LARGE ARTERY STIFFNESS EFFECTS ON CEREBROVASCULAR FUNCTION AND COGNITIVE DECLINE

by

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The pathophysiology of Alzheimer's Disease (AD) is still poorly understood despite its prevalence. Epidemiological studies have shown a link between increased large artery stiffness (LAS) and the development of neurodegenerative diseases such as AD. Increased LAS is a common age-related change in the vasculature and is primarily caused by the increased fragmentation and degradation of elastin. To assess the contribution of LAS on cerebrovascular, neuroinflammation, and cognitive function, a mouse model haploinsufficient in elastin ($Eln^{+/-}$) was employed in young (6 months) and old (24 months) mice. LAS was measured in vivo using pulse wave velocity. Cognitive function was assessed using Morris water maze test of spatial memory. Cerebrovascular function was investigated by assessing endothelial-dependent and independent dilation using ex vivo pressure myography experiments. Last, neuroinflammation was assessed through gene expression from cortex samples. Contrary to previous research establishing $Eln^{+/-}$ mice as a model of LAS, higher arterial stiffness was measured in wild-type mice compared to $Eln^{+/-}$ mice in both young ($p<0.0005$) and old ($p<0.05$) age groups. An impairment in spatial memory with age was observed in $Eln^{+/-}$ mice ($p<0.0005$), but not wild-type mice. A decline in endothelial-dependent dilation, measured by maximal dilation to Acetylcholine (ACh), was observed with age in both $Eln^{+/-}$ mice ($p<0.05$) and wild-type mice

($p<0.0005$), however endothelial-independent dilation remained unaffected. A greater expression of the gene expression of the cytokine interleukin-1β (IL1β) was observed in both *Eln*$^{+/-}$ mice ($p<0.0001$) and wild-type mice ($p<0.0001$) with age. Additionally, wild-type but not *Eln*$^{+/-}$ mice had greater gene expression of another cytokine tumor necrosis factor α (TNFα) ($p<0.05$) with age. These results suggest that age is a primary risk factor for reducing cerebrovascular function, cognitive function, and higher neuroinflammation. Conclusions about the effects of LAS cannot be drawn since the *Eln*$^{+/-}$ model failed to display arterial stiffness.

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Introduction

Neurodegenerative Diseases

Neurodegenerative diseases are a group of diseases characterized by a progressive loss of neurons, accompanied by a decline in cognitive and other neurological functions. Despite significant research on these diseases, the exact disease mechanisms and effective interventions for treating these diseases are still lacking. The most prevalent neurodegenerative disease is Alzheimer's disease (AD), which is the seventh leading cause of death in the United States.[1] AD is an age-related disease with prevalence rates increasing from 5% in people aged 65 to 74, to 33.4% in people aged 85 and older.[2] Finding effective treatments for AD is of particular importance as the number of Americans aged 65 and older is projected to increase from 58 million in 2022 to 82 million by 2050.[3] The slow, unrelenting nature of AD places an unparalleled burden on the healthcare system and caregivers, highlighting the urgent need for research

Cardiovascular Disease

Cardiovascular disease (CVD) is the number one cause of death in the United States and, like AD, has increasing prevalence with age.[4] Several studies have shown a link between CVD and the development of AD and other related dementias. A longitudinal study of older adults showed those with a history of CVD had significantly higher rates of AD when compared to those without a history of CVD.[5] Further, people with a history of CVD have worse cognition when compared to those without a history of CVD.[6] In a five year follow up, patients who had subclinical CVD at the onset had a 30% greater risk for substantial cognitive decline. This

indicates that cardiovascular changes begin occurring prior to the onset of neurological deficits. This identifies CVD as a potential target for preventing the development of AD or other dementias.

Large Artery Stiffness and Aging

Many cardiovascular changes occur with age, contributing to the development of CVD. A common change is an increased stiffening of large elastic arteries. Epidemiological studies in humans have shown an age-dependent increase in arterial stiffness of large elastic arteries.[7] This change can be attributed to age-related vascular remodeling. One of the drivers of this remodeling is the fragmentation and reduction of elastin content that occurs with age.[8] Elastin is the dominant protein in the vascular extracellular matrix, contributing to a vessel's capacity to stretch and recoil. Production of new elastin protein terminates in infancy, requiring mature elastin to last a lifetime.[8] However, a lifetime of arteries stretching with each heartbeat contributes to the fatigue and eventual fracture of elastin. With decreased elastin, collagen takes over as the dominant matrix protein in vessels which is 100-1000 times stiffer than elastin.[9] The response of large arteries to blood flow is connected to the cardiac cycle. During systole the heart contracts, pumping blood through the arterial system increasing pressure. Healthy elastic arteries provide the first line of defense against high systolic blood pressure, absorbing the force by stretching to accommodate blood flow. This response results in a downstream reduction in blood pressure fluctuations. However, stiffer large arteries are unable to dampen pulse pressure. This results in high amplitude pressure waves that propagate downstream into peripheral microcirculation which are not designed to handle high pulse pressure.[10] Increases in large artery stiffness have been shown to correlate reductions in cerebral blood flow, endothelial

dysfunction,[11] and worse global cognition,[12] identifying arterial stiffness as a potential driver of cerebrovascular dysfunction and cognitive decline.

Cerebrovascular Dysfunction

Pressure-dependent myogenic responses in downstream cerebral resistance arteries can further become impaired.[13] Resistance arteries, unlike elastic arteries, utilize endothelial-dependent dilation to accommodate blood flow. The endothelium, the most internal layer of arteries, senses the increased pressure and signals to the smooth muscle layer to reduce myogenic tone, resulting in vasodilation. Molecularly, the shear stress on endothelial cells leads to an increase in endothelial nitric oxide synthase, which produces nitric oxide (NO).[14] The NO then diffuses to the smooth muscle layer where it acts as a signaling molecule initiating vasodilation. However, as arterial stiffness increases the pulse pressure delivered to cerebral resistance increases. The high pulse pressure causes cyclic strain on the endothelial cells leading to an increased production of reactive oxygen species (ROS).[15] ROS are highly reactive molecules which in excess can cause widespread damage, termed oxidative stress. In the endothelium, ROS readily react with NO reducing its availability. With reduced NO, the endothelium can't properly communicate with the smooth muscle layer impairing endothelial-dependent dilation (EDD).[16] Under normal conditions cerebral resistance arteries regulate blood flow resulting in a relatively constant, low-pressure blood flow to the microcirculation. However, when EDD is impaired, turbulent, high-pressure blood flow can propagate further down the vascular tree.

The brain microvasculature is unique from other tissues as it is highly regulated to maintain the blood-brain barrier (BBB). The BBB allows for the selective transport of essential nutrients and other solutes to the brain while protecting it from other potentially harmful

substances. To maintain this separation, endothelial cells are linked together by protein complexes, called tight junctions, that allow for selective permeability. It has been observed however, that when the microvasculature is exposed to high shear stress it results in a reduction of tight junction markers,[17] leaving the brain susceptible to cerebral microbleeds and the disruptions in the BBB.[18] A leaky BBB can trigger immune responses, by activating glial cells that release proinflammatory and neurotoxic factors, including ROS.[19] Chronic activation of these glial cells can lead to persistent neuroinflammation which has been linked to increased neuronal damage and degeneration.[20,21]

Overtime, the cerebral microvasculature undergoes hypertrophic remodeling in response to high pressure.[22] The result is microvasculature that has a much smaller lumen diameter and has higher resistance. This remodeling limits the damaging blood flow that can reach the microvasculature, however it also contributes to reduced overall cerebral blood flow.[23] Reductions in cerebral blood flow have been linked with impaired cognitive function and may further contribute to the development of neurodegenerative diseases.[24]

Animal Model

To investigate the downstream effects of large artery stiffness, a mouse model haploinsufficient in elastin protein ($Eln^{+/-}$) was employed. These mice have a deletion in one copy of their elastin encoding gene, resulting in an approximately 30% reduction in arterial elastin content compared to wild-type mice ($Eln^{+/+}$)[25]. Previous studies have established that young $Eln^{+/-}$ mice display large artery stiffness, as measured by increased pulse wave velocity, and elevated systolic blood pressure.[26] Thus, this model was employed to model arterial stiffness caused by age-related reductions in elastin content.

Gap in Knowledge

The mechanistic understanding of how LAS contributes to the development of cerebrovascular dysfunction and cognitive decline is still lacking, identifying this as an important area of research. The insights from this study will aid in our understanding of the role of LAS in cerebrovascular dysfunction and cognitive decline. Further, the variable of age was examined to determine how long-term exposure to LAS in the presence or absence of other age-related factors differed. It was hypothesized that greater cerebrovascular dysfunction and cognitive decline will be observed in mice deficient in elastin and that age will exacerbate these changes.

Methods

Animals

Male and Female Wild-type C57BL/6 with heterozygous knockout mutation in Elastin gene ($Eln^{+/-}$) and Wild-type littermates ($Eln^{+/+}$) were studied. Mice were studied at 6-7 months of age or at 24 months. All mice were fed a normal chow diet consisting of food and water ab libitum and were housed in an animal care facility on a 12/12-hour light-dark cycle at 24 °C. Investigators were blinded to groups when analyzing data. All animal procedures conform to the Guide to the Care and Use of Laboratory Animals[27] and were approved by the Institutional Animal Care and Use Committees at the University of Oregon.

Pulse Wave Velocity

Aortic stiffness was measured by in vivo pulse wave velocity (PWV) as previously described.[28,29] Mice were anesthetized by 2% inhaled isoflurane in 100% oxygen and situated on a temperature-controlled heating pad (37 °C) to maintain body temperature. Electrodes were placed on the distal parts of the limbs to record ECG information, while 20 MHz Doppler transducers were placed on the aortic arch and abdominal aorta. Indus Doppler Flow Velocity System (Webster, Texas) was used to record and analyze data. The time for each beat to travel from aortic arch to abdominal aorta was measured and was divided by the distance between the two locations to determine the PWV value. Two researchers analyzed each Doppler recording and inter-observer confidence intervals were measured, and PWV measurements with confidence intervals over 15% were reanalyzed by a third researcher.

Morris Water Maze

The Morris Water Maze cognitive test was used to assess hippocampal-dependent spatial learning and memory. A circular pool was filled with water and chalk to give the water an opaque appearance. The pool was divided into 4 imaginary quadrants and a hidden platform was placed in the middle of a quadrant slightly below the opaque water surface. Mice relied on spatial cues in the room to find the hidden platform.

Mice underwent a 3-day training period, consisting of 2 sessions per day, spaced 4 hours apart. Each session consisted of 3 sixty-second trials with 10 minutes of rest between trials. Mice were placed into the pool facing the wall in 9 different locations around the pool, with the locations changing each trial. Mice were given sixty seconds to navigate the pool. If they found and remained on the platform for 3 seconds they were promptly removed, if not they were directed to the platform after the sixty seconds.

On the fourth day, a probe trial was performed, followed by a beacon trial. For both trials the mouse was placed in the quadrant opposite to the target quadrant. In the probe trial the platform was lowered so mice were unable to stand on the platform. Mice were given sixty seconds to navigate the pool and the number of times the mouse crossed into the target quadrant or platform area was recorded. In the probe trial the platform was raised again, and a flag was added to mark the location of the platform.[30]

To reduce stress, mice were habituated to the testing room in individual, fresh cages with a white noise background and heating pads 1 hour before the experiment.

Accelerated Rotarod Test

The rotarod test was used to assess motor coordination. This test utilized a rotarod machine (47650 Rotarod NG, Ugo Basile, Gemonio, Italy) consisting of a rotating rod that gradually accelerates. The test occurred over the course of two days and mice were placed in fresh, individual cages with a white noise background and given an hour to habituate before the experiments. On the first day of testing the mice are placed onto a rod rotating at a constant 4 revolutions per minute (rpm) for 90 seconds. If the mouse fell off before they could complete the 90-second trial , they were placed back onto the rod until they could complete the trial. On the second day the mice were placed on the rotarod which accelerated from 4 rpm to 40 rpm over a 5-miute trial. Mice ran on the rod until they fell off or the trial was over. This was repeated for a total of three trials, with 10 minutes of rest in between.

Cerebrovascular Reactivity

Posterior cerebral arteries were collected for ex-vivo pressure myography experiments to assess endothelial-dependent dilation. Arteries were dissected and cannulated onto glass micropipettes in a myograph chamber (DMT Inc., Denmark) containing a physiological salt solution. All arteries were preconstructed with phenylephrine (1 to 6 μM) to obtain a 20-40% preconstruction of starting luminal diameter. Dose responses to increasing concentrations of acetylcholine (ACh, 10^{-9} to 10^{-4} M) were recorded. Ach is an endothelial-dependent vasodilator that activates endothelial nitric oxide synthase (eNOS), leading to the production of nitric oxide and subsequent vasodilation. Afterwards the vessels were incubated in N omega-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 0.1 mm) for 30 minutes. Following incubation another dose response to ACh was recorded following the same protocol. Incubating the vessel in L-

NAME blocks eNOS activity, thereby providing insight about the contribution of nitric oxide to endothelial-dependent dilation. Following this dose responses to sodium nitroprusside (SNP, 10^{-10} to 10^{-4} M) were recorded following preconstruction with PE (1 to 6 μM) to test endothelium-independent dilation. SNP acts directly on the smooth muscle layer, by releasing NO independent of eNOS.

Passive Stiffness

Passive arterial stiffness was measured ex vivo in the posterior cerebral and carotid arteries. Passive stiffness was measured following a 60-minute incubation in a calcium-free solution to eliminate the contribution of myogenic tone. The resting lumen diameter and medial wall thickness was recorded in response to increasing pressures from 5 to 100 cmH_2O (3.7-73.5 mmHg) in 5-cmH_2O increments. Stress-strain curves, β-stiffness, and elastic modulus were calculated from this.

Gene Expression

In samples of the cortex, mRNA gene expression was quantified. Superoxide dismutase (Sod) 1, 2, 3; Il-1β, TNFα, and NADPH oxidases (Nox2) were measured in the cortex through qPCR. RNA was isolated from samples using a standard protocol using QIAzol and RNeasy Micro Kits (Qiagen, Hilden, Germany) and quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed to produce cDNA with a Qiagen QuantiTect Reverse Transcription Kit. The cDNA samples underwent real-time qPCR using Thermo Fisher PowerUp System (Thermo Fisher Scientific, Waltham, MA). mRNA expression

was calculated using the $2^{-\Delta\Delta CT}$ method, mRNA (18s) was used as a housekeeping gene control, and values were normalized to the young, wild-type group.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 10. A two-way analysis of variance (ANOVA) was used to determine the interaction effects and independent effects of age and genotype. In the case of a significant F-value, post-hoc analyses were performed using a Tukey correction for preplanned comparisons. The non-normal data was transformed using Y=ln(Y) before analysis. Significance was set at $p<0.05$, and values are represented as mean ± SEM. Outliers were identified as a z score >2 and were removed from the data set.

Results

Morris Water Maze and Rotarod

All groups displayed a gradual learning curve over the six training sessions (**Figure 1A**). Some differences were observed between young and old mice of the same genotype, but these differences weren't observed by the last training session. During the probe trial, there was a main effect of age ($p<0.0001$) when comparing latency to platform (**Figure 1B**), which was significant between young $Eln^{+/-}$ and old $Eln^{+/-}$ mice. Further there was a main effect of age ($p=0.0004$) and an interaction effect between age and genotype ($p=0.0021$) in the number of platform crossings (**Figure 1C**). This was observed in a significantly reduced number of platform crossings in old $Eln^{+/-}$ mice compared to young $Eln^{+/-}$ mice. During the beacon trial, swimming velocity was assessed to account for differences in motor function (**Figure 1D**). There was a main effect of age ($p=0.0001$) on swimming velocity where velocity was slower in older mice. This was significant between young and old mice of the same genotype. To further assess motor coordination, the accelerating rotarod test was employed (**Figure 1E**). A main effect of age ($p<0.0001$) was observed, showing a decline in motor coordination between young and old mice of the same genotype.

Collectively these results indicate old mice had impaired spatial learning and memory compared to young mice, but this was only significant in $Eln^{+/-}$ mice. Further, motor function was not different across genotypes in mice of the same age, indicating the results observed in $Eln^{+/-}$ mice were not due to differences in motor function compared to $Eln^{+/+}$ mice.

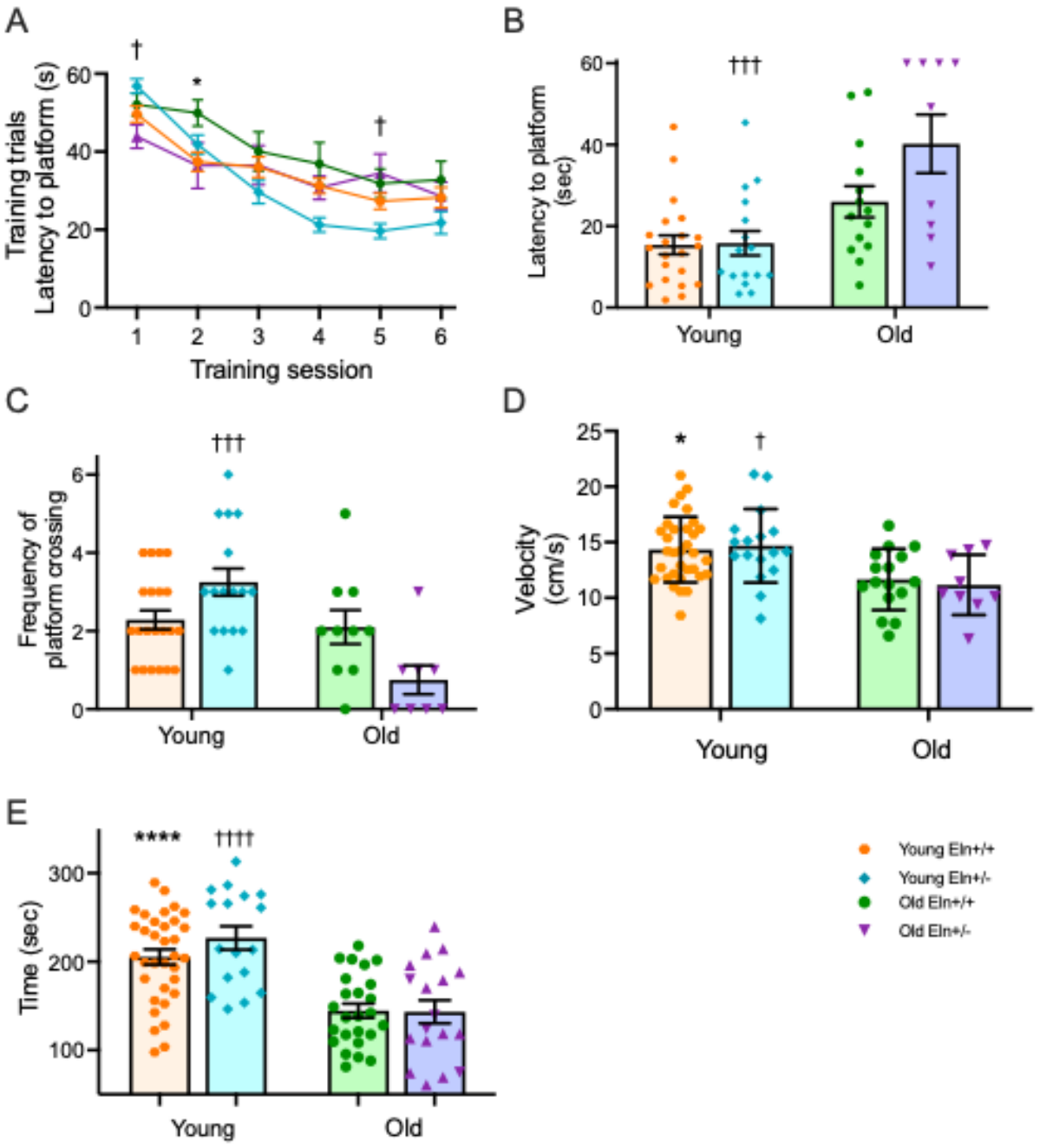

Figure 1. Spatial Learning and Motor Coordination

A-D Morris water maze test of spatial learning and memory, **E** accelerating rotarod test of motor coordination. **A** Time taken to reach platform (latency) across six training sessions. Young $Eln^{+/-}$ mice had a quicker latency than old $Eln^{+/-}$ mice during training sessions 1 and 5 ($p<0.05$); and young $Eln^{+/+}$ mice had faster latency than old $Eln^{+/+}$ mice during training session 2 ($p<0.05$). **B, C** Probe trial results, a significantly faster latency to platform and a fewer number of platform

crossings were observed in old *Eln*$^{+/-}$ mice compared to young *Eln*$^{+/-}$ mice ($p<0.005$). **D** Beacon trial velocity, a significantly slower swimming velocity was observed between young and old mice of the same genotype ($p<0.05$). **E** Significantly less time was spent on rotarod between young to old mice of the same genotype ($p<0.0001$). * = young *Eln*$^{+/+}$ to old *Eln*$^{+/+}$, † = young *Eln*$^{+/-}$ to old *Eln*$^{+/-}$, ‡ = old *Eln*$^{+/+}$ to old *Eln*$^{+/-}$. All values are mean ± SEM.

Pulse Wave Velocity

A main effect of genotype ($p<0.0001$) and age ($p=0.0021$) was observed, but no interaction effect. *Eln*$^{+/+}$ mice regardless of age had significantly higher PWV, indicating higher arterial stiffness, than *Eln*$^{+/-}$ mice of the same age (**Figure 2**).

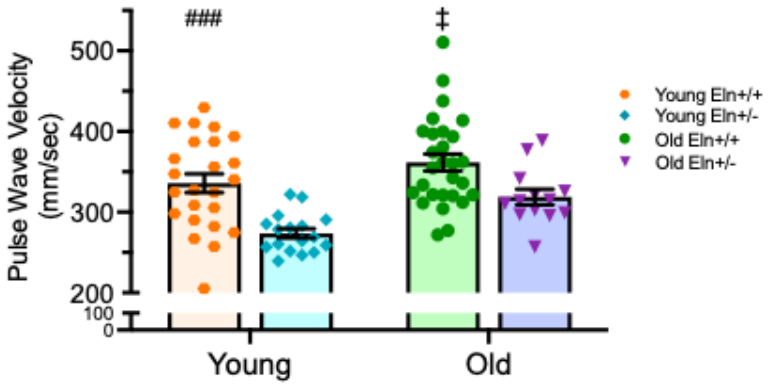

Figure 2. In-Vivo Large Artery Stiffness

Pulse Wave Velocity. *Eln*$^{+/-}$ mice had significantly lower PWV than *Eln*$^{+/+}$ mice of the same age (young, $p<0.005$) (old, $p<0.05$). # = young *Eln*$^{+/+}$ to young *Eln*$^{+/-}$, ‡ = old *Eln*$^{+/+}$ to old *Eln*$^{+/-}$. All values are mean ± SEM.

Cerebrovascular Reactivity and Passive Stiffness

Young mice had significantly better endothelial-dependent dilation, as measured by vasodilation to ACh, compared to old mice of the same genotype (**Figure 3A**). There were no significant differences in dilation to ACh after incubation in L-NAME indicating the differences observed are due to a decrease in NO bioavailability. Further, young mice had a significantly higher maximal dilation to ACh than old mice of the same genotype (**Figure 3B**), with a main effect of age observed ($p<0.0001$). There were no differences in responses to SNP (**Figure 3C**), indicating endothelial-independent dilation was unaffected. These results indicate there was impaired endothelial cerebrovascular function with age, but no effect of genotype.

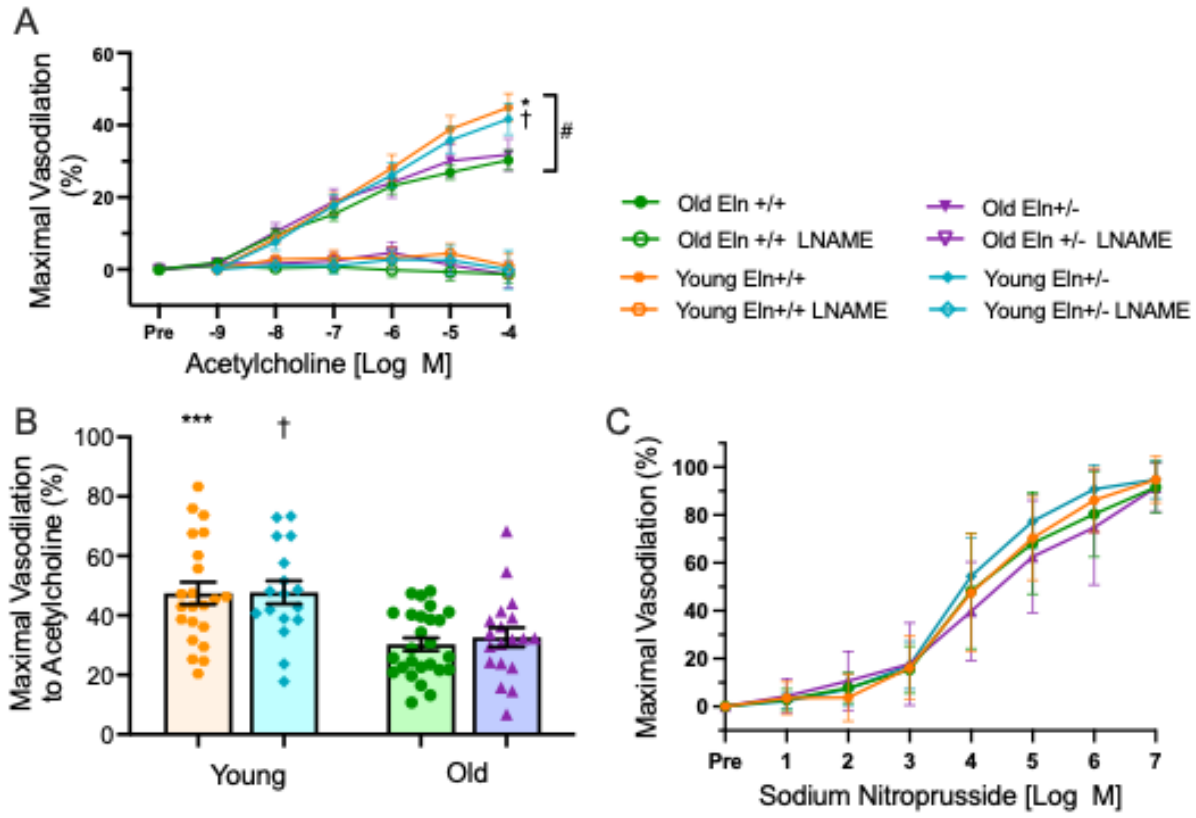

Figure 3. Pressure Myography Cerebrovascular Reactivity

A Dose response curve to increasing concentrations of Acetylcholine (ACh) with or without L-NAME. ACh vs. L-NAME + ACh was significant across all groups ($p<0.05$). **A,B** young mice had

a significantly higher maximal vasodilation to ACh than old mice of the same genotype (*Eln*$^{+/+}$, p<0.005) (*Eln*$^{+/-}$, p<0.05). **C** Dose response curve to increasing concentrations of sodium nitroprusside (SNP). No significant differences observed in these results. * = young *Eln*$^{+/+}$ to old *Eln*$^{+/+}$, † = young *Eln*$^{+/-}$ to old *Eln*$^{+/-}$, # = ACh to ACh + L-NAME. All values are mean ± SEM.

Next, passive stiffness of the PCA and carotid arteries was measured. In the PCA, there was a trend towards higher arterial stiffness with age, this was significant when comparing young *Eln*$^{+/-}$ to old *Eln*$^{+/-}$ mice (**Figure 4A**). There were no differences observed in carotid artery arterial stiffness (**Figure 4B**). Passive arterial stiffness was higher with age, but not genotype, in cerebral arteries, but not carotid arteries.

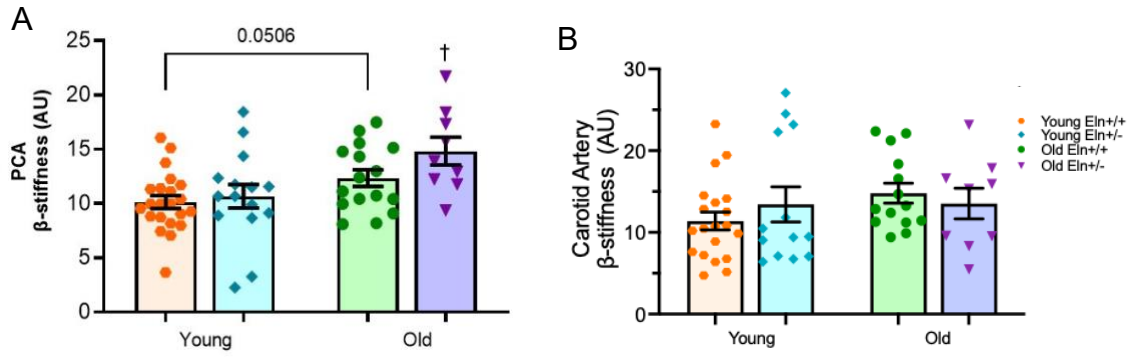

Figure 4. Ex-Vivo Arterial Stiffness

β-stiffness measure of arterial stiffness. In the **A** posterior cerebral artery (PCA) there was a significant difference in stiffness observed between young *Eln*$^{+/-}$ mice and old *Eln*$^{+/-}$ mice (p<0.05). There was a trend towards higher stiffness in young *Eln*$^{+/+}$ compared to old *Eln*$^{+/+}$, but this was not significant. In the **B** carotid artery there were no significant differences in stiffness. † = young *Eln*$^{+/-}$ to old *Eln*$^{+/-}$. All values are mean ± SEM.

Gene Expression

Cerebral artery mRNA expression was measured to examine differences in pro-oxidant enzymes, inflammatory signaling, and antioxidant defenses (**Figure 5**). NADPH oxidase 2 (NOX2) is an enzyme that generates ROS and measuring NOX2 can give insight into the comparative concentrations of ROS producers between groups. NOX2 was elevated in old $Eln^{+/+}$ mice compared to young $Eln^{+/+}$ and old $Eln^{+/-}$ mice (**Figure 5A**), This may suggest a higher potential for ROS production with age but a lower potential with $Eln^{+/-}$ genotype.

TNFα and IL1β are proinflammatory cytokines that are higher with neuroinflammation. TNFα was higher in old $Eln^{+/+}$ mice compared to young $Eln^{+/+}$ mice (**Figure 5B**), with a main effect of age ($p=0.0013$) but not genotype. IL1β was higher with age in both genotypes and was in old $Eln^{+/+}$ mice compared to old $Eln^{+/-}$ mice (**Figure 5C**). There was a main effect of age ($p<0.0001$) and genotype ($p=0.0056$), but no interaction effect. This indicates neuroinflammation, as measured by TNFα and IL1β, higher with age and IL1β lower with $Eln^{+/-}$ genotype.

Superoxide dismutase (SOD 1,2,3) are important antioxidant enzymes that protect cells from oxidative stress by converting the highly reactive superoxide into less reactive hydrogen peroxide. SOD1 was higher in old $Eln^{+/+}$ mice compared to old $Eln^{+/-}$ mice (**Figure 5D**) with a main effect genotype ($p=0.0008$), but not age. SOD2 was higher in old $Eln^{+/+}$ compared to young mice of the same genotype (**Figure 5E**), with a main effect of genotype ($p=0.006$), but not age. SOD3 was higher in old $Eln^{+/+}$ mice compared to old $Eln^{+/-}$ mice (**Figure 5F**), with a main effect of genotype ($p=0.0283$), but not age. Collectively SOD enzymes were higher with $Eln^{+/+}$ genotype, but not age except for SOD2 in $Eln^{+/+}$ mice.

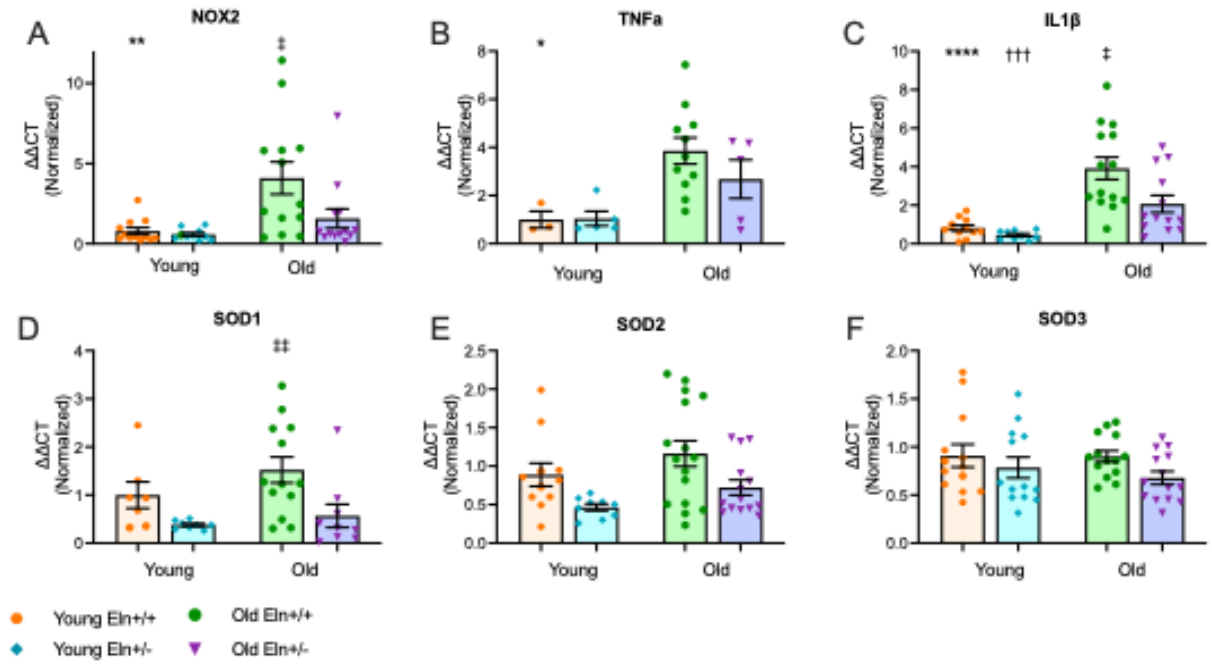

Figure 5. Gene Expression

Cortex mRNA gene expression normalized to young *Eln*$^{+/+}$ mice. Statistical significance calculated using Y=ln(Y) transform of data. **A** NADPH oxidase 2 (NOX2), **B** tumor necrosis factor α (TNFα), **C** interleukin-1β (IL1β), and **E** superoxide dismutase 2 (SOD2) were higher in old *Eln*$^{+/+}$ mice compared to young *Eln*$^{+/+}$ mice. **C** IL1β was higher in old *Eln*$^{+/-}$ mice compared to young *Eln*$^{+/-}$ mice. Further, **A** NOX2, **C** IL1β, **D** superoxide dismutase 1 (SOD1), and **F** superoxide dismutase 3 (SOD3) ($p<0.05$) were higher in old *Eln*$^{+/-}$ mice compared to old *Eln*$^{+/-}$ mice. * = young *Eln*$^{+/+}$ to old *Eln*$^{+/+}$, † = young *Eln*$^{+/-}$ to old *Eln*$^{+/-}$, ‡ = old *Eln*$^{+/+}$ to old *Eln*$^{+/-}$. All values are mean ± SEM.

Discussion

Millions of Americans live with AD or other forms of dementia[2], and with the population of adults over 65 expected to grow by nearly 50% by 2050[3], the need for the development of effective treatments is critical. Recent studies in humans have shown that increased arterial stiffness, measured by PWV, is correlated with an increased risk for developing mild cognitive impairment[31] or conversion of mild cognitive impairment into clinical dementia.[32] This identifies arterial stiffness as a potential therapeutic target to prevent or delay the onset of cognitive decline and dementia. One age-related change that contributes to the increase in arterial stiffness is the increased fragmentation and degradation of elastin protein.[33] This results in an overall decline in arterial elastin content, which can be observed in humans over 65 years of age.[34] This study sought to understand how LAS, caused by decreased elastin content, contributes to cerebrovascular dysfunction and cognitive decline. To do so, a mouse model with a heterozygous deletion in the elastin coding gene, $Eln^{+/-}$, was employed to mimic to condition of decreased elastin content with age.

Arterial Stiffness and Cerebrovascular Function

Despite prior studies indicating increased arterial stiffness in $Eln^{+/-}$ mice compared to wild-type mice, we did not observe that effect.[26,35,36] PWV experiments, an in vivo measurement of arterial stiffness, indicated $Eln^{+/+}$ mice actually had higher arterial stiffness than $Eln^{+/-}$ mice. Further, ex vivo passive stiffness experiments showed no significant differences in carotid artery stiffness. This is contrary to previous studies that have shown $Eln^{+/-}$ mice as young as 6 months display elevated arterial stiffness compared to $Eln^{+/+}$ mice.[26] Passive arterial stiffness in the PCA

was elevated with age in *Eln*$^{+/-}$ mice and a similar, but not significant, trend was seen with age in *Eln*$^{+/+}$ mice.

Age, but not genotype, had an effect of decreasing cerebrovascular function through impaired EDD. After incubating the vessels in L-NAME, a NOS inhibitor, no differences were observed in EDD. Further, there were no differences in dose responses to SNP, which supplies NO directly to the smooth muscle layer. Collectively these results indicate the differences observed in EDD were due to reduced NO bioavailability.

Cognitive Decline

Evidence of accumulating brain damage due to AD can first be observed in the hippocampus and entorhinal cortex.[37] These regions both have important functions in spatial learning and memory, which when impaired can present as disorientation and poor spatial navigation.[38] To investigate this the MWM test for spatial learning and memory was employed. Overall, there was an effect of age in increasing latency and decreasing the number of platform crossings, however there was also a decline in swimming velocity with age which could account for some of the differences observed. There was only a significant difference between the young *Eln*$^{+/-}$ and old *Eln*$^{+/-}$ groups. Motor function as measured by swimming velocity and time spent on the accelerating rotarod was almost identical between genotype groups of the same age, indicating the *Eln*$^{+/-}$ genotype has decreased spatial learning and memory with age.

Gene Expression

ROS are highly reactive compounds that can aberrantly react with other molecules causing widespread damage. The two main producers of ROS in endothelial cells are the

mitochondria and enzymes, like NOX2, that produce superoxide, a ROS, as byproducts. Prior research has indicated NOX2 expression levels is significantly increased with age.[39] NOX2 is particularly prevalent in the central nervous system and in cerebral endothelial cells.[40] Increased expression of NOX2 in endothelial cells is linked to endothelial dysfunction as the superoxide produced can react with NO and inhibit endothelial-dependent dilation. This dysfunction in the endothelial cells can further contribute to NOX2 activation in other brain cells, contributing to widespread damage.[40] The results indicate NOX2 enzyme expression was higher with age, but lower with $Eln^{+/-}$ genotype. This could provide an explanation for the impaired endothelial-dependent dilation with age observed in the pressure myography experiments. However, gene expression alone provides an incomplete picture of ROS production. The results indicate an upregulation of NOX2 enzymes with age, but this doesn't provide any information about the quantity of NOX2 enzymes present, or the relative amounts of ROS production. Further protein expression experiments need to be conducted to gain draw conclusions about the burden of ROS producers.

Nest, superoxide dismutase (SOD) levels were assessed to examine antioxidant defense systems. There are three versions of SODs located in different cellular locations that catalyze the conversion of superoxide into the less reactive ROS, hydrogen peroxide. SOD is particularly important in preserving vascular function as hydrogen peroxide, unlike superoxide, doesn't react with NO, preserving endothelial function. In quantifying SOD levels, a main effect of $Eln^{+/+}$ genotype in increasing SOD levels was observed, with no effect of age. The higher levels observed in wild-type mice may indicate impaired antioxidant defenses in $Eln^{+/-}$ mice. It is however possible SOD levels are increased in $Eln^{+/+}$ mice because they have higher rates of ROS production. A persistent increase in ROS production could contribute to a more robust

antioxidant defense system.[41] In measures of ex-vivo NO endothelial-dependent dilation, *Eln*$^{+/+}$ mice had almost equivalent function to *Eln*$^{+/-}$ mice of the same age. This indicates that the increased SOD production in *Eln*$^{+/+}$ may counteract the increased ROS generation to preserve endothelial-dependent dilation. The results indicate *Eln*$^{+/-}$ could have impaired antioxidant defenses, however this does not correlate with impaired endothelial function.

Gene expression of two pro-inflammatory cytokines, TNFα and IL1β, were measured. These cytokines have important functions in mediating innate immune responses to pathogens, however with age, tissue damage can contribute to persistent increases in inflammatory responses causing further damage.[42] These cytokines can act directly on mitochondria to increase ROS production or signal for increased expression of NOX to generate ROS as a byproduct.[43] Both TNFα and IL1β had a main effect of age in increasing expression and IL1β further had an effect of genotype where wild-type mice had higher IL1β. This indicates cytokine production increases with age consistent with an age-dependent increase in neuroinflammation. Further, as seen in NOX2 expression wild-type mice had higher expression, it is possible the higher expression of TNFα and IL1β is what is contributing to the increased NOX2 seen with wild-type genotype.

Limitations

The mouse model employed failed to show evidence of increased arterial stiffness. As a result, we are unable to conclude the effects observed in cognitive function and gene expression are correlated with LAS. It is possible the effects of reduced elastin content during development could have contributed to genotype differences observed, rather than an impact of arterial stiffness. The differences in arterial stiffness results between this study and previous studies

using this model could be due to possible differences in housing, diet, or a possible genetic drift away from the LAS phenotype.

The MWM test results could be confounded by stress levels. The water acts as an aversive stimulus to motivate the mice to learn the location of the exit platform. It has been demonstrated however that this test raises levels of corticosterone,[44] a stress hormone. This is a fine balance as both too little and too much stress can be deterrents to learning.[45] Further, there are other confounding factors with age that could impair the ability to find the platform. This includes declining motor function and visual acuity[46] that occur with age. Differences in motor coordination are accounted for by recording swimming velocity during the beacon trial and through the accelerating rotarod test, however we have no method to account for differences in visual acuity.

Future Directions

There are ongoing immunohistochemistry experiments to analyze the arterial wall composition of cross-sections of the aorta and cerebral arteries. These experiments will provide more context about elastin and collagen content, as well as information about wall thickness which is correlated with increased arterial stiffness. Considering the model failed to show arterial stiffness, as measured by PWV and passive stiffness, these experiments will be important to confirm whether arterial elastin content was reduced in $Eln^{+/-}$ as previously described, or if genetic drift away from the LAS phenotype has occured.[36] Further, this data could provide important insights about arterial remodeling in $Eln^{+/-}$ mice that may account for the reduced arterial stiffness observed. Additionally, western blotting experiments for protein expression will

be carried out so that comparisons between gene expression and protein expression can be evaluated.

A further next step would be to investigate why the *Eln*$^{+/-}$ model failed to show LAS. Investigating potential epigenetic, environmental, or dietary influences could provide insight into the contradictory results observed in this study. Furthermore, studying a different model of LAS could be beneficial. Another potential transgenic mouse model of LAS is mice deficient fibrillin-1, *Fbn1*$^{C1039G/+}$, which is a model of Marfan Syndrome. Fibrillin-1 is an important scaffolding protein in the extracellular matrix of arteries that allows for the deposition of elastin during development.[47] Studies have established these mice have increased aortic stiffness meaning they could be a potential model for LAS. Other non-transgenic models such as carotid artery calcification or transverse aortic constriction could provide information about short term impacts of high pulse pressure in the brain.

Conclusion

A mouse model deficient in elastin protein was used to investigate the impacts of arterial stiffness on cerebrovascular and cognitive functions. Despite previous research establishing LAS in this mouse model, we did not observe signs of increased arterial stiffness both in vivo and ex vivo. However, a number of age-dependent results were obtained including a decrease in spatial learning, motor coordination, and endothelial-dependent dilation; and an increase in ROS and cytokine production and antioxidant defense systems.

Effective treatments to prevent or stop the progression of AD is still lacking. Future research into the mechanisms that contribute to the development of dementia and the contributions of cardiovascular aging are necessary to identify potential therapeutic targets. The results of this study will provide important insights about how best LAS can be modeled in rodents.

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