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# Early Life Stress and Barrier Dysfunction of the Blood-Brain Barrier

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# EARLY LIFE STRESS AND BARRIER DYSFUNCTION OF THE BLOOD-BRAIN BARRIER

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## ABSTRACT

Early Life Stress (ELS) refers to any process or event of chronic and/or sever stress that occurs early in an individual's development; this can be spurred by environmental or behavioral cues and helps predict inflammation later[1] in life. ELS is associated with increased risk of cardiovascular disease (CVD) and other chronic conditions like depression. However, many of the physiological processes that lead to increased risk of chronic illness later in life have yet to be established or examined. The ELS mouse model is used to examine the unknown mechanistic links between childhood stress and risk for chronic disease in adulthood. To generate the mouse model for ELS, we combine maternal separation with early weaning (MSEW).

A preliminary study by the Pollock and Harms labs showed an increased number of brain-associated macrophages (BAMs) in the ventral mid-brain of MSEW mice compared to normally reared (NR) mice. However, whether the BAMs migrated to this region from another part of the brain or through the vasculature remains unknown.

Endothelial dysfunction is a possible mechanism linking ELS to CVD[2]. Since the Blood-Brain Barrier (BBB) is composed of endothelial cells, it should be examined for pathological signs of degradation like vascular permeability. To

establish whether ELS induces increased vascular permeability in the brain, a fluorescent carbohydrate, FITC-Dextran, was intravenously injected into the MSEW and NR mice and the intensity of the marker was measured at the BBB and compared between the two ELS groups.

**If vascular permeability in the brain is increased in the MSEW group then I hypothesize that Early Life Stress in mice leads to increased Blood-Brain Barrier permeability in a sex-dependent manner.**

**Keywords**: Early Life Stress, Blood-Brain Barrier, Vessel Permeability, Inflammation, Endothelial dysfunction

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# CHAPTER 1: INTRODUCTION

## Early Life Stress

Stress is defined as a change in homeodynamic balance[1] and requires an organism to respond to physical, mental, or emotional pressure. Excessive stress exposure especially during highly developmental stages of life (early childhood) may lead to an altered homeodynamic state and impact many physiological processes[1]. Early Life Stress (ELS) is a term used to describe environmental or behavioral stress in animal models which is synonymous to the term, Adverse Childhood Experiences (ACEs) used to depict early life stress in humans[3]. ELS, ACEs, childhood maltreatment, and early life adversity are vocabulary used to reference trauma experienced at a young age. ACEs are associated with negative health outcomes later in life and are divided into three categories: abuse, neglect, and household dysfunction [3; 4]. Exposure to early life adversity is linked to increased risk for developing chronic diseases like cardiovascular disease (CVD) and neuropsychiatric disorders such as depression[4]. Childhood maltreatment has an estimated annual economic burden of $2 trillion in the United States[5] with 40% of adults reporting ACE exposure[1].

Chronic inflammation is a biomarker for chronic diseases and has been examined as a mechanistic link between ELS and negative health outcomes[3]. Proinflammatory markers such as C-reactive protein (CRP), interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α have been positively associated with trauma exposure[6]. Presence and

quantification of these markers during childhood can then be used to predict potential risks of developing adverse health problems later in life[6]. However, many of the physiological processes between ELS and the long-lasting negative effects have yet to be established. The red question marks in **Figure 1** illustrate the unknown mechanistic links between ELS and adverse outcomes in adulthood.

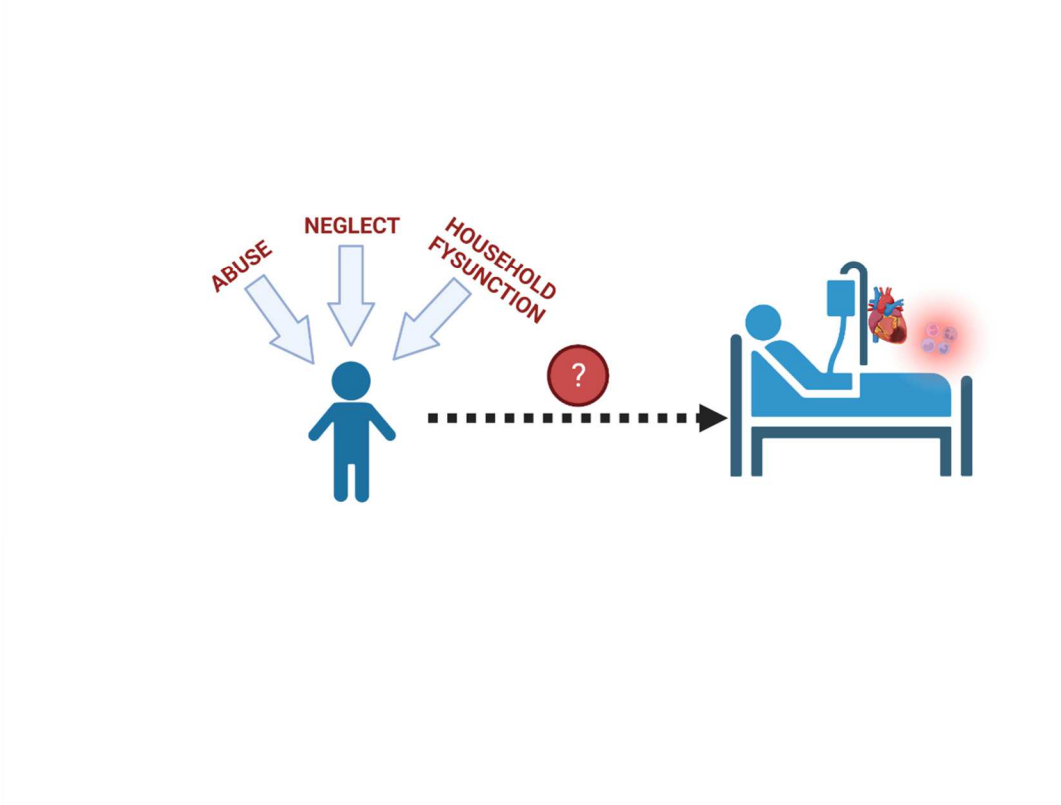


**Figure 1**: **Schematic of Early Life Stress**. ACEs such as abuse, neglect, and household dysfunction have been linked to increased risk of chronic conditions and adverse outcomes later in life, but many of the mechanistic links leading to the negative health outcomes have yet to be established. This schematic shows the unknown physiological mechanisms between childhood adversity and increased risk of chronic illness in adulthood such as cardiovascular disease. Figure adapted from McPherson, Et. al., 2019,[3]. Created with BioRender.com

## Early Life Stress, HPA Axis, and the Immune System

The hypothalamic-pituitary-adrenal (HPA) axis is a mechanism that mediates the body's response to stress and develops from infancy to early adulthood[1]. There is evidence that childhood maltreatment is associated with neuroendocrine and immunological abnormalities involving the HPA axis and are thought to mediate the development of a proinflammatory phenotype in adults[7]. Acute stress activates the secretion of proinflammatory cytokines to promote a robust immune response against harmful substances. Eventually the threat should be terminated and proinflammatory cytokines should stimulate the secretion of glucocorticoids which signal to the (HPA) axis to end the immune response[8]. However, ELS has been associated with insufficient glucocorticoid signaling resulting in a reduced ability to regulate the HPA axis[8] and control the inflammatory response.

IL-6 is an inflammatory cytokine that has been examined in several ELS studies and is known to stimulate the HPA axis to sustain a chronic immune response[1]. One study showed that healthy adults who experienced childhood maltreatment had higher levels of IL-6 than those healthy adults who did not experience ELS[7]. This depicts that ELS is a risk factor for systemic inflammation in adulthood[1; 7; 8] which is a risk factor for other chronic illnesses such as depression and cardiovascular disease[3].

## Early Life Stress and Sex Differences

Many ELS studies with rodents were only conducted in males, so it is unclear whether early life adversity affects males and females differently[9]. Female rats were not used in studies because they were shown to be more resilient to the maternal separation protocol compared to male rats[10]. The combined MSEW protocol was established for

mice because they require more stress than rats to attain changes in behavior[11]. Even with the MSEW protocol, the male mice had increased anxiety in adulthood compared to females. However, female humans are at an increased risk for developing stress-associated diseases[12], so female rodents should not be excluded from studies.

## Cardiovascular Disease and Early Life Stress

Cardiovascular Diseases (CVDs) are a group of disorders that affect the heart and blood vessels and are the leading cause of death worldwide. There is a strong relationship between ELS and CVD, but the molecular processes linking the two are still not fully understood. The CDC has reported that approximately 1.9 million cases of heart disease cases could be avoided by preventing ACE exposure[13].

The primary function of the cardiovascular system is to transport nutrients and oxygen to all parts of the body. It is composed of vessels lined with endothelial cells that support tissue homeostasis and maintain cardiovascular health[14; 15]. Vascular endothelial dysfunction is a mechanism that promotes hypertension and CVD[14] . In a previous study, MSEW induced endothelial dysfunction in male mice which may provide a mechanistic link between ELS and CVD[2]. Although the molecular mechanisms between inflammation and CVD have not been clearly defined, multiple studies have suggested that chronic, low-grade inflammation is evident in hypertension[16]. Research with ELS and rodents have revealed increased levels of inflammatory cytokines including IL-1β and IL-6. One possible mechanism for ELS-induced hypertension risk[3] is that vascular dysfunction contributes to disease propagation by disrupting the endothelial barrier and may promote the extravasation of immune cells into tissues leading to inflammation[15].

## The Blood-Brain Barrier

The Blood-Brain Barrier (BBB) is made of the blood vessels that vascularize the central nervous system (CNS). These vessels are made of endothelial cells that express tight junction proteins and maintain CNS homeostasis by tightly regulating the movement of ions, molecules, and cells between the blood and the brain parenchyma[15]. By having a strong barrier for the brain, neuronal tissue is protected from toxins, pathogens, inflammation, injury, and disease[17]. Unlike the other tissues in the body, even slight inflammation in the brain can lead to dangerous swelling known as edema and impact neuronal function. Barrier dysfunction leads to hyperpermeable vessels in the BBB and contributes to disease progression by allowing entry of immune cells and other foreign molecules into the neuronal tissue. Increased vascular permeability in the brain has been associated with neurological diseases like stroke, multiple sclerosis (MS), and neurodegenerative disorders[17].

## The Blood-Brain Barrier and Cardiovascular Disease

Both the autonomic nervous system and renin-angiotensin systems are being examined as contributors to the pathogenesis of hypertension[16]. Circulating leukocytes, proinflammatory cytokines, and angiotensin II are thought to downregulate endothelial tight junction proteins in the BBB[7; 16]. In one study, leakage of 10kDa fluorescent dextran was observed in hypertensive rats and was localized to the central autonomic nuclei including the paraventricular nucleus of the hypothalamus (PVN), but not in other regions like the amygdala or somatosensory cortex[16]. It also showed that Ang II tagged with a fluorescent marker could enter these regions suggesting that peripherally circulating factors can enter a leaky BBB[16]. However, it is unknown if the BBB

disruption was mediated by Ang II. The specific processes underly BBB hyperpermeability and CVD are not fully understood, but **Figure 2** depicts a proposed mechanism and positive feedback loop of BBB disruption and hypertension through inflammatory and autonomic processes.

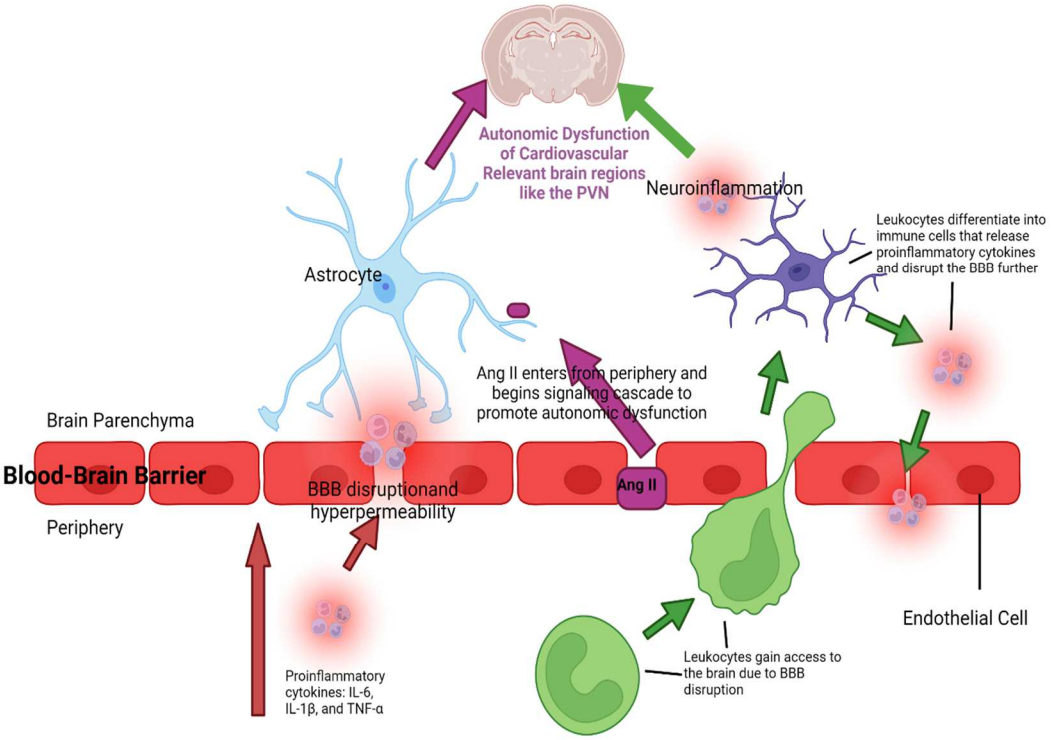


**Figure 2**: **Proposed mechanism of BBB disruption and Cardiovascular Disease: Red arrows:** Circulating proinflammatory cytokines disrupt the BBB and increase vessel permeability. **Purple Arrows:** Periphery Ang II disrupts the endothelial cells of the BBB and enters the brain parenchyma to directly act on astrocytes and initiate a signaling cascade that will lead to autonomic dysfunction in cardiovascular relevant regions like the PVN. **Green Arrows**: Circulating leukocytes enter through the BBB and differentiate into immune cells that will release proinflammatory cytokines that contribute to neuroinflammation and further disrupt the BBB. Figure adapted from Setiadi et al., 2018, Figure 1[16]. Created with Biorender.com.

## Brain Anatomy

ELS leaves the HPA axis vulnerable and affects stress response later in life[1] and the principal effectors of the stress response are found in the paraventricular nucleus (PVN) of the hypothalamus[18]. therefore the PVN of the hypothalamus is one structure that will be assessed for vascular permeability in MSEW mice. Due to the relationship between ELS and CVD, the cardiovascular relevant regions of the brain will be examined as well. This also includes the PVN, and another structure known as the Subfornical Organ (SFO). The hippocampus is a bigger neuronal structure than the PVN and can be used as a marker to detect the PVN's location in the brain. The hippocampus (HPC) is a limbic structure that contributes to the regulation of the HPA axis and has a significant effect on behavioral responses to stress[18]. Therefore, the HPC will also be observed for BBB permeability in MSEW mice. In a previous study with salt sensitive male rats, the BBB of the HPC was disrupted leading to cognitive defects[16].

The PVN could be observed in sections with the SFO or the hippocampus depending on the tissue collected. **Figure 3** depicts the ideal sections for examination of the brain regions we are interested in assessing for vascular permeability. Each fluorescent brain image corresponds to its reference images from the *Allen Mouse brain Atlas*.

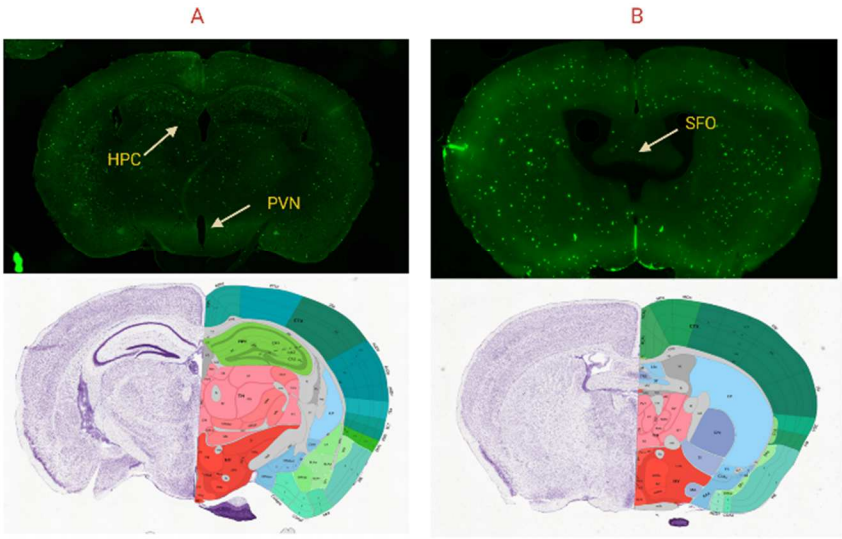


**Figure 3: Brain Sections and Regions used for ELS assessment: 3A** depicts a brain section with the Hippocampus (HC) and Periventricular hypothalamic nucleus (PVN) regions. **3B** depicts a brain section containing the Subfornical Organ (SFO). Both fluorescent images were taken at a magnification of 10x. Both Reference Images are from the *Allen Mouse brain Atlas*.

## Preliminary Data

Our preliminary studies with MSEW mice may indicate a relationship between vessel permeability and ELS. Our Lab compared the permeability of vessels in the brains of older MSEW male mice with their NR controls. The mice were approximately 24 weeks old. The fluorescent imaging showed increased permeability in certain brain vessels located in the ventral midbrain, including the cardiovascular regions, PVN and SFO (**Figure 4**). After staining the ventral midbrain with Ionized Calcium-binding adaptor molecule 1 (Iba1), a microglia/macrophage-specific calcium-binding protein, the scans indicated an increase of Brain Associated Macrophages in the PVN and SFO of the 24-

week MSEW mice (**Figure 4**). The infiltration of BAMs could result from barrier dysfunction in the vessels of the brain. However, these preliminary studies were done with an intracerebroventricular injection (ICV). ICV is a method of drug administration that is meant to bypass the blood-brain barrier with an injection directly into the vessels of the brain. The protocol that we plan to implement will instead use an intravenous injection which will allow us to observe the extravasation of FITC-dextran molecules across the BBB and analyze the effect of ELS on vessel permeability. The preliminary data indicate that ELS may contribute to vessel permeability in the MSEW model of 24-week male mice compared to the NR mice and promote the infiltration of BAMs.

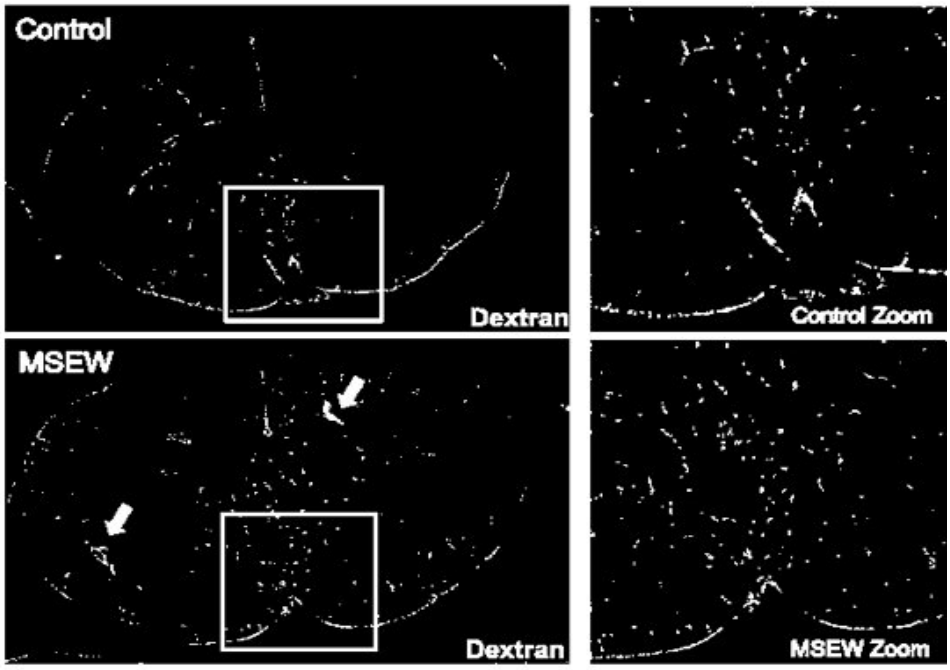


**Figure 4: Preliminary data indicating a relationship between ELS and permeability:** Control (top panel) and MSEW (bottom panel) were bilaterally injected with FITC-dextran. The images were not quantified, but show a potential relationship between ELS and BBB permeability. Brain associated macrophages (BAMs) are observed at a higher volume in the ventral mid-brain containing the cardiovascular-relevant regions (PVN and SFO) in the MSEW panel.White dots depict brain associated macrophages (BAMs). White boxes are zoom inserts and white arrows indicate potential increased BBB

permeability in the MSEW mice. These scans were done in collaboration with the Pollock and Harms labs.

## Mouse Model of Early Life Stress

Studies were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Animals*. C57BL/6J mice were used for our study and are an established mouse strain used for ELS studies[19]. Mice were given two weeks to adapt to the facility before breeding. Male and female mice were paired together for 17 days before males were removed. Females were then observed each day for signs of pregnancy and to properly record the date of birth (postnatal day (PD) 0) of their pups. Pregnant females or parent females are referred to as dams. Litters were assigned to control groups (Normal Rear (NR)) or Maternal Separation and Early Weaning (MSEW) groups. NR pups were not disturbed and remained with their dam until PD21 for normal weaning. MSEW pups were undisturbed from PD0-PD1. Starting on PD2 until PD5, the pups were separated from their dam for 4 hours every day at the same time (0900 hour (h) to 1300 h). From PD6-PD16 the pups were separated for 8 hours every day (0900 h to 1700 h). On PD17, the pups were weaned early from their dam. During separation hours, the pups did not have access to food, water, warmth, or stimulation. **Figure 5** compares the MSEW protocol to the NR protocol. Male and female mice were both used in this study. Note that male and female pups are separated on weaning day which is dependent on the group they are assigned (PD17-MSEW or PD21-NR).

The combination of Maternal Separation and Early Weaning provides an effective model for early life neglect without causing damage to the pups[19]. Mice that have

undergone the MSEW protocol have increased anxiety-like and depressive behaviors which are often observed in humans who experienced early life adversity[3].

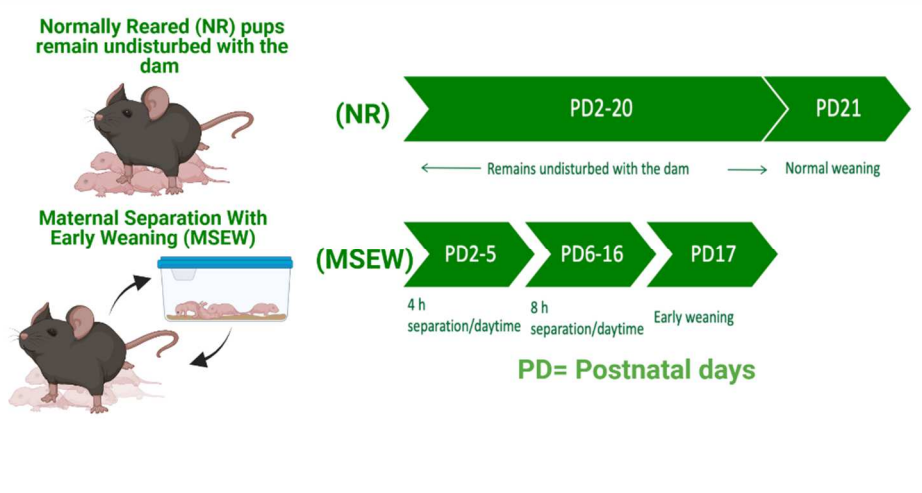


**Figure 5**: **Comparison of NR and MSEW protocol**: This illustration depicts and compares the NR and MSEW protocols. NR litters are undisturbed and weaned normally. However, MSEW litters are separated for 4 h (PD2-PD5), then 8 h (PD6-16), and are weaned early on PD17.

## Hypothesis

Although there is a strong relationship between ELS and negative health outcomes, the mechanistic links leading to these long-lasting adverse effects have not been thoroughly investigated, especially their links to CVD[3]. A preliminary study by the Pollock and Harms labs showed an increased number of brain-associated macrophages (BAMs) in the ventral mid-brain of MSEW mice compared to normally reared (NR) mice. However, whether the BAMs migrated to this region from another part of the brain or through the vasculature remains unknown. Since endothelial dysfunction is a possible mechanistic process linking ELS to CVD[2] then the endothelial cells at the BBB should be examined for barrier dysfunction. By putting mice through the MSEW protocol, we can

induce behavioral and anxiety phenotypes similar to humans who experienced childhood adversity[19] and observe physiological processes including activity at the BBB to establish whether ELS induces increased vascular permeability in the brain and possibly leads to the extravasation of BAMs. **I hypothesize that Early Life Stress in mice leads to increased vascular permeability in the brain in a sex-dependent manner. To test our hypothesis, we proposed two aims:**

**SPECIFIC AIM 1. Establish a protocol for the assessment of blood-brain barrier dysfunction in MSEW and control mice.** We developed a brain permeability assay that used a fluorescence marker, Fluorescein Isothiocyanate-Dextran (FITC-dextran), to observe barrier dysfunction. FITC-dextran is an exogenous carbohydrate that allowed us to quantify BBB permeability by measuring fluorescent intensity and comparing the two ELS groups, MSEW and NR, to one another**.**

**SPECIFIC AIM 2. To determine whether MSEW induces increased vascular permeability in the brain in a sex-dependent manner.** We used the Blood-Brain Barrier permeability protocol on both female and male MSEW mice to compare barrier dysfunction in a sex-dependent manner. After FITC-dextran was injected into both female and male MSEW and NR mice via retro-orbital injection, the brains were perfused and sectioned to include the relevant cardiovascular regions, PVN and SFO. Fluorescent intensity was measured in these regions to quantify differences in barrier dysfunction between female and male MSEW and NR mice.

This study examines the effect of ELS on the vasculature of the brain and provides insight into possible physiological mechanisms that contribute to the burden of ELS.

# CHAPTER 2: METHODS

## FITC-Dextran Troubleshooting

Due to the time commitment of the MSEW protocol (**Figure 5**), I wanted to optimize the use of Fluorescein Isothiocyanate-Dextran (FITC-dextran) using mice from a different protocol. I also needed to determine what size dextran to attach to FITC during future BBB permeability experiments. I wanted to find a positive control that would potentially disrupt the BBB in a similar mechanism as proposed in **Figure 3**. Lipopolysaccharide (LPS) has been known to induce inflammation in the brain through activation of pro-inflammatory cytokines and microglia which are the brain's resident macrophages[20], therefore I used LPS as my positive control. The first and second optimization experiments with LPS were used to determine whether 70 kDa FITC-dextran or 4 kDA FITC-dextran were better suited for BBB permeability assessment.

### Development of the Blood-Brain Barrier Permeability Protocol

To develop the Blood-Brain Barrier Permeability Protocol, I needed to determine five additional factors: **1)** Type of intravascular injection (IV) **2)** Concentration of FITC-dextran that should be used **3)** Time of circulation after intravascular delivery **4**) Age of mice to collect data from and **5)** Timeline between injection and microscopy analysis.

**Type of Intravascular Injection (IV)**: The two possible methods for IV delivery in our lab are the lateral tail vein injection and the retro-orbital injection of the venous

sinus. I needed to practice both to determine which method served the protocol most efficiently.

**Concentration of FITC-Dextran:** To determine whether 400mg/kg or 200mg/kg is a better concentration for the analysis of vascular permeability in Early Life Stress models, I performed an optimization experiment to compare which of the two concentrations led to better fluorescent imaging in their brain sections.

**Time of Circulation after intravascular delivery:** To determine whether 2 minutes or 10 minutes is a better circulation time after FITC-dextran administration, I performed an optimization experiment to determine which circulation time led to better fluorescent imaging in their brain sections.

**Age of Mice to Collect:** To determine what age of mice to study for examining BBB permeability, I examined the preliminary data which used 24-week mice. I was concerned that the potential increase observed in BBB permeability in the MSEW mice could have been influenced by their older age. BBB leakage severity is age dependent and older individuals may experience more barrier dysfunction[21] which could have contributed to the increase in BAMs observed in the MSEW mice. I wanted to focus on younger mice instead to decrease the effect of aging on BBB permeability.

**Timeline between Injection and Microscopy Analysis:** To create a timeline for the BBB permeability experiments, I need to establish a schedule that is manageable and can be repeated between cohorts. The timeline must be short enough that FTIC does not decay.

## Perfusion Fixation and Tissue Collection

The perfusion step is important for clear visibility and accurate analysis at the BBB and was provided by the Harms Lab. The advantage of directly perfusing fixative through a live circulatory system is to ensure that the fixative agent can reach every tissue in the organism at approximately the same time unlike the immersion fixation technique[22]. This proves that FITC-dextran only has 10 minutes to permeate the endothelial barrier before being flushed out. I performed the perfusion protocol on one cohort before realizing that I needed a partner who was faster and could effectively perfuse the brains because I was too slow and affecting the time of circulation.

The perfusion protocol begins once the 10-minute systemic circulation of FITC-dextran is complete. The mouse is quickly put under anesthesia again and secured to the surgical plane. The ribcage containing the heart is dissected open so a butterfly syringe can be used to puncture the left ventricle while scissors cut the right atrium. This allows the person perfusing to administer their substances while using the heart to their advantage. The left ventricle is still pumping the substance of choice to the entire vasculature without recycling any of the old material through the right atrium.

Approximately 20 mL of Phosphate Buffered Saline (PBS) with heparin is used as a wash before the fixative is added to remove blood cells and prevent clotting from occurring. Then about 15 mL of 4% Paraformaldehyde (PFA) is pushed through the cardiovascular system to preserve the tissues and cells[22]. Once the mouse shows signs of

stiffness, tissue fixation is complete. The head is removed using a pair of scissors and then the brain is carefully dissected from the skull.

Since the brain contains many tiny vessels, I submerge them for two additional hours in 4% PFA once they are removed from the skull. During this step, I cover the conical tubes with aluminum foil to prevent light exposure. After two hours, the brains are transferred to a 30% sucrose solution in PBS and stored for 24-48 hours or until the solution has diffused into the brain causing it to sink[22]. Sucrose is used as a cryoprotectant to prevent ice crystal formation during the freezing step used in sectioning.

**Sectioning and Mounting Protocol**

The Section and Mounting protocol were taught to me by the Harms Lab. Brains are frozen with dry ice and placed on a chilled platform where a microtome is used to cut each section of the brain 40 microns think. Since we are searching for increased permeability throughout the entire brain, each part of the brain is cut. All the sections for one brain are stored evenly between 12 wells containing a 50% PBS: glycerol solution. This solution allows for the sections to be stored until they can be mounted on a slide and examined.

The technique for Mounting Free-Floating Sections takes patience and practice. Ideally, each of the 12 wells should contain all the free-floating sections that make up a whole brain. Using a brush and a mounting solution (Tris Buffered Saline (TBS)), the

sections are floated onto a slide. Once all the sections are mounted, I will add a coverslip and mounting medium-Vectashield (150 µl).

## Microscopy

The BBB permeability protocol timeline requires analysis to be done within a week of the injection date (**Figure 6;7**). To discourage any bias during the microscopy process, the mounted slides are given to a colleague and relabeled for a blinded analysis. All slides are imaged using a digital Keyence microscope which captures fluorescent images using a GFP (Green Fluorescent Protein) laser. Each section is imaged at 10x magnification with the same exposure to ensure consistency among cohorts.

## Image Processing

To quantify the fluorescent markers on the brain sections, a statistical software known as ImageJ (Java) and FIJI (Fiji is Just ImageJ) is used. I remain blinded during image processing. Using ImageJ, I invert the image to a black and white copy and convert it to an 8-bit-image. Using the polygon tool, I outline my area of interest which is the entire brain section containing the sections that I am interested in. Additionally, I measure a part of background area. I take the mean gray value of both the area of interest and background area then subtract the background area from my area of interest. The Mean Gray Value is the average gray value within a selection and is the sum of the gray values of all the pixels in the selection divided by the number of pixels[23]. The mean gray value indicates the brightness of a pixel and detects the fluorescent intensity from the FITC-dextran[23]. Once I have processed every image, I allow myself to organize the results back into their respective groups: MSEW versus NR (male and female).

## Statistical Analysis

For statistical analysis, I export the mean gray values into GraphPad Prism which is a software used regularly in the scientific community to visualize and test for significance in data. Groups are organized by MSEW or NR protocol and can further be divided or combined by cohorts, genders, or various variations of the two. The female mice cannot be used in statistical analysis alone because of the low yield of NR female mice (n=2). However, the female mice can be combined with other male mice to compare BBB permeability in MSEW mice relative to NR mice. I performed two-sided t-tests to determine if there were significant differences in FITC-dextran intensity between MSEW mice and NR mice. A two-sided student t-test is used to compare a control (NR) to an experimental (MSEW). By examining the cohorts alone and then together, I can determine which parts of the brain are more permeable due to (ELS) and whether sex may influence permeability.

# CHAPTER 3 RESULTS

## The Blood-Brain Barrier Permeability Protocol

**FITC-Dextran Troubleshooting:** FITC-dextran is an exogenous carbohydrate that allows for the assessment of BBB permeability through quantification of fluorescence markers observed in tissue once they have diffused from the vasculature. Previous experiments have utilized Evan's Blue to examine BBB permeability. However, unbound molecules of Evan's Blue can still diffuse into neuronal tissue regardless of if there is a disrupted BBB[24] which is not a fair assessment of barrier dysfunction. Unlike Evan's blue which attaches to an endogenous protein, albumin (68 kDa)[25], FITC can be attached to any size dextran which can give insight on the severity of barrier dysfunction if necessary[24]. FITC-dextran is sensitive to light and will lose its fluorescent capabilities if exposed to prolonged brightness[24]. Anything involving FITC should be done in the dark and materials should be covered when possible.

Positive control mice (n=4) were injected with a intraperitoneal injection (IP) of 80mg/kg of LPS[20], while the controls (n=5) were injected with saline 24 hours before FITC-dextran injection. At 24 hours, each mouse regardless of group was injected by tail vein injection with FITC-dextran at 80mg/kg. There was no significant reason that these parameters were chosen, and the mice were sacrificed without time of circulation being a concern. I did not expect to see any fluorescent markers in the saline/FITC control since

internal inflammation was not meant to occur. However, I did expect to see fluorescence in the LPS/FITC group, but it was not observed. I realized that I had used a dextran, 70 kDa, which was too large of a molecule to pass through the BBB[24].

The second optimization experiment was performed using the same protocol as before (positive control: n=3, control: n=3), but with the smallest dextran instead (4 KDa). FITC-dextran was detected in the LPS/dextran group, but not quantified. It was not observed in the saline/dextran group. The purpose of troubleshooting the FITC-dextran was to gain information on which size dextran to use and to determine if permeability could be detected in the brain parenchyma. Now that we had this information, I could begin developing a BBB permeability protocol

**Type of Intravascular Injection:** To study vascular permeability, we needed to observe the movement of molecules from blood vessels to tissues. This meant that FITC-dextran had to be delivered intravascularly to the mice. I had started my optimization experiments with the tail vein injection but realized that I may have been causing distress to the mice during my repeat attempts to inject into the tail vein. The stress of the mechanical restraint and the high failure rate of the lateral tail vein injection convinced me to change my method of IV delivery to the retro-orbital injection instead. According to one protocol, a right-handed operator should find it easiest to administer to the right-orbital sinus of the mouse[26] which was the case for me. All my mice have been administered FITC-dextran to the right-orbital sinus to maintain consistency between cohorts. An inhalant anesthetic is used during the injection. Mice successfully stayed asleep unlike when the tail vein method was used. One factor that I had to be aware of

with retro-orbital injections was for the volume to not exceed 150µl[26] which leads me to the second factor: concentration of FITC-dextran.

**Concentration of FITC-Dextran:** My third optimization experiment used 18-week MSEW (n=5) and NR (n=6) males who had undergone the gut permeability assay every 4 weeks. I examined gut permeability using FITC-dextran where I used 400mg/kg of FITC-dextran with an injection volume of 100mg/mL in PBS. The gut permeability assay requires FITC-dextran delivery as a food bolus and relies on gut absorption to enter the vasculature. Since IV injections enter the bloodstream immediately, I decided to test what halving the concentration of FITC-dextran would do to half the mice. The groups were MSEW/200mg (n=3), MSEW/400mg (n=2) and NR/200mg (n=3), NR/400mg (n=3). Unfortunately, during experiment three, I was still attempting the tail vein injection and did not properly inject the FITC-dextran into the vasculature of most of the mice. I was only able to compare images between a few mice and deemed the results inconclusive. However, I began to understand that the concentration of FITC-dextran did not matter if the injection amount was normalized to body weight[24] to maintain consistency across injections. I decided to proceed with a 400mg/kg concentration going forward since it had been established in previous permeability experiments.

**Time of Circulation after intravascular delivery:** The fourth optimization experiment compared LPS positive controls with saline controls (n=4 for both groups) to determine circulation time of FITC-dextran after injection. The findings from previous experiments determined the other valuables: 4 kDa and 400 mg/kg, so this was the last variable that needed to be optimized. Although it takes an adult mouse approximately two minutes for blood to circulate the entire vasculature, I wanted to test a longer time point

ensure enough time for the molecule to diffuse into the smaller vessels of the brain. Additionally, I anticipated that it would be difficult to perform multiple sacrifices at once with only two minutes between injection and perfusion. Two minute and 10 minutes timepoints were compared and are shown in **Figure 6.** The 10-minute circulation seems to allow FITC-dextran more time to circulate and diffuse into the neuronal tissue. However, the intensity of FITC-dextran was not quantified in either image. The 10-minute time point will be used in the BBB permeability protocol.

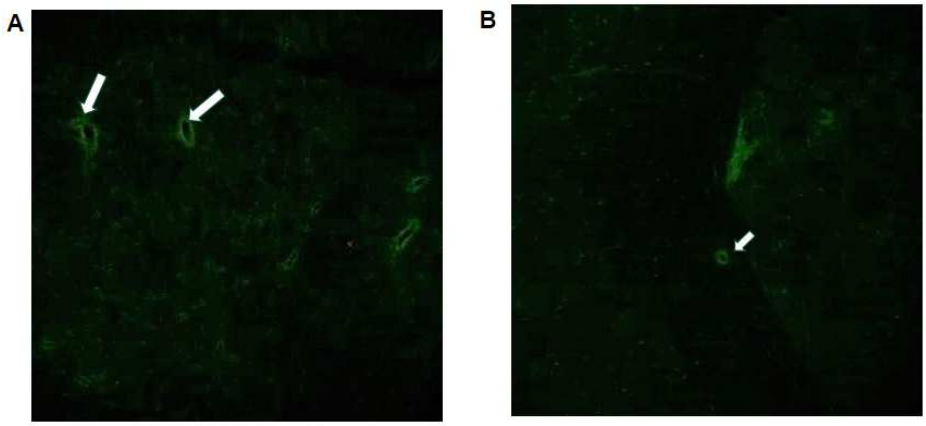


**Figure 6**: **Optimization Experiment for Circulation Time: A.** Circulation time of 10 minutes **B.** Circulation time of 2 minutes. These images depict more fluorescence (green) in **A**. However, the FITC-dextran was not quantified. Both **A** and **B** were LPS positive control mice injected via tail vein with 3-5kDa at 400mg/kg. Arrows point to vessels. Images were taken at 10x in the Striatum of the brain.
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**Age of Mice to Collect**: In previous optimization experiments, I was not concerned with the age of the mice because I wanted to focus on developing a protocol to assess vessel permeability in ELS. Once the protocol was developed, I needed to decide on what age mice to study. I did not want to begin the study of BBB dysfunction in ELS using older mice who are expected to have more barrier permeability already. BBB leakage severity is age dependent with older individuals experiencing more barrier dysfunction[21]. Unlike older mice, 12-14-week mice have just entered the human equivalent of early

adulthood[27]. If increased BBB permeability in MSEW mice occurs, it is unlikely that the BBB dysfunction is a result of older age rather than endothelial dysfunction. However, examining age dependent vessel permeability could be a future study.

**Timeline between Injection and Microscopy analysis:** There are varying results to how long a slide with immunofluorescent material will last. FITC-Dextran is sensitive to light and its fluorescent signaling will decay eventually which will affect ImageJ analysis. Consistency between ELS cohorts must be maintained to properly compare fluorescent intensity in neuronal tissues. A difference is fluorescent intensity is evident between the sections that were analyzed 1 week after FTIC-dextran injection compared to 6 weeks later (**Figure 7**). The mean grey value (MGV) of **Figure 7A** (11.436) is almost double of the MGV of **Figure 7B** (6.233) indicating a decay in the fluorescent intensity of FITC.

**Figure 8** is the timeline for the BBB permeability experiments and is an overview of the entire Blood-Brain Barrier Permeability Protocol.

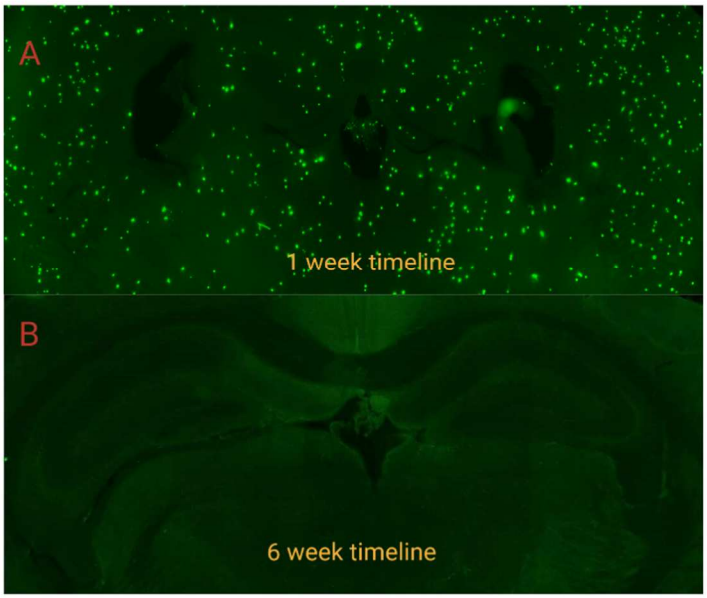

**Figure 7**: **The Effect of Time on Fluorescence Intensity:** Both **A** and **B** depict the Hippocampal region of the brain and the mean grey value (MGV) has been calculated using ImageJ. **A.** This section was analyzed within a week of FITC-dextran injection and has an MGV=11.436 **B**. This section was analyzed 6 weeks after FITC-dextran injection and has an MGV=6.233 The images are both taken at 20x magnification.

## FITC-Dextran Analysis

Once the protocol for the assessment of Blood-Brain Barrier had been practiced and optimized (**Figure 8**), MSEW mice at the 14- week time point could finally be used. 3 cohorts were examined using this protocol.

**Cohort 1** will not be included in the results but was instrumental in practicing the **Figure 8** protocol completely and using the retro-orbital injection and Keyence microscope for the first time**. Figure 7B** is a cohort 1 section and cannot be analyzed properly due to the decay of fluorescence in the neuronal tissue. Both male (MSEW:4) and females (MSEW:3, NR:4) were used.

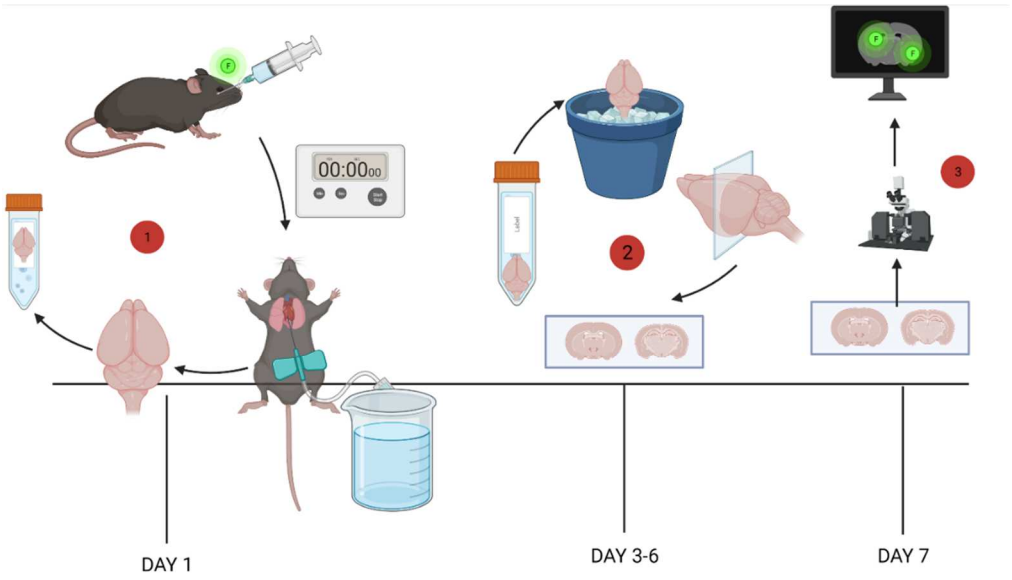

**Figure 8: The Blood-Brain Barrier Permeability Protocol and Timeline: (1)** Day 1 includes the injection of FITC-dextran, perfusion protocol, and brain dissection. **(2)** After 48 hours have passed in the sucrose solution, Days 3-6 include sectioning and mounting the free-floating sections. **(3)** On Day 7, the sections are imaged by a fluorescent microscope. Created with Biorender.

**Cohort 2** contained males only (MSEW:4, NR:6) and allowed us to finally quantify and compare fluorescence intensity between MSEW and NR mice. Sections containing the SFO and PVN were imaged, and fluorescence intensity was measured using ImageJ. There were significant differences between the male MSEW group and NR group when comparing fluorescent intensity of brain tissue in the two groups.

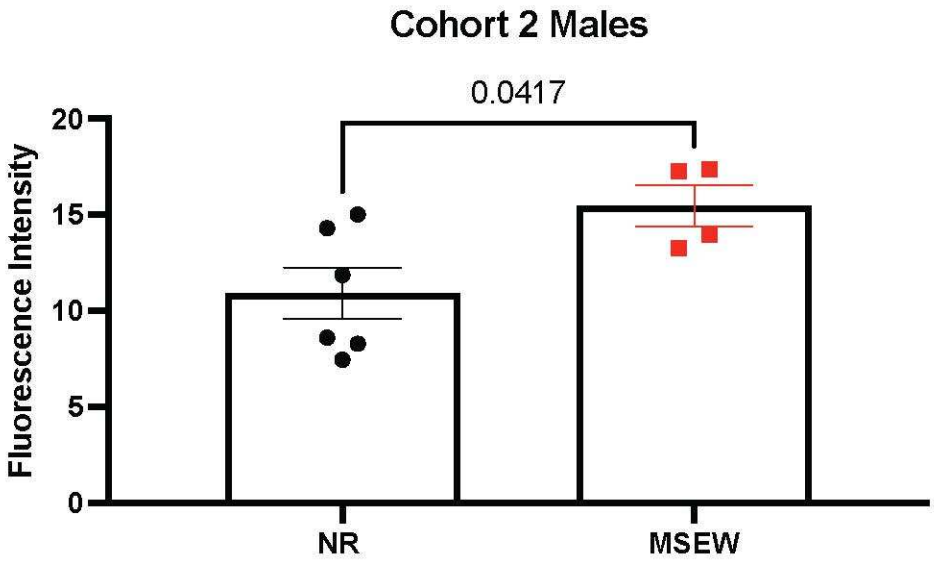


**Figure 9: Cohort 2: Males**: The sections containing the PVN or SFO in 14-week male MSEW (n=4) and NR mice (n=6) were examined. The fluorescence intensity in male MSEW mice is significantly more than in NR mice. $P < 0.05$. A two-sided student t-test was used.

**Cohort 3** contained both males (MSEW: 6, NR: 6) and females (MSEW:4, NR:2). However, one of the NR female mice had hydrocephalus and had to be excluded from the study, so the n=2 of the NR females makes it impossible to perform a statistical study alone. The same sections containing the regions of the brain associated with ELS and CVD (SFO, PVN, HPA-axis-HPC) [3; 7; 10] were collected and imaged for analysis. Unlike the males in cohort 2, no significant differences in fluorescent intensity were observed in cohort 3 males.

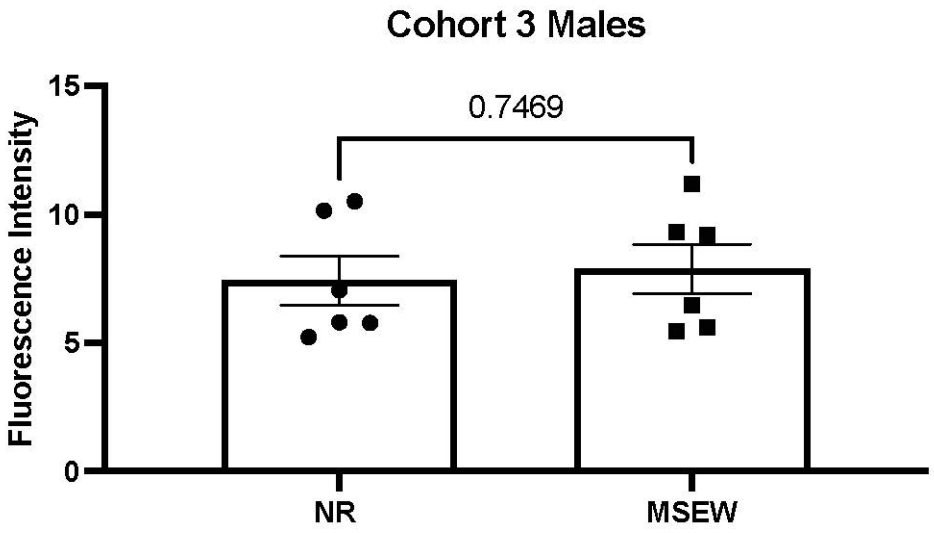


**Figure 10: Cohort 3: Males**: The sections containing the PVN or SFO in 14-week male MSEW (n=6) and NR mice (n=6) were examined. No significance observed. A two-sided student t-test was used.

Since the female mice could not be analyzed alone, I added them to the cohort 3 males where absolutely no significant difference was observed between the NR and MSEW groups. The addition of the females increased the P value from 0.7469 to 0.9898. Unlike the male mice, the female mice show little variability in their groups (MSEW and NR) and trend lower for fluorescence intensity.

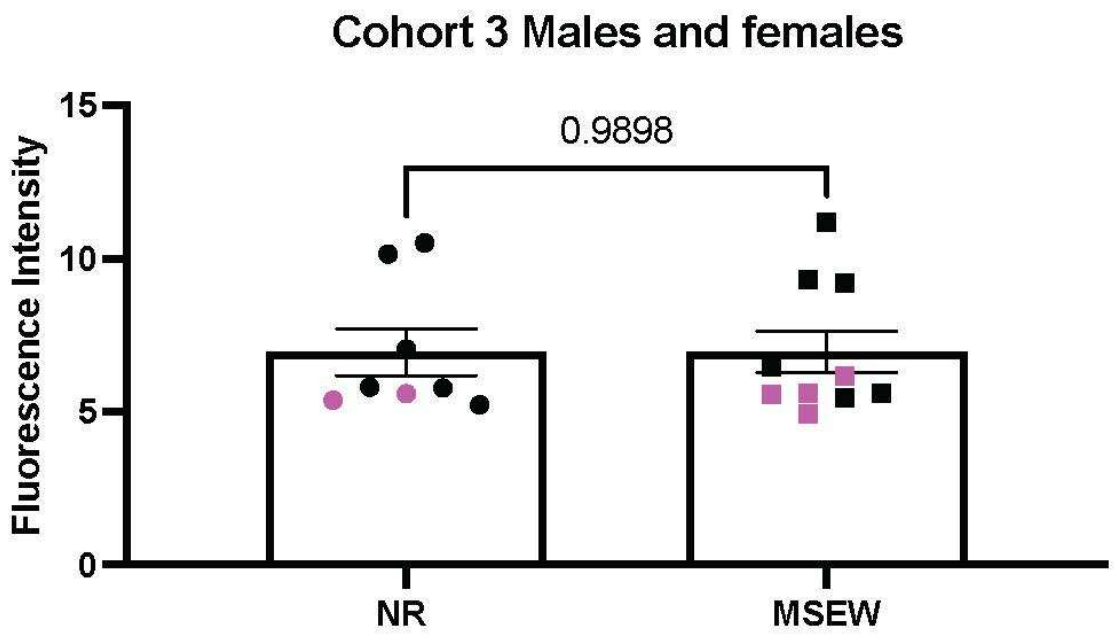


**Figure 11: Cohort 3: Males and Females**: The sections containing the PVN or SFO in 14-week male and female MSEW (n=10) and NR (n=8) mice were examined. No significance observed. A two-sided student t-test was used for analysis.

Replacing the cohort 3 females with the cohort 2 males reduced the P value to 0.3055. Although it is not yet significant, I continue to observe the male phenotype of MSEW mice with the highest fluorescent intensity. However, combining the two male cohorts (MSEW:10, NR: 12) has revealed high variability of fluorescence intensity within the groups.

To examine the effect of early life stress on fluorescence intensity regardless gender, all cohorts and genders were combined and did not reveal any significant findings.

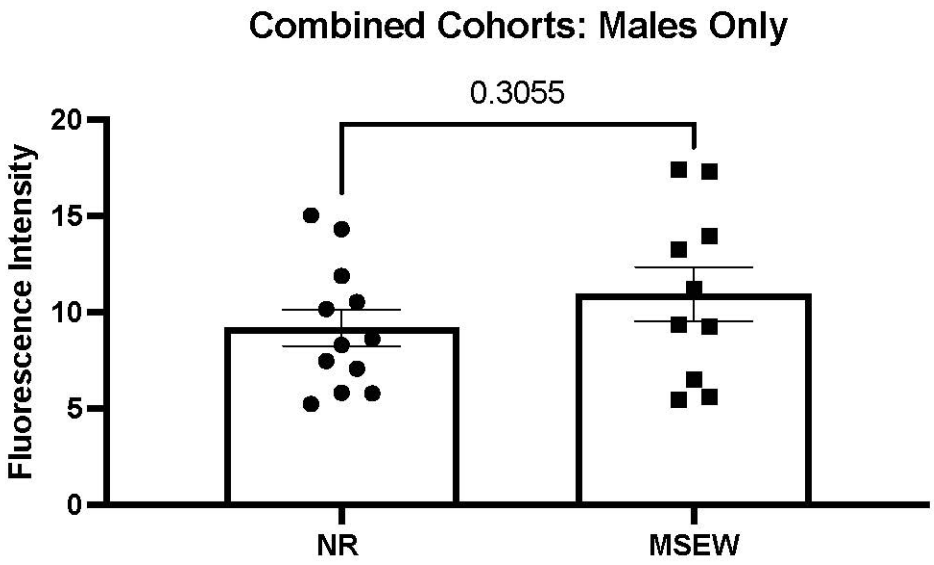


**Figure 12: Cohort 2 and 3: Males**: The sections containing the PVN or SFO in 14-week male MSEW (n=10) and male NR (n=12) mice were examined. No significance observed. A two-sided student t-test was used for analysis.

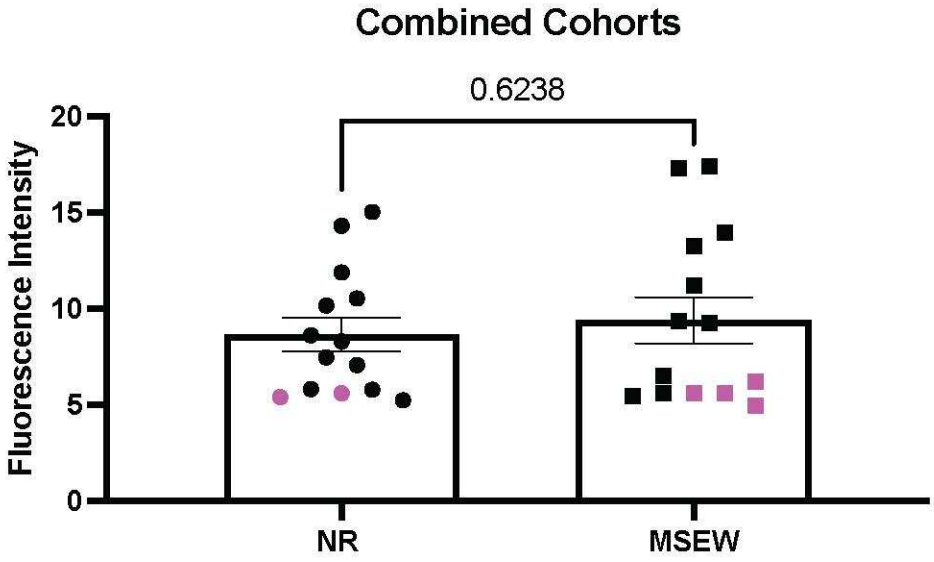


**Figure 13: All cohorts and genders combined**: The sections containing the PVN or SFO in 14-week male and female MSEW (n=14) and NR (n=14) mice were examined. No significance observed. A two-sided student t-test was used for analysis.

## CHAPTER 4 DISCUSSION

After using the Blood-Brain Barrier Permeability protocol on three different cohorts, it seems to be an effective way of measuring vascular permeability in the brain. By using FITC-dextran as a marker for permeability, we can measure fluorescence intensity at the BBB which provides insight on barrier function.

A significant difference between MSEW and NR permeability was only observed in cohort 2 males (n=10) (**Figure 8).** These MSEW had higher fluorescence intensity compared to its NR group. However, we did not see the same results in the cohort 3 male mice (**Figure 9**). The MSEW mouse model is used because it is an effective and consistent model for early life stress. Mice who have undergone the protocol have increased anxiety-like and depressive behavior across multiple litters of mice[19]. I do not believe the differences in permeability between cohort 2 and 3 was a result of the MSEW mouse model because both cohorts were treated the same. However, like any living organism, mice respond to their environment which is why it is important to create a similar experience between cohorts. Both cohort 2 and 3 were transported to the Harms lab on the day of their sacrifice. Transporting the mice was an additional stressor and the mice were not given time to acclimate to their new environment, but this variable was consistent in both cohorts.

Two events happened differently for cohort 3 which could possibly explain the variability in permeability observed. Plasma was collected from cohort 3 at the request

of another scientist, but not from previous cohorts. FITC-dextran was injected and in circulation for 10 minutes before the mice were anesthetized for tissue collection. Normally, the mice are perfused at 10 minutes, but blood from cohort 3 mice was collected before the perfusion. We collected blood using the retro-orbital bleed for the first cage of mice (NR males: n=6) but determined this was not an effective or quick method. For the other mice, blood was collected before perfusion using a cardiac puncture. Not only was the collection of plasma a different factor between cohorts 2 and 3, but the plasma collection changed between the groups from cohort 3. The NR males were the only group in cohort 3 that had plasma collected by the retro-orbital injection which could have influenced their results since FITC-dextran was in circulation longer than 10 minutes. The second event was the placement of the dissected brains in 30% sucrose/PBS into the -20°C freezer instead of the 4°C refrigerator for 36 hours. The purpose of the sucrose is to diffuse into the brain and act like a cryoprotectant to keep ice crystals from forming in the brain during the sectioning method which requires freezing the brain. They had been in the freezer for 36 hours before I realized this mistake. I moved them to the correct refrigerator but had to wait an additional 48 hours for the brains to sink. They still sunk which was a promising sign of the sucrose diffusing into the brain. However, while I was sectioning and mounting, I noticed the brain sections were more brittle compared to previous cohorts. The brittleness caused breaks in the sections and made it harder to mount the sections in one piece. During imaging, the sections had tears which could have influenced the fluorescence detected.

To detect a difference in fluorescence between groups (MSEW, NR) of female mice required a minimum of 3 mice per group. However, I only had 2 NR female

mice(n=2), so I could not compare the female groups. My original hypothesis predicted that ELS would lead to increased permeability in a sex-dependent manner, but I cannot perform statistical analysis on the females therefore it cannot be determined if vascular permeability is sex-dependent. I was expecting to see more permeability in male MSEW mice compared to female MSEW mice since female mice are more resistant to the ELS protocol[21]. When I combined the fluorescent results of the cohort 3 female mice with the cohort 3 male mice **(Figure 10**), there was no difference in permeability detected between the MSEW and NR group. In future experiments, I hope to have a greater number of female mice, so I can perform ANOVA (analysis of variance) analysis. A two-way ANOVA test is a statistical test that would allow us to compare two factors at once which means we could investigate permeability between MSEW and NR while also investigating how sex influences permeability.

The project was meant to establish a protocol for measuring permeability at the BBB and then to use that protocol to determine if ELS promotes barrier dysfunction in a sex-dependent manner. More experiments need to be performed to determine if hyperpermeability occurs in the BBB of mice who have experienced ELS. If increased permeability is detected in mice who experience ELS, then we can begin to examine the cause of the hyperpermeability. Both endothelial dysfunction[2] and chronic inflammation[3; 4] have been observed in ELS and could be possible explanations for the increased vascular permeability of the BBB. To test whether systemic inflammation or endothelial dysfunction leads to hyperpermeability, we could eliminate inflammation by introducing an anti-inflammatory to the mice. If the MSEW mice continue to have increased BBB permeability, then we could hypothesize that endothelial dysfunction is leading to

hyperpermeability which promotes systemic inflammation by allowing molecules and pathogens to cross the BBB. Another possibility for this area of research is to examine the severity of BBB dysfunction by introducing different size FITC-dextran molecules[24; 25].

Additional experiments need to be performed to establish whether MSEW induces increased vascular permeability in the brain and a dysfunctional blood-brain barrier (BBB). By determining the effect of ELS on the vasculature of the brain, it will provide insight into possible physiological mechanisms that contribute to the burden of ELS.

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