) and Leica LAS software (Leica). Images were analyzed using FIJI (ImageJ, NIH, Bethesda, MD). The arterial structure was traced, excluding the lumen and peripheral tissue. The mean gray value was measured for each image, and the mean gray value of the no primary control section was subtracted from the mean gray value of the sections incubated with the primary antibody. Then, the average mean gray value of the phosphorylated eNOS incubated sections were divided by the average mean gray value of the eNOS incubated sections.

Iba-1

Mice were euthanized and then perfused with saline in order to remove blood from the vasculature. All brains were embedded in paraffin. 20 μm coronal sections were sliced and placed on microscope slides. A primary antibody that binds to Iba1 was added, and then a secondary antibody that was polymerized with horseradish peroxidase and DAB (3,3 – diaminobenzidine) was added. Slides were imaged using a Leica DMi1 Microscope (Leica) and Leica LAS software (Leica). For each animal, images were taken in the entorhinal cortex, hippocampus, and thalamus if corresponding sections were present. Images were analyzed using FIJI (ImageJ, NIH). Images were stain separated using H DAB color deconvolution. For the images in the brown channel (which represents Iba1), a threshold value of 171 was used to increase accuracy. These images were analyzed for % area and cell count. For images in the blue channel (which represents other cells), a threshold value of 160 was used. These images were analyzed for cell count.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism. For cerebral artery endothelial function tests, group differences were determined by repeated-measures ANOVA. Otherwise, group differences were compared by one-way ANOVA and unpaired t-tests. All data is presented as mean ± SEM. A post-hoc Tukey's multiple comparisons test was used to determine group differences if ANOVA results were significant. Significance was set at $p < 0.05$.

Results

Novel Object Recognition Test

Open Field Test

Time spent in the center of the empty arena during the open field phase of the novel object test is indicative of anxiety levels. Age and large artery stiffness did not lead to significantly different anxiety levels among the three groups ($p > 0.05$, Figure 4).

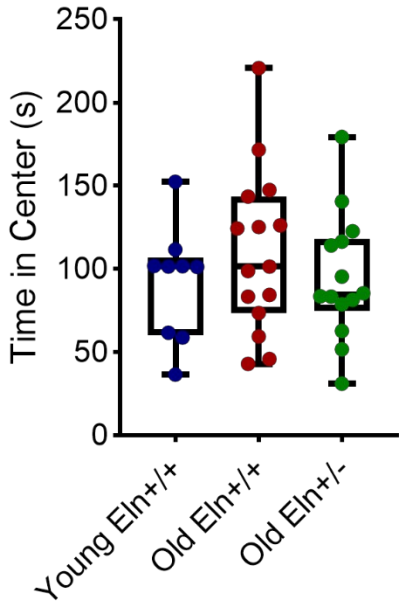

Figure 4

Time spent in the center of the arena during the open field test for Young Eln+/+ (n = 9), Old Eln+/+ (n = 15), and Old Eln+/- (n = 14) mice. Time spent in the center is not significantly different between groups ($p > 0.05$).

Novel Object Recognition Test

The total time spent exploring objects in the novel object recognition phase is calculated by adding the time spent exploring the novel object (T_n) to the familiar object (T_f). There were no significant differences in total exploration time between groups, indicating that exploratory behavior of the mice does not confound novel object recognition results ($p > 0.05$, Figure 5)

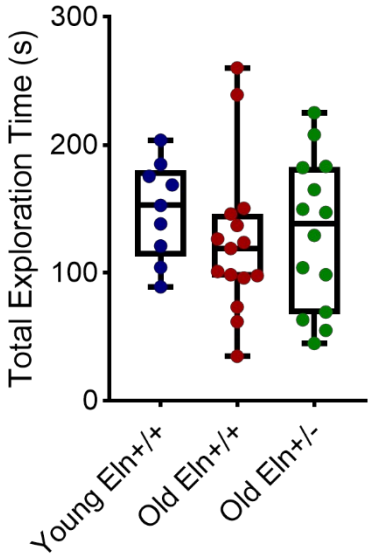

Figure 5

Total time spent exploring both objects during the novel object recognition phase for Young Eln+/+ (n = 9), Old Eln+/+ (n = 15), and Old Eln+/- mice (n = 14). Time spent exploring objects is not significantly different between groups ($p > 0.05$).

The discrimination index, which is calculated as the difference between the time spent exploring the novel object (T_n) and the familiar object (T_f) divided by total time is a measure of how well a mouse can discriminate the novel object from the familiar object. Therefore, it is an accepted measure of memory.

$$\text{Discrimination Index} = \frac{T_n - T_f}{T_n + T_f}$$

There were no significant differences in the discrimination index among any of the three groups ($p > 0.05$, Figure 6).

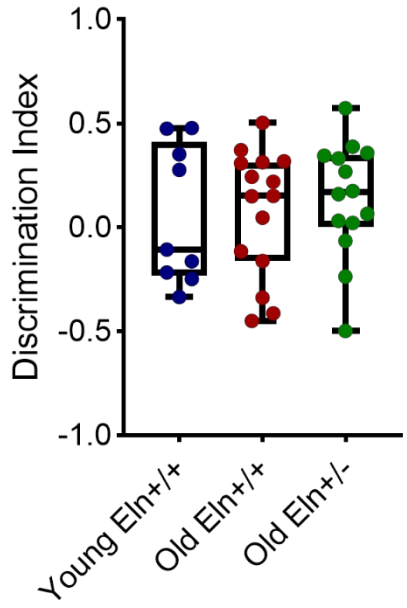

Figure 6

Discrimination index for Young Eln+/+ (n = 9), Old Eln+/+ (n = 15), and Old Eln+/- (n = 14) mice, calculated using results of the novel object recognition test. Discrimination index is not significantly different between groups ($p > 0.05$)

Accelerating Rotarod Test

Motor coordination, indicated by the time spent on the rod during the accelerating rotarod test, did not significantly differ between the three groups ($p > 0.05$, Figure 7).

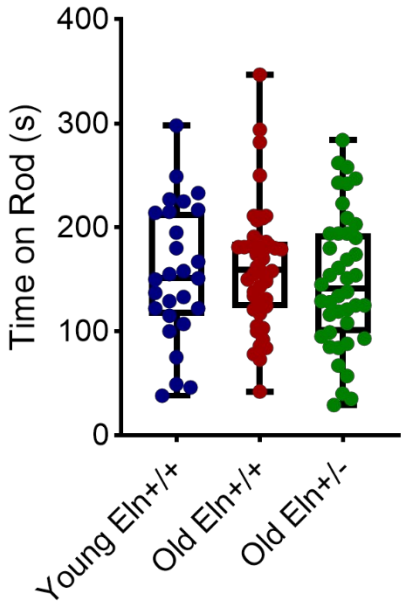

Figure 7

Time spent on the rod during the accelerating Rotarod test for Young Eln+/+ (n = 9), Old Eln+/+ (n = 15), and Old Eln+/- (n = 14) mice. Time spent on the rod is not significantly different between groups ($p > 0.05$).

Cerebral Artery Endothelial Function

In the posterior cerebral arteries, maximal vasodilation to the endothelium-dependent dilator ACh was 27% lower in Old Eln+/- than Old Eln+/+ mice ($p < 0.05$, Figure 8). In addition, maximal vasodilation to ACh was 40% lower in Old Eln+/+ mice than Young Eln+/+ mice ($p < 0.05$, Figure 8).

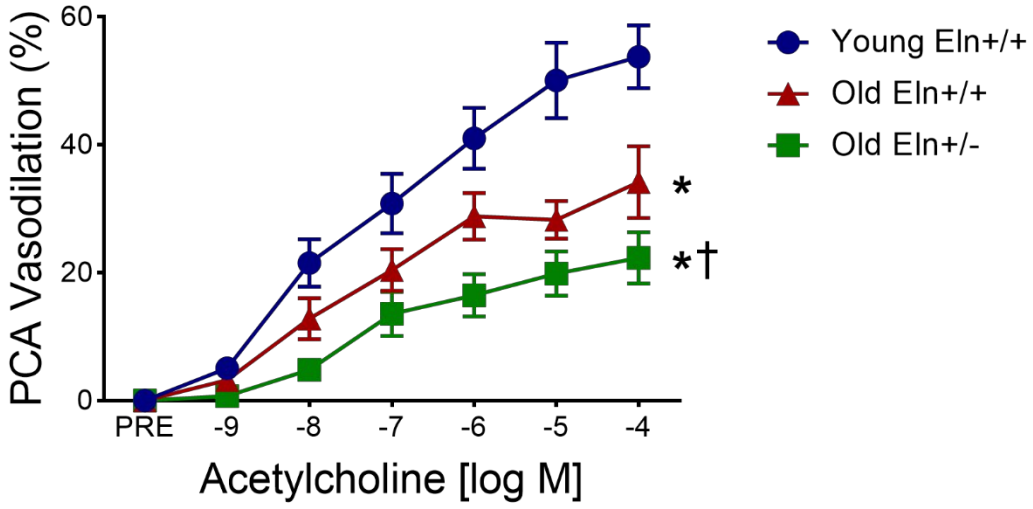

Figure 8

Endothelium-dependent dilation to acetylcholine, measured *ex vivo* in the posterior cerebral arteries in Young Eln+/+ control (n = 9), Old Eln+/+ (n = 8), and Old Eln+/- (n = 8) mice. *p < 0.05 vs Young Eln+/+, †p < 0.05 vs Old Eln+/+

The addition of L-NAME (the eNOS inhibitor) led to decreased endothelium-dependent vasodilation in the PCAs among all groups, compared to the value without L-NAME ($p < 0.05$, Figure 9). The maximal dilations with L-NAME were similar among all groups ($p > 0.05$, Figure 9).

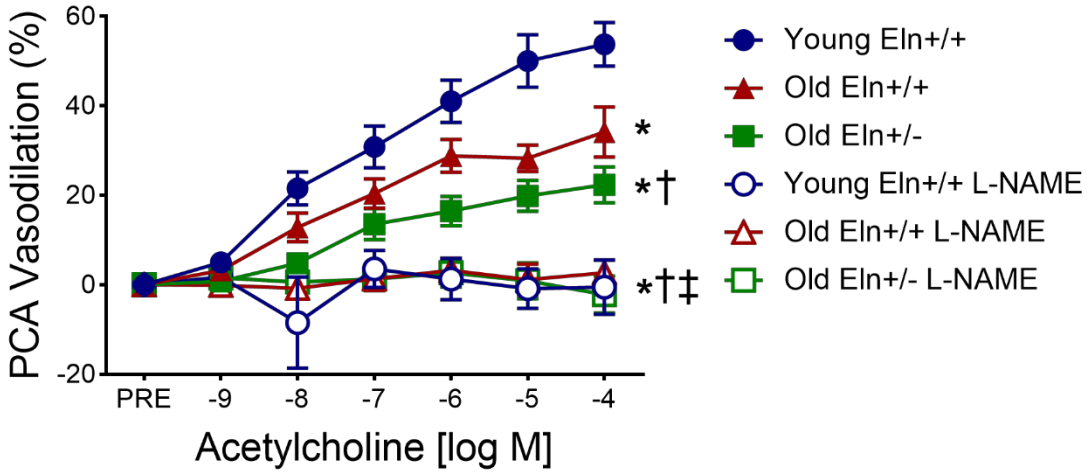

Figure 9

Endothelium-dependent dilation to acetylcholine in the absence or presence of the eNOS inhibitor L-NAME, measured *ex vivo* in the posterior cerebral arteries in Young Eln+/+ (n = 9), Old Eln+/+ (n = 8), and Old Eln+/- (n = 8) mice. *p < 0.05 vs Young Eln+/+, †p < 0.05 vs Old Eln+/+, ‡p < 0.05 vs Old Eln+/-

The response to sodium nitroprusside (SNP), the endothelial independent dilator, was not significant among the three groups (p > 0.05, Figure 10).

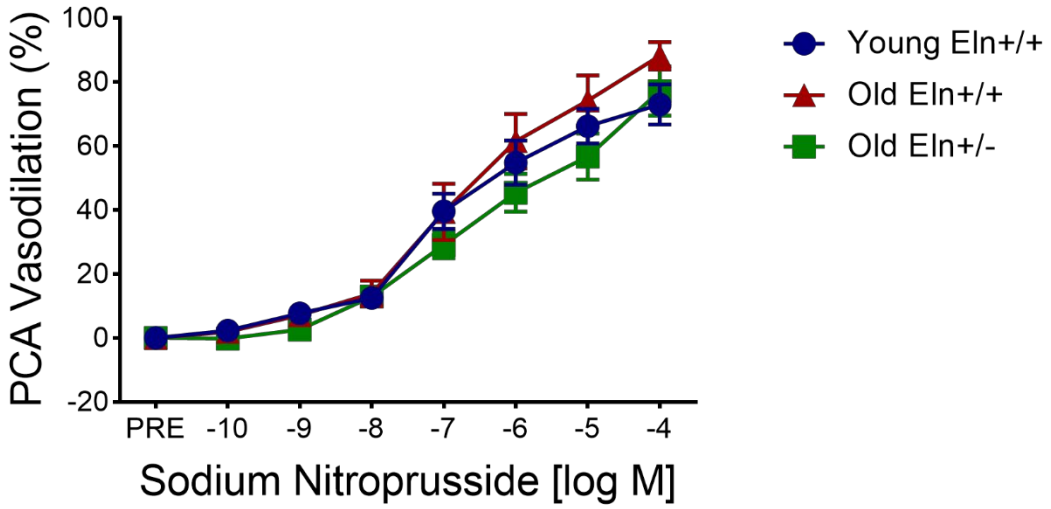

Figure 10

Endothelium-dependent dilation to sodium nitroprusside, measured *ex vivo* in the posterior cerebral arteries in Young Eln+/+ (n = 9), Old Eln+/+ (n = 8), and Old Eln+/- (n = 8) mice. Endothelium-dependent dilation is not significantly different between groups ($p > 0.05$).

Immunofluorescence

eNOS & phosphorylated-eNOS

Old Eln+/- mice had a significantly higher Thr 495 phosphorylated-eNOS/eNOS ratio in the MCA compared to the Old Eln+/+ mice, indicating more eNOS uncoupling ($p < 0.05$, Figure 11)

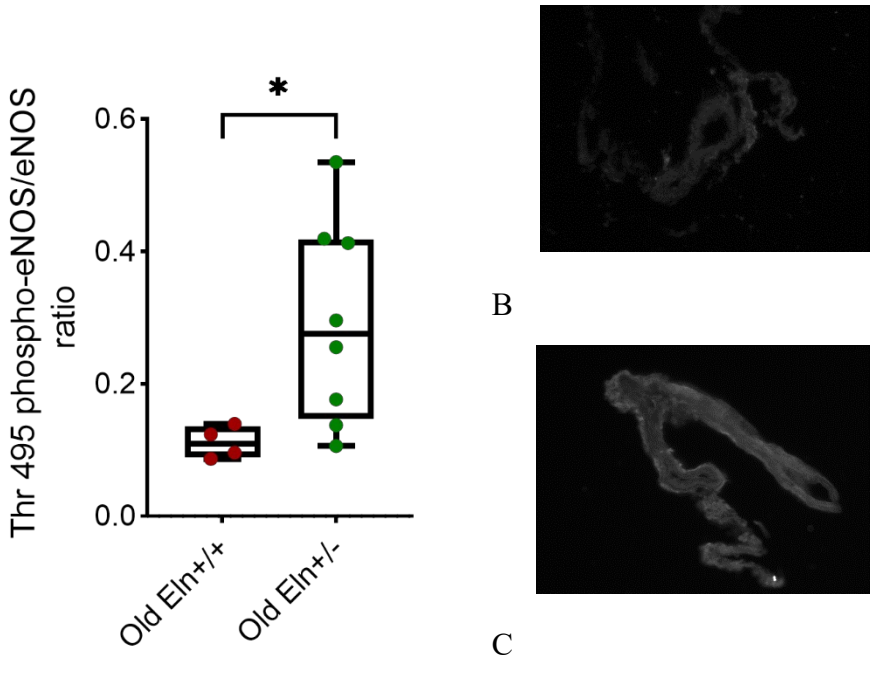

Figure 11

A. Thr 495 phosphorylated-eNOS/eNOS ratio in the MCA for Old Eln+/+ (n = 4) and Old Eln+/- (n = 8) mice. B. A representative image of Thr 495 phosphorylated-eNOS in Old Eln+/+ mice. C. A representative image of Thr 495 phosphorylated-eNOS in Old Eln+/- mice. *p < 0.05.

Iba1

Old mice had a significantly higher percentage of area of the entorhinal cortex taken up by microglia than young mice, indicated by the higher % Iba1 positive area ($p < 0.01$, Figure 12).

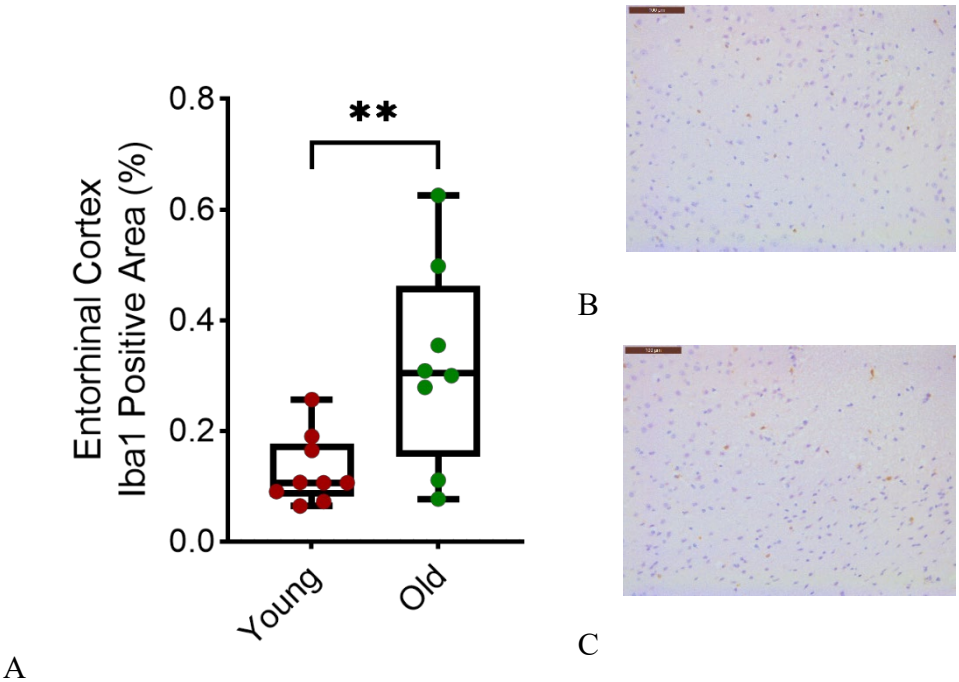

Figure 12

A. Percent of area positive for Iba1 in the entorhinal cortex of young (n = 19) and old mice (n = 15). B. A representative image of the entorhinal cortex of a young mouse. C. A representative image of the entorhinal cortex of an old mouse. **p < 0.01.

Old mice also had a significantly higher percentage of area of the hippocampus taken up by microglia compared to young mice, indicated by the higher % Iba1 positive area ($p < 0.0001$, Figure 13).

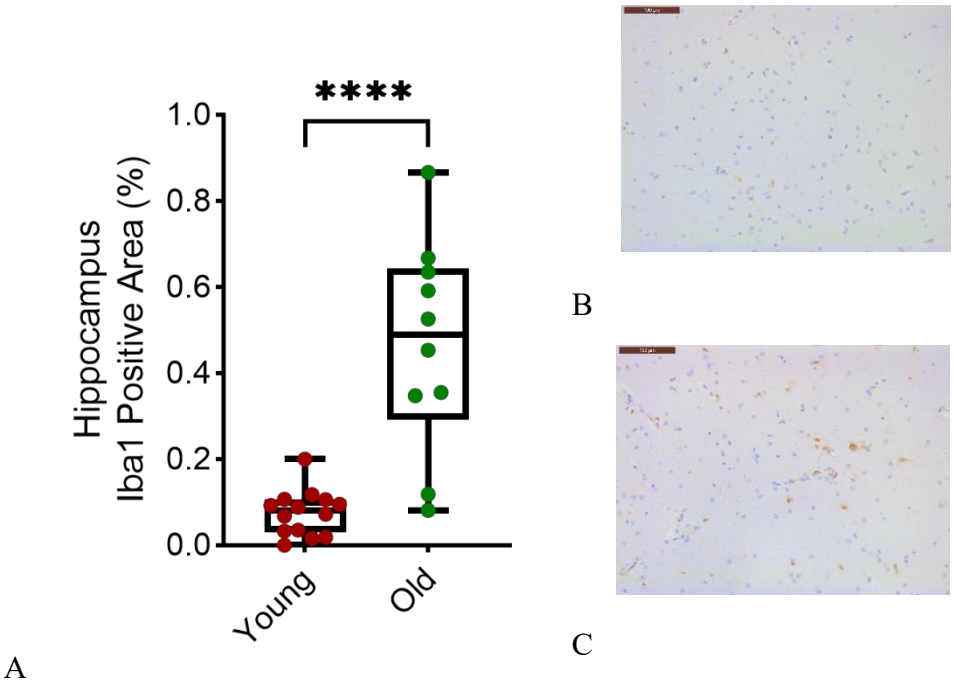

Figure 13

A. Percent of area positive for Iba1 in the hippocampus of young (n = 19) and old (n = 15) mice. B. A representative image of the hippocampus of a young mouse. C. A representative image of the hippocampus of an old mouse. ****$p < 0.0001$.

In addition, old mice had a significantly higher percentage of area of the thalamus taken up by microglia compared to young mice, indicated by the higher % Iba1 positive area ($p < 0.0001$, Figure 14).

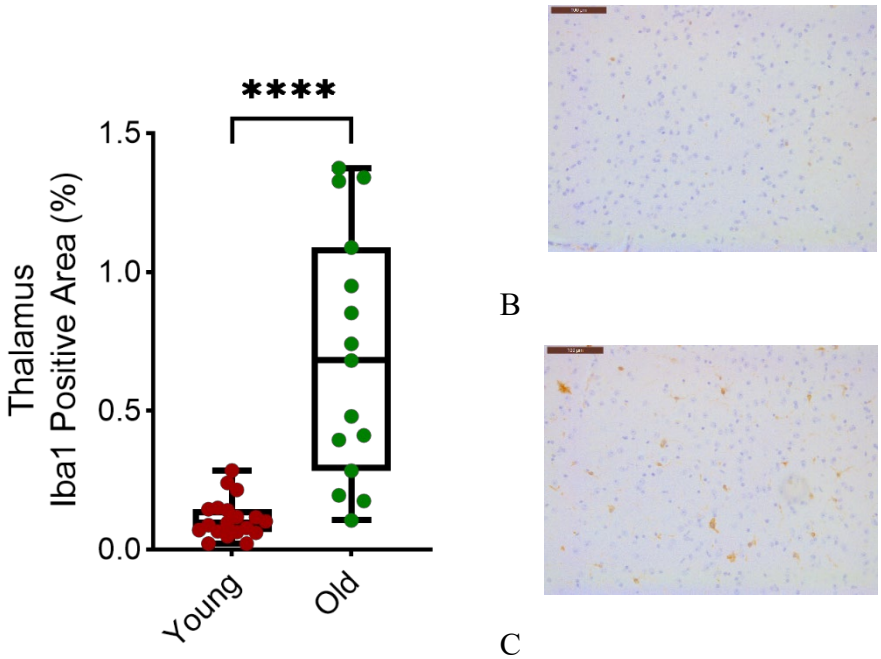

Figure 14

A. Percent of area positive for Iba1 in the thalamus of young (n = 19) and old (n = 15) mice. B. A representative image of the thalamus of a young mouse. C. A representative image of the thalamus of an old mouse. ****$p < 0.0001$.

When comparing Eln+/+ and Eln+/- mice, there was no significant difference in the percent of area positive for Iba1 in any of the brain regions measured. However, in the entorhinal cortex there was a trend towards significance, with Eln+/- mice having greater area positive for Iba1 ($p = 0.08$, Figure 15).

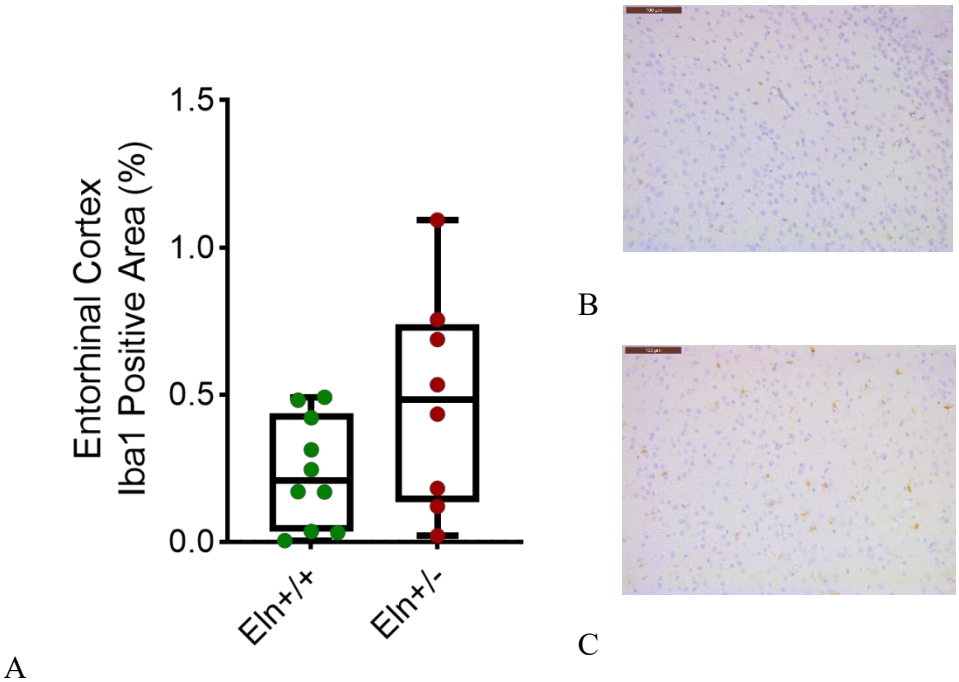

Figure 15

A. Percent of area positive for Iba1 in the entorhinal cortex of Eln+/+ (n = 8) and Eln+/- (n = 13) mice. B. A representative image of the thalamus of a Eln+/+ mouse. C. A representative image of the thalamus of an Eln+/- mouse. p = 0.08.

Discussion

Age related diseases such as cardiovascular diseases and dementia affect millions of people nationwide. Despite impacting many patients and caretakers, so far there has been no effective cure to treat dementia. However, recent work has shown that the aging cardiovascular system may play a role in the development of Alzheimer's disease and other kinds of dementia. One change of the aging vascular system is increased large artery stiffness, which can cause downstream damage to smaller arteries and surrounding tissue. Large artery stiffness with age has been shown to be associated with the conversion to dementia (Rouch et al., 2018), but the causal role has not yet been fully studied. This study aimed to assess the combined impact of age and large artery stiffness. Mice with a heterozygote deletion of the elastin gene were used as the model of large artery stiffness. Contrary to our hypothesis, Old Eln+/- mice did not have impairments in memory, as assessed by the novel object discrimination index, nor impairments in motor coordination, as assessed by the accelerating Rotarod test. However, cerebral arteries of Old Eln+/- mice had significantly lower vasodilation compared to the other groups. While old mice had higher levels of inflammation in the brain compared to young mice as expected, Eln+/- only had a trend towards significantly higher levels of inflammation than Eln+/+ mice. Therefore, the data suggests that large artery stiffness and age leads to decreased cerebral artery endothelial function and possibly increased inflammation, but these changes do not lead to impaired cognitive function.

Old Eln+/- mice do not have impairments in cognitive function

There was no significant difference in the novel object discrimination index between Old Eln+/- mice and Old Eln+/+ mice, contrary to what was expected. As previous studies have indicated that large artery stiffness is associated with poorer cognition in humans (Rouch et al., 2018), it would be expected that old elastin haploinsufficient mice should have impaired memory. Furthermore, Old Eln+/+ and Old Eln+/- mice did not have impaired performance on the novel object recognition test compared to Young Eln+/- mice. However, previous studies have indicated that old mice should have impaired discrimination index compared to young mice (Kushwaha et al., 2020; Bevins et al., 2006). It is possible that the inter time interval of 4 hours was too long for even young mice to remember the objects as several other studies use an inter time interval of 2 hours (Wang et al., 2020). As mice in all groups had roughly the same amount of total exploration time, it does not seem that a lack of exploration behavior is responsible for the results seen in the discrimination index. Also, there were no group differences in motor coordination, judged by the performance on the accelerating Rotarod test. Once again, these results are contrary to prior studies that have indicated that old mice have impaired motor coordination compared to young mice (Hamieh et al., 2021). However, the lack of significant differences for the Rotarod test indicate that the novel object recognition results are not confounded by a lack of motor coordination in some of the groups.

Old Eln+/- mice have impaired cerebral artery endothelial function

In response to increasing dose concentrations of ACh (the endothelium-dependent dilator), the pre-constricted PCA from the Old Eln+/- mice dilated

significantly less compared to the Old Eln+/+ artery, indicating impaired endothelial function. In addition, arteries from the Old Eln+/+ mice had impaired endothelial function compared to the Young Eln+/+ arteries. This data falls in line with previous studies that have found that young mice with large artery stiffness have decreased cerebral artery endothelial function compared to young control mice (Walker et al., 2015). Results also match previous studies that have found that old mice have impaired cerebral artery endothelial function compared to young mice (De Silva et al., 2018).

The addition of L-NAME leads to significantly lower vasodilation among all groups due to the loss of production of the vasodilator NO. Addition of L-NAME leads to dose response curves that are similar among all the groups. These results put together indicate that the contribution of NO to vasodilation was significantly smaller in the Old Eln+/- group compared to the Old Eln+/+ group to begin with, because the drop in vasodilation with the addition of L-NAME was smallest in the Old Eln+/- group. Additionally, the contribution of NO to vasodilation was significantly smaller in the Old Eln+/+ group compared to the Young Eln+/+ group. Therefore, it can be concluded that the drop in vasodilation due to age and large artery stiffness is caused by lower NO bioavailability.

Eln+/- mice have higher levels of eNOS uncoupling

Through immunofluorescence of eNOS and the Thr 495 phosphorylated eNOS isoform, MCAs of Old Eln+/- mice were shown to have a higher phosphorylated-eNOS/eNOS ratio than MCAs of Old Eln+/+ mice. As the Thr 495 phosphorylated eNOS isoform produces less NO but more superoxide, higher levels of the Thr 495 phosphorylated eNOS isoform indicate greater levels of eNOS uncoupling. Uncoupled

eNOS is more likely to creates higher amounts of ROS, which creates conditions of greater oxidative stress. These results match previous studies that have found that levels of oxidative stress, as measured by nitrotyrosine, are increased in young elastin haploinsufficient mice compared to young wildtype mice (Walker et al., 2015). eNOS uncoupling due to endogenous inhibitors such as asymmetric dimethylarginine has even been shown to exacerbate cognitive dysfunction (Choi et al., 2020). Therefore, eNOS uncoupling in cerebral arteries is one possible mechanism by which large artery stiffness leads to cognitive and cerebrovascular deficits in mice.

Eln+/- mice trend towards having more neuroinflammation

As hypothesized, old mice had significantly higher % Iba1 positive area in the entorhinal cortex, hippocampus, and thalamus. These results are in line with previous studies that have found increased levels of microglia in the hippocampus of aged rats (Spencer et al., 2017). These results help establish changes to certain brain regions with age that are implicated in dementia. The hippocampus and thalamus, brain structures with multiple roles in learning and memory, have been implicated in the development of Alzheimer's disease and other kinds of dementia (Moodley et al., 2014; Moustafa et al., 2017).

Eln+/- mice had a higher % Iba1 positive area in the entorhinal cortex compared to Eln+/+ mice. However, this data is not significant ($p = 0.08$). As Iba1 is a marker of microglia, immune cells of the brain that can cause inflammation, greater levels of microglia indicate greater levels of inflammation. These results are important as the entorhinal cortex is a region of the brain that communicates with the hippocampus and plays a significant role in various memory pathways. Changes to the entorhinal cortex

such as inflammation and cell loss are also implicated in early stages of Alzheimer's disease (Khan et al., 2014; Hauss-Wegrzyniak et al., 2002).

Limitations

There are certain limitations in this study that are important to note. First, due to several issues a Young Eln+/- group of mice could not be included. Therefore, it is more difficult to isolate the effects of just large artery stiffness compared to age and large artery stiffness. Due to the small group sizes, it was not possible to run gene expression tests to determine levels of pro-inflammatory and anti-inflammatory proteins. Also, the discrepancy of group sizes for the eNOS and phosphorylated eNOS immunofluorescence is something that should be fixed in future studies. Furthermore, not enough female mice were included in this study to determine sex differences with aging and large artery stiffness. Also, this study used immunofluorescence of eNOS and phosphorylated-eNOS. While the ratio of phosphorylated-eNOS to eNOS can indicate conditions in which the production of reactive oxygen species is more likely, it does not indicate the actual level of oxidative stress. Therefore, immunofluorescence of nitrotyrosine, a direct marker of oxidative stress, would indicate more about the role of reactive oxygen species in large artery stiffness. Furthermore, multiple different sets of mice were included, which makes it difficult to match results of one experiment with another.

Conclusion

The goal of this study was to identify the combined effect of age and large artery stiffness on cognitive function, oxidative stress, and inflammation. Old mice with large artery stiffness have impaired cerebral artery endothelial function due to the molecular mechanism of reduced nitric oxide bioavailability. Old mice with large artery stiffness also had higher but not significant levels of neuroinflammation in the entorhinal cortex. Old elastin haploinsufficient mice also had a higher phosphorylated-eNOS/eNOS ratio in the middle cerebral artery, indicating more eNOS uncoupling and conditions with a higher likelihood of reactive oxygen species production. Contrary to the hypothesis, old mice with large artery stiffness did not have impairments in cognitive function compared to both old and young control mice.

Further studies are needed to identify more inflammatory mechanisms that could be involved with age and large artery stiffness. Future studies should also include a Young Eln+/- control group in all tests to further elucidate the causes of impairments in Old Eln+/- mice. Finally, studying even smaller vessels of the brain such as arterioles and capillaries is necessary to fully understand the effects of large artery stiffness on the brain vasculature.

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