SEX- AND AGE-DEPENDENT WESTERN-DIET INDUCED BLOOD-BRAIN BARRIER DYSREGULATION AND RELATIONSHIP TO BEHAVIOR, HYPERGLYCEMIA, BODY WEIGHT, AND MICROGLIA

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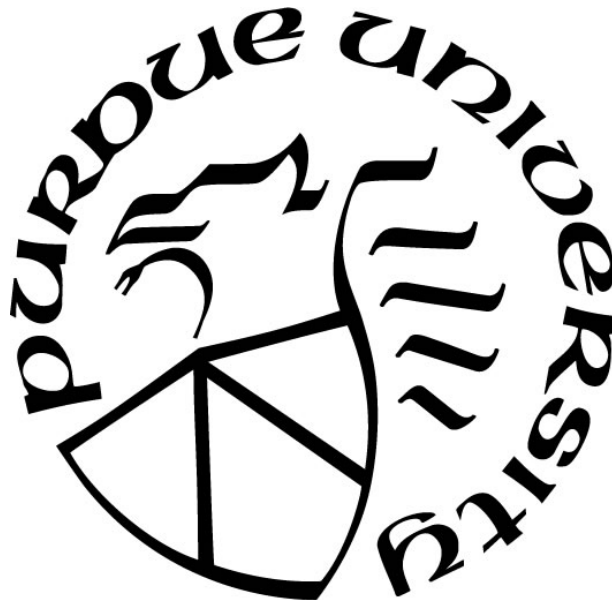
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Este doctorado está dedicado a mi familia y a mi comunidad porque ustedes me enseñaron que 'si se puede.' Gracias por todos sus sacrificios y por su apoyo, porque mis logros son un reflejo de su fortaleza y de lo que somos capaces cuando tenemos oportunidad y apoyo. This dissertation is also dedicated to those that encouraged me to find work-life balance during graduate school and to invest in other aspects of life that bring me joy. I am particularly grateful for my friends and colleagues in the climbing community and All In Alpine family.

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LIST OF ABBREVIATIONS

ANOVA (Analysis of variance)

Blood-brain barrier (BBB)

BW (body weight)

Chow (CH)

Glucose tolerance test (GTT)

Glucose transporter 1 (GLUT1)

Hippocampus (HIP)

Hypothalamic-pituitary-adrenal axis (HPA axis)

Ionized calcium binding adaptor molecule (IBA1)

Medial prefrontal cortex (mPFC)

Ovariectomized (OVX)

PBS (phosphate buffer saline)

PFA (paraformaldehyde)

Prefrontal cortex (PFC)

ROI (region of interest)

SEM (standard error mean)

Western diet (WD)

ABSTRACT

There has been a rapid shift in food environment of Western cultures that has increased consumption of diets high in fat and sugar, which have imparted negative effects on metabolic and neurocognitive health. There is also building evidence that the adverse effects of Western diet (WD) are different in males and females, such that males are impacted more at an earlier age and females are impacted later in life. The underlying biological mechanisms linking WD and neurocognitive health are often associated with energy dysregulation or neuroinflammation. WD disrupts glucose homeostasis and causes low grade inflammation in the body, and these can impact the brain by disrupting the blood-brain barrier (BBB). The BBB is the microvasculature found throughout the entire brain that tightly regulates what compounds get into the brain to ensure optimal neuronal function. WD disrupts the BBB, however, the effects of WD on BBB integrity in females and younger individuals remain largely unknown. Based on the metabolic and behavioral effects of WD, we hypothesized that the effects are age- and sex- specific. To test this, we gave male and female rats access to a WD for 8-10 weeks starting in juvenile period (post-natal day 21) or in adulthood (post-natal day 75), then measured body weight, behavior, glucose tolerance, the density of two different markers of BBB integrity. We also measured density of resident immune cells (microglia) to assess the relationship between inflammation and BBB integrity. First, we focused on the impact of hyperglycemia on the BBB since elevated glucose alters glucose transporter 1 (GLUT1). We found sex- and age- specific decreases in GLUT1 density in the prefrontal cortex and hippocampus—two brain regions commonly associated with neurocognitive impairments associated with WD. Correlational comparisons between WD and chow (CH) animals also found that the typically relationship between glucose tolerance and GLUT1 in the PFC and hippocampus were overall disrupted in WD animals. Second, we measured the leakage of albumin, a blood protein, since WD depletes the tight junctions that would typically prevent albumin from entering the brain and triggering a neuroinflammatory response. We did not find an increase in albumin density in WD animals, however, we found a main effect of age which offers insight to differential susceptibilities to BBB leakage. Third, we focused on inflammation and found that WD did not impact microglia density in our experiments, nor did it correlate with GLUT1, albumin, or behavior. Collectively, our findings support the hypothesis that the impact of WD on the BBB is sex- and age- specific, suggest that WD does not increase leakage of large

compounds such as albumin, and highlights the nuanced relationships between WD, metabolic disruption, behavioral deficits, and neuroinflammation.

BACKGROUND

Western Diet

There has been a relatively rapid shift in food options in Westernized cultures, namely higher availability of refined sugars and processed fats. The consumption of these food options is increasingly observed in children and teenagers as well as adults. Children and adolescents in the United States (ages 2-18) are over consuming hypercaloric, low nutrient foods, typically in the form of grain desserts, pizza, soda, and other high fat and high sugar foods (Reedy & Krebs-Smith, 2010). Given the high consumption of these types of food, the 2020-2025 Dietary Guidelines for Americans recommend children ages 2-18 limit their intake of added sugars and saturated fats to 10% of calories consumed (Jin, 2016). Despite these recommendations, NHANES data from 2013-2016 report that people on average exceed sugar and fat intake limit, and when increased portion sizes are taken into account, it is estimated that people 2-18 years of age overconsume sugar and fat by 64% and 73% respectively (Bowman et al., 2017). Non-adherence to these guidelines is also observed in adults, and the high consumption of ultra-processed foods is associated with poor cardiovascular health (Zhang et al., 2021). These dietary patterns are alarming, given that they contribute to the increased rates of overweight and obesity, which are attributed to the development of diabetes, heart disease, and premature death (Orpana et al., 2010). Also alarming, are the increasing rates of overweight and obesity in children.

As of 2013 approximately a third of people under 20 in the US were overweight or obese, and they are predicted to remain overweight or obese through the remainder of their lives (Simmonds et al., 2016). It is also projected that in 2048, about 58% of today's children will develop obesity by the age of 35—a drastic increase from the current worldwide adulthood average of 33% (Ng et al., 2014; Ward et al., 2017). Collectively, this is creating a major healthcare crisis in the United States, as the more common metabolic determents (such as insulin resistance, atherosclerosis, or non-alcoholic fatty liver disease) are lifelong and irreversible. Fortunately, there are some effective therapeutic treatments to increase life longevity, however high WD consumption is also linked to neurological disorders that also negatively impact quality of life (Bray & Macdiarmid, 2000).

WD, Cognition, and Mental Health During Development

WD is associated with impaired cognitive function and susceptibility to symptoms of affective disorders. For example, metabolic dysregulation and obesity during childhood and adolescence are associated with impaired visuospatial organization and mental ability, and lower scores on working memory, attention, psychomotor efficiency, and mental flexibility (Li et al., 2008; Yau et al., 2014). Also, a longitudinal study evaluating dietary patterns in individuals that develop attention-deficit/hyperactive disorder (ADHD) found an association between WD and ADHD diagnosis (Howard et al., 2011). This is supported by a cross-sectional study in Norway that found hyperactivity, mental distress, and conduct problems among adolescents with high sugar consumption (Lien et al., 2006). Diet composition and quality is also associated with symptoms of depression and anxiety disorders in children and adolescents in measures used to assess mental health (such as the Child Behavior Checklist and Short Mood and Feelings Questionnaire) (Jacka et al., 2010; Khalid et al., 2016; Weng et al., 2012). These adverse effects of WD on cognition and affect are also observed in adults. For example, there are relationships between obesity, hyperglycemia, and high blood pressure, and poor performance on tests of visual organization, memory, concept formation, verbal fluency, and the Wechler adult intelligence scale (Dye et al., 2017; M. Elias et al., 2005; P. Elias et al., 1997). Studies of high fat and high sugar consumption have also found an association between poor diet and impaired cognitive function (Akbaraly et al., 2009; Edwards et al., 2011; Kalmijn et al., 2004; Nabb & Benton, 2006; Papanikolaou et al., 2006). When it comes to mental health and psychiatric disorders, evidence suggests that high consumption of WD increase risk of depression and bipolar disorder diagnosis (Hryhorczuk et al., 2013; Jacka et al., 2011; Sánchez-Villegas et al., 2011, 2012). Further, rates of depression in diabetes patients are twice the rates of nondiabetic patients (odds ratio 2.9) (Anderson et al., 2001). There is also an association between high-anxiety individuals and high sugar consumption (Kose et al., 2021), and anxiety symptoms also correlate with poor eating habits in middle aged adults (Masana et al., 2019).

Sex Differences in Neurocognitive Health

It is also important to consider how WD may be impacting males and females differently. For example, childhood obesity in Western countries is more common in boys than girls (Shah et

al., 2020). ADHD is also more common in boys than girls (Mowlem et al., 2019), although the association between poor diet and anxiety/depression is the opposite (Jacka et al., 2010; Quek et al., 2017). In adulthood, obesity continues to have a strong association with cognitive decline in men and depression in adult women (Elias et al., 2005) but type 2 diabetes impacts cognition in men and women at similar rates (Devore et al., 2009; Elias et al., 2005). Also, the prevalence of depression in diabetes patients is higher in women (28% women, 18% men) (Anderson et al., 2001) and the association between WD and depression and anxiety is greater in women (Masana et al., 2019; Pan et al., 2012). Studies that look at females specifically, have also found strong associations between obesity and poor diet and depression and anxiety (Jacka et al., 2010, 2011; Pan et al., 2012). Collectively, this may suggest that WD is impacting the brain differently in males and females of different ages—where males are generally more susceptible to adverse effects of WD in relation to obesity, the impact on the brain may be different in males and females, and the adverse effects on females may rely on other factors less attributed to body mass index. These potential sex differences may be attributed to gonadal hormones, and the more specific biological mechanisms linking WD to negative consequences on cognition and mental health can be studied using animal models.

Animal Models of WD

There are numerous animal studies using dietary manipulations to model what is observed in humans, and we can use these models to further understand how WD can impact behavior (Bortolin et al., 2018; Hariri & Thibault, 2010; Hintze et al., 2018; Small et al., 2018). The negative consequences of WD on behavior during adolescence have been reviewed recently by Tsan and colleagues (2021) to outline how it can disrupt learning and memory, reward-motivated behavior, and social behavior (Tsan et al., 2021). They conclude that early life WD consumption can set the stage for long-lasting neurocognitive impairments. The effects of WD in adult animals have also been recently reviewed and despite some conflicting reports associated with differences in behavior tasks, the majority of research suggests that WD does impart negative effects on cognitive behavior (Abbott et al., 2019). WD during adulthood also contributes to the development of anxiety of depressive- and anxiety- like behaviors in rodent models (Andre et al., 2014; De Macedo et al., 2016; Dutheil et al., 2015; Sharma & Fulton, 2013; Yu et al., 2021). The development of these models that are analogous to human behavior are crucial to understanding the underlying

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biological mechanisms contributing to the associations made between WD and cognitive and mental health.

It is worth noting that most studies are only done on males. Of 70 papers that were reviewed in Tsan (2021) for behavioral effects of WD in adolescence, only 10 of them included females (Tsan et al., 2021). Of those 10, four assess anxiety-like behavior and two found effects only in males (Kim et al., 2018; Maniam & Morris, 2010) one found effects in males and females (Lalanza et al., 2014), and the fourth paper only assessed females and found differences only when animals were stressed (da Costa Estrela et al., 2015). Five studies assessed learning and memory and of those, three found worse outcomes in males than females (Abbott et al., 2016; Buyukata et al, 2018; Hwang et al., 2010), one found the same effects in both sexes (Underwood & Thompson, 2016), and one only studied females and found differences (Klein et al., 2016). Two studies assessed reward-motivated behavior and found similar effects in males and females (Carlin et al., 2016; Frazier et al., 2008). Of the 16 studies reviewed for effects on cognition in adults, only two evaluated females (Abbott et al., 2019). Those studies, along with others, have found that WD impaired cognitive function in adult females (Dharavath et al., 2019; Molteni et al., 2004; Pratchayasakul et al., 2015). Collectively, this may suggest that males are more susceptible to the adverse behavioral effects of early WD, and that it increases susceptibility in females via mechanisms associated with gonadal hormones.

Studies that focus specifically on understanding sex differences in the metabolic effects of WD (such as obesogenic phenotype, development of diabetes, and hyperlipidemia) have found that there are age- and sex- specific effects of WD, and the patterns are similar to what is observed in the general population (Huang et al., 2020; Krishna et al., 2016). For example, females are more resistant to developing obesity at an earlier age, but this resiliency decreases during adulthood, and in older age, they show worse outcomes than males (Hwang et al., 2010; Kramer et al., 2018; Kruse et al., 2019; Lindqvist et al., 2006; Manrique et al., 2013; Robison et al., 2020; Salinero et al., 2018). These differences are attributed to the role of estradiol, and is supported by evidence from studies that ovariectomized (OVX) females and evaluate metabolic differences between OVX females, intact females, and males (Litwak et al., 2014; Yonezawa et al., 2012). These have found that OVX females have the same susceptibility to WD as males, and that susceptibility decreases in females that have estradiol replacement (Stubbins et al., 2012; Zafirovic et al., 2017). OVX studies have also found that estradiol plays a critical role in regulating food-reward behavior

and regulating food intake, such that decreasing estrogen increases sucrose seeking behavior and disrupts typical eating patterns when compared to females that only undergo sham surgery (Omotola et al., 2019; Richard et al., 2017). WD also more readily induces depressive-like behavior in OVX females than sham or OVX females that have replaced estradiol (Boldarine et al., 2019). This suggests that estrogen may be neuroprotective, and the metabolic and neurological decline patterns observed in females may be due to decreasing ovarian hormones (Bake et al., 2009; Bake & Sohrabji, 2004; Brann et al., 2007; Kuruca et al., 2017; Lewis et al., 2010; Sohrabji, 2007).

The more commonly studied mechanisms underlying the effects of WD on behavior are focused on neuroendocrine systems and the hypothalamus—the brain area that directly senses hormone changes that occur during metabolic stress and modifies behavior accordingly. The hypothalamus communicates with the pituitary and adrenal glands, and these endocrine networks are known as the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis has received a lot of attention because it is a primary method in which hormone signals, such as corticosterone, estrogen, leptin, appetitive hormones communicate with the brain to communicate physiological stress, metabolic stress, and coordinate reproductive development (Makris et al., 2021; Rutters et al., 2012). For example, WD consumption disrupts the hypothalamic-pituitary-adrenal (HPA) axis by altering feedback networks that regulate stress response and modulate homeostasis (Chen & Su, 2013). Studies on sex differences in response to WD have also focused on the HPA axis, given that this is where reproductive hormones can communicate directly with the brain through the hypothalamus, and disrupting reproductive hormones can impact other endocrine and inflammatory signals (Vasconcelos et al., 2016). Research on the neuroendocrine system have provided major insight as to how WD can disrupt communication signaling between the body and the brain, however less is known about how WD is disrupting other brain regions that regulate cognitive and affective behavior such as the prefrontal cortex and the hippocampus (López-Taboada et al., 2020).

Prefrontal Cortex

Neurological and behavioral disorders linked to WD and obesity during development, are associated with brain regions that regulate executive function, cognition, and reward processing such as the prefrontal cortex (PFC) (Francis & Stevenson, 2013). The prefrontal cortex is critical for behavioral control, self-regulation, and reward decision-making, yet does not reach full

maturity until about 20 years of age in humans (Gogtay et al., 2004). Clinical studies involving patients with affective disorders have found metabolic abnormalities in the prefrontal cortex that are associated memory and behavior modulation in patients with mental health disorders (Pennington et al., 2008). Animal studies have found that diet high in fat and sugar during early life disrupts cellular function in the mPFC and contributes to behavioral deficits associated with low sociability, anxiety, and impaired cognition (Kanoski et al., 2007; Labouesse et al., 2017; Yaseen et al. 2019). For example, males with early WD develop neuropathological behavioral phenotypes and are associated with modulation in dendritic spine density, cellular metabolic stress, glial dysfunction that prevents the clearance of senescence cells, and decreases in brain-derived neurotrophic factor (Kanoski et al., 2007; Kruse et al., 2019; Rincel et al, 2018; Yang et al, 2020).

There also seem to be sex-specific differences in reward seeking behavior with early-life access to sucrose-rich diet where females have an exacerbated preference later in life, where males have a dampened preference compared to same-sex control groups (Reichelt et al., 2016). Further, there is evidence that females with access to hypercaloric foods during adulthood also develop disordered neuronal function associated with altered reward decision making and are less likely to modulate their food intake (Sinclair et al., 2019). Maturation of the mPFC is dependent on sex hormones, and there are reports that disrupting androgen and ovarian hormones during development can be detrimental to normal brain development (Markham et al., 2013). Collectively, this suggests that the mPFC plays a crucial role in mediating the behavioral differences associated with WD and the associations between obesity and cognitive and affective regulation (De Macedo et al, 2016; Geiger et al., 2009; Morris et al., 2015; Reichelt, 2016).

Hippocampus

WD is also associated with a broad range of derangements in the hippocampus that are associated with behavioral deficits (Jacka et al., 2015). Like the PFC, the hippocampus does not fully develop until adulthood, making children and adolescents susceptible to the negative hippocampal consequences of poor diet (Gómez & Edgin, 2016). For example, obesity and metabolic syndrome are associated with lower hippocampal volume, and compromised development of white matter, which may be irreversible later in life (Yau et al, 2012, 2014). This is also seen in animal studies that give male rats WD from weaning and observe changes in neurogenesis, glutamatergic neurotransmission, dendritic spine morphology, and decreases in

brain derived neurotropic factor (Boitard et al., 2012; Del Olmo & Ruiz-Gayo, 2018; Kanoski et al., 2007; Lindqvist et al., 2006). These hippocampal changes are particularly observed in the dentate gyrus, the part of the hippocampus that projects to other parts of the hippocampus for memory formation, and the part of the brain where neurogenesis happens (Ferreira et al., 2018).

A study comparing sex differences in response to early WD found that neurological and behavioral deficits were worse in males than in females (Hwang et al., 2010). For example, females are resistant to metabolic changes associated with WD when directly compared to males (Underwood & Thompson, 2016), and the neurological disruptions observed also differ (Hwang et al., 2010; Kim et al., 2018; Maniam & Morris, 2010). This seems to be unique to early life diet exposure, because deleterious effects of WD on the hippocampus are exacerbated in females when diet exposure starts in adulthood (Molteni et al., 2002; Robison et al., 2020). In females, the adverse effects of WD seem to be associated with low estradiol—for example OVX females had disrupted mitochondrial and synaptic function in the hippocampus compared to sham (Pratchayasakul et al., 2015). There are numerous compelling explanations for these differential effects, and among them are hypothesis that neuronal deficits associated with WD are attributed to energy dysregulation and inflammation in the brain, and that sex- and age- differences are due to developmental susceptibility and anti-inflammatory properties of female reproductive hormones. This is paradoxical, as the brain is "immune privileged" and most triggering compounds typically have limited access to the brain (Cazettes et al., 2011; Davalos et al., 2012). One hypothesis is that WD alters the complex neurovascular interface in charge of maintaining this homeostasis and immune privilege, known as the blood-brain barrier (BBB) (Hay et al., 2015; Perry, 2004). This is observed in several brain regions that regulate behavior (Lussint et al., 2012).

Blood-Brain Barrier

The BBB dynamically regulates brain homeostasis and communicates peripheral signaling to the brain and ensures an optimal environment for healthy neuronal function (Carolina et al., 2014). The BBB, or neurovasculature, is a ubiquitous mesh-like network of vessels that provide blood flow to all regions and equate to ~15-20m^2 of endothelial surface area in an adult brain (Abbott et al., 2006). Videos of the complexity this neurovascular network is linked in Todorov (Todorov et al., 2020). This complex network of endothelial cells is supported by astrocytes and pericytes to create a neurovascular unit. End (Andreone et al., 2015) othelial cells are sealed by

tight junction proteins that prevent toxins and plasma proteins from entering the brain (Huber et al., 2001; Knisel & Wilburg, 2000; Lussint et al., 2012). In a healthy state, the BBB effectively regulates the brain's energy and prevents toxins and plasma proteins from entering the central nervous system. These functions become compromised in pathological conditions. In several neurological disorders, there are decreases or morphological changes of these proteins can compromise the tight seal that is essential in regulation and result in increased permeability, or "leakage", of toxins and/or blood compounds into the central nervous system (Cheng et al., 2018; Patel & Frey, 2015; Rosenberg, 2012). BBB dysfunction is a known contributor of microglia dysregulation and could be contributing to the neuroinflammatory and neurological changes observed with WD (Abrahamson & Ikonomovic, 2020; Haruwaka et al., 2019; Rhea et al., 2017; Thurgur & Pinteaux, 2019).

Emerging evidence suggests that metabolic disorders alter the BBB and compromise the brain's immune-protected and homeostatic state and disrupts healthy neuronal function (Van Dyken & Lacoste, 2018). For example, chronic consumption of a WD has shown to disrupt vascular homeostasis, and deplete tight junction proteins, particularly in the hippocampus (Davidson et al., 2013; Freeman & Granholm, 2012; Hagrave et al., 2016). This would increase the leakage of small proteins that typically do not enter the central nervous system, and contribute to a neuroinflammatory response (Davidson et al., 2012; Kanoski et al., 2010; Salameh et al., 2019). There is also evidence that WD disrupts vascular homeostasis by decreasing glucose influx, limiting energy availability, this depriving neurons of a healthy extracellular environment (Barros et al., 2017; Winkler et al., 2015). The BBB may be particularly sensitive if WD induces hyperglycemia (Prasad et al., 2014; Rom et al., 2019). All of these may be contributing to the neuronal dysfunction observed in the PFC and hippocampus of animals consuming WD that demonstrate behavioral deficits.

There is limited work about the effects of WD on the BBB in early life, however there are several studies that have shown that BBB insult can have long-lasting effects. For example, leakage of blood proteins into the central nervous system is seen in longitudinal evidence where women with a higher body mass index have a higher albumin ration in cerebrospinal fluid later in life (Gustafson et al., 2007). This is also observed in animal models where WD cause changes in expression of tight junction proteins (Banks et al., 2006). The weakening of these tight junctions increases diffusion of circulating compounds may be contributing to the neuroinflammatory

response observed after long term consumption of WD (Banks et al, 2006; Davidson et al., 2012, 2013; Hargrave et al., 2016). Both of these deleterious effects of WD on the BBB are associated with behavioral deficits (Davidson et al., 2013; Hargrave et al., 2016). Further, studies manipulating gonadal hormones to understand their neuroprotective properties, have found that OVX females increased BBB leakage—an effect not found in OVX females with estradiol replacement (Bake & Sohragji, 2004; Kuruca et al., 2017). This provided some insight into how females are resistant to neurological insult that are typically observed in males, particularly around adolescence when estradiol is highest than at any other age.

Despite evidence that small molecules can cross the BBB more readily after WD and that this is a potential cause for sex differences observed at different ages, there are several gaps that still need to be addressed. For example, less is known about 1) what happens to the BBB when WD consumption starts early in life, 2) what specific compounds are crossing and how they can potentially alter neuronal function in the PFC and hippocampus, 3) whether vascular energy regulation occurs in the PFC as well as the hippocampus in adults, and whether this also occurs in females. It is crucial to fill these gaps to provide more insight about the different ways WD can disrupt the BBB, and whether this is associated with the sex- and age- specific metabolic and behavioral patterns that are observed.

Glucose Transporter 1 (GLUT1)

The mammalian brain relies heavily on glucose as a primary source of energy, such that it consumes 20% of the circulating glucose supply despite only being 2% of body weight (Mergenthaler et al., 2013). Glucose gets metabolized into ATP, through oxidation and glycolysis, which is crucial for cell maintenance and production of neurotransmission (Ashrafi & Ryan, 2017). GLUT1 is a glucose transporter found in the brain, mostly in endothelial cells that make up neurovasculature that is crucial in providing the brain fuel for optimal neuronal function. Limited GLUT1 may result in limited energy to the brain, which contributes to neuronal dysfunction and is associated with cognitive and affective disorders (Barros et al., 2017; Sullivan et al., 2019).

Glucose transporters can be classified into two subcategories—sodium-glucose co-transporters that actively transport glucose against a concentration gradient, and facilitative transporters that diffuse glucose down a concentration gradient. (Pragallapati & Manyam, 2019). GLUT1 is a facilitative transporter present on cell membranes. It is a hydrophobic protein (54 kDa)

that transports hexose molecules (sugars) such as glucose, galactose, mannose, glucosamine, and ascorbic acid. The crystal structure of GLUT1 was described in 2014 (Deng et al., 2014) and the more commonly recognized structure of GLUT1 consists of a single polypeptide chain with 492 amino acids organized as 12 transmembrane alpha helix fragments. Of the 12 fragments, 8 create a cavity with a glucose binding site that glucose transports through, and the remaining 4 serve as structure support. When no glucose is bound to the cavity, the cavity opening is oriented outward to allow glucose binding, then fragments orient inward to release glucose into the cell. However, there is some disagreement about the structural function of GLUT1 and some groups believe there are two import binding sites and multiple export points (Cao et al., 2021).

GLUT1 is encoded by the gene SLC2A1 and is the most widely distributed transporter. Low GLUT1, such as observed in genetic mutation of SLC2A1, results in pathological neuronal changes and cognitive deficits (De Giorgis et al., 2019). Vascular GLUT1 reduction also exacerbates BBB dysfunction and degeneration in the cortex and the hippocampus [133]. Under normal conditions, GLUT1 increases in response to low circulating glucose, and decreases when glucose is high to compensate and maintain energy homeostasis. This is disrupted in several brain region, including the cortex and hippocampus, with chronic hyperglycemia (Duelli et al., 2000; Pardridge et al, 1990). GLUT1 also decreases with WD consumption in males, and is also linked to hyperglycemia (Hargrave et al., 2015; Jais et al., 2016). Although the majority of this BBB research is done in adults, this is also observed in males particularly when the exposure starts in early life (Cordner et al, 2019).

There are few WD and GLUT1 studies that include females, however, there is some evidence that early life hyperglycemia differentially affects presence of GLUT1 across brain regions. One study found no differences in the cortex of mice that had pharmacologically induced diabetes early in life, and only slight decrease of GLUT1 in the hippocampus (Vorbrodt et al., 2001). These differences between studies in males and females may be due to the sexually dimorphic patterns in GLUT1 development. Where males have low hypothalamic GLUT1 early in life compared to adulthood, females have more abundant GLUT1 expression early on (Kelly et al, 2014). Although this has only been evaluated in the hypothalamus, there is no evidence that this is unique to the hypothalamus and may be occurring in the hippocampus and prefrontal cortex as well. This would suggest that WD stunts the GLUT1 surge that is observed in males, and that high expression in females is protected during these early stages. That protection of glucose

homeostasis seems to decrease with aging, and females become more vulnerable to insult (Ding et al., 2013). Additionally, juvenile females are more likely to be resistant to diet-induced hyperglycemia which supports that BBB protection is systemically modulated (Hwang et al., 2010; Salinero et al., 2018). Hyperglycemia could downregulate GLUT1, cause depletion of brain glucose, deprive neurons of a healthy extracellular environment, and increase cell death—which could also contribute to diet-induced microgliosis (Cornford et al., 1995; Duelli et al., 2000; Jurcovicova, 2014; Pardridge et al., 1990. This could be occurring in a sex- and age- specific manner, and could also help explain the neuroimmune and neurocognitive differences observed in response to WD.

BBB Leakage

WD depletes tight junction proteins that regulate BBB permeability (Freeman & Granholm, 2012; Kanoski et al., 2010). This depletion facilitates the leakage of small compounds into the brain, and may be a causal mechanism for the neuroinflammatory response observed in response to WD. For example, there is increased diffusion of tracers such as Evans Blue and sodium fluorescein in animals that consume WD (Hargrave et al., 2015, 2016). Studies using tracers have increased our understanding of how WD impacts the brain, however, they are exogenous compounds which limits our understanding of what compounds can get into the brain to impart negative effects on neuronal function. Because of this limitation, other studies have assessed the amount of albumin in the brain, and have found that WD can increase the amount of this blood protein (de Aquino et al., 2019). Albumin serves as a proxy for blood proteins that can enter the brain and disrupt behavior. For example, albumin can serve as a proxy for the leakage of fibrinogen, which is a blood clotting protein that elevated in patients with metabolic disorders associated with WD (Imperatore et al., 1998; Lou et al., 1998; Solá et al, 2007). This protein is not found in the brain, unless there is a breach at the BBB (Baeten & Akassoglou, 2011). Leakage of these proteins stimulate an inflammatory response and contribute to the pathology of neurological disorders such as multiple sclerosis, Alzheimer's disease, other disorders associated with neurodegeneration (Petersen et al., 2018). Fibrinogen's leakage into the BBB and ability to stimulate an immune response, may also be contributing to neuroimmune response associated with WD (Cortes-Santeli et al., 2012; Merlini et al, 2019; Ryu & McLarnon, 2009). Fibrinogen, in particular, has recently shown to stimulate microglia-mediated neuron alterations and is a protein that could be

contributing to behavioral deficits (Davalos et al., 2012; Imperatore et al., 1998; Melini et al, 2019). Patients with obesity also have elevated levels of circulating fibrinogen, and this is associated with neuroinflammation (Cazettes et al., 2011). There is limited evidence on how WD affects fibrinogen levels after early life, however, there is evidence that BBB damage and fibrinogen leakage can persist long after insult (Hay et al., 2015).

Also, WD is associated with increased systemic inflammation, which can permeate into the brain more readily with BBB depletion, and increase inflammatory markers in the central nervous system (Anty et al., 2006; Perry, 2004; Roher et al., 2012; Sweat et al., 2008; Wang et al., 2017). Attenuating systemic inflammation seems to decrease inflammation in the central nervous system, and potentially improve neurological function (Jeon et al., 2012; Mulder et al., 2001). Further, OVX studies evaluating the effects of estradiol on systemic and neuro inflammation have found that estradiol plays a protective role, and steroid hormones can modulate the neuroinflammatory response to diet (Ludgero-Correia et al., 2012; Vasconcelos et al., 2016). One hypothesis explaining this relationship is that depletion of tight junctions increases permeability of inflammatory markers into the brain to triggers microglia reactivity (Bhattacharya et al., 2016; Jones & Raison, 2016; Miller & Raison, 2016; Varatharaj & Galea, 2017). Because females would benefit from anti-inflammatory benefits of ovarian hormones, there may be fewer cytokines entering the central nervous system to cause disruption and this would help explain the sex and age differences observed in response to WD.

Neuroinflammation

Neuroinflammation is generally described as an inflammatory response in the central nervous system (DiSabato et al., 2016). The cells in charge of mediating this response are called microglia. They clear cell debris and dead cells (phagocytosis), prune synapses, and play a role in neurogenesis (DiSabato et al., 2016; Gemma & Bachstetter, 2013; Ziv et al., 2006). Their under or over activation can result in dysregulation of neuron maintenance and neurotransmitter signaling which contribute to the neurological changes observed under metabolic stress (Davidson et al., 2013; Dorfman & Thaler, 2015; Janssen et al., 2016; Molteni et al., 2002). Neuroinflammation is also associated with the pathophysiology of cognitive and affective disorders (Bhattacharya et al., 2016; O'Brien et al., 2007; Vancampfort & Stubbs, 2017). Consumption of a WD can also alter neuroinflammatory processes such as altered microglia

morphology in the PFC of adult males and females (Bocarsky et al., 2015; Veniaminova et al., 2020), and abundance of microglia and expression of inflammatory markers in the hippocampus (Beilharz et al., 2016; Gzielo et al., 2017; Pistell et al., 2010). WD consumption during early life and adulthood also alters neuroinflammatory processes such as immune sensitivity in the hippocampus (Boitard et al., 2014; Sobesky et al., 2014). This diet-induced inflammation is associated with impaired cognition, anxiety, and anhedonia in adult (Dutheil et al., 2015; Jeon et al., 2012; Pistell et al., 2010), as well as juveniles and adolescents (Almeida-Suhett et al., 2017; Andre et al., 2014; Wu et al., 2018). The impact of WD on neuroinflammation is reviewed in Spencer et al (2017), and they conclude that it is important to understand how WD contributes to neuroinflammation observed in tandem with negative effects on cognition and emotion in order to prevent or treat comorbid neurological conditions that often surface with metabolic disorders (Spencer et al., 2017).

Sex differences in neuroinflammatory response to WD have also been reported in adolescents and adults (Daly et al., 2020; Kang et al., 2014; Lainez et al., 2018). There is also evidence that estradiol attenuates diet-induced microgliosis, suggesting that neuroinflammatory mechanisms can be one of the underlying factors of the sex differences reported in brain and behavior (Butler et al., 2020). Neuroinflammation in females also tends to get worse with aging, and is associated with the sex differences in cognitive disorders, such as Alzheimer's, that occur more often in women later in life (Brann et al., 2007; Ratnakumar et al., 2019; Teixeira et al., 2019). Despite these associations, it is unclear how the brain can be impacted by WD since it is supposed to be protected from peripheral insult and most pro-inflammatory compounds have limited access to the brain (Liebner et al., 2018; Miller et al, 2013). One hypothesis is that WD can disrupt the blood-brain barrier (BBB)—one of the interfaces between the brain and the body—to cause neurocognitive impairments (Janssen et al., 2016; Kealy et al., 2018; Najjar et al., 2013). A flow chart of our rationale is illustrated in Figure 1.
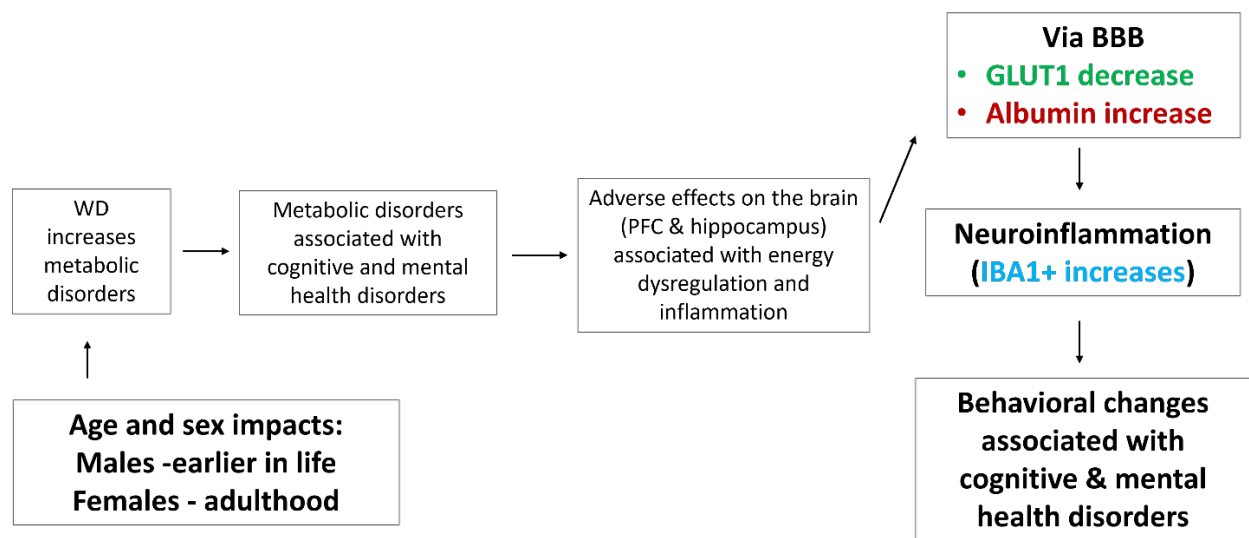
Figure 1. Rationale flowchart. Links between sex- and age- dependent WD-induced metabolic disorders and neurocognitive behavior.

EXPERIMENTS

WD-Induced Changes in Body Weight, Glucose, and Behavior

WD increases body weight, causes hyperglycemia, and disrupts neurotypical behavior in a sex- and age-dependent manner. This is supported by experiments that compare metabolic effects of diet-induced obesity in male and female juvenile and adult rodents (Salinero et al., 2018), as well as epidemiological data suggesting prevalence of childhood obesity is higher in boys than in girls (Shah et al., 2020). In this experiment we aimed to replicate those findings to then assess their relationship with BBB integrity. Our *working hypothesis* was that a diet high in fat and sugar, or Western diet (WD), will increase body weight gain and fasting glucose, impair glucose tolerance, and cause behavioral deficits. To test this hypothesis, we gave male and female rats access to a WD for 8 weeks starting at juvenile period or adulthood in the form of a standardized diet consisting of 45% fat, 35% carbohydrates, and 20% protein (Research Diets: D12451), as well as access to a sucrose bottle with 20% sucrose solution to also model the high consumption of sugar beverages in children and adolescents (Lien et al., 2006). Body weights were compared from the start to the end of the experiment for total body weight gain. We also ran a glucose tolerance test at 8 weeks to measure glucose levels at 15, 30, 60, and 120 minutes after an i.p. glucose challenge. Area under the curve (AUC) was calculated as our measure of hyperglycemia. We used Y Maze testing for spontaneous alternations as a behavioral proxy for neurocognitive impairment and also reported total arm entries during the test.

We expected to see a main effect of age, sex, and diet on body weight, glucose tolerance, and behavior. We specifically predicted greater body weight gain and higher glucose in adults, males, and WD animals. We also predicted that there will be an interaction of age, sex, and diet such that WD males have higher body weight gain and glucose than WD females during juvenile exposure, and WD females will be higher than WD males during adulthood. This would be consistent with literature suggesting males are more susceptible to metabolic effects of early life WD, and that females are more susceptible later in life. We also expected to see changes in behavior, specifically a decrease in spontaneous alternations in WD animals, particularly in males that start WD during juvenile period and in females that start WD during adulthood. This would be indicative of behavioral deficits associated with WD-induced hippocampal disruption.

GLUT1 Downregulation in Male and Female Juveniles and Adults Consuming WD

WD decreases GLUT1 in males, and this decrease in GLUT1 has been attributed to hyperglycemia. This relationship has only been assessed in adult males, despite there being evidence that males are more susceptible to insult earlier in life, and females also develop diet-induced hyperglycemia. Decreased GLUT1 impairs energy regulation in the brain, and may contribute to neuronal detriments and behavioral deficits associated with WD. In these experiments, we aimed to expand on the hypothesized relationship between WD and GLUT1 in brain regions affected by WD. Our *working hypothesis* was that susceptibility to GLUT1 downregulation is higher in males during juvenile period and females in adulthood because of different susceptibility windows to metabolic disruption. To test this, we used immunofluorescence to measure GLUT1 density in the mPFC and hippocampus after 8 weeks of WD starting either in juvenile period or in adulthood. Glucose tolerance and GLUT1 were correlated to assess relationship between hyperglycemia and disrupted energy regulation in the brain.

We predicted to see a main effect of diet, sex, and age. More specifically, we predicted to see lower GLUT1 in the mPFC and hippocampus of WD animals compared to CH. We also expected juvenile WD males to have lower GLUT1 than juvenile WD females, and adult WD females to have lower GLUT1 than adult WD males. This would support our hypothesis that diet induced GLUT1 downregulation is sex- and age- specific, and that the disruption is in areas associated with cognitive and behavioral deficits. We also expected GLUT1 to negatively correlate with glucose AUC in GTT, as this would support the premise that energy dysregulation is associated with hyperglycemia.

BBB Leakage in Male and Female Juveniles and Adults Consuming WD

WD increases the leakage of circulating compounds into the brain. Evidence of BBB leakage suggests a new mechanism of injury that could alter neuronal function and contribute to behavioral deficits associated with WD. Despite this evidence, those experiments have only been done in adult males and less is known about age- or sex- specific effects of WD on BBB leakage. Further, these studies use exogenous dyes that are not typically circulating in the body, so it remains unclear what compounds are entering the brain to contribute to neuronal dysfunction. Here we proposed to measure the presence of albumin as a proxy of BBB leakage. Albumin is a blood

protein that is typically not found in the brain and one that can contribute to neurological dysfunction. We measured albumin in the hippocampus because that is where previous diet studies have assessed BBB leakage, as well as the mPFC given that it is a brain region that may also be impacted during development. Our *working hypothesis* was that BBB leakage in the mPFC and hippocampus will be higher in males with access to WD early in life and females in adulthood because females show fewer adverse neurological and behavioral deficits early in life. To test this, we used immunofluorescence to measure albumin density in the mPFC and hippocampus after 8-10 weeks of WD starting either as juveniles or adults. We predicted a main effect of age, diet, and an interaction between sex, age, and diet. Specifically we expected to see more albumin in the mPFC and hippocampus of WD animals, as well as higher albumin in juvenile WD males than juvenile WD females, and higher albumin in adult WD females than adult WD males. This would support the hypothesis that diet-induced BBB leakage of circulating compounds is sex- and age-specific, and that the disruption is in areas associated with neuronal and behavioral deficits.

Diet-Induced Changes in Microglia

WD alters microglia in males, and BBB dysfunction is a mechanism that could potentially trigger microgliosis. Despite this, little is known about the relationship between the two and less is known about whether these effects are sex- and age- specific. Further, most studies aiming to understand this relationship use methods such as PCR for overall expression and Western blotting for total protein that do not offer the specificity to build strong causal hypotheses between diet-induced BBB dysfunction and microgliosis. In the following experiments, we aimed to understand age- and sex- specific changes in microglia in response to WD, and assess the relationship between microglia, BBB leakage, and GLUT1 dysfunction. Our *working hypothesis* is that WD will alter microglia in a sex- and age- dependent manner since that would be consistent with neurological changes observed by other research groups, and we hypothesize that microgliosis will be correlated with BBB dysfunction. To test this, we quantified IBA1+ density in mPFC and hippocampus and correlated these with albumin and GLUT1. Significant correlations would support the hypothesis that diet-induced BBB dysregulation contributes to diet-induced neuroinflammation.

METHODS

WD Protocol

Diets high in fat and high in simple carbohydrates are commonly used to induce metabolic disruption associated with deleterious effects of high fat high sugar consumption [204]. In these experiments, WD consists of *ad libitum* access to pellet diet consisting of 45% fat, 35% carbohydrates, and 20% protein (Research Diets: D12451) and access to a bottle of 20% sucrose solution in addition to a bottle containing normal water. Animals consuming WD are compared to animals consuming regular chow (CH) diet that only have access to water. An experimental timeline is shown in Figure 2.

Figure 2. Target experimental timeline. Animals were assigned to either CH or WD at weaning (post-natal day 21) in Juvenile group, or after maturity (post-natal day 75) in Adult Group. Animals had *ad libitum* access to their assigned diet for 8 weeks then were behaviorally tested in Y Maze, glucose tested in a glucose tolerance test (GTT), and then sacrificed to measure density of GLUT1, albumin, and IBA1+ in the prefrontal cortex (PFC) and hippocampus.

Experimental Groups

Juvenile Group

Male and female Long Evans rats ($N = 16$ per sex) were bred in the lab, weaned at day 21, singly housed in wire cages (24.5x18x18cm) at controlled temperature (20.5 +/-1 degree Celsius) and humidity (55-65%), and consistent 12hr light/12hr dark cycle. They then were assigned to one of two diet groups. Chow (CH) animals had access to standard chow diet (Teklad 2018) and WD animals had access to previously described WD (CH = 8 per sex, WD = 8 per sex). Animals had *ad libitum* access to their respective diets from post-natal day 21 through the remainder of the

8-week experiment (day 75). In rats, postnatal day 21 is the equivalent to approximately 2 years of age in humans (Kanoski & Davidson, 2011).

Adult Group

Lab-bred male and female Long Evans rats ($N = 16$ per sex) will start diet at day 75 after being weight matched and randomly assigned to CH ($n = 8$ per sex) or WD ($n = 8$ per sex). Animals were singly housed in wire cages (24.5x18x18cm) at controlled temperature (20.5 +/-1 degree Celsius) and humidity (55-65%), and consistent 12hr light/12hr dark cycle. Rat postnatal day 75 is the equivalent of young adulthood in humans (Sengupta, 2013). They remained on their respective diets for 8 weeks (day 135) until behavior testing and glucose tolerance test. They remained on their diet for an additional 10 days than juvenile group due to an unforeseen delay in acquiring drugs used for euthanasia.

Outcome Measures

Body Weight

Body weight was measured three times a week to evaluate effectiveness of WD protocol. We analyzed weekly body weight (g) and total body weight gain (end weight-start weight/start weight *100). Body weight gain for correlations was calculated as percent control for each WD group (WD body weight gain/average body weight gain of respective CH group * 100).

Y-Maze

The Y maze is a behavioral test used to evaluate hippocampal damage in rats. It is a spatial recognition task that has shown to be altered with WD (Labouesse et al., 2017). In this experiment, the Y maze was used as a proxy for behavioral changes reported in rodents consuming WD. Neurotypical animals have "spontaneous alternations" in the test-- that is that they more frequently revisit a new arm of the apparatus rather than the arm they had just been in (Gudapati et al., 2020). In this test, rats were habituated to the testing room for 10 minutes, then placed in the center of a Y maze apparatus with 3 wooden arms (1m long and 20cm wide) radiating 120 degrees apart. Illustration of apparatus is shown in Figure 3. A ceiling mounted camera recorded the rat during

testing for 5 minutes. Then animals were returned to their home cage and apparatus was cleaned between animals. Videos were scored manually to evaluate arm entry patterns. Total number of arm entries were recorded as well as the number of times the animal entered the new arm of the apparatus (spontaneous alternations). We report total number of entries and total spontaneous alternation arm entries as measures of locomotor activity, as well as percent of arm entries that were spontaneous alternations (spontaneous alternation/total arm entries x 100). One animal was excluded from analysis due to nail injury and another was excluded for escaping the apparatus during testing.
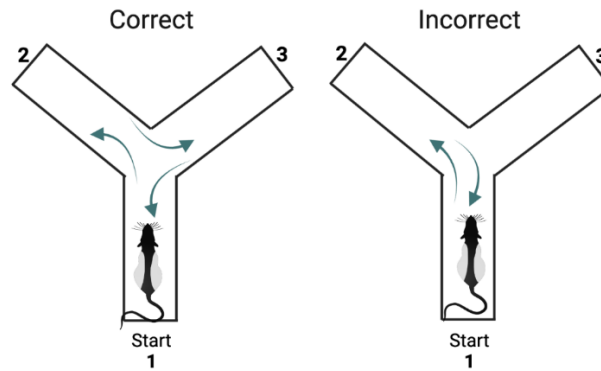
Figure 3. Y Maze behavioral test. Correct spontaneous alternation is illustrated where animals complete a full rotation of 3 novel arms. Incorrect rotation is illustrated where animal returns to arm it was in previously.

Intraperitoneal Glucose Tolerance Test (GTT)

Animals were fasted overnight for 16 hours, then fasting blood glucose was be measured in duplicate using Nova Max glucose meters and via tail prick. After baseline fasting glucose is recorded, animals were i.p injected with 1.5 g dextrose/kg of body weight (dextrose concentration 0.2g/mL). Blood glucose was then measured 15, 30, 45, 60, and 120 minutes after glucose load. Values were plotted across time, and the area under the curve was calculated to determine glucose

tolerance. One animal was excluded from GTT AUC calculations because of a missing value during the 120 minute timepoint.

Immunofluorescent (IF) Staining

Animals were sacrificed via beuthanasia overdose to collect brain for IF staining. We perfused using ice-cold PBS and 4% PFA, then whole brains were extracted and post-fixed in 4% PFA for 24 hours before they are switched to 20% sucrose solution. Tissue was sectioned into 30 micron sections and stored in cryopreservant. Three to five mPFC and hippocampal sections were chosen for staining of ALB, GLUT1, IBA1+, and counterstained with DAPI (Table 1).

Once sections are chosen, they were washed 3 times with 0.1% Triton-X in PBS for 10 minutes, then incubated with 3% Triton-X for 30 minutes. Then we incubated with goat blocking solution for 3 hours, incubated with primary antibodies for albumin (anti-chicken, Abcam: ab106582, 1:500), GLUT1 (anti-mouse IgG2, Abcam: ab40084, 1:500), and IBA1 (anti-rabbit, Wako: 019-19741, 1:500) for 16 hours, washed with 0.1% Triton-X three times for 10 minutes, incubated with secondary antibodies for 2 hours, washed again three times for 10 minutes in 0.1% Triton-X before mounting. We cover slipped using Invitrogen Prolong Gold Antifade mounting medium then sealed the cover slip with clear nail polish. Tissue was processed and imaged as 8 sets of 8 with n=1 per group to counterbalance any variance during staining and imaging.

Table 1. Immunofluorescence Markers

Marker	Antibody	Manufacturer	Host	Dilution	Block	Secondary
BBB leakage	Albumin	Abcam: ab106582	Chicken	1:500	Goat	647
Glucose Transport	GLUT1	Abcam: ab40084	Mouse IgG2	1:500	Goat	488
Microglia	IBA1+	Wako: 019-19741	Rabbitt	1:500	Goat	405

IF Microscopy

Images were acquired at 20x using a Leica THUNDER Imager 3D Tissue using a Leica K5 microscope camera. During imaging, regions of interest were selected using slide scan features and 2D focus was adjusted per region to acquire single images on 405, 488, 568, and 647 channels. Microscope settings were the same across all images, and THUNDER instant computational

clearing was used for instant removal of background. Cleared images were then exported as .lif files then quantified using FIJI (FIJI is Just Image J, 1.53p). Representative immunofluorescence images of GLUT1, albumin, IBA1+, and merged channels of the mPFC are shown in Figure 4A-D, and hippocampus in Figures 5A-D.

IF Quantification

IF images were quantified using FIJI macros. Lif files were imported in Bio-Formats as Hyperstacks for all 4 channels. Regions of interest were outlined and images were split into 4 channels converted to .tif files. Thresholding and densitometry was performed for each channel using batch macros. Masks of all quantified fluorescence were saved and reviewed for consistent thresholding. Total section area was quantified by saturating GLUT1. Macros codes and image thresholds can be found in Table 2. Immunofluorescence density was determined in mm^2 by calculated by dividing total staining area by section area. Representative images of GLUT1, albumin, IBA1+, and quantified area masks of the mPFC are shown in Figure 6A-C, and hippocampus in Figures 7A-C.

Statistical Analysis

Three-way ANOVA was used to analyze main effects of diet (levels: WD and CH), sex (levels: male and female), and age group (levels: juvenile and adult) using R Studio (version 4.1.2). Pre-planned comparisons of each WD group to their respective CH group were carried out using Fishers LSD test. Further post-hoc tests were corrected in Tukey HSD test. Figures report individual values, mean ± SEM, main effects, and significant interactions. (a) denotes effect of diet, (b) denotes effect of sex, and (c) defects of age group. Significance will be set at $p < 0.05$.
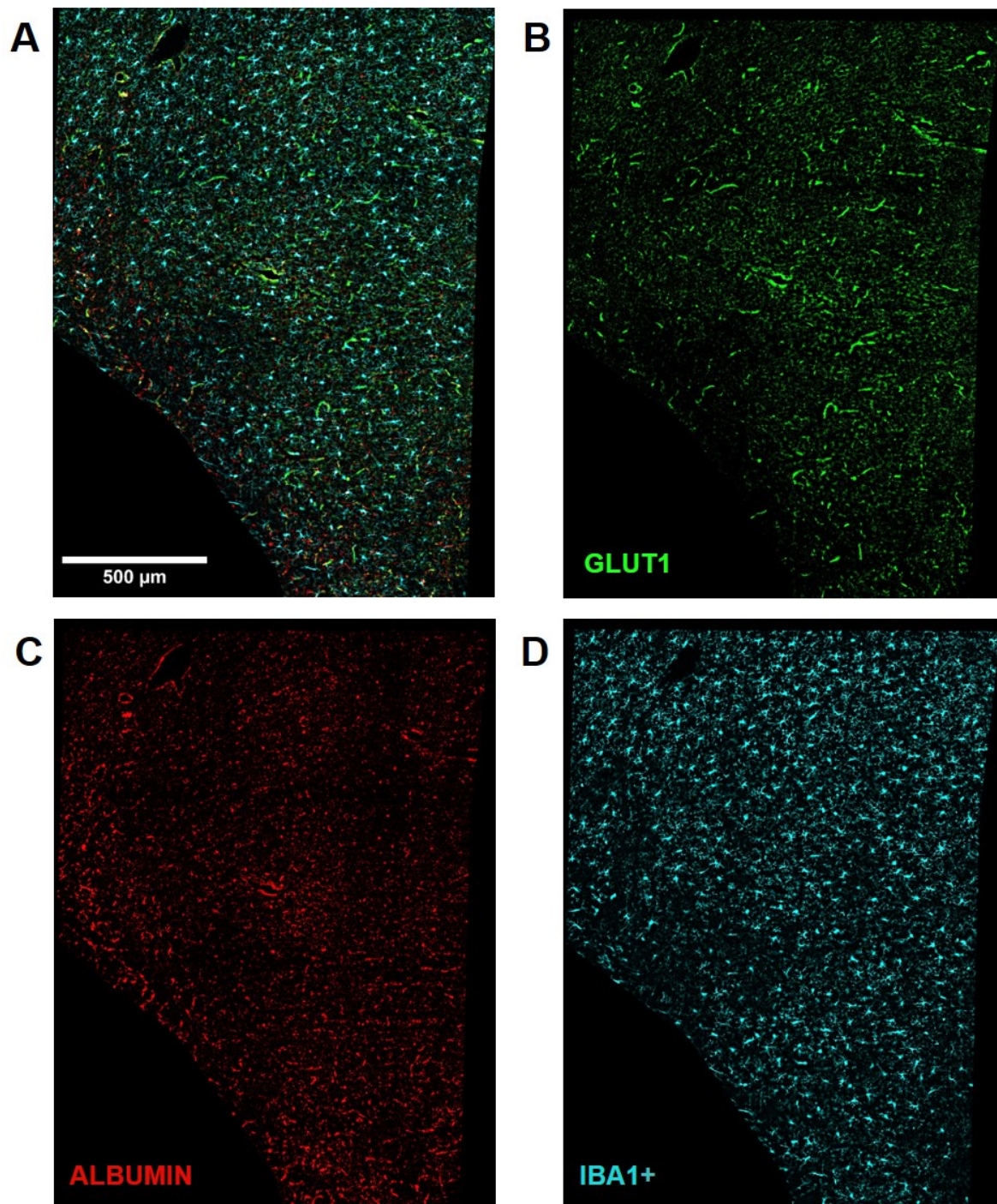
Figure 4. Representative immunofluorescence PFC images. (A) Merged GLUT1, ALB, IBA1+
(B) GLUT1 (C) Albumin (D) IBA1+.
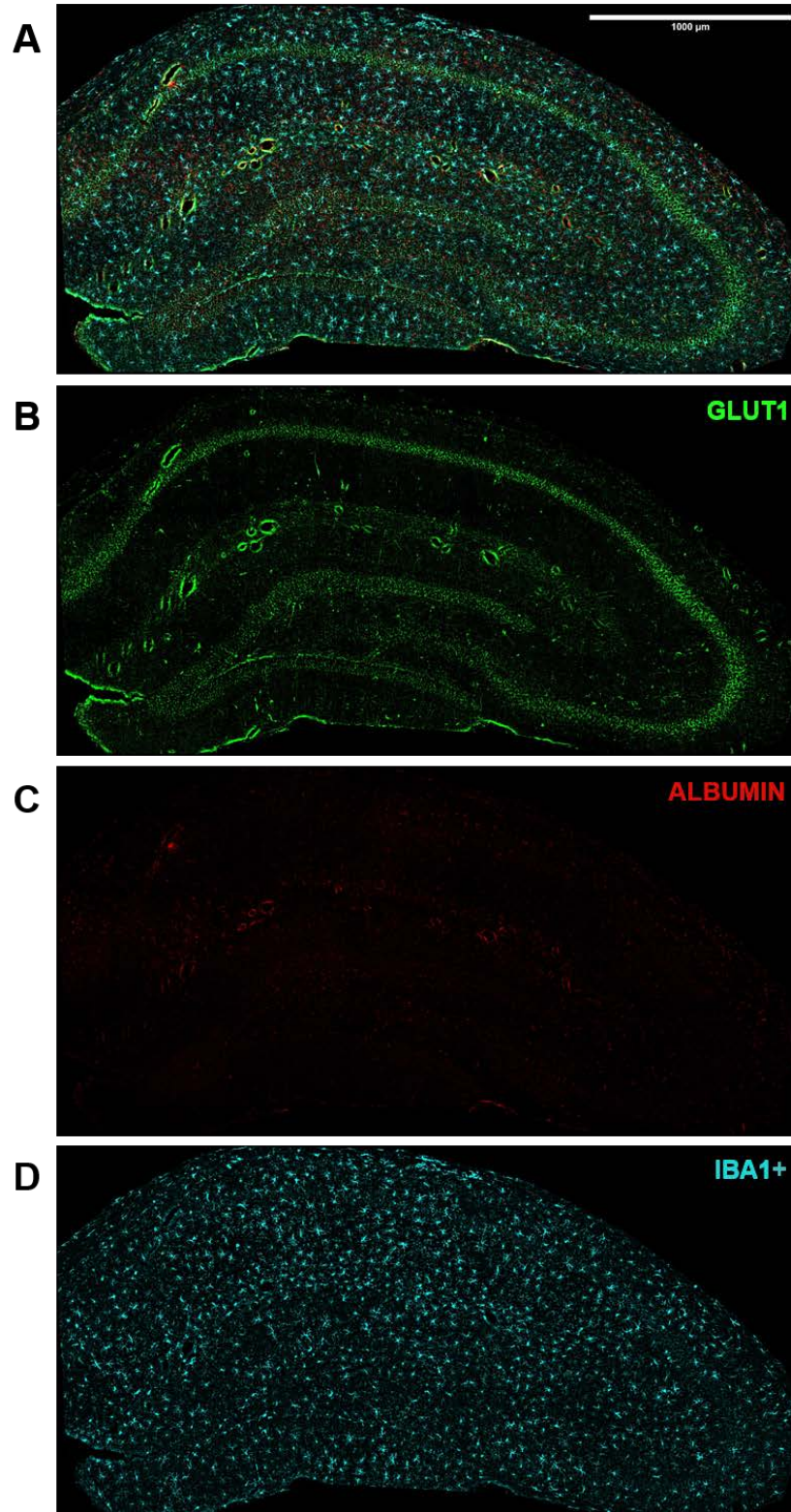
Figure 5. Representative immunofluorescence hippocampal images. (A) Merged GLUT1, ALB, IBA1+ (B) GLUT1 (C) Albumin (D) IBA1+.

Table 2. FIJI Macros Codes

Purpose	Code
Convert .lif files to .tiff and split 4 channels after outlining region of interest (run macros image by image) Output: folder for split channel images	run("Clear Outside", "stack"); resetMinAndMax(); run ("Crop") output=getDirectory ("Save files") title= getTitle() saveAs ("tiff", output + getTitle() + "ALL") run("Split Channels"); saveAs ("tiff", output + getTitle() + "DAPI") close() saveAs ("tiff", output + getTitle() + "ALB") close() saveAs ("tiff", output + getTitle() + "IBA1") close() saveAs ("tiff", output + getTitle() + "GLUT1") close()
Measuring area of section (batch) Input: folder with GLUT1 images Output: folder to save AREA mask images	run("Maximum...","radius=15"); setThreshold(160, 65535); run("Convert to Mask"); run("Analyze Particles...", " show=Masks display summarize");
Measuring albumin (batch) Input: folder with ALB images Output: folder to save ALB mask images	run("Auto Crop"); idOrig = getImageID( ); run("32-bit"); run("Duplicate..." + "idOrig + dup") idDuplicate = getImageID(); run("Gaussian Blur...", "sigma=3"); selectImage(idOrig); run("Gaussian Blur...", "sigma=10"); imageCalculator("Subtract create", idDuplicate, idOrig); selectImage(idDuplicate) close(); selectImage(idOrig) close(); run("Scale to Fit");

Table 2 continued

Purpose	Code

	```//run("Threshold...");```
	```setAutoThreshold("Triangle dark");```
	```setOption("BlackBackground", false);```
	```run("Convert to Mask");```
	```run("Analyze Particles...", "size=0-Infinity show=Masks summarize");```

Measuring IBA1
(batch)
Input: folder with IBA1 images
Output: folder to save IBA mask images
	```run("Auto Crop");```
	```idOrig = getImageID( );```
	```run("32-bit");```
	```run("Duplicate..." + "idOrig + dup")```
	```idDuplicate = getImageID();```
	```run("Gaussian Blur...", "sigma=3");```
	```selectImage(idOrig);```
	```run("Gaussian Blur...", "sigma=100");```
	```imageCalculator("Subtract create", idDuplicate, idOrig);```
	```selectImage(idDuplicate) close();```
	```selectImage(idOrig) close();```
	```run("Scale to Fit");```
	```//run("Threshold...");```
	```setAutoThreshold("Triangle dark");```
	```setOption("BlackBackground", false);```
	```run("Convert to Mask");```
	```run("Analyze Particles...", "size=0-Infinity show=Masks summarize");```

GLUT1
(batch)
Input: folder with GLUT1 images
Output: folder for GLUT1 mask images
	```run("Auto Crop");```
	```idOrig = getImageID( );```
	```run("32-bit");```
	```run("Duplicate..." "idOrig + dup"))```
	```idDuplicate = getImageID();```
	```run("Gaussian Blur...", "sigma=3");```
	```selectImage(idOrig);```

Table 2 continued

Purpose	Code
	run("Gaussian Blur...", "sigma=20");
	imageCalculator("Subtract create", idDuplicate, idOrig);
	selectImage(idDuplicate) close();
	selectImage(idOrig) close();
	run("Scale to Fit");
	//run("Threshold...");
	setAutoThreshold("Triangle dark");
	setOption("BlackBackground", false);
	run("Convert to Mask");
	run("Analyze Particles...", "size=0-Infinity show=Masks summarize");
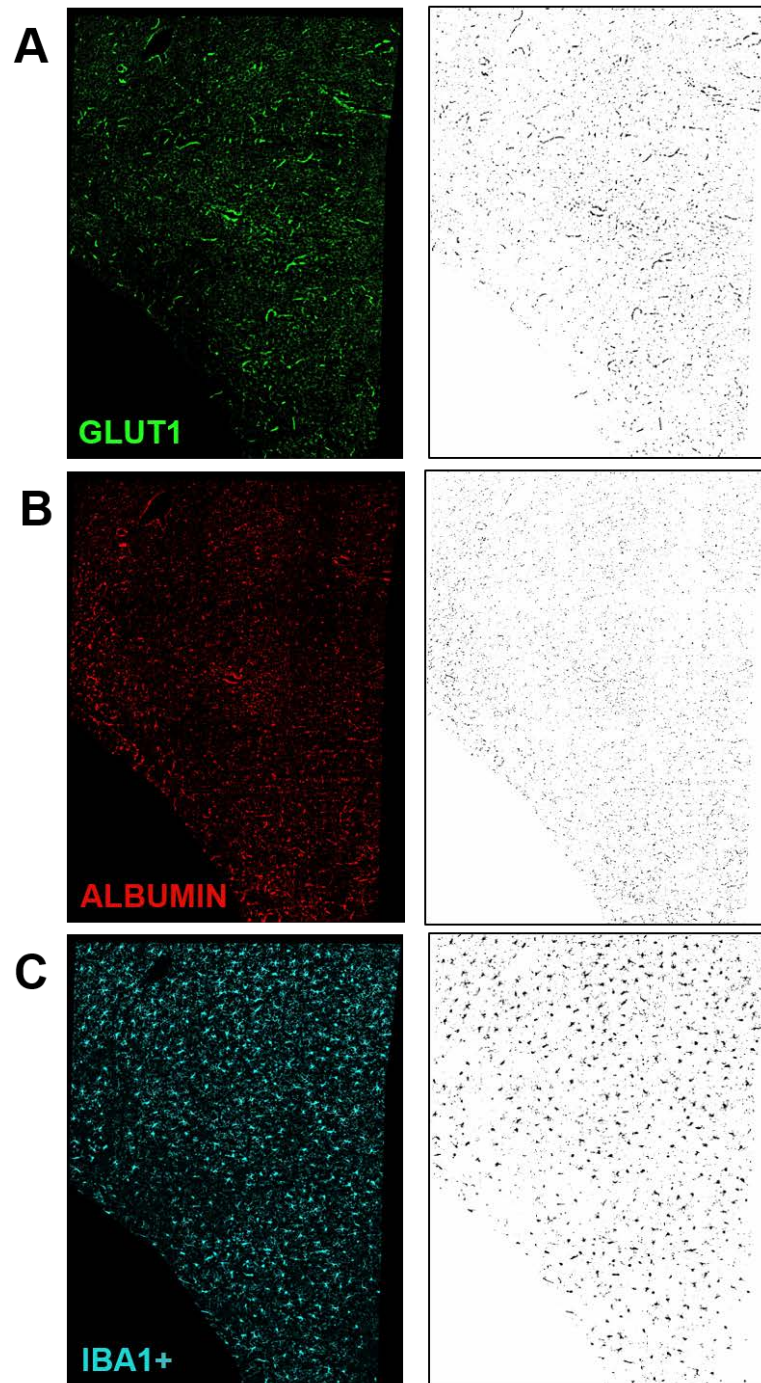
Figure 6. Representative immunofluorescence PFC image quantification. (A) GLUT1 (B) Albumin (C) IBA1+.
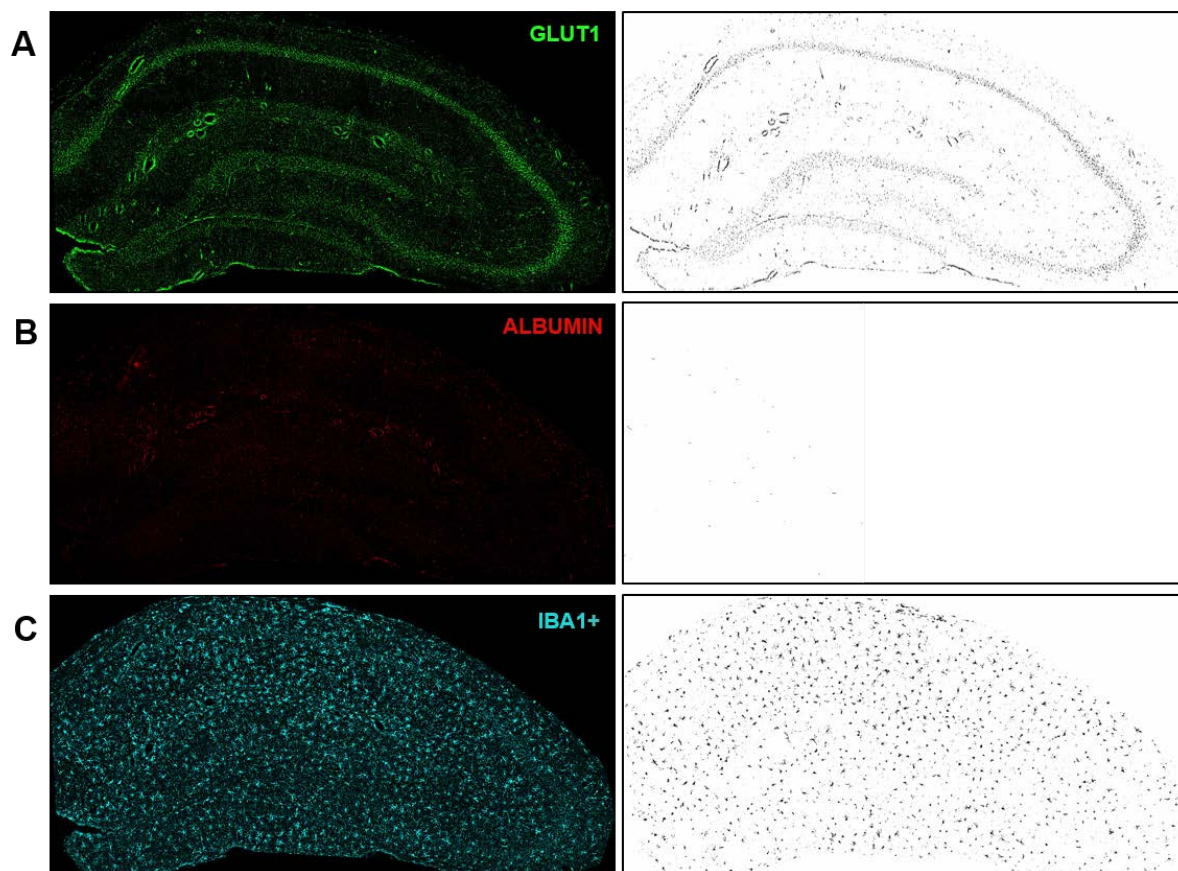
Figure 7. Representative hippocampal image quantification. (A) GLUT1 (B) Albumin (C) IBA1+.

RESULTS

Body Weight

WD increased body weight in juvenile and adult male and female groups compared to CH ($F_{1,56} = 55.504$, $p < 0.0001$). We also found a main effect of age ($F_{1,56} = 693.599$, $p < 0.0001$) where animals that started diet as juveniles gained more body weight than those that started as adults and a main effect of sex ($F_{1,56} = 397.011$, $p < 0.0001$). There was an interaction between age and sex ($F_{1,56} = 33.689$, $p < 0.0001$) where body weight gain was contingent upon age and sex. In our planned comparisons of each WD group to CH, we found that juvenile males gained more weight than juvenile CH ($p < 0.0001$), juvenile females gained more than juvenile CH ($p = 0.02$), adult male WD gained more body weight than adult male CH ($p < 0.0001$), and adult WD females gained more than adult CH females ($p = 0.0003$). Body weight and body weight change are illustrated in Figure 8.
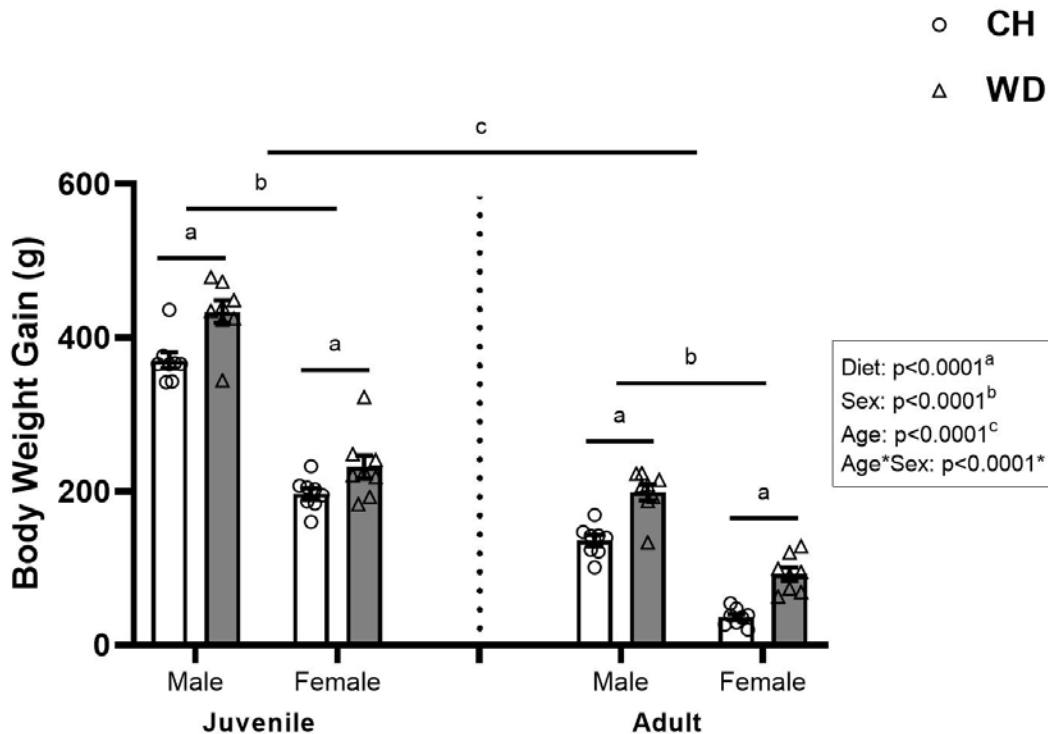
Figure 8. Body weight after 8-9 weeks on Western diet. (A) Body weights in juvenile and adults from start of diet exposure to end. (B) Mean ± SEM and individual values are shown.

Y Maze

Total Arm Entries

WD did not significantly impact total arm entries in Y maze apparatus compared to CH ($F_{1,54} = 2.71$, $p = 0.105$). There was also no effect of sex ($F = 0.013$, $p = 0.91$), however, we found a main effect of age ($F_{1,54} = 9.183$, $p = 0.004$) such that juvenile group had more entries than adult group. There were no significant interactions. There were no significant differences in post-hoc analyses. Results are illustrated in Figure 9A.

Percent Spontaneous Alternations

WD did not significantly impact behavior in spontaneous alternations test ($F = 0.2$, $p = 0.656$). We also found no effect of sex ($F = 0.579$, $p = 0.45$) or age ($F = 0.007$, $p = 0.933$) and no interactions. There were also no significant differences in our planned comparisons between individual WD groups compared to CH. Juvenile WD males were not different than juvenile CH males ($p = 0.714$), adult WD males were not different than adult CH males ($p = 0.847$), juvenile WD females were not different than juvenile CH females ($p = 0.436$), and adult WD females were not different than adult CH females ($p = 0.65$). Results are illustrated in Figure 9B.
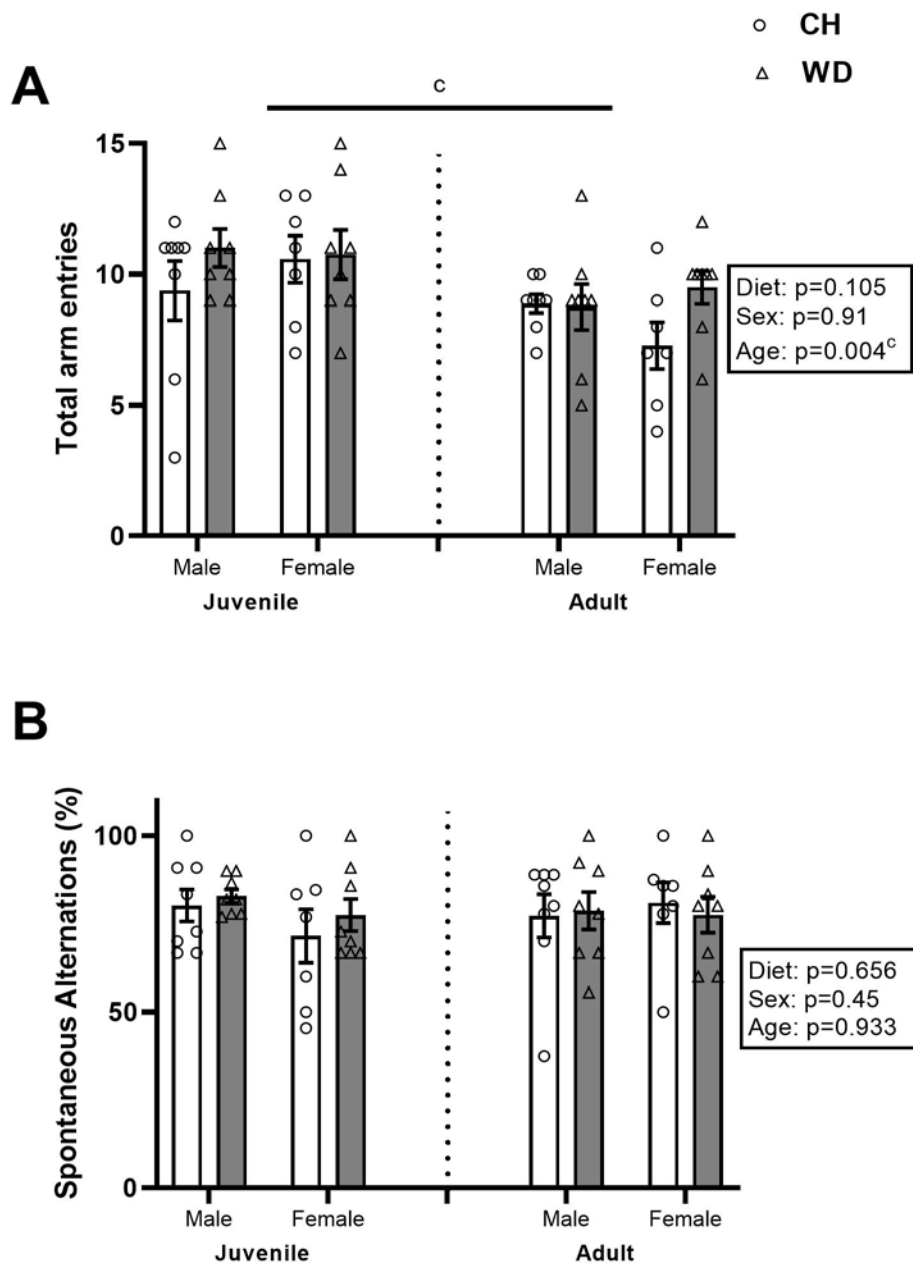
Figure 9. Y Maze behavior after 8 weeks on Western diet. (A) There was a main effect of age in total arm entries during behavior test, such that animals in the juvenile group had more arm entries than the adult group. (B) There were no effects of WD, age, or sex on percent of spontaneous alternations in Y Maze test. a: $p < 0.05$. Mean $\pm$ SEM and individual values are shown.

Hyperglycemia

Fasting Glucose

WD increased fasting glucose compared to CH ($F_{1,55} = 18.216$, $p < 0.0001$). We also found a main effect of sex ($F = 38.91$, $p < 0.0001$), such that males had overall higher fasting glucose than females, and a main effect of age ($F_{1,55} = 9.268$, $p = 0.004$) such that animals in the juvenile group had overall higher fasting glucose than adults. In our planned comparisons of each WD group to CH, we found that juvenile males had higher fasting glucose than juvenile CH ($p = 0.002$) as did adult male WD compared to adult CH males ($p = 0.003$). Juvenile females did not have higher fasting glucose compared to CH ($p = 0.782$), and adult WD females did not have higher glucose than adult CH females ($p = 0.057$). Fasting glucose is illustrated in Figure 10A.

Glucose Tolerance Test

WD altered area under the curve (AUC) in glucose tolerance test compared to CH ($F_{1,55} = 29.935$, $p < 0.0001$). There was no effect of age ($F_{1,55} = 0.102$, $p = 0.7512$), however there was a main effects of sex ($F_{1,53} = 27.402$, $p < 0.0001$) where males had higher glucose during GTT than females. And there was an interaction between age and sex ($F_{1,55} = 4.072$, $p = 0.0485$) suggesting that response to glucose tolerance test was age- and sex- dependent. In our planned comparisons of each WD group to CH, we found that juvenile males had higher glucose response than juvenile CH ($p = 0.0005$) as did adult male WD compared to adult CH males ($p = 0.0004$). Juvenile females did not have higher fasting glucose compared to CH ($p = 0.1439$), however adult female WD have higher glucose than adult CH females ($p = 0.0474$). Glucose tolerance curves and AUC are illustrated in Figures 10B and 10C.

A Fasting Glucose

○ CH
△ WD

Diet: p=0.0001[a]
Sex: p<0.0001[b]
Age: p= 0.004[c]

B Juvenile / Adult

-○- CH Male
-△- WD Male
-●- CH Female
-▲- WD Female

C Glucose Tolerance

Diet: p<0.0001[a]
Sex: p<0.0001[b]
Age: p= 0.751
Age*Sex: p=0.049*
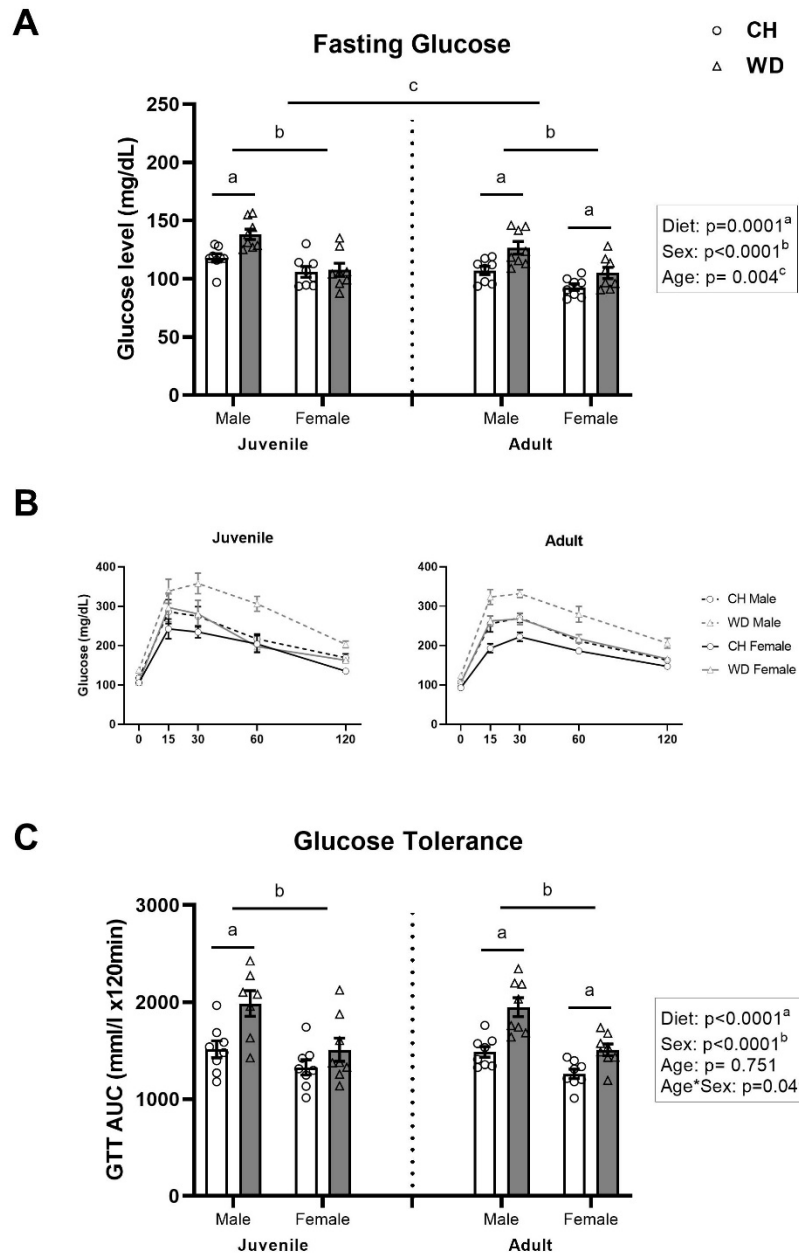
Figure 10. Glucose after 8 weeks on Western diet. (A) Fasting glucose was greater in juveniles than adults (c), males than females (b), and in juvenile and adult WD males and adult females compared to their respective CH groups (a). (B) Glucose response during glucose tolerance test in juvenile and adult groups. (C) Area under the curve during GTT. Glucose response was greater in males than females, and some WD groups were greater than their respective CH group. a, b, c: $p < 0.05$. Mean ± SEM and individual values are shown.

GLUT1 Results

GLUT1 Immunofluorescence PFC

WD decreased GLUT1 density in the PFC compared to CH ($F_{1,56}$= 4.086, p= 0.048). There was also a main effects of age ($F_{1,56} = 31.059$, $p < 0.0001$) such that animals in the juvenile group had lower GLUT1 than those in the adult group. There was no main effect of sex ($F_{1,56} = 1.581$, $p = 0.214$) or any interactions. Our planned comparisons show that juvenile WD males did not differ from juvenile CH males ($p = 0.253$) and adult WD males did not differ from CH males ($p = 0.799$). Juvenile WD females also did not differ from juvenile CH females ($p = 0.276$), however adult WD females did have lower GLUT1 than adult CH females ($p = 0.0458$). Results are illustrated in Figure 11A.

GLUT1 Immunofluorescence Hippocampus

WD did not overall decrease GLUT1 density in the hippocampus compared to CH ($F_{1,56} = 0.791$, $p = 0.3775$). There were also no main effects of age ($F_{1,56}$= 0.016, p= 0.8994) or sex ($F_{1,56} = 0.011$, $p = 0.9159$). However, there was a significant interaction between age, sex, and diet ($F_{1,56} = 4.809$, $p = 0.0325$) suggesting diet, age, and sex specific differences in GLUT1 density. Planned comparisons showed no specific differences between WD and respective CH groups. Juvenile WD males were not different than juvenile CH males ($p = 0.187$), adult WD males were not different than adult CH males ($p = 0.446$), juvenile WD females were not different than juvenile CH females ($p = 0.594$), and adult WD females were not different than adult CH females ($p = 0.086$). There were also no significant differences in post-hoc comparisons. Results are illustrated in Figure 11B.

A Prefrontal Cortex
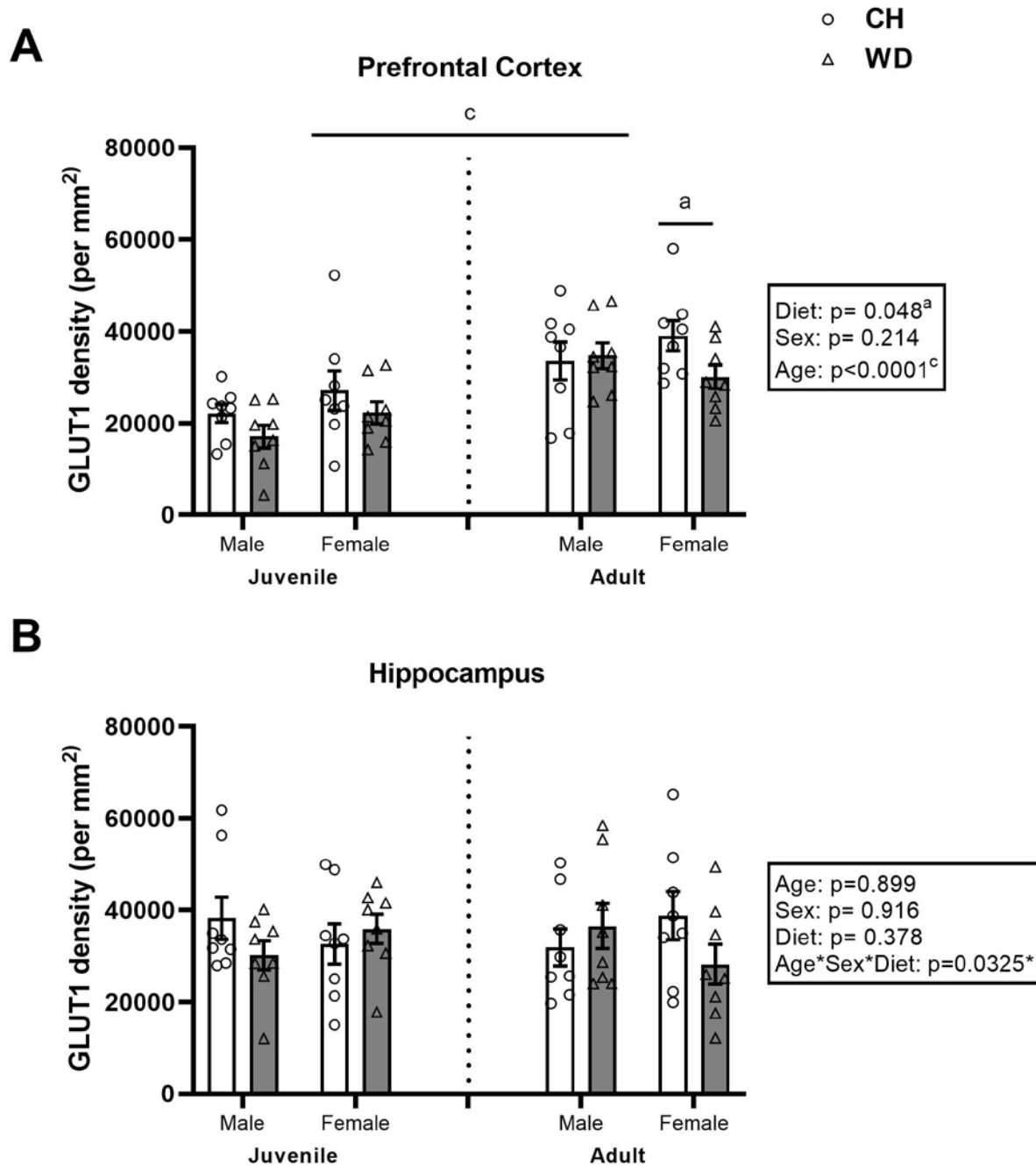
B Hippocampus

Figure 11. GLUT1 Immunofluorescence. (A) Adult group had higher GLUT1 density in the PFC than juvenile group (c). WD animals had lower GLUT1 density than CH, particularly in adult females (a). (B) Decrease of hippocampal GLUT1 density was contingent upon age, sex, and diet.(*). a, c, *: $p < 0.05$. Mean ± SEM and individual values are shown.

GLUT1 Density, GTT, and Behavior

We correlated average GLUT1 density to GTT AUC to evaluate the relationship between hyperglycemia and GLUT1 dysregulation in the brain. We found that GLUT1 was negatively correlated with GTT AUC in CH animals (Pearson's r = -0.36, p = 0.023) but not in WD animals (Pearson's r = 0.071, p = 0.35). Correlations illustrated in Figure 12.
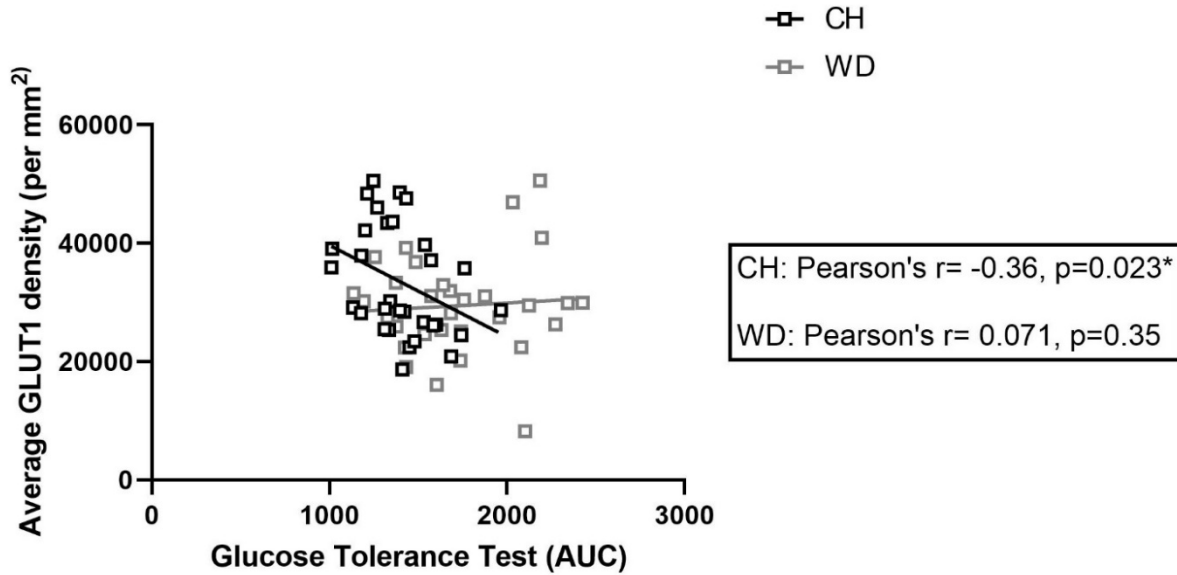
Figure 12. Correlation of GLUT1 and Glucose Tolerance. Average GLUT1 was negatively correlated with GTT response in CH consuming groups. No significant correlation was observed in animals on WD for 8 weeks. Pearson's r and p values shown. *p < 0.05.

We correlated total arm entries in Y Maze to PFC GLUT1 density to evaluate the relationship between locomotor activity and PFC GLUT1 dysregulation. We found that total arm entries were not correlated with GLUT1 density in CH (Pearson's r = -0.177, p = 0.17), or WD animals (Pearson's r = -0.029, p = 0.438). Correlations illustrated in Figure 13A.

We also correlated percent of spontaneous alternations to hippocampal GLUT1 to evaluate relationship between hippocampal-dependent behavioral deficits and hippocampal GLUT1 dysregulation. We found that percent of spontaneous alternations was not correlated with hippocampal GLUT1 density in CH animals (Pearson's r = -0.108, p = 0.285), however it was correlated in WD animals (Pearson's r = 0.418, p = 0.018). Correlations illustrated in Figure 13B.

Figure 13. Correlation of Y Maze behaviors and GLUT1. (A) No significant correlations between Y Maze arm entries and GLUT1 in the PFC. (B). Percent of spontaneous alternations positively correlated with GLUT1 in WD animals and not CH. *$p < 0.05$. Pearson's r and p values shown.

Albumin Immunofluorescence

WD did not increase albumin in the PFC compared to CH ($F_{1,56}$ = 1.257, p = 0.2669). However, we found a main effect of age ($F_{1,56}$ = 4.918, p = 0.0307), such that the juvenile group had higher albumin than the adult group. We found no effect of sex ($F_{1,56}$ = 0.169, p = 0.6822) and no interactions. Planned comparisons showed no specific differences between WD and respective CH groups. Juvenile WD males were not different than juvenile CH males (p = 0.449), adult WD males were not different than adult CH males (p = 0.449), juvenile WD females were not different than juvenile CH females (p = 0.549), and adult WD females were not different than adult CH females (p = 0.486). There were also no significant differences in post-hoc comparisons. Results are illustrated in Figure 14A.

WD did not increase albumin in the hippocampus compared to CH ($F_{1,56}$ = 1.396, p = 0.2425). We also found no effects of age ($F_{1,56}$ = 3.248, p = 0.0769), or sex ($F_{1,56}$ = 2.705, p = 0.1056) and no interactions. Planned comparisons showed no specific differences between WD and respective CH groups. Juvenile WD males were not different than juvenile CH males (p = 0.115), adult WD males were not different than adult CH males (p = 0.901), juvenile WD females were not different than juvenile CH females (p = 0.734), and adult WD females were not different than adult CH females (p = 0.332). There were also no significant differences in post-hoc comparisons. Results are illustrated in Figure 14B.

Albumin Density, Body Weight, and Behavior

We correlated average albumin density to body weight change (%CTL) to evaluate the relationship between diet-induced obesity and BBB leakage in the brain. We found brain albumin was not correlated with differences in body weight in CH (Pearson's r = 0.01, p = 0.477) or in WD animals (Pearson's r = -0.161, p = 0.188). Correlations illustrated in Figure 15.
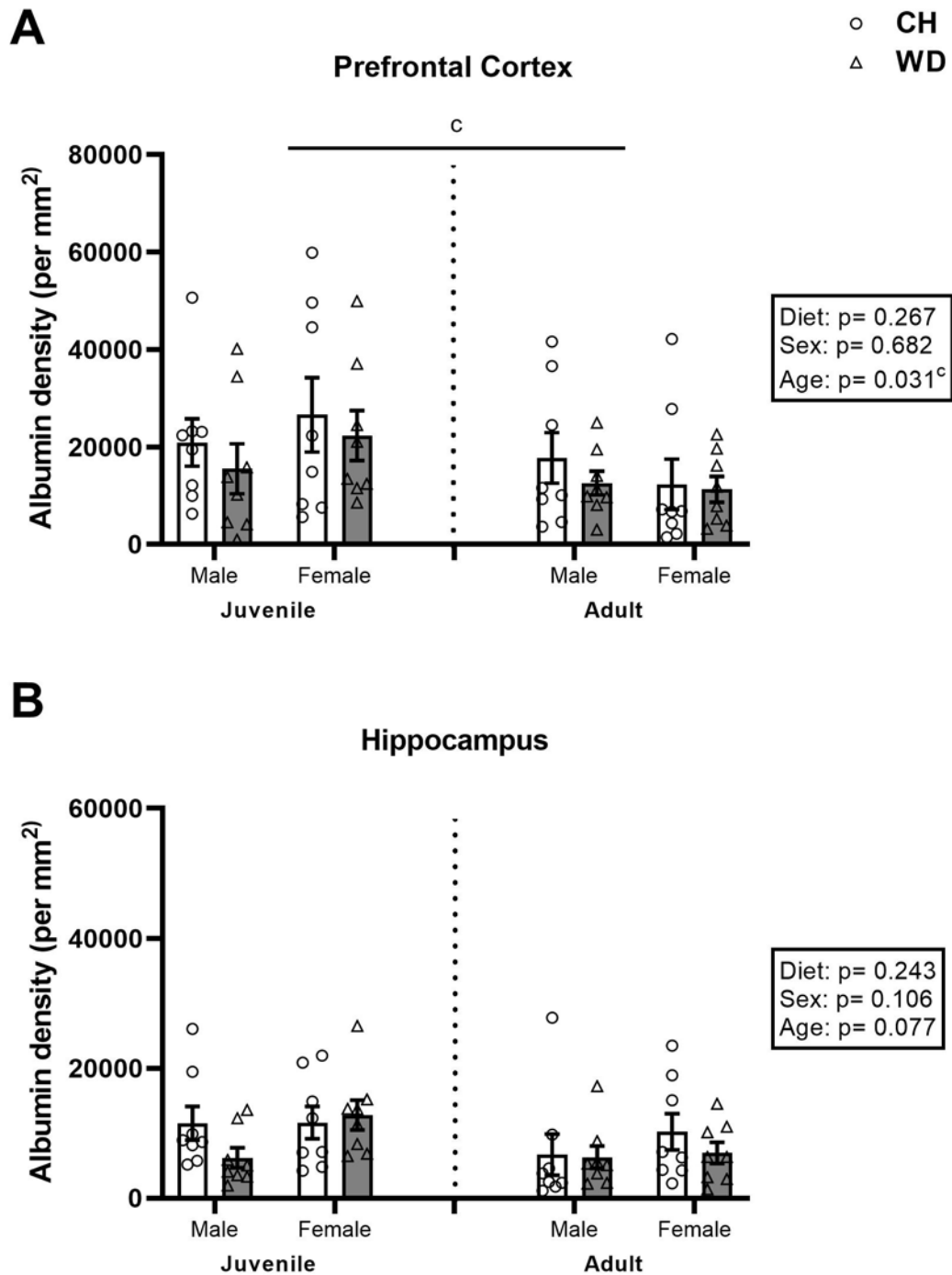
Figure 14. Albumin Immunofluorescence. (A) Juvenile group had higher albumin density in the PFC than adult group (c). (B) There were no effects of age, sex, or diet after 8-10 weeks on WD in the hippocampus. a: $p < 0.05$. Mean $\pm$ SEM and individual values are shown.
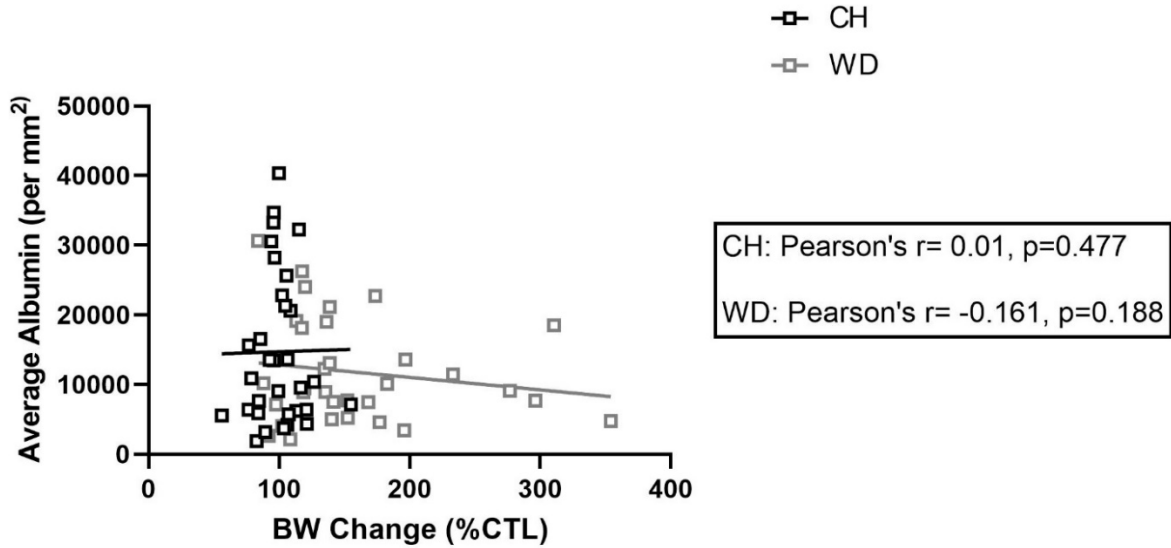
Figure 15. Correlation of albumin and body weight change. No significant correlations were observed in CH or WD animals. Pearson's *r* and *p* values shown.

We correlated total arm entries in Y Maze to PFC albumin density to evaluate the relationship between locomotor activity and PFC BBB leakage. We found that total arm entries were not correlated with albumin density in CH (Pearson's $r = 0.073$, $p = 0.35$), or WD animals (Pearson's $r = 0.052$, $p = 0.389$). Correlations illustrated in Figure 16A.

We also correlated percent of spontaneous alternations to hippocampal albumin to evaluate relationship between hippocampal-dependent behavioral deficits and hippocampal BBB leakage. We found that percent of spontaneous alternations was not correlated with hippocampal albumin density in CH animals (Pearson's $r = 0.061$, $p = 0.748$) or WD animals (Pearson's $r = 0.203$, $p = 0.267$). Correlations illustrated in Figure 16B.

A

Y Maze Arm Entries vs PFC albumin density (per mm^2), with legend CH and WD.

CH: Pearson's r= 0.073, p= 0.35

WD: Pearson's r= 0.052, p=0.389

B

Spontaneous Alternations (%) vs HIP albumin density (per mm^2).

CH: Pearson's r= 0.061, p=0.748

WD: Pearson's r= 0.203, p=0.267
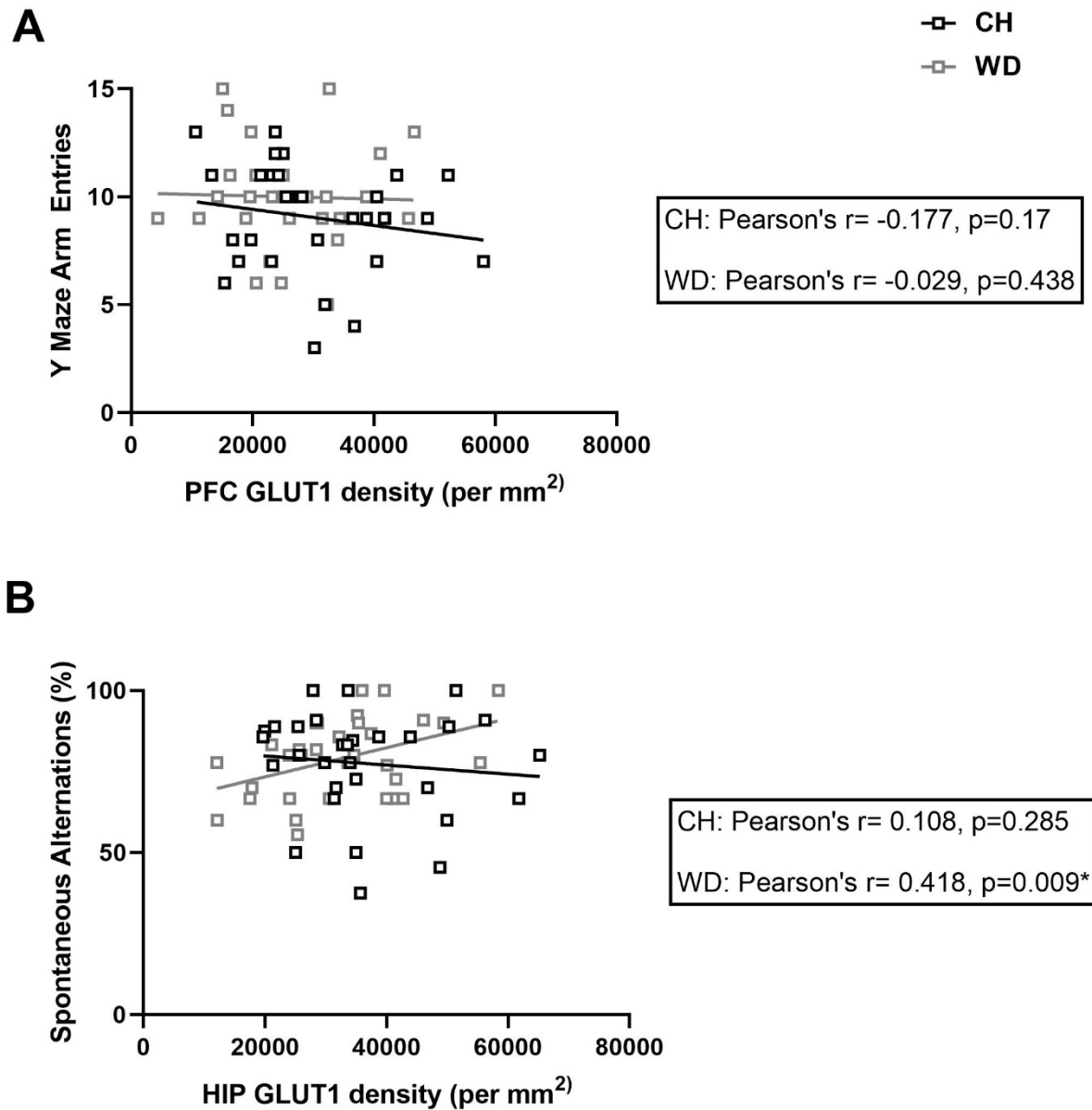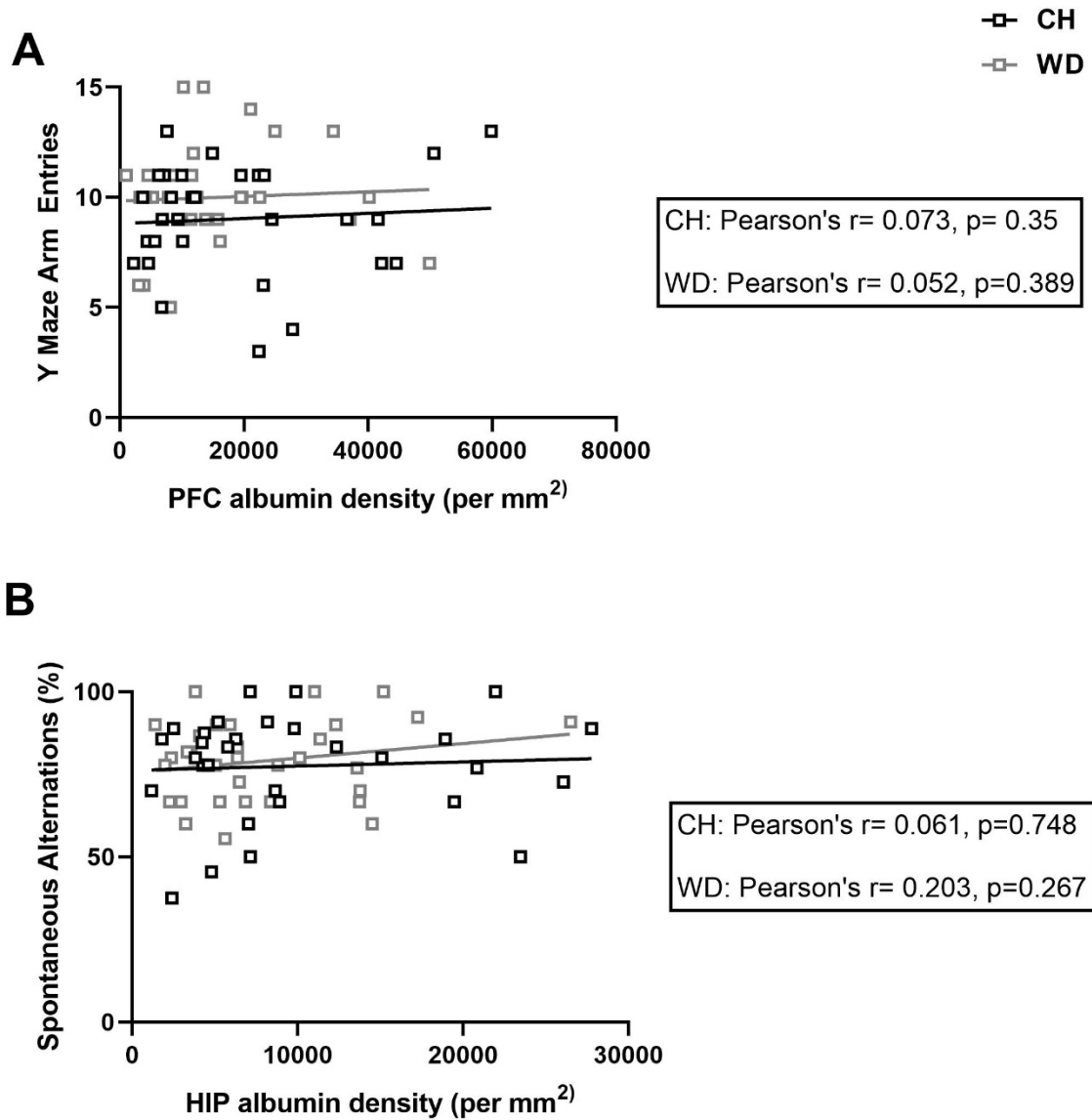
Figure 16. Correlation of Y Maze behaviors and albumin. (A) No significant correlations between Y Maze arm entries and albumin in the PFC. (B). No significant correlations between spontaneous alternations and albumin density in the hippocampus. Pearson's *r* and *p* values shown.

IBA1+ Results

IBA1+ Immunofluorescence

WD did not increase IBA1+ in the PFC compared to CH ($F_{1,56} = 0.235$, $p = 0.6297$). There was also no main effect of age ($F_{1,56} = 0.498$, $p = 0.4834$) or sex ($F_{1,56} = 0.154$, $p = 0.6964$). However, there was an interaction between age and sex, suggesting differential IBA1+ density between males and females at different ages. Planned comparisons showed no specific differences between WD and respective CH groups. Juvenile WD males were not different than juvenile CH males ($p = 0.809$), adult WD males were not different than adult CH males ($p = 0.81$), juvenile WD females were not different than juvenile CH females ($p = 0.453$), and adult WD females were not different than adult CH females ($p = 0.789$). There were also no significant differences in post-hoc comparisons. Results are illustrated in Figure 17A.

WD did not increase IBA1+ in the hippocampus compared to CH ($F_{1,55} = 3.273$, $p = 0.0759$). We did find a main effect of age ($F_{1,55} = 6.901$, $p = 0.0111$) such that the adult group had more IBA1+ than juvenile group. We also found a main effect of sex ($F_{1,55} = 6.158$, $p = 0.0162$) such that females had higher IBA1+ than males. There were no interactions. Planned comparisons showed no specific differences between WD and respective CH groups. Juvenile WD males were not different than juvenile CH males ($p = 0.234$), adult WD males were not different than adult CH males ($p = 0.385$), juvenile WD females were not different than juvenile CH females ($p = 0.094$), and adult WD females were not different than adult CH females ($p = 0.126$). Post-hoc comparisons showed that juvenile females had more IBA1+ than juvenile males ($p = 0.047$), and adult males also had higher IBA1+ than juvenile males ($p = 0.034$). Results are illustrated in Figure 17B.

Correlation of IBA1+ to GLUT1

We correlated IBA1+ density in the PFC to GLUT1 density in the PFC to evaluate the relationship between energy dysregulation and neuroinflammation in CH and WD animals. We found that IBA1+ did not correlate with GLUT1 in CH (Pearson's $r = 0.242$, $p = 0.091$), however we did find a positive correlation between IBA1+ and GLUT1 in WD animals (Pearson's $r = 0.31$, $p = 0.042$). Results are illustrated in Figure 18B.

A

Prefrontal Cortex

○ CH
△ WD

Diet: p= 0.63
Sex: p= 0.696
Age: p= 0.483
Age*Sex:p=0.026*

B

Hippocampus

Diet: p= 0.076
Sex: p= 0.016[b]
Age: p= 0.011[c]
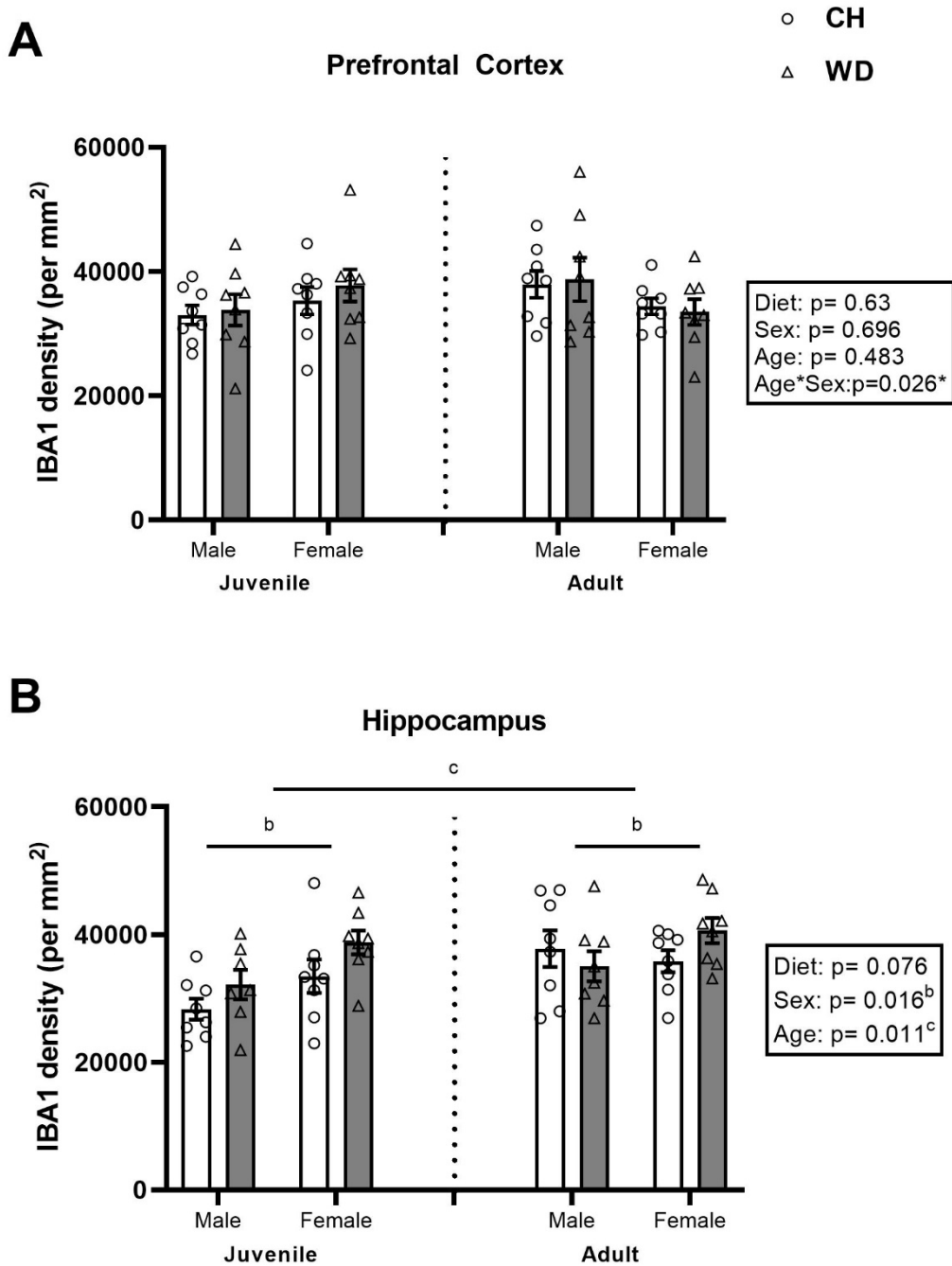
Figure 17. IBA1+ Immunofluorescence. (A) In the PFC, microglia density was age- and sex-dependent. (B) In the hippocampus, the adult group had greater IBA1+ density than juvenile group (c), and females had greater density than males (b). a, b, *: $p < 0.05$. Mean ± SEM and individual values are shown.

A

PFC IBA1 density (per mm^2)

CH: Pearson's r= 0.242, p= 0.091

WD: Pearson's r= 0.31, p= 0.042*

PFC GLUT1 density (per mm^2)

CH
WD

B

HIP IBA1 density (per mm^2)

CH: Pearson's r= -0.101, p=0.29

WD: Pearson's r= 0.07, p=0.354

HIP GLUT1 density (per mm^2)
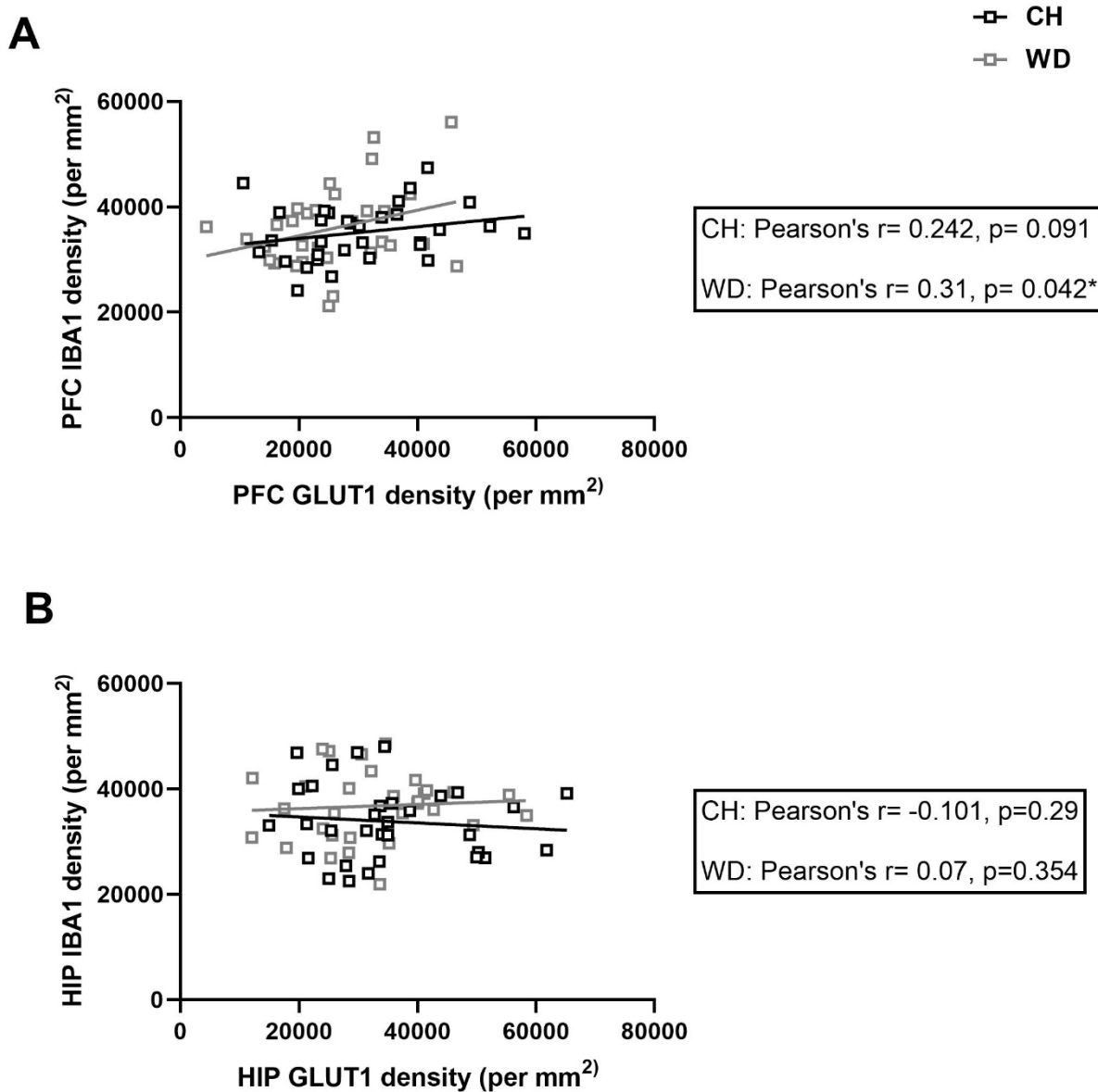
Figure 18. IBA1 and GLUT1 correlation. (A) IBA1 density in the PFC correlates with GLUT1 density in WD animals and not CH. (B) There were no correlations between IBA1 and GLUT1 in the hippocampus in CH or WD animals. Pearson's *r* and *p* values shown. *$p < 0.05$.

We correlated IBA1+ density in the hippocampus to GLUT1 density in the hippocampus to evaluate the relationship between energy dysregulation and neuroinflammation in CH and WD animals. We found that IBA1+ did not correlate with GLUT1 in CH (Pearson's r = -0.101, p = 0.29) or WD animals (Pearson's r = 0.07, p = 0.354). Results are illustrated in Figure 18B.

Correlation of IBA1+ to Albumin

We correlated IBA1+ density in the PFC to albumin density in the PFC to evaluate the relationship between BBB leakage and neuroinflammation in CH and WD animals. We found that IBA1+ does positively correlate with albumin in CH (Pearson's r = 0.466, p = 0.004), however this correlation was not significant in WD animals (Pearson's r = 0.272, p = 0.066). Results are illustrated in Figure 19A.

We correlated IBA1+ density in the hippocampus to albumin density in the hippocampus to evaluate the relationship between energy dysregulation and neuroinflammation in CH and WD animals. We found that IBA1+ did not correlate with albumin in CH (Pearson's r = -0.25, p = 0.084) or WD animals (Pearson's r = 0.111, p = 0.275). Results are illustrated in Figure 19B.

Correlation of IBA1+ to Behavior

We correlated total arm entries in Y Maze to PFC IBA1+ density to evaluate the relationship between locomotor activity and PFC neuroinflammation. We found that total arm entries were not correlated with IBA1+ in CH (Pearson's r = 0.103, p = 0.293), or WD animals (Pearson's r = -0.097, p = 0.299). Correlations illustrated in Figure 20A.

We also correlated percent of spontaneous alternations to hippocampal IBA1+ to evaluate relationship between hippocampal-dependent behavioral deficits and hippocampal neuroinflammation. We found that percent of spontaneous alternations was not correlated with hippocampal IBA1+ density in CH animals (Pearson's r = 0.077, p = 0.686) or WD animals (Pearson's r = -0.075, p = 0.687). Correlations illustrated in Figure 20B.

A

PFC IBA1 density (per mm^2)

CH: Pearson's r= 0.466, p= 0.004*

WD: Pearson's r= 0.272, p= 0.066

PFC albumin density (per mm^2)

□ CH
□ WD

B

HIP IBA1 density (per mm^2)

CH: Pearson's r= -0.25, p= 0.084

WD: Pearson's r= 0.111, p= 0.275

HIP albumin density (per mm^2)
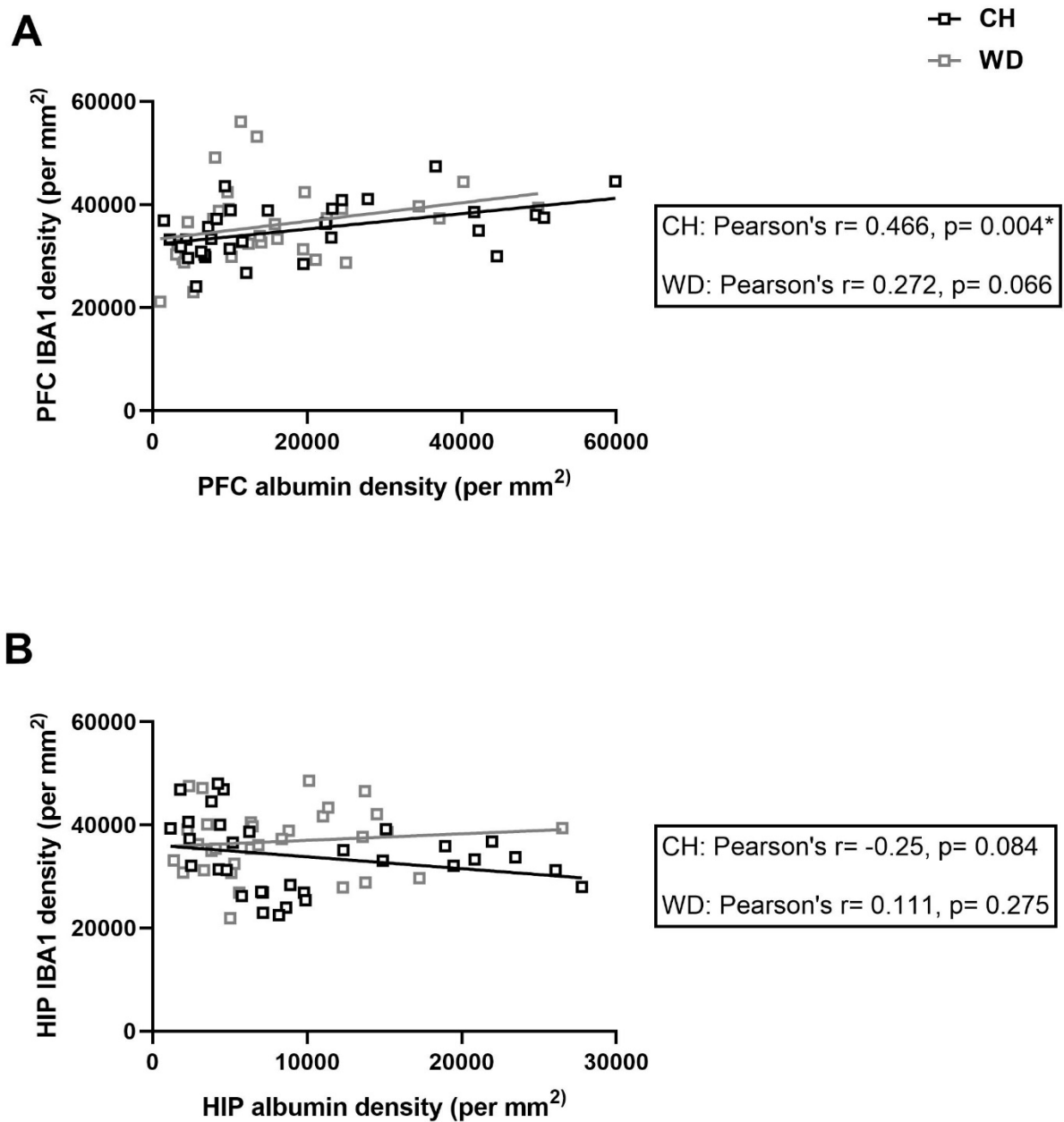
Figure 19. IBA1 and albumin correlation. (A) IBA1 density in the PFC correlates with albumin density in CH animals but not WD. (B) There were no correlations between IBA1 and albumin in the hippocampus in CH or WD animals. Pearson's *r* and *p* values shown. *$p < 0.05$.

A

CH
WD

Y Maze Arm Entries

PFC IBA1 density (per mm^2)

CH: Pearson's r= 0.103, p= 0.293

WD: Pearson's r= 0.052, p=0.389

B

Spontaneous Alternations (%)

HIP IBA1 density (per mm^2)

CH: Pearson's r= 0.077, p=0.686

WD: Pearson's r= -0.705, p=0.687
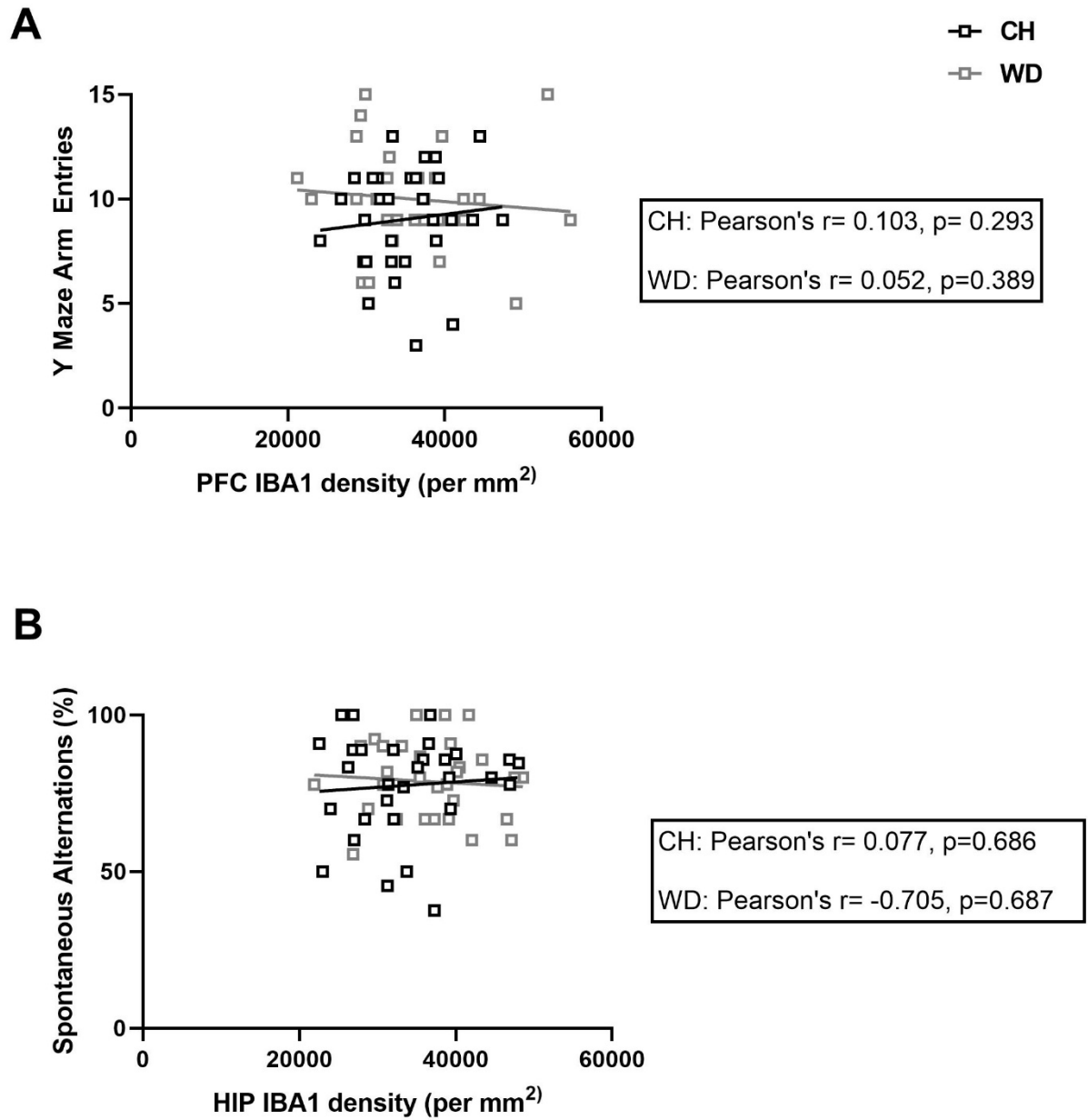
Figure 20. Correlation of Y Maze behaviors and IBA1 density. (A) No significant correlations between Y Maze arm entries and IBA1 in the PFC. (B). No significant correlations between spontaneous alternations and IBA1 density in the hippocampus. Pearson's *r* and *p* values shown.

DISCUSSION

Body Weight, Hyperglycemia, and Behavior

We examined the effects of 8-10 weeks of WD in males and females starting either during juvenile period (post-natal day 21) or adulthood (post-natal day 75) on body weight, glucose tolerance, and behavior. We found that our results are consistent with what is reported in the literature (Salinero et al., 2018). WD increased body weight in males and females, and the effect on females was greater when the diet started in adulthood than during juvenile period. This supports the premise that WD differentially impacts metabolism according to age and sex. We did not, however, observe diet-induced cognitive deficits. Although there are many studies that report behavioral deficits after WD, there are also many that do not—including some that report no differences in Y-Maze after longer exposure to WD (Kaczmarczyk et al., 2013). Despite this, manystudies still find diet-induced neurological changes despite there being no differences in behavioral tasks (Abbott et al., 2019; Tsan et al., 2021). Neurological changes precede behavioral changes; therefore diet exposure may not have been long enough to result in behavioral deficits.

Further, Y maze is a proxy for severe hippocampal impairments on spatial memoryand 8 weeks on WD may not have been enough to reach that level of impairment, or behavior test may not have been sensitive enough to capture subtle changes in neuronal function. For example, some have observed diet-induced behavioral changes in a radial arm maze—which is a more complex task that encompasses not just simple hippocampal-dependent spatial memory but also motivation and learning (Hargrave et al., 2016; Kanoski et al., 2007). Other tasks such as novel object recognition or fear conditioning may have offered more insight as to the cognition-related behavioral deficits associated with WD, tasks such as sucrose preference test would have offered more insight on PFC-dependent behaviors, and tests such as elevated plus maze or open field test would have offered more insight to depression- and anxiety- like behaviors. Here we were limited to a single behavioral test that offered a narrow insight to the functional neuronal changes that may be happening in response to WD. Other behavior testing would be beneficial in identifying how diet-induced body weight gain and hyperglycemia impact brain and behavioral function.

Sex- and Age-Specific Decrease in GLUT1

WD overall decreased GLUT1 density in the PFC, which supports our hypothesis that WD impacts the BBB across age and sex in brain regions that take longer to develop. We also found that GLUT1 was higher in the adult group than juvenile group, which also supports our premise that insult at an earlier age has long term consequences. The age-specific effects may also be due to confounding factors of experimental age and diet duration (see "confounding factors" section below). However, these findings are important because although we found no behavioral differences in hippocampal-dependent Y maze performance, PFC disruption during development alters cognitive control, risk taking behavior, and decision making (Francis & Stevenson, 2013). Decreased GLUT1 in the PFC may also be contributing to decreases in dendritic spine density, cellular metabolic stress, and decreases in brain-derived neurotrophic factor that are reported by other groups (Kanoski et al., 2007; Kruse et al., 2019; Rincel et al., 2018; Yang et al., 2020), as well as age-sensitive behavioral differences in reward-seeking behavior (Carlin et al., 2016; Frazier et al., 2008). Although we found no main effects of WD on GLUT1 in the hippocampus, we did find an interaction between diet, age, and sex which also supports our hypothesis that effects of WD on GLUT1 are age- and sex- specific.

Correlations Between Hyperglycemia, GLUT1, and Behavior

We also assessed the relationship between glucose tolerance and average GLUT1 in rats on WD diet. We found a negative correlation between GLUT1 and GTT in CH-consuming rats, but not WD-consuming rats. This was initially surprising given that the adverse effects of WD on GLUT1 are suggested to be due to long term downregulation of GLUT1 in response to diet-induced hyperglycemia. Our findings challenge that assumption and suggest that GLUT1 decrease is not directly related to glucose levels. GLUT1 is instead associated with a general disruption of glucose regulation of vascular GLUT1, and inability to adapt and regulate the amount of glucose circulating glucose that gets into the brain. This non-linear relationship between hyperglycemia and and GLUT1 density in WD animals is also supported by studies that find a decrease in GLUT1 after several weeks on WD, then see an increase more long term (Tomassoni et al., 2020). We did, however, find a significant relationship between hippocampal GLUT1 and deficits in Y Maze spontaneous alternations in WD consuming animals, again suggesting that although WD did not

disrupt GLUT1 or behavior overall, it did have a negative impact on both of these factors in the same animals.

Out findings also suggests that although some WD animals have similar GLUT1 density to CH animals, they may be functioning differently under different conditions. For example, GLUT1 density in our experiments was measured in non-fasting and non-metabolically challenged conditions several days after GTT. Assessing the relationship between GTT and GLUT1 during a metabolic challenge or at a closer timepoint may further elucidate how WD-induced hyperglycemia disrupts GLUT1 density. A way to test this would be either to correlate fasting glucose at the time of sacrifice or test WD-induced dysregulation of GLUT1 via hyperglycemia by sacrificing animals at each timepoint after glucose load (fasting, 15min, 30 min, 60 min, 120min) to detect differential responses between WD-consuming animals and CH.

It is challenging to identify what other contributing factors could be disrupting the relationship between GLUT1 and glucose levels in WD animals because the mechanistic relationship between GLUT1 in the brain and hyperglycemia are largely unexplored. Studies exploring the genetic relationships between GLUT1, hyperglycemia, and neurocognitive decline identified several genes and mechanisms that could be explored further (Leão et al., 2020). In their GeneCards analysis, the genes noteworthy markers that surfaced were GLUT-3, insulin, alanine, hexokinase 1, 2, and 3, glucokinase, amyloid precursor protein, apolipoprotein E, microtubule-associated protein tau, phosphofructokinases, and granulins. These are markers that involve the feedback mechanisms of glucose sensing and energy metabolism within the CNS, therefore the GLUT1 dysregulation we observe may be more related to BBB changes occurring in response to energy dysregulation within the CNS and less with GLUT1 downregulation in response to hyperglycemia like it has been hypothesized.

Age, Body Weight, and BBB leakage

We found no evidence of exacerbated albumin leakage in WD consuming animals in the PFC or hippocampus. Our hypothesis was based on literature using NaFl as a proxy, and the molecular difference in these compounds may be the reason for our null findings. While NaFl may diffuse into the brain when tight junctions are depleted due to WD consumption, the tight junction depletion may not be significant enough to allow larger blood protein diffusion. Also, our WD exposure was approximately 60-70 days, which may not be long enough to significantly increase

BBB leakage (Hargrave et al., 2016). However, we did find a main effect of experimental age which does support our premise that animals are more susceptible to insult earlier in life than in adulthood.

We also found no correlation between body weight change and average albumin in the brain. This challenges the assumption that diet-induced obesity increases BBB leakage. This also suggests that BBB leakage in response to WD is more related to specific metabolic impairments than phenotypical characteristics. For example, atherosclerosis is highly associated with BBB leakage because vascular hardening and plaque buildup happens in the brain as well (Methia et al., 2001). This causes vascular ruptures and micro-bleeding in the brain and contributes to neurocognitive deficits, and strongest evidence for this is in the use of APOE knock out mice in diet research as well as in Alzheimer's disease (Hafezi-Moghadam et al., 2007; Najjar et al., 2013). Collectively, our albumin-related findings suggest that 8-10 weeks of WD alone does not increase BBB leakage of albumin and that any variance observed was not associated with obesogenic phenotype.

IBA1+ and BBB Integrity

We measured IBA1+ density in the PFC and found a sex and age interactions due to developmental differences in IBA1+ (Schwarz et al., 2012), and no effects of WD. Although we did not find evidence of diet-induced changes in microglia density, WD is also associated with morphological changes or changes in number of microglia that are not always captured by measuring density (Bocarsly et al., 2015; Daly et al., 2020). We also evaluated the relationship between BBB integrity and neuroinflammation in animals that were on WD for 8-10 weeks starting either during juvenile period or adulthood by correlating BBB markers (GLUT1 and albumin) with our marker for neuroinflammation (IBA1+). We found no correlations between GLUT1 and IBA1+ in the PFC or hippocampus of WD or CH animals. However, we did find a correlation between albumin and IBA1+ in the PFC of CH animals but not WD. This relationship was not observed in the hippocampus.

There are several explanations to our findings. For example, we did not observe an overall increase in IBA1+ density in response to WD, nor did we observe significant BBB albumin leakage, therefore our range may be too narrow to detect a relationship between the two. That is, the IBA1+ and albumin density in our experiments are less pathological than other studies have reported

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which limits our ability to assess the relationship between BBB leakage and neuroinflammation in pathological conditions. Second, the GLUT1 dysregulation observed in WD animals may not be pro-inflammatory after 8-10 weeks on diet and there are other molecular mechanisms by which GLUT1 impairments can disrupt neuronal function. For example, disrupted energy homeostasis could alter astrocyte function, or it could alter insulin function in the brain (Arnold et al., 2018; De Felice et al., 2014). WD can disrupt astrocyte function or alter energy metabolism in the brain, and cause behavioral deficits linking hyperglycemia to neurocognitive health independent of microglia (Li et al., 2018).

Differentiating Nutrition, Obesity, & Metabolic Disorders

Understanding the impact of WD on metabolic and mental health is challenging because there are many factors at play that each warrant more specific research questions. For example, it is challenging to determine if adverse effects of WD are due to specific nutritional differences or the other myriad adverse effects associated with Westernized diets. Here, we use a diet high in fat that mainly consists of lard, and many groups have attributed negative consequences of WD patterns specifically to high consumption of saturated fats (Freeman & Granholm, 2012), however our WD also has high sugar in solid and liquid form and some groups have attributed negative consequences to high sugar consumption (Kose et al., 2021). This combination makes it difficult to isolate what specific aspects of the diet are driving the results because to test the specific dietary components, all other macronutrient and micronutrient components would need to be matched, and that was not the case in this study although it is possible to obtain diets that are more closely matched. We were aware of these interpretational limitations and designed our analyses to focus more on the diet as a metabolic stressor than the specific nutritional components of our chosen WD. More specifically, we focused on evaluating the relationships between the metabolic outcomes in response to the diet. We decided that correlating with diet-induced metabolic changes to offer more insight and focus more on the link between metabolic *consequences* of WD and links to BBB integrity and dysregulation than the specific dietary components themselves. Assessing the specific relationship with hyperglycemia and body weight gain helped us understand what consequences were attributed to GLUT1, BBB leakage, IBA1+ and behavior even though we were not able to isolate what specific part of our WD contributed to the metabolic changes. Ultimately, we still faced the challenge of understanding whether the hyperglycemic state was induced by

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specific macronutrient changes, nutritional changes, or endocrine changes resulting from the diet change—and more specific dietary experiments with more closely matched diets would be needed.

Further, consuming highly palatable energy dense foods also results in overconsumption of calories and it is challenging to determine whether adverse effects are due to the changes in dietary macronutrient composition, or generally overconsuming calories. Differentiating between effects of diet composition & hypercaloric consumption can be isolated using pair feeding studies, where experimental diet groups are fed the same number of calories as a control group. This allows experimenters to test the adverse effects of the dietary components themselves. Pair feeding also comes with its own limitations. WD may be altering appetitive mechanisms and limiting caloric intake may cause additional stress unrelated to the diet itself, then experimenters face the challenge of identifying whether adverse effects are due to the diet, or the stress of not having *ad libitum* access to food so they can feed until they are satiated.

It is also important to note that although we evaluated the relationship between hyperglycemia and body weight with behavior and BBB changes, we were limited to relatively mild pathological conditions. First, the hyperglycemia levels we evaluated are more analogous to pre-diabetic stages than fully diabetic, and fully insulin resistant conditions may result in more specific GLUT1 alterations. Although we did not fully induce a pathological hyperglycemic state, these data elucidate how the relationships between circulating glucose levels and glucose transport start changing in pre-diabetic conditions and warrants further research in understanding how the relationship continues to change as insulin resistance develops. Second, we only evaluated group body weight change as our proxy of obesogenic conditions and did not differentiate between animals that developed an obesogenic phenotype in response to WD and those that may have been diet resistant (Shin, et al., 2010). Evidence suggests that about one third of animals that have access to high-fat high-sugar diets do not develop characteristics and studies that focus specifically on obesogenic phenotypes typically exclude animals that do not gain significantly more weight.

Other Metabolic & Endocrine Consequences of WD

In these experiments, we focused on the impact of WD on two mechanisms of disruption of the BBB however, WD may have impacted metabolic & endocrine function in other ways that could have impacted behavior. Here, we only evaluated the relationship between GLUT1 and hyperglycemia, however the observed dysregulation suggests that other diet-induced metabolic

dysregulation may be at play. It would be worth correlating other diet-related factors (such as lipids, insulin, body weight, adiposity), to further elucidate what specific diet-induced disruptions contribute to GLUT1 dysregulation and associated relationship to Y Maze behavior.

For example, we did not measure adiposity although adipose tissue is an obesity-related measure associated with neuroendocrine and chronic low-grade inflammation observed in response to WD. This measure could have further elucidated the relationship between obesity and behavior beyond just body weight. These also could have offered further details the negative results observed in IBA1+, which was our measure of neuroinflammation where we found no differences. Measuring other circulating compounds such as lipids, or hormones such as insulin or leptin would provde further insight into the relationship between other specific diet-induced changes related to metabolic pathologies can impact the brain.

Potential Confounding Factors

There are several confounding factors that could be influencing our findings. First, there is evidence that stress also imparts adverse effects on the BBB, and that these effects may also be age-dependent. This could be a contributing factor to the main effect of age observed in albumin leakage. All animals that started WD during juvenile period were individually housed at PND 21-25. This is a significant early life stressor that the adult group does not experience since they are group housed before they start the experiment at PND 75. The potential interaction between early life stress and WD are supported by the literature (Solarz et al., 2021). It is challenging to remove this confounding factor however, because group housing animals for diet studies limits the ability to accurately measure food intake. Another potential factor between juvenile and adult groups is that these were run at separate times and adult group remained on WD for an additional week than juvenile after GTT and behavior. Experimenters had to delay animal sacrifice due to unforeseen delayed access to euthanizing agent, and this may have impacted impact some of the age differences we found in the brain. This may have also introduced some discrepancies in the correlation between GTT and brain markers in adults because the measures were not recorded in the same timeline as in juveniles.

COVID-19 Context

The majority of this dissertation work was completed during the COVID-19 pandemic that started in spring 2020 and caused a year-long lab shut down where no animal experiments were run. Consequently, we resorted to building on previous experiments to maximize the use of archived brain tissue. This limited some aspects of the experimental design. For example, only Y Maze had been run in animals from archived brain tissue, so we were unable to test for PFC-dependent behaviors or other behavioral deficits associated with WD such as sociability, anxiety-like behavior, or other learning tasks (Tsan et al., 2021). We also only had $n = 8$ per group, which may have left us insufficiently powered. To address this group size limitation, follow up analyses could be carried out to calculate eta squared and determine effect sizes and optimal group size for follow up experments. The pandemic also resulted in long-term issues with supply availability, which contributed to delay in drugs and supplies needed to sacrifice animals in a fully consistent timeline. Inconsistencies across experimental timelines also limited our ability to compare age groups (e.g. WD juvenile males compared to WD adult males) like we originally planned because age ended up not being the only factor that were different between groups. We continued with planned comparisons between CH and WD groups, however, because compared groups were run at the same time.

COVID-19 also impacted research on the links between metabolic disorders, inflammation, and mental health on a greater scale. First, it highlighted the need to understand how metabolic disorders increase susceptibility to immune insults in an age and sex specific manner because people with underlying conditions related to metabolic disorders (such as diabetes, cardiovascular disease, and obesity) were at greater risk of hospitalization (O'Hearn et al., 2021). Second, there were many "long COVID" symptoms and long term consequences once patients recovered from immediate symptoms of the virus--- and some of these were related to neurocognitive health decline (Berenguera et al, 2021). This highlighted the need to understand how inflammation in the body can impact the brain to disrupt mental and cognitive function. Third, COVID brought about a lot of physical and social isolation, which highlighted the importance of having a strong social support network to develop resiliency to stress and adverse effects on mental health (Hossain et al,, 2020). A global pandemic on a highly contagious respiratory virus impacted not just the execution of the research reported in this dissertation but will also impact the next several decades

of research related to metabolism, body-brain interactions, and mental health because there are many links yet to be understood.

Future Directions in Diet & Obesity Research in Mental Health

Diet and obesity research is an incredibly nuanced area, not only because of the complexity of the environment that researchers try to model in pre-clinical settings to isolate different aspects of a Westernized environment. We have discussed the difficulty in isolating nutrition from obesity and metabolic disorders and have offered several approaches to differentiating between these and how they can uniquely impact the brain to contribute to BBB changes and neurocognitive changes—however, the truly impactful direction is one that considers a broader scope not a narrower one. That is, it is important to consider the full environment (not just the medical one) in which people with metabolic disorders exist in to truly understand how comorbid mental health disorders develop. Even further, it's important how these environments are different across age and sex to truly understand the best approach to treating mental health disorders that are comorbid with diet-induced metabolic disorders.

As biomedical researchers there is a strong emphasis understanding the specific overlapping causal mechanisms linking diseases to develop novel pharmacological and therapeutic treatments. This approach can become reductionist because the cause of the comorbidity may not be treatable by a single biological target like a medical model of disability suggests. The medical model of disability focuses on the symptomatic pathology that exists in an individual and aims to correct it. This is flawed because the causes of symptomatic pathology may not just be a biological one but a social one as well because people with metabolic disorders often live with more stress in their lives than individuals who are not having to constantly adapt to an environment designed for people who do not have metabolic disorders. For example, people with overweight or obesity face extra social stress in the form of bullying or negative self-image due to cultural ideas of beauty that are imposed by society (Dion-Albert et al., 2022; Goering, 2015). Another example is people living with diabetes and the additional life stress of monitoring glucose and having more limited food options to manage their glucose levels, in addition to navigating the stigma surrounding diabetes. The extra life stress of navigating a Westernized environment while managing metabolic disorders can be a major contributing factor to comorbid mental health disorders (Sartorius, 2018).

Although the differential development of metabolic disorders is driven by sex hormones (Clark et al., 2022), our negative results and lack of relationship with body weight also suggest that the link to phenotypic obesity is more complex in humans. Phenotypically overweight and obese individuals, particularly women, girls, and transgender folks face many social challenges in addition to medical that are not captured by most WD animal experiments. For example, children that are overweight or obese are more likely to get bullied (Mamum et al., 2013), teenagers experience negative self-image due to weight and size (Reel et al., 2015), and women are more likely to engage in dieting due to body dissatisfaction (Von Soest & Wichstrøm, 2009). If these individuals are already biologically susceptible to developing cognitive and mental health disorders because of metabolic status, the interaction with social stress may be the determining factor in whether they develop neurological and behavioral disorders. In Figure 21 we propose a revised rationale that includes the social model and suggests that while metabolic disorders can biologically increase susceptibility to mental health disorders by priming neuroendocrine and neuroimmune mechanisms to be hypersensitive to stress—the additional stress itself may be what leads to the development of comorbid disorders. This would align with our results and suggests that understanding the interaction between metabolic disorders and stress are the key to understanding the best combined approach to addressing comorbid physiological and mental health disorders (Ee et al., 2020). The role of the BBB is particularly important to understand in this interaction between diet-induced metabolic disruption and behavior because chronic stress and chronic social defeat have also shown to increase BBB leakage and disrupt energy and neuroimmune regulation (Dion-Albert et al., 2022; Solarz, 2021, Najjar et al., 2013; Menard et al., 2017). The field of diet & obesity research would greatly benefit from combining dietary models with stress models to increase translational impact and implications.
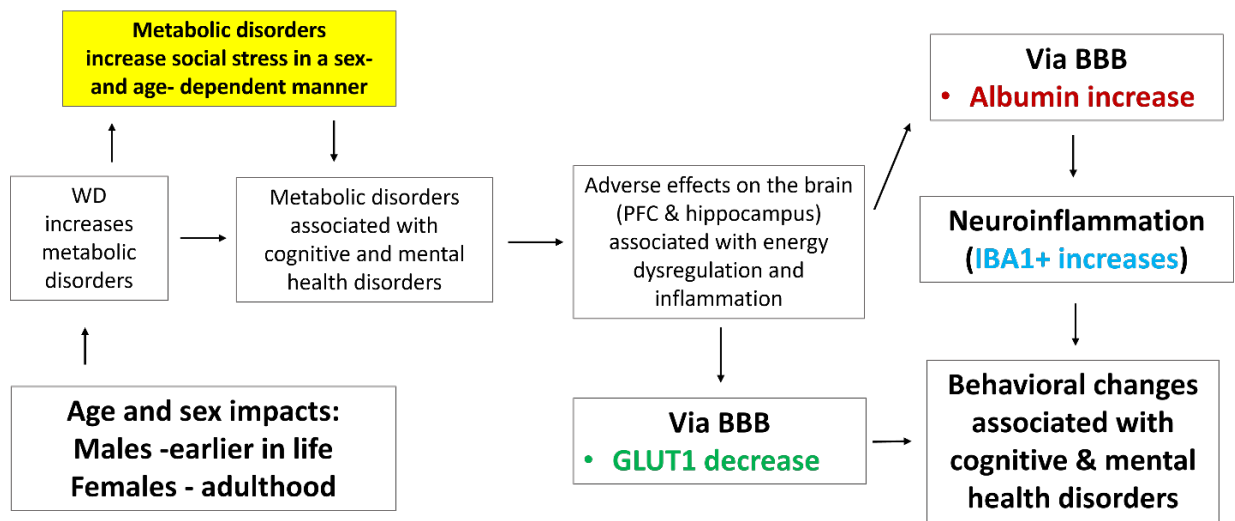
Figure 21. Summary of revised rationale. Including the role of social stress and adding inflammation-independent BBB dysregulation.

CONCLUSION

WD is associated with myriad negative effects on metabolic and neurocognitive health. It is associated with impaired cognitive development and poor mental health, and it can disrupt healthy neuronal function in the PFC and the hippocampus. WD patterns are observed starting at a very young age, which is alarming given that the PFC and hippocampus are not fully developed and disruptions early in life may have long term consequences. Further, there is building evidence that the adverse effects of WD are different in males and females, such that males are impacted more at an earlier age and females are impacted later in life. This is supported by behavioral and neurological data, and research on the underlying biological mechanisms seem to be associated with neuroinflammation. WD can disrupt microglia function and can contribute to the neurological changes associated with WD. This is paradoxical because the brain is supposed to be protected from inflammatory insult via the BBB, however WD may be impacting the BBB itself. WD impacts the BBB by inducing hyperglycemia and dysregulating glucose transporters in neurovasculature. Elevating circulating glucose downregulates transporters, such as GLUT1, and potentially deprive neurons of the energy they need to function properly. This could trigger a chronic neuroinflammatory response during prolonged WD consumption. Diet-induced BBB dysfunction is also associated with the physical breakdown of the BBB by depletion that allow the entry of circulating compounds that typically do not enter the brain. This leakage can also trigger a chronic neuroinflammatory response in microglia. Despite these connections and the evidence that the impact of WD is sex- and age-specific as well, there has been limited research on how WD can disrupt the BBB in both sexes and at different ages.

These experiments aimed to fill the knowledge gap between the sex and age differences observed in behavior and neuronal deficits, and neuroinflammation. We hypothesized that WD would impart adverse effects in males earlier in life, and in females during adulthood because of different susceptibility windows. Our findings on the effects of WD on GLUT1 support this hypothesis. We found that diet decreased GLUT1 density in the PFC, and that it differentially decreased GLUT1 density in the hippocampus according to age of diet access and sex. Further, our correlational analysis of glucose tolerance results and GLUT1 density also suggest that WD results in GLUT1 dysregulation. We also found that GLUT1 correlated with performance in spontaneous alternation task, however, we did not find an association between GLUT1

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dysregulation and IBA1+ in the PFC or hippocampus. This suggests that either energy dysregulation precedes neuroinflammation or disrupts neuronal function and behavior independent of inflammatory mechanisms. We also found that albumin leakage in the PFC was overall higher in juvenile experimental group which supports our premise that the PFC is more sensitive to insult earlier in life.

These experiments used triple labeling to test association between BBB glucose homeostasis, BBB leakage, and neuroinflammation in males and females—and are novel in many ways. These three markers have not been assessed together, nor have their relationships been tested despite all being part of a larger hypotheses on how WD causes BBB breakdown and contributes to neuroinflammation and neurocognitive dysfunction. These experiments also expands the body of literature aiming to understand sex differences not only in response to WD, but also general sex differences. For example, there is very limited work in understanding the development of the BBB, and it is currently unknown whether males and females have different BBB developmental patterns. The comparison of age across control groups lays a foundation for studies on the organizational sex differences of BBB development. These immunofluorescence protocols could be used by other researchers aiming to understand different types of BBB dysregulation and how it can contribute to neurological dysfunction. Further, our approach to include correlational analyses has provide greater insight to the specific mechanisms that contribute to neuroinflammation. This consequently challenges the assumed relationships between the BBB, WD, behavior, body weight, and microglia.

It is important to further understand these relationships because diet-related disorders and neurocognitive disorders are significantly impacting the quality of life of more and more people worldwide starting at a very young age. Our findings contribute more insight as to how WD disrupts energy regulation at the BBB in relation to hyperglycemia, however, we were limited in our ability to evaluate the role that diet-related disorders play in neuroinflammation. WD did not induce neuroinflammation or disrupt behavior the way many groups report, so we are revisiting our hypothesis to suggest that WD, particularly diet-induced obesity, may be increasing susceptibility to a wide range of neuroinflammatory insults related to cognition and mental health rather than causing it directly. A revisited rationale is illustrated in figure 21 that includes our findings and the role of social stress on the development of neurocognitive disorders. Neuroinflammatory insults can come in many forms such as chronic stress, or even COVID—both of which also reflect the gender bias reported in cognitive and mental health disorders. It is also

important to consider sex differences in neurological and behavioral disorders outside of the medical model of disability because our social environment can play an equal, if not larger role, in the development of comorbid metabolic and mental health disorders.. This interaction between metabolic stress and social environment will be crucial in fully understanding the sex- and age-dependent responses to Western diet, and in promoting a combined approach to improve the quality of life of individuals with comorbid disorders.

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