

THE ROLE OF NUTRITION IN BRAIN DEVELOPMENT AND HEALTH

by

STEPHANIE DUBROF

(Under the Direction of Hea Jin Park)

ABSTRACT

Brain development and health are influenced by nutrients that modulate neurochemical pathways, functional connectivity, and recovery mechanisms. Nutritional interventions, including functional foods rich in essential nutrients and bioactive compounds, support neurodevelopment, mitigate oxidative stress, and promote brain resilience. This dissertation investigates how nutritional and pharmacological interventions, including compounds derived from natural sources, influence brain outcomes using a translational pig model. Study 1 examined the effects of perinatal docosahexaenoic acid (DHA) supplementation on specialized pro-resolving lipid mediators (SPMs) in offspring brains. Perinatal DHA status has previously been associated with enhanced cognitive performance in offspring, potentially mediated by its anti-inflammatory properties. SPMs, derived from DHA, play a crucial role in resolving inflammation and have recently been identified as modulators of cognitive function. DHA supplementation elevated SPM levels in the prefrontal cortex and cerebellum and revealed associations between prefrontal SPMs and cerebellar network functional activation. These findings support a mechanism by which maternal DHA intake enhances neurodevelopment via SPMs. Study 2 expanded the focus

from a single compound to a whole-food approach, investigating perinatal egg yolk supplementation. Egg yolk provides a complex nutrient matrix, including amino acids, fatty acids, vitamins, minerals, and other bioactives linked to infant brain development. Maternal supplementation increased executive and cerebellar functional activity, enhanced hippocampal and cerebellar fiber lengths, and improved behavioral outcomes in offspring. This supports the role of a whole-food based approach to perinatal nutrition in promoting functional neurodevelopment. Study 3 shifted focus to brain injury recovery, evaluating the antioxidant therapeutic CMX-2043 in a pig model of traumatic brain injury (TBI). Treatment altered antioxidant enzyme activity in a route-specific manner, where intravenous administration enhanced liver catalase and brain SOD activity, while subcutaneous delivery increased brain catalase activity. Elevated brain catalase activity correlated with improved MRI-based recovery measures, including reduced lesion volume, midline shift, and swelling and atrophy. Together, these studies highlight the therapeutic potential of both functional foods and bioactive compound-based interventions in supporting brain development and recovery. The findings from this dissertation offer translational insights into how targeted nutrition and therapies can enhance neurodevelopmental outcomes and inform strategies to optimize brain health across the lifespan.

INDEX WORDS: DHA, SPECIALIZED PRO-RESOLVING MEDIATORS, POLYUNSATURATED FATTY ACID METABOLITES, LIPIDOMICS, EGG YOLK, NEURODEVELOPMENT, MAGNETIC RESONANCE IMAGING, FUNCTIONAL MAGNETIC RESONANCE IMAGING, COGNITIVE FUNCTION, BRAIN HEALTH, BRAIN INJURY, CATALASE, OXIDATIVE STRESS, BRAIN RECOVERY

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STEPHANIE DUBROF

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by

STEPHANIE DUBROF

Major Professor:

HEA JIN PARK

Committee:

ALEX ANDERSON

TODD R. CALLAWAY

NIKOLAY M. FILIPOV

Electronic Version Approved:

Ron Walcott

Vice Provost for Graduate Education and Dean of the Graduate School

The University of Georgia

May 2025

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CHAPTER 1

INTRODUCTION

Early brain development and long-term brain health are influenced by a variety of external factors, with nutrition playing a fundamental role in shaping neurodevelopmental trajectories [1-4]. Indeed, various essential nutrients and non-essential bioactive food compounds have gained increasing attention for their ability to support brain function [5,6] through mechanisms including the reduction of oxidative stress [7,8], modulation of neurochemical pathways [3,9], and facilitating synaptic plasticity [10], ultimately promoting cognitive function [3,10,11] and neuroprotection [8,12] in both healthy and injured brains. This dissertation explores how early-life nutrition shapes neurodevelopment and examines the potential of antioxidant-based interventions in supporting brain repair after injury.

The perinatal period is a critical window for neurodevelopment. During this period, fetal and neonatal neurodevelopmental outcomes are influenced by maternal nutrition status [4,13-21]. Docosahexaenoic acid (DHA) is an omega-3 fatty acid and essential for brain structure and function, contributing to neuronal growth, myelination, and synaptic plasticity [19,21-24]. DHA has been well investigated for its role in early brain development and cognitive outcomes, but the mechanism in which it exerts these benefits remains unclear [25,26]. One potential mechanism involves the conversion of DHA into specialized pro-resolving mediators (SPMs), a novel family of fatty acid-derived metabolites that play a crucial role in regulating neuroinflammation and may ultimately impact cognitive function [27-31]. Pregnancy is characterized by heightened

systemic and placental inflammation, with established mechanistic links between maternal inflammation and an increased risk of neurodevelopmental conditions in offspring [32-34]. This implicates a potential role for SPMs in mitigating inflammation while simultaneously supporting cognitive outcomes. Evaluating DHA-derived metabolites within the brain will further elucidate how DHA status directly influences cognitive function and neurodevelopmental trajectories. A single nutrient, such as DHA, provides targeted effects through its conversion into specific bioactive metabolites that regulate inflammation and cognitive processes, while a nutrient- and bioactive-rich whole food offers a complex food matrix that can broadly support multiple aspects of neurodevelopment. Eggs are a nutrient-dense food commonly recommended during the perinatal period due to their essential macronutrients and bioactive compounds that support fetal and maternal health [35]. While key egg yolk nutrients like choline, lutein, fatty acids, and amino acids have been extensively studied [36-39], research on perinatal supplementation of egg yolk as a whole food remains limited. Investigating how maternal dietary supplementation of a single nutrient or of a whole food impacts offspring brain function is crucial for understanding the role of perinatal nutrition in shaping early cognitive outcomes.

Managing external factors to support brain health is critical not only during healthy neurodevelopment but also following injury, when restoring and preserving brain function becomes essential. Traumatic brain injury (TBI) is a major public health concern, leading to long-term cognitive deficits due to oxidative damage and inflammation followed by the primary injury [40-45]. Oxidative stress negatively impacts brain health by exacerbating neuronal damage and impairing cognitive function, thus strategies that regulate oxidative stress hold promise for preserving brain function. Regulation of oxidative stress using antioxidants have the potential to improve outcomes following brain injury [47,49,53-55]. Therefore, identifying effective

antioxidant-based interventions is crucial for enhancing neuroprotective mechanisms and promoting optimal recovery outcomes in the aftermath of neurological injury.

To explore the impact of both nutritional and therapeutic interventions on brain health, this dissertation utilizes a translational pig model, which offers distinct advantages over other models in neuroscience research [56-58]. Pigs have neuroanatomical and physiological similarities to humans, including comparable brain size, cortical folding, white matter composition, and neurodevelopmental timelines [57,59-62]. Their gyrencephalic brain structure more closely resembles the human brain than the lissencephalic brains of rodents, allowing for better modeling of higher-order cognitive functions and network connectivity [63-65]. Additionally, the pig's extended gestational and postnatal development period allows for controlled perinatal nutritional interventions that mimic human maternal-offspring dietary influences, providing a robust platform for studying nutrient impacts on early-life brain development and cognitive outcomes [58,60,61,66]. In studies of early brain development, pig models have effectively demonstrated that prenatal and postnatal dietary interventions can influence brain maturation, modulate neurotransmitter synthesis, and subsequently shape cognitive function and behavioral outcomes [26,67,68]. For example, one study effectively investigated how diets containing deficient or adequate levels of linoleic acid influenced specific neurotransmitter profiles in the brains of neonatal piglets [67]. These studies have provided critical insights into the mechanisms through which early nutrition can shape neurodevelopmental trajectories in humans. Similarly, pig models have also been extensively employed to investigate neurological injury and recovery [69-74]. Due to similarities in brain anatomy, vascularization, and inflammatory responses, pig models of TBI have enabled precise examination of injury-induced pathologies and assessment of therapeutic efficacy [65,72,73,75]. Pigs have been utilized to assess functional responses and

deficits following TBI, with outcome measures often including clinically relevant modalities such as magnetic resonance imaging (MRI), behavioral assessments, and biomarker analyses [69,71,72]. These characteristics make the pig an ideal model for investigating both the developmental impact of early-life nutrition and the therapeutic potential of antioxidant interventions in brain injury recovery, facilitating more accurate translational applications to human health, as exemplified in this dissertation.

This dissertation is structured into a literature review and three experimental studies. The literature review in Chapter 2 examines how early-life nutrition influences cognitive outcomes, integrating human and animal studies to explore the roles of placental function, breastfeeding, energy intake in shaping neurodevelopment. Chapter 3 details a study that examines how perinatal DHA supplementation influences SPMs using a sow and piglet dyad model to provide insight into the potential mechanistic links between DHA status and early cognitive outcomes. Chapter 4 presents a study that investigates the effects of maternal egg yolk supplementation on functional brain activity and behavioral outcomes in piglet offspring to propose how whole food supplementation during critical developmental periods may influence neurodevelopmental trajectories. While Chapters 3 and 4 focused on nutritional interventions during healthy early brain development, Chapter 5 shifts the focus to therapeutic interventions aimed at mitigating damage and supporting recovery following traumatic brain injury. The study in Chapter 5 explores the impact of a novel antioxidant therapeutic on oxidative stress markers and brain recovery in a pig model of TBI, highlighting the critical role of oxidative stress regulation in supporting neuroprotection and optimizing functional recovery outcomes. Together, these studies provide insight into the potential of functional foods and therapeutic interventions in optimizing brain development, function, and resilience.

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CHAPTER 2

REVIEW OF THE LITERATURE

Early nutritional programming for cognition: how the placenta, breastfeeding, energy intake shape brain development

INTRODUCTION

The developing fetus and infant rely on maternal nutrient delivery systems to support the rapid growth and maturation of the brain during critical periods of neurodevelopment [1-4]. The concept of “nutritional programming” suggests that early-life nutrition exerts long-term effects on cognitive function and brain health, mediated by epigenetic regulation, cellular signaling pathways, and developmental plasticity [5]. In utero fetal neurodevelopment depends on the placenta as the primary interface for nutrient transfer, regulating the supply of essential macronutrients necessary for neuronal proliferation, differentiation, and synaptogenesis [1,6-14]. Infant neurodevelopment continues postnatally with breastfeeding, which serves as a complex behavior that acts as a mechanism that has been shown to influence cognitive outcomes in offspring [1,12-18]. Studies consistently link breastfeeding, when compared to formula feeding, to improved language, memory, and executive function in offspring, highlighting its role in early brain development [15,19-25].

Energy availability is a fundamental requirement for proper neurodevelopment, as the brain is one of the most metabolically demanding organs during early life [26]. In early postnatal life, the brain accounts for up to 60% of total energy expenditure, and disruptions in energy availability, whether through maternal undernutrition, overnutrition, or metabolic disorders, can

alter neurodevelopmental trajectories and impair cognitive outcomes [2,26,27]. Energy is derived from macronutrients, carbohydrates, proteins, and fats, which are the energy-yielding nutrients that not only fuel physiological processes but also serve as critical structural and functional components in the developing brain [13,28,29]. Carbohydrates are the principal energy source for the brain, fueling neuronal metabolism and supporting synaptic plasticity [4,26,30]. Proteins provide amino acids essential to hippocampal formation [31], neurotransmitter synthesis, neurotrophic signaling, thus impacting cognitive processes [13,29]. Fats, particularly polyunsaturated fatty acids, contribute to neurotransmitter production [13], myelination, membrane fluidity, and synaptic efficiency, all of which are essential to higher-order cognitive function [2,32,33]. The balance of these macronutrients is crucial for supporting early neurodevelopmental processes, and disruptions in their intake may have lasting effects on cognition and brain function [27,34]. Furthermore, given the brain's disproportionately high metabolic demands during early life and development, elucidating the role of each macronutrient in shaping neurodevelopment and cognition is essential for understanding how early nutritional exposures influence long-term cognitive potential.

Epidemiological studies provide compelling evidence that highlights a prominent association of perinatal nutrition with cognitive and developmental outcomes. Observational studies and clinical trials have highlighted the role of early nutritional status in supporting optimal neurodevelopment [35]. Complementary to epidemiological studies, animal models have provided mechanistic insights into how nutrients influence cognitive development. These studies enable the exploration of cellular and molecular pathways, such as the role of protein in hippocampal neurogenesis [36,37], a process fundamental to shaping cognitive outcomes. Studies using animal models that explore the associated cognitive outcomes generally involve widely

extended standard tasks that assess for hippocampal-dependent learning and memory processes, which include the Morris water maze, Barnes maze, and novel object recognition tests.

This literature review synthesizes current knowledge on the impact of early-life nutrition status on cognitive outcomes, integrating findings that are complimented by mechanistic insights from animal models. By focusing on placental function, breastfeeding, energy intake, and energy-yielding macronutrient metabolism, this review provides a targeted exploration of how early nutritional exposures shape neurodevelopment. It also identifies gaps in the literature, emphasizing the need for future research that bridges mechanistic and translational perspectives. The findings have critical implications for clinical practice, public health policy, and the development of targeted nutritional interventions to optimize early brain development and lifelong cognitive potential.

Placental development and nutrient transfer

The placenta serves as the critical interface between the mother and the developing fetus, orchestrating the transfer of nutrients necessary for fetal growth and development [6,12,38,39]. Its role is especially significant for brain development, as the fetal brain requires a continuous supply of specific nutrients during critical periods of neurogenesis, synaptogenesis, and myelination [8,9,39,40]. The placenta is highly sensitive to perturbations, which can disrupt placental gene expression, downstream function, and placental signaling, thus influencing neurodevelopmental programming [7-9,38,39]. Seminal research by Jones et al. has provided experimental evidence supporting the hypothesis that the placenta acts as a nutrient sensor [10]. Interestingly, Broad and Keverne proposed that the placenta adapts to short-term food deprivation to protect fetal brain development, particularly the hypothalamus during midgestation [41]. Their findings suggest that maternal nutrient deprivation disrupts synchronized

gene expression between the placenta and hypothalamus, triggering increased protein degradation in the placenta and compensatory upregulation [9,41]. In mice, the placenta-specific deletion of the insulin-like growth factor (IGF) 2 transcript led to intrauterine growth restriction (IUGR), disrupted the balance between fetal nutrient demands and placental supply, and resulted in increased anxiety-like behaviors in offspring [42]. Levitt et al. demonstrated that the placenta is a primary source of serotonin for the developing murine hypothalamus between gestational days 10.5 and 15.5, synthesizing serotonin from maternal metabolites [11]. Serotonin influences neuronal proliferation and axonal outgrowth during early development [43], suggesting that the placenta, as a source of serotonin, could regulate brain development. Serotonin plays a key role in the maturation of neural circuits involved in emotional regulation in mice [44], and polymorphisms in serotonergic genes are associated with depressive symptoms in humans [45], thus alterations in placental serotonin may effect cognitive outcomes. These findings support an association of nutritional status, placental responses, and offspring behavioral changes.

Preeclampsia, a hypertensive disorder of pregnancy characterized by high blood pressure and signs of organ dysfunction, has been associated with impaired placental function and altered nutrient transfer, potentially impacting fetal brain development and cognitive outcomes [46,47]. This condition is linked to placental insufficiency, resulting in reduced oxygen and nutrient delivery to the fetus, which can lead to IUGR and fetal hypoxia [48,49]. Studies suggest that preeclampsia may disrupt the supply of key neurodevelopmental nutrients, including oxygen, glucose, and amino acids, which are essential for neuronal proliferation, synaptogenesis, and myelination [46,47]. Preeclampsia is also associated with increased oxidative stress and systemic inflammation, both of which have been implicated in neurodevelopmental alterations [46,48]. Infants born to mothers with preeclampsia are at greater risk for cognitive and behavioral

impairments later in life, including lower IQ scores, attention deficits, and an increased likelihood of neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) [48,50-52]. For example, neurodevelopment assessed in a cohort of children born from severe preeclamptic pregnancies revealed that, on average, they did not reach appropriate cognitive milestones by three years of age [52]. Studies in animals have demonstrated that preeclampsia-like conditions lead to fetal brain structural abnormalities, including reduced cortical thickness and altered hippocampal connectivity [53], and diminished performance in cognitive tests [54]. In a rodent model of preeclampsia, offspring had increased glucocorticoid receptor expression in the hippocampus compared to controls, suggesting highly activated glucocorticoid signaling, which has been previously associated with preeclampsia and physiological stress responses [39,54]. Furthermore, offspring demonstrated significantly longer escape latencies than controls in the Morris water maze, indicating that the upregulated expression of hippocampal glucocorticoid receptors may contribute to the underlying mechanism [54]. Given these findings, preeclampsia represents a significant prenatal factor that may compromise placental nutrient transfer, underscoring the need for continued research into its long-term effects on neurodevelopment and cognitive outcomes.

Breastfeeding

Breastmilk is uniquely designed to meet infants' nutritional and developmental needs, providing essential energy, nutrients, and bioactive components that support brain development and ultimately cognitive outcomes [15,55,56]. Beyond its composition, breastfeeding also represents a complex feeding behavior that extends the influence of maternal physiology into the postnatal period. The first form of milk produced is known as colostrum, which provides essential factors for immune development [55-59] and primes the gut-brain axis, which is

increasingly recognized for its role in neurodevelopment [59,60]. Breastmilk contains hormones such as cortisol [61], leptin [62], and IGF [18], which can influence brain development and behavior [61-64]. For example, IGF promotes neuronal growth and myelination [64,65], while leptin has been implicated in hippocampal plasticity and cognitive function [66-68]. The presence of these hormones in breastmilk underscores its importance as a dynamic source of bioactive compounds essential for healthy neurodevelopment and long-term cognitive outcomes. Several studies suggest that longer durations of breastfeeding is associated with improved neurocognitive outcomes in childhood and into later life [69-71]. For example, longitudinal studies have demonstrated that prolonged breastfeeding is correlated with higher scores on intelligence quotient (IQ) tests, better academic performance, and enhanced executive function during childhood and adolescence compared to shorter breastfeeding durations or exclusive formula feeding [72,73]. Both prospective and retrospective studies have identified an association between shorter breastfeeding duration and an increased risk of developing ADHD in childhood [74-77], suggesting that components within breastmilk may influence attentional regulation and behavior control in offspring. Observational studies consistently demonstrate that breastfed infants perform better on cognitive tests compared to formula-fed infants, particularly in language, problem-solving, and memory domains [23-25,78-80]. These advantages are often attributed to the unique composition of breastmilk, including its fatty acid and human milk oligosaccharide content, which support gut-brain communication and neurodevelopment [79-84].

Animal models have demonstrated that breastfeed and maternal behaviors that coincide with this period, such as licking, grooming, and arched-back nursing postures, can have an influence on offspring brain development [85-88]. Rodent offspring that experienced prolonged arched-back nursing, as well as licking and grooming, displayed elevated NMDA receptor subunit expression

in the hippocampus and enhanced long-term potentiation, indicating improved synaptic plasticity compared to those with less exposure [86]. This was coupled with a superior performance in the novel object recognition test and Morris water maze, suggesting that increased periods of nursing may improve spatial learning and memory by enhancing NMDA-mediated neuroplasticity in the hippocampus [86,87]. A study by Liu et al. found similar results in rats, where offspring of mothers that engaged in more frequent nursing, licking, and grooming exhibited higher expression of hippocampal NMDA receptor subunits and BDNF mRNA, along with increased cholinergic innervation of the hippocampus [88]. These neurobiological changes were accompanied by enhanced performance in the Morris water maze, indicating improved cognitive function [88]. Early weaned male mice offspring also had decreased BDNF protein expression and neurogenesis in the hippocampus compared to normally-weaned mice [85].

These findings suggest that the breastfeeding period, along with the associated maternal behaviors, represents a critical window in early development that plays a key role in shaping neurodevelopment and cognitive outcomes. The physical interactions associated with breastfeeding influence hippocampal plasticity, synaptic connectivity, and long-term potentiation, contributing to enhanced memory and learning. Studies with animal models emphasize that variations in maternal caregiving behaviors during the breastfeeding period can lead to measurable differences in neurobiological markers and cognitive performance. Future research should explore the mechanisms underlying these behavioral influences, as well as how early maternal-infant interactions during breastfeeding shape long-term neurodevelopmental trajectories.

Energy intake

Adequate calorie and total energy intake during the perinatal period is essential to support the rapid growth and maturation of the brain, which is particularly metabolically demanding during

fetal and early postnatal stages [26,89]. Energy intake plays a fundamental role in brain development, providing the necessary resources to support neurogenesis, synaptogenesis, and myelination, all of which are essential for cognitive function [26], and the high energy requirements of the brain is fulfilled by a constant transport of nutrients via the blood-brain barrier [26,90]. Glucose, derived from dietary carbohydrates, serves as the brain's primary energy source, fueling processes such as neurotransmitter synthesis, neuronal signaling, and synaptic remodeling [26]. A more in depth discussion into the role of glucose will be covered in a later section. During early postnatal development, the brain undergoes rapid maturation, with significant growth in axons, dendritic arborization, synapse formation, and myelination, all of which contribute to heightened metabolic needs. Brain energy demands peak between ages 2 and 3, aligning with maximal synapse formation and myelination, emphasizing the critical role of adequate energy intake during this period to support cognitive development.

Caloric deficiency during early life, as seen in conditions such as IUGR or childhood undernutrition, has been consistently associated with impaired cognitive outcomes [91]. Epidemiological studies reveal that prenatal caloric restriction is linked to reduced brain size, altered cortical development, and lower cognitive performance in offspring [92]. For example, data from the Dutch Hunger Winter study demonstrated that individuals exposed to caloric restriction in utero exhibited lower IQ and higher rates of cognitive dysfunction later in life [93]. Similarly, inadequate caloric intake during early childhood is associated with deficits in attention, memory, and executive function. Stunting, a marker of chronic undernutrition [94], has been shown to correlate with reduced school performance and lower cognitive test scores [94-97]. For instance, the Cebu Longitudinal Health and Nutrition Survey in the Philippines revealed that children with lower energy intake in infancy had reduced cognitive performance, indicated by a

diminished IQ, at school age [98]. Similarly, a study from the MAL-ED cohort, spanning multiple countries, highlighted that children with recurrent episodes of growth faltering due to undernutrition performed poorly on cognitive assessments measuring language development and non-verbal reasoning [99-103]. These findings emphasize that insufficient caloric intake not only hampers physical growth but also critically impairs cognitive development, with effects that can persist in later life stages.

Caloric sufficiency not only provides the energy required for basic cellular processes but also facilitates the synthesis of essential macromolecules and the development of complex neural networks [104,105]. To study the effects of maternal undernutrition, pregnant rabbits were subjected to a 70% reduction in basal food intake beginning at day 22 of pregnancy [106]. This resulted in reduced birth weight, where offspring exhibited impaired neurobehavioral performance. In rhesus macaques models, perinatal caloric restriction has been shown to compromise fetal cerebral development, including widespread postnatal reductions in white matter fractional anisotropy and reduced gray matter cerebral volume [107], which is associated with observed diminished learning abilities [108,109]. Studies in malnourished rat pups demonstrated decreased hippocampal neurogenesis [36,110] and impaired long-term potentiation [36,111-113]. Perinatal caloric deficiency has also been associated with disruptions in BDNF signaling [111], a key regulator of neuronal survival [114], differentiation [115], and synaptic modulation [116,117]. Paredes et al. demonstrated that BDNF deficiency in the neonatal hippocampus of rats led to behavioral changes, emphasizing how early BDNF disruptions due to caloric insufficiency can have enduring epigenetic and cognitive consequences [118]. Behavior assessments in rodent models show that these structural and neurodevelopmental abnormalities, including to neurochemical systems, correlate with impaired performance in tasks measuring

spatial memory, such as the Morris water maze, and deficits in problem-solving and social interaction paradigms [119-124]. For example, malnourished rat pups impaired spatial learning and memory behaviors in the Morris water maze which was accompanied by decreased density in the hippocampus of neuronal cells positive with nitric oxide synthase, an enzyme involved in neuronal survival and synaptic plasticity [121]. These findings emphasize the critical role of adequate nutrition in supporting neurodevelopmental processes that underpin cognitive functions.

On the other hand, caloric excess and maternal obesity during the perinatal period may serve as a critical factor in shaping offspring cognition and mental health. A review by Shook et al. revealed that children born to mothers with obesity during pregnancy exhibited higher rates of cognitive impairments, including difficulties with executive function, attention regulation, and language development [125]. This was seen in a Swedish population-based prospective pregnancy-offspring cohort study that followed children until the age of 5 and found that maternal pre-pregnancy overweight and obesity were associated with increased inattention symptoms in childhood [126]. Supporting these findings, Norr et al. demonstrated that elevated maternal BMI was associated with poorer cognitive outcomes in offspring, particularly in areas related to behavioral regulation and problem-solving skills, observed as early as the infant stage and persisting into early childhood [127]. Furthermore, an analysis of data from participants in Project Viva, a prospective longitudinal pre-birth cohort study, demonstrated that maternal obesity was linked to lower visual-motor integration scores in children, a key predictor of academic achievement and cognitive development [128]. These findings highlight consistent associations between maternal obesity and early cognitive deficits in offspring, including attention problems, executive dysfunction, and reduced academic-related cognitive skills.

Studies using animal models have been conducted to explore how excess caloric intake during the perinatal period affects brain development, synaptic plasticity, and cognitive function in offspring. Rivera et al. examined the effects of a perinatal high-caloric diet on prefrontal cortex function in a rat model, assessing anxiety responses and glutamatergic and GABAergic receptor expression, and it revealed that male offspring exposed to the diet both prenatally and post-weaning exhibited increased anxiety-like behaviors linked to glutamate receptor overexpression in the prefrontal cortex [129]. Early life overfeeding in rodents impaired episodic-like memory [130] and spatial memory performance, which is coupled with reduced ability to regulate neurons and microglia in the context of a learning task [131]. These findings provide strong evidence that excessive caloric intake during critical developmental windows can have lasting consequences on cognitive and emotional regulation in offspring.

Acceptable Macronutrient Distribution Range

The Acceptable Macronutrient Distribution Range (AMDR) provides dietary guidelines for the proportion of daily caloric intake derived from carbohydrates, proteins, and fats, primarily aimed at promoting overall health and reducing the risk of chronic disease [132]. A longitudinal study utilizing data from the Eunice Kennedy Shriver National Institute of Child Health and Human Development examined macronutrient intake among pregnant women in the U.S and assessed if it was within AMDR for pregnant women [132]. The findings revealed that while most women met the AMDR of 10-35% for protein, approximately one-quarter of these women consumed carbohydrates below the AMDR of 45–65%, and nearly half exceeded the AMDR for total fat intake of 20–35% [132]. Although deviations from the AMDR have been extensively studied in relation to long-term health outcomes [133], their specific implications for fetal neurodevelopment remain unclear, highlighting the need for further research in this area.

However, as the aforementioned research has demonstrated, both insufficient and excessive intake of energy-yielding macronutrients during early life can significantly impact brain development and cognitive outcomes [89]. Understanding how deviations from the AMDR during critical developmental periods impact cognitive and neurological outcomes over time is essential. Future research should focus on refining macronutrient recommendations tailored to the unique metabolic demands of early neurodevelopment.

Carbohydrates

Carbohydrates serve as the primary energy source for the developing brain, playing a crucial role in neurodevelopment, neurotransmitter synthesis, and cognitive function during early life [134-137]. In the human brain, glucose, which is derived from dietary carbohydrates, is the primary energy source, and the developing brain has a disproportionately high glucose requirement, with up to 60% of the total energy intake in infancy dedicated to brain function [135-137]. Glucose transport across the blood-brain barrier is mediated by glucose transporter proteins (GLUT) [136,138], and disruptions in glucose availability have been coupled to cognitive deficits [136]. Beyond energy metabolism, glucose supports critical neurodevelopmental processes such as neuronal proliferation [136,139], differentiation [139-141], and synaptic formation and transmission [136,142]. Adequate glucose availability ensures proper myelination, a process crucial for efficient signal transmission between neurons, particularly in the rapidly growing fetal and neonatal brain [136,142]. Moreover, glucose metabolism plays a vital role in neurotransmitter synthesis, as it provides the fuel and the precursors for key neurotransmitters, including glutamate, essential to learning, memory, and executive function [30,143].

Gestational diabetes mellitus (GDM) is a pregnancy-related metabolic disorder characterized by elevated blood glucose levels due to impaired cellular glucose uptake [136,144,145]. It is widely

recognized as a risk factor for adverse developmental outcomes, as excessive maternal glucose exposure can disrupt fetal growth and neurodevelopment [136,144-146]. Intrauterine exposure to abnormal glucose levels has been linked to potential adverse effects on cognitive and behavioral development, though some findings are conflicting [144,147]. A study using diffusion tensor imaging (DTI) found that infants born to mothers with gestational diabetes exhibited white matter abnormalities, which were associated with impaired neurocognitive performance compared to hyperglycemia-unexposed controls [148]. Additionally, a study of 5,126 mother-child pairs found that eight-year-old children born to mothers with GDM had lower verbal IQ scores on the Wechsler Intelligence Scale for Children-Third Edition [149], whereas another study of 785 mother-child pairs reported higher scores in learning, verbal ability, and long-term retrieval among children of mothers with GDM [147]. Differences in cognitive assessment tools, such as the Kaufman Assessment Battery for Children-Second Edition (KABC-II) and the Kohs Block Design Test, may contribute to these inconsistencies. Two systematic reviews concluded that the relationship between maternal diabetes and offspring cognitive development remains unclear [144,146], emphasizing the need for large-scale prospective studies that account for potential confounders.

There is increasing evidence in animal models that support high maternal intake of glucose and GDM can inhibit fetal neuronal development and impair cognition in offspring. Vuong et al. found that maternal obesity associated with gestational diabetes in rats led to offspring neuroinflammation, increased pro-inflammatory cytokines (IL-1 β , TNF- α), reduced hippocampal synaptophysin expression, and impaired recognition memory [150]. Another study found a significant reduction in hippocampal size and cellularity in the offspring of diabetic rats, suggesting that elevated maternal glucose levels during neurodevelopment increase neuronal

vulnerability, potentially contributing to learning and memory impairments [151]. Offspring of diabetic rats have shown heightened apoptosis of hippocampal neurons, particularly in regions essential for learning and memory [152], and a decrease in insulin-like growth factor 1 receptor levels was observed in the cerebellum of rats born to diabetic mothers, reinforcing the idea that maternal diabetes negatively impacts motor and cognitive development in offspring [153]. A rodent study of maternal diabetes has been shown to decrease hippocampal BDNF expression, increase TNF- α levels, impair cellular proliferation, and enhance apoptosis in offspring, contributing to neurodevelopmental deficits [154]. Similarly, reduced hippocampal BDNF levels observed in offspring of obese mothers have been linked to cognitive impairments, highlighting how metabolic disturbances during pregnancy can disrupt key pathways of brain homeostasis [155]. Collectively, these findings suggest that elevated maternal glucose levels during pregnancy can impair fetal brain development by disrupting neuronal growth, increasing neuroinflammation, and altering key neurotrophic signaling pathways, ultimately leading to cognitive and motor deficits in offspring.

Dietary fiber, particularly prebiotic fibers, plays a crucial role in shaping brain function by modulating the gut microbiota and influencing neuroinflammatory pathways [134]. The microbiota-gut-brain axis is an emerging area of research demonstrating how dietary components influence cognition through microbial metabolites, immune signaling, and neurotransmitter modulation [156,157]. Prebiotic fibers support beneficial gut bacteria, which produce short-chain fatty acids (SCFAs) like acetate, propionate, and butyrate, playing a crucial role in cognitive function and brain health [157]. Epidemiological studies involving maternal fiber intake and its impact on offspring brain development and function are limited, but there is some evidence to suggest that a low-fiber diet during the perinatal period may impair cognitive outcomes in

offspring [158]. Data analyzed from the Japan Environment and Children's Study, an ongoing nationwide prospective birth cohort study, revealed that low maternal dietary fiber intake was associated with a higher risk of delayed communication, problem solving, fine motor, and personal-social skills compared to the highest intake group [158]. Another study in humans found that maternal serum levels of propionate and butyrate were closely correlated with those in umbilical cord blood, suggesting that maternal SCFA bioavailability may influence fetal neurodevelopment [159]. Supporting this, a mouse study showed that offspring of mothers on a low-fiber diet exhibited motor impairments, learning and memory deficits, increased anxiety-like behavior, and altered hippocampal glutamate receptor expression, which were linked to gut microbiome shifts and reduced SCFA levels [159]. Butyrate supplementation during gestation, however, mitigated these effects, likely by downregulating hippocampal histone deacetylase 4 expression [159]. It has also been demonstrated in a mouse model that a high-fiber diet mitigates maternal obesity-induced cognitive dysfunction in offspring via the gut-brain axis [156].

Proteins

Proteins contribute to the structural integrity and functionality of neurons, glial cells, and the extracellular matrix, and adequate protein intake during early life is therefore crucial for supporting cognitive development. Sloan et al. has previously reported that both low and high protein intake during pregnancy has a quadratic relation with fetal growth [160]. Experimental studies suggest that children born to mothers who consumed high-protein diets and energy-rich beverages exhibited superior intellectual abilities, including enhanced information processing, numeracy, and vocabulary, compared to control children [161]. An examination of early protein intake on preterm-born children found that increased protein consumption in the first weeks of life was associated with enhanced functional connectivity between the thalamus and the default

mode network, which are integral to cognitive processing [162]. This increased connectivity was associated with higher processing speed and visual-motor skills at age seven, suggesting a direct impact of early protein intake on longer-term neurocognitive outcomes [162]. One study investigating maternal macronutrient intake on offspring cognitive and behavioral outcomes at age four found that child externalizing behavior was positively trending with log-transformed maternal protein intake, but this was not statistically significant [89]. There seems to be a gap in evidence pertaining to human subjects and maternal protein intake, thus, additional studies are warranted to further substantiate a connection.

Animal models have shown that protein restriction during the gestation and lactation period impairs the micro-structure of the fetal brain, altering cortical and hippocampal development [163-167]. These structural changes are associated with deficits in neuronal proliferation and dendritic arborization, critical processes for establishing neural networks [168]. Neurotrophic growth factors such as BDNF are critical to optimal neurodevelopment, and levels of BDNF have been observed to be decreased in mice and rat offspring from protein-deficient mothers [167,169-171]. Protein restriction during fetal development has also been shown to alter hippocampal neurogenesis [36,37], leading to reduced levels of insulin-like growth factor [167], as well as decreases in brain and neuronal volume [172] in rodent models. These physiological impairments translate into cognitive deficits, as protein restriction has been shown to correlate with diminished cognitive and behavioral function in offspring [173-175]. Studies in rodents indicate that a maternal low-protein diet during pregnancy and lactation impairs associative learning and reduces motivation in the offspring [175]. These findings underscore that perinatal protein restriction disrupts key neurodevelopmental processes, such as neuronal proliferation, dendritic arborization, and neurotrophic signaling, leading to structural brain impairments that

manifest as cognitive deficits in both animal models and epidemiological studies, reinforcing the critical role of adequate early protein intake for lifelong cognitive health.

Fats

Lipids are fundamental to brain development, serving as structural components of neuronal membranes, regulators of synaptic function, and precursors for signaling molecules that influence cognitive processes [176-178]. The brain is composed of nearly 60% fat, with long-chain polyunsaturated fatty acids (PUFAs), playing critical roles in neurogenesis, myelination, and synaptic plasticity [81,177,179,180]. Additionally, lipids contribute to myelin sheath formation, facilitating rapid signal transmission between neurons, which is crucial for cognitive function and information processing [177,181,182]. Beyond structural roles, lipid metabolism influences inflammatory signaling in the brain, with an imbalance in fatty acid composition linked to neuroinflammation and cognitive dysfunction [176,183-186]. Given the brain's reliance on lipids for both structural integrity and functional regulation, adequate fat intake during early life is essential for optimizing neurodevelopment and cognitive outcomes.

The type and quantity of maternal dietary fat intake can significantly shape neurodevelopmental trajectories, impacting memory, learning, and behavioral outcomes [187]. A high fat diet is typically comprised of saturated fats, and the Western-style diet generally falls into this category. Research suggests that children born to mothers with high-fat, Western-style diets during pregnancy exhibit poorer cognitive performance [128,188], lower IQ scores [189,190], and increased risk of neurodevelopmental disorders such as ADHD [188,191-196]. A study using data from the Generation R cohort revealed that maternal consumption of diets high in fats during pregnancy was associated with lower IQ in school-aged children [197]. Additionally, maternal obesity, which is often linked to high-fat dietary patterns, has been correlated with

altered brain functional connectivity [198-200]. Studies indicate that maternal high-fat intake contributes to gestational diabetes [145,201], which has been linked to poorer cognitive function [146,202] and increased neuropsychiatric risk in offspring [201,203], as mentioned earlier in this discussion. Studies also indicate that early consumption of diets high in saturated fats lead to slower cognitive processing speeds and a compromised capacity to flexibly modulate during cognitive tasks [204].

Maternal obesity is commonly simulated in animal models by feeding adult females a high fat diet (HFD), thus, investigations into the effects of maternal obesity and diet marked by increased fat intake on offspring cognition are commonly interchangeable [125,191,196]. Maternal high-fat diet influences both prenatal and postnatal neurodevelopment in offspring, leading to molecular, cellular, and structural brain alterations observed across fetal, juvenile, and adult stages in mice born to HFD-fed dams [191]. Male and female rodent offspring from mothers fed a HFD diet exhibited impaired long-term memory as evidenced through a reduced preference for novel objects in the novel object recognition test [156,184,205] and a reduced preference for a stranger mouse in the three-chamber test [156,206,207]. Offspring exposed to a HFD during the perinatal period exhibited decreased retention times, higher escape latencies, and less time spent in the target quadrant of the Morris water maze, suggesting diminished spatial memory capacity [155,186,205,208-210]. Other studies on maternal HFD and offspring cognition have yielded mixed results. One report found that rat offspring from HFD-fed dams had better performance in the Morris water maze [211] and spatial memory task [185] compared to control rats, as well as enhanced spatial memory in young piglet offspring exposed to HFD during the perinatal period [212,213]. These discrepancies may relate to timing of dietary exposure, sex, and exact age of offspring during the testing period. The cognitive deficits in offspring from HFD-fed dams

seemingly stems from underlying mechanisms within the hippocampus, prefrontal cortex, and other integral areas of the brain. Offspring exhibited decreased myelination in the prefrontal cortex [184], reduced hippocampal BDNF production [155,205] and impaired neurogenesis, leading to delayed spatial learning [155]. However, some cognitive deficits did not persist into adulthood, suggesting that the effects of maternal high-fat diets on exploratory behaviors may be most pronounced during early development [155]. Maternal and postweaning HFD also downregulated expression of plasticity-related genes in the hippocampus which was coupled with impaired spatial learning and memory [186,208]. These findings suggest that maternal high-fat diet exposure can significantly influence offspring cognitive outcomes, likely through alterations in hippocampal and prefrontal cortex function, neurogenesis, and synaptic plasticity. While deficits in spatial memory and object recognition are commonly observed, inconsistencies in results highlight the role of factors such as sex, timing of dietary exposure, and age at testing. Some cognitive impairments appear transient, indicating a potential window for recovery, but the long-term impact of maternal HFD on neurodevelopment warrants further investigation.

Research on the role of monounsaturated fatty acids (MUFAs) in early neurodevelopment and cognitive outcomes remains limited. Most studies have focused on oleic acid, which is produced endogenously but also obtained through dietary sources [214]. Oleic acid functions as a neurotrophic factor in early development, facilitating neuronal differentiation, neurite extension, neuronal migration, and synapse formation [215-218]. Another MUFA, nervonic acid, is a constituent of white matter lipids and plays a crucial role in myelin biosynthesis [219,220]. Nervonic acid is a natural component of breastmilk, implicating its role in nervous system development [220]. One study found that supplementation with MUFAs, including oleic, nervonic, and lignoceric acids, improved social interaction at one month and enhanced mental,

psychomotor, and behavioral development at 6, 10, and 18 months in preterm infants [219].

Despite these findings, the role of MUFAs in early neurodevelopment remains underexplored, warranting further research to clarify their specific contributions to cognitive outcomes and brain maturation.

Polyunsaturated fatty acids (PUFAs) can primarily be found in fish, nuts, seeds, and oils [2]. PUFAs and their derivatives are essential for optimal myelination, membrane development and function, and synapse function [3]. Docosahexaenoic acid (DHA) comprises 90% of the brain's omega-3 PUFAs, and it is derived from alpha-linolenic and linoleic acids, with other omega-3 and omega-6 fatty acids regulating its levels to support brain function [2,4]. Epidemiological studies suggest that maternal consumption of long-chain PUFAs during pregnancy is associated with cognitive and neural benefits in offspring, particularly in older children [161]. Low maternal DHA levels have been associated with an increased risk of altered neural development, while sufficient DHA intake has been linked to a lower likelihood of low visual acuity in infants [177]. Maternal adrenal acid levels, and omega-6 fatty acids, have also been identified as a key predictor of neural development, even in the absence of DHA deficiency [177]. Research suggests that maternal oily fish consumption during pregnancy correlates with higher stereo acuity in children at 3.5 years, likely due to DHA's role in the maturation of visual processing [178]. Normal maternal omega-3 levels have been associated with a lower risk of reduced IQ [178], better mental processing at age 4 [221], better sequential processing at age 7 [222], and improved verbal abilities [32]. Experimental studies also show that children of mothers who consumed diets rich in cod liver oil or DHA-containing oils during pregnancy scored higher on cognitive assessments [178]. Maternal DHA supplementation has been positively associated with improved infant outcomes at birth, particularly in reducing latencies of visual-evoked responses and

enhancing neuromotor development [33]. However, despite these findings, a meta-analysis by Gould et al. reported no significant differences in cognitive, language, or motor development between supplemented and non-supplemented groups, highlighting the need for additional research [223].

Studies using animal models have provided valuable insights into the role of PUFAs in early cognition that complement findings from human research. Inadequate maternal intake of omega-3 fatty acids in a primate model has been shown to decrease functional connectivity [224] and impair neurotransmitter metabolism, learning, and visual function [2]. Moreover, maternal omega-3 deficiency has been linked to reduced neuronal cell body size in key brain structures such as the parietal cortex and hypothalamus, with potential long-term consequences for neurogenesis, dendritic arborization, myelination, and synaptogenesis [225]. Learning deficits due to omega-3 deficiency are found in rats using the Morris water maze [226,227], and performance has been observed to be restored to control levels with extended repletion [226]. These findings were replicated using the Barnes maze, a less stressful escape task that does not require swimming, where omega-3-deficient rats showed significantly poorer performance, particularly when the escape path was reversed [183]. This impairment, which requires suppressing previously learned information and developing new strategies, highlights deficits in hippocampal-dependent learning and frontal cortex function [183]. Another study showed that rats fed an omega 3-deficient diet through pregnancy and lactation led to impaired learning in avoidance tests, which was coupled with significantly lower levels of noradrenaline in the cerebral cortex, hippocampus, and striatum [228]. Conversely, excessive maternal PUFA intake may also negatively impact offspring, as studies in mice and rats have associated high PUFA exposure with reduced hippocampal neurogenesis, altered stress responses, and anxiety-like behaviors, such as

thigmotactic behavior [229]. Furthermore, while the PUFA arachidonic acid indirectly supports brain development by regulating synaptic plasticity, long-term potentiation, and spatial learning via prostaglandin synthesis, docosapentaenoic acid (n-6) cannot replace DHA in promoting neurite outgrowth and formation [2,230,231]. Together, these findings highlight the delicate balance required in maternal PUFA intake, as both deficiencies and excesses can have lasting implications for offspring neurodevelopment and cognitive function.

CONCLUSION

This literature review provides evidence that underscores the critical influence of early-life nutritional status on neurodevelopment and cognitive outcomes, emphasizing the specific influence of placental function, breastfeeding, and energy-yielding macronutrient intake. The placenta serves as the primary conduit for fetal nutrient supply, regulating the availability of essential substrates that drive key neurodevelopmental processes for the developing baby. Postnatally, breastfeeding extends the impact of maternal physiology through bioactive compounds and behavioral interactions that support brain maturation and cognitive function in offspring. Energy intake, derived from macronutrients, is fundamental for providing both the metabolic fuel and structural components integral to brain development. While research has extensively explored the detrimental effects of imbalanced macronutrient intake, including maternal obesity, gestational diabetes, and protein malnutrition, future studies are warranted to elucidate optimal macronutrient distribution for neurodevelopmental benefits.

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CHAPTER 3

THE EFFECT OF PERINATAL SUPPLEMENTATION OF DHA ON SPECIALIZED PRO- RESOLVING LIPID MEDIATORS IN THE BRAIN OF OFFSPRING ¹

¹ Dubrof S, Zukaitis J, et al. The effect of perinatal supplementation of DHA on specialized pro-resolving lipid mediators in the brain of offspring. Submitted to the *Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids*, 3/14/2025.

ABSTRACT

The perinatal period is a critical time for fetal neurological development which depends on omega-3 polyunsaturated fatty acids (PUFA) for fundamental processes. Omega-3 PUFA, including docosahexaenoic acid (DHA), are precursors to a novel class of bioactive metabolites called specialized pro-resolving mediators (SPMs), which have been suggested to have a dual purpose in mitigating neuroinflammation while simultaneously supporting cognitive outcomes, implicating a role in offspring neurodevelopment. DHA is evidenced for its role in early brain development, but the underlying mechanism it exerts its cognitive benefits remain unclear. Pregnant sows were fed a control diet (CON; n=6) or a diet with DHA (n=6, 75 mg DHA/kg BW/day) from gestation through lactation. At weaning, piglets (n=2/sow) underwent resting state-functional magnetic resonance imaging (rs-fMRI) to assess brain functional activation. Subsequently, brain tissue from prefrontal cortex, cerebellum, and hippocampus were collected from piglets. Tissue DHA and eicosapentaenoic acid (EPA)-derived SPMs were quantified using LC-MS. Levels of SPMs were higher in the brains of piglets from DHA-fed sows, particularly in the prefrontal cortex and cerebellum, compared to control piglets. Additionally, a distinct association of several prefrontal SPMs with activation of the cerebellar functional network was marked in the piglet offspring. The findings highlight a potential for SPMs to function as mediators for neurodevelopmental programming, through contributing to inflammation resolution and neuronal connectivity. This work underscores the importance of maternal nutrition in shaping offspring brain health and lays the groundwork for targeted interventions leveraging the benefits of DHA and its bioactive metabolites.

INTRODUCTION

The perinatal period is critical for fetal neurological development and relies heavily on polyunsaturated fatty acids (PUFAs) [1-3]. Docosahexaenoic acid (DHA), the predominant omega-3 PUFA in the brain, comprises over 40% of the omega-3 content in neuronal phospholipids and accumulates throughout cortical expansion and maturation during development [4, 5]. Since *de novo* PUFA synthesis is absent, DHA supplementation is recommended for pregnant mothers to promote endogenous production and enhance its incorporation into the developing brain [6]. Post-natal DHA accumulation in infants is primarily supported via breastmilk, with compelling evidence indicating that maternal dietary DHA intake consistently reinforces its incorporation into the offspring's brain [7, 8].

Omega-3 PUFA, including DHA, are precursors to a novel family of bioactive metabolites known as specialized pro-resolving mediators (SPMs) [9-11]. SPMs contribute to the crosstalk between glial cells and neurons, as they affect microglia activation [9-11], which can alter the course of events from a pro-inflammatory to an anti-inflammatory status, thus giving it their neuroprotective effects and the potential to influence neurodevelopment. Recently, SPMs have garnered attention for their biological function, with numerous *in vivo* and *in vitro* studies, as reviewed by Weylandt et al. [12] and Serhan et al. [3, 13, 14], highlighting their potent pro-resolving and anti-inflammatory properties. Pregnancy is associated with an increase in systemic and placental inflammation [15], and mechanistic links between maternal inflammation and the risk of neurodevelopmental conditions in offspring have been established [16, 17]. Several SPMs have been detected in the placenta, indicating the potential for placental inflammation to be mediated via the resolution-promoting and inflammation-dampening mechanisms that are characteristic of SPMs [15, 18]. High concentrations of SPMs and SPM pathway markers have

also been quantified in human breastmilk [19]. Moreover, human breast milk isolates accelerated resolution of acute inflammation and infection *in vivo* with isolated human leukocytes, implicating a biological role of SPMs in early neonatal development [20]. Recent studies have identified correlations between cognitive assessments and SPM levels in the cerebrospinal fluid of patients with Alzheimer's disease [21], while postmortem analyses have revealed reduced SPM levels in hippocampal tissue of those with the disease [22]. Notably, in a mouse model of Alzheimer's disease, treatment with SPMs mitigated cognitive decline while also downregulating levels of pro-inflammatory proteins and cytokines in favor of anti-inflammatory ones [23]. These findings collectively suggest that SPMs play a dual role in mitigating neuroinflammation while simultaneously supporting cognitive outcomes.

DHA has been well investigated for its beneficial role in early brain development and influencing cognitive outcomes, but the underlying mechanism through which it exerts its cognitive benefits remain unclear [6, 24]. One potential mechanism may involve the metabolism of DHA into SPMs and SPM pathway markers, and evidence suggests that endogenous synthesis of SPMs may be amplified by increasing substrate supply via dietary supplementation of DHA [18, 25].

The pig is proposed as an effective translational model for studying the effects of maternal nutritional influence on offspring neurodevelopment, given its physiological and anatomical similarities to humans [26]. Both pigs and humans possess a gyrencephalic brain, a feature that enhances neurological complexity by influencing brain activation and function [27]. Additionally, both pig and human brains consist of over 60% white matter [28, 29]. Pigs and humans exhibit similar brain growth patterns, with rapid brain development occurring from the last trimester through lactation in humans and from 50 days pre-farrowing to 40 days post-birth

in pigs [30, 31]. The nutritional needs of piglets also resemble those of human infants [32]. These key parallels make the pig an ideal model for our study.

This study investigated the vertical transfer of perinatally supplementing DHA on SPMs in various brain regions of piglet offspring. A previous study from our laboratory found that perinatally supplementing DHA modulated functional network activation in the brain of offspring [24]. This study further explores the potential association between SPMs and functional network activation in the brain. By exploring this potential mechanistic link, we seek to provide greater insight into how DHA influences cognitive outcomes.

METHODS

Animals and study design

Cross-bred healthy Landrace sows (n=12) were assigned to the DHA (DHA, n=6) or control group (CON, n=6) after accounting for parity and body weight. Sows were fed their corresponding diets from day 70 of gestation and throughout lactation. At farrowing, 1 male and 1 female piglet (CON, n=12; DHA, n=12) nearest the litter's average birth weight were selected to undergo testing for data collection. At weaning (21 days), the selected piglets (n=24) were sacrificed for brain tissue collection. This study was conducted in accordance with the University of Georgia Institutional Animal Care and Use Committee guidelines (Animal Use Protocol: A2021 01-026).

All sows were fed a basal diet (2 kg/day during gestation; ad libitum during lactation) that met the metabolic requirements of maternal health and maximized fetal growth according to the National Research Council's recommendations for swine nutrient requirements (**Table 1**) [33]. DHA sows were supplemented 75 mg/kg BW/day of algae-derived DHA (contains 44.6% DHA, life'sDHA S40, DSM Nutritional Products, Inc., Kingstree, SC, USA) mixed into the basal diet.

The dosage of DHA was chosen based on our previous study and other findings that this amount of DHA effectively exerts beneficial health impacts on pregnant sows and piglets [24, 34]. CON sows were fed an isocaloric basal diet mixed with safflower oil (Jedwards International Inc., Braintree, MA, USA), rich in omega-6 fatty acids, primarily linoleic acid (68.46%), with negligible omega-3 content [35].

Fatty acid analysis of colostrum

Colostrum was collected within 12 hours of farrowing from the same teats of all sows. Fatty acid composition analysis was performed using combined gas chromatography-mass spectrometry (GC-MS) as described previously by Patel et al. [36]. Briefly, one weighted drop of colostrum sample was heated with 1 M methanolic HCl in a sealed tube for 14 h at 80 °C. After removal of the solvent, samples were dissolved in water and chloroform. The chloroform phase was transferred to a new tube, concentrated to dryness under nitrogen, and redissolved to make a 10-mg/ml solution in hexane that was injected into the Agilent 7890A GC instrument (Santa Clara, CA, USA).

SPM analysis in brain tissue

At weaning (21 days), study piglets were euthanized via CO₂ asphyxiation. Brains were resected and coronally divided into 2-mm-thick slices using a piglet brain slicer (Zivic Instruments, Pittsburgh, PA, USA). Fresh brain tissue slices were individually placed on microscope slides and flash frozen in liquid nitrogen. Micropunches of prefrontal cortex, cerebellum, and hippocampus were acquired from frozen brain tissue slices using a stereotaxic atlas of the porcine brain as reference [37]. Samples were stored in –80 °C until analysis.

Brain tissue samples were subjected to LC-MS analysis using methods previously described [38, 39]. Briefly, the internal standard-spiked samples were loaded onto C18 cartridges, washed

with 15% methanol in water and hexane, and vacuum-dried. Cartridges were eluted with methanol, and the eluate was dried under nitrogen. The residue was dissolved in 50 μ l methanol/25 mM ammonium acetate (1:1) for LC-MS analysis. HPLC was performed on a Prominence XR system (Shimadzu, Kyoto, Japan). The eluate was introduced to a QTRAP5500 mass analyzer (AB Sciex, Framingham, MA, USA) (negative ion mode) and analyzed for lipid mediators, using optimized collision energies (18-35 eV) and exit potentials (7-10 V). Spectra for each peak were confirmed using Enhanced Product Ion scans. Data was collected with Analyst 1.6.2 (AB Sciex, Framingham, MA, USA) and quantified using MultiQuant (AB Sciex, Framingham, MA, USA). Internal standard signals were used for normalization, recovery, and relative quantitation of each analyte.

The measured DHA-derived metabolites included protectins, maresin, D-series resolvins (RvD1), and hydroxydocosahexaenoic acids (HDHAs). The EPA-derived metabolites analyzed were E-series resolvins (RvE1) and hydroxyeicosapentaenoic acids (HEPEs).

Functional Magnetic Resonance Imaging

Previously, our lab reported that perinatally supplementing DHA altered brain functional activation in piglet offspring as assessed through resting-state functional magnetic resonance imaging (rs-fMRI) [24]. Piglets in the current study also underwent rs-fMRI at weaning (postnatal day 21) as previously described with modifications on data analysis [40-42]. Briefly, the data was corrected for head motion and slice timing, then registered to a reference template [41]. A binary mask created with 3D Slicer (v4.11, 2021) was applied to remove non-brain signals from the data. The independent components (ICs), representing baseline functional activation maps, were generated using group independent component analysis on CON piglets. FSL's dual regression function was then applied to reconstruct ICs for each individual. Pearson

correlations were calculated for eight resting-state networks: executive control, cerebellar, visual, sensorimotor, auditory, default mode, salience, and basal ganglia networks.

Statistical analysis

Data is expressed as mean $\pm$ S.E.M. and analyzed using RStudio (Version 2023.03.0+386, R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism (Version 10.1.0, GraphPad Software, Inc.; San Diego, CA, USA). Treatment effects were investigated using a linear mixed-effect model to control for sex as a fixed effect and for maternal (sow) as a random effect. Significance was determined at a significance level of 0.05.

RESULTS

Fatty acid composition of colostrum

The overall fatty acid profile of the colostrum samples (n=12) contained high relative percentage levels of palmitic acid (C16:0, 23.5542 ± 0.6692), oleic acid (C18:1, 34.2708 ± 0.8039), and linoleic acid (C18:2, 27.5320 ± 2.0313), which is similar to that of human colostrum [43]. DHA-supplemented sows (n=6) had significantly greater amounts of DHA (C22:6, undetected and 0.7217 ± 0.3593 for CON and DHA, respectively) and docosapentaenoic acid (DPA; C22:5, 0.1000 ± 0.000 and 0.1480 ± 0.0634 for CON and DHA, respectively) compared to CON (n=6) sows ($p < 0.0001$), demonstrating that the maternal DHA intake vertically transferred from colostrum to piglet offspring. Omega-6 and omega-3 PUFAs are competitively metabolized by the same set of enzymes, thus, supplementing an omega-3 PUFA such as DHA often results in diminished levels of omega-6 PUFAs [44]. Indeed, relative percentage levels of linoleic acid, an omega-6 PUFA, were lower in DHA-supplemented sows compared to CON sows (C18:2; 32.4800 ± 1.4891 and 26.5583 ± 1.2400 for CON and DHA, respectively; $p = 0.0121$).

Docosahexaenoic (DHA)-derived specialized pro-resolving mediators

Protectins

The results showing the effect of perinatal DHA supplementation on DHA-derived protectin family metabolites in the brain of offspring are shown in Figure 1. Protectin D1 (PD1) is biosynthesized from DHA and has been observed to exhibit potent neuroprotective actions in the brain and retina [45]. PD1 levels in the prefrontal cortex were 115.63% higher in piglets from DHA-fed sows compared to CON piglets ($p=0.0443$), while there was no difference observed in the cerebellum or hippocampus ($p>0.05$) (**Figure 1A**). Protectin DX (PDX) is a positional isomer of PD1, sharing some overlapping but distinct effects on specific target cells [45]. Aspirin triggered-PD1 (AT-PD1) is an epimer of PD1 that also displays comparable potent protective bioactions [45]. With DHA supplementation, PDX levels in the cerebellum were again significantly higher compared to CON piglets ($p=0.0177$). There was no difference in the prefrontal cortex or hippocampus ($p>0.05$) (**Figure 1B**). No significant difference were observed for AT-PD1 across the three brain regions ($p>0.05$) (**Figure 1C**). These results suggest a region-specific increase in protectin metabolites, chiefly in the prefrontal cortex and cerebellum, in response to perinatal DHA supplementation.

The distribution of protectin metabolites in the prefrontal cortex, cerebellum, and hippocampus in CON piglets was analyzed (**Figure 1D**). PDX and AT-PD1 levels were higher in the cerebellum compared to both the prefrontal cortex ($p<0.0001$ and $p=0.0007$ for PDX and AT-PD1, respectively) and the hippocampus ($p<0.0001$ and $p=0.0006$ for PDX and AT-PD1, respectively). PD1 had no regional differences ($p>0.05$). This indicates a potential regional preference for PDX and AT-PD1 accumulation in the cerebellum.

Hydroxydocosahexaenoic acids

HDHAs are DHA-derived pathway markers for protectins, resolvins, and maresins [46]. DHA-supplemented piglets exhibited markedly higher levels of the five HDHA metabolites, including 4-, 7-, 13-, 14-, and 17-HDHA, in the prefrontal cortex and cerebellum compared to CON piglets ($p < 0.05$) (Figure 2A-E). Noticeably in the cerebellum of DHA piglets, 17-HDHA levels were particularly higher, measuring 3.4 times higher than piglets in the CON group (Figure 2E). Levels of HDHA metabolites within the hippocampus were not different between groups ($p > 0.05$). These results show that perinatal DHA supplementation selectively increased HDHA metabolites in the prefrontal cortex and cerebellum of offspring, suggesting a region-specific enhancement of these DHA-derived metabolites in response to maternal DHA intake.

Regional distribution of HDHA metabolites varied (Figure 2F). 4-HDHA, 7-HDHA, and 14-HDHA were higher in both the prefrontal cortex ($p = 0.0051$, $p = 0.0048$, and $p < 0.0001$, respectively) and cerebellum ($p = 0.0238$, $p = 0.0028$, and $p = 0.0029$, respectively) compared to the hippocampus. Conversely, 13-HDHA was higher in the hippocampus relative to the prefrontal cortex ($p = 0.0041$). 17-HDHA showed no significant regional differences in the three brain regions ($p > 0.05$). This data reflects regional variability, which may signify distinct metabolic or functional roles of these HDHA metabolites in early brain development.

Maresin and D-series Resolvin 1

Maresins are anti-inflammatory mediators that exhibit protection against nervous system disorders and oxidative stress [46]. DHA piglets exhibited a non-significant trend of higher Maresin1 in the prefrontal cortex and cerebellum compared to CON piglets ($p > 0.05$) (**Figure 3A**). The regional distribution of Maresin1 showed significantly higher levels in the prefrontal cortex compared to the hippocampus ($p = 0.0061$), implying an accumulation of Maresin1 in this brain region (**Figure 3B**).

No significant treatment effect of DHA supplementation on RvD1 levels amongst the three brain regions was observed ($p>0.05$) (**Figure 4A**). RvD1 was highest in the prefrontal cortex compared to both the cerebellum and hippocampus ($p=0.0049$ and $p=0.0061$, respectively) (**Figure 4B**), presenting a similar trend to Maresin1 of a distinct accumulation of this metabolite in the prefrontal cortex.

Eicosapentaenoic (EPA)-derived specialized pro-resolving mediators

Hydroxyeicosapentaenoic acids

Metabolites in the HEPE family demonstrated comparable trends as the DHA-derived metabolites in response to perinatal DHA supplementation. DHA-supplemented piglets showed significantly higher levels of 5-HEPE, 12-HEPE, and 18-HEPE in the prefrontal cortex and cerebellum compared to CON piglets ($p<0.05$) (**Figure 5A-C**). Interestingly, hippocampal 12-HEPE was also higher in DHA piglets ($p=0.0437$) (**Figure 5B**).

Regional distribution of the HEPE metabolites varied amongst the three brain regions (**Figure 5D**). For 5-HEPE and 18-HEPE, levels were significantly higher in the prefrontal cortex relative to the cerebellum ($p=0.0055$ and $p=0.0234$, respectively), with both metabolites seemingly lowest in the cerebellum amongst the three brain regions. By contrast, 12-HEPE was highest in the cerebellum compared to both the prefrontal cortex and hippocampus ($p=0.0019$ and $p=0.0008$, respectively).

E-series Resolvin 1

Perinatal DHA supplementation minimally influenced levels of RvE1, except for in the hippocampus, where it was unexpectedly lower compared to CON piglets ($p=0.0415$) (**Figure 6A**). Regional distribution of RvE1 in the prefrontal cortex with respect to the cerebellum and

hippocampus ($p < 0.0001$ and $p = 0.0016$, respectively) (**Figure 6B**), following a similar trend to the aforementioned 5-HEPE and 18-HEPE metabolites.

Association of specialized pro-resolving mediators with functional network activation

The rs-fMRI analysis in the current study revealed similar trends in results as previously reported [24]. An appreciable but non-significant ($p > 0.05$) increase in functional activation was observed for DHA piglets compared to CON piglets in the cerebellar network ($r = 0.3461 \pm 0.0154$ and $r = 0.3816 \pm 0.0183$ for CON and DHA, respectively; $p > 0.05$) and visual network ($r = 0.2993 \pm 0.0151$ and $r = 0.3199 \pm 0.0200$ for CON and DHA, respectively; $p > 0.05$). On the other hand, functional activation in the sensorimotor network were significantly lowered in DHA piglets compared to CON piglets ($r = 0.2840 \pm 0.0110$ and $r = 0.2450 \pm 0.0127$ for CON and DHA, respectively; $p = 0.0485$), which is consistent with previously reported findings for this network. Functional activations in the executive, auditory, default mode, salience and basal ganglia networks did not show any cross-group differences ($p > 0.05$).

An analysis of the association between SPMs and functional network activation revealed significant correlations for several metabolites (**Figure 7**). Cerebellar network activation was positively associated with PD1 in the prefrontal cortex ($r = 0.5193$, $p = 0.0093$) (**Figure 7A**). 4-HDHA ($r = 0.3312$, $p = 0.3312$), 7-HDHA ($r = 0.4501$, $p = 0.0273$), and 13-HDHA ($r = 0.2192$, $p = 0.0210$) in the prefrontal cortex also showed significant positive correlations with cerebellar network activation (**Figure 7D**). Similarly, prefrontal levels of Maresin1 and 5-HEPE were positively correlated with cerebellar network activation ($r = 0.6794$, $p = 0.0003$ and $r = 0.4578$, $p = 0.0245$ for Maresin1 and 5-HEPE, respectively) (**Figure 7E and F**). Interestingly, 5-HEPE in the prefrontal cortex was negatively correlated with sensorimotor network activation ($r = -0.4209$, $p = 0.0405$). SPMs in the cerebellum and hippocampus showed no significant correlations with

the eight functional networks in the brain of piglets ($p>0.05$). These results indicate that certain SPMs in the prefrontal cortex may be associated with functional network activation at this early stage of brain development.

DISCUSSION

This study is the first to examine the effects of perinatal DHA supplementation on SPMs across brain regions in offspring. Notably, piglets from DHA-fed sows exhibited higher SPM levels, particularly in the prefrontal cortex and cerebellum, compared to the CON group. Additionally, several prefrontal SPMs were distinctly associated with cerebellar functional network activation. These findings provide novel insights into SPMs during the perinatal period and suggest a potential mechanism linking DHA intake to cognitive outcomes.

While there is limited evidence on the presence and role of SPMs during the perinatal period, the available studies imply that these bioactive lipid mediators may play a critical role in maternal and fetal health [9, 11]. Previously, Mozurkewich et al. found that supplementing DHA-rich fish oil to pregnant women, resulted in a positive association of DHA-derived SPM pathway markers, including 4-HDHA, 14-HDHA, and 17-HDHA, with DHA levels in maternal blood [25]. Similarly, maternal omega-3 fatty acid supplementation significantly increased 17-HDHA and 18-HEPE in both the rat and human placenta compared to control groups [18, 47]. The presence of SPM pathway markers points toward an overall increase in basal production of their metabolites. Indeed, we observed that heightened HDHAs and HEPes was concomitant with increased levels of some SPMs, resembling what was previously observed in the rat placenta [18]. Potent SPMs have been observed to exhibit a short half-life, which can make detecting and quantifying in archived samples challenging [48]. This observation may explain the pronounced changes in SPM pathway markers, such as HDHAs and HEPes, following DHA

supplementation, as well as the higher number of samples with undetectable levels of resolvins and protectins compared to their pathway markers. Conversely, the lack of change in certain SPMs, like hippocampal RvE1, may reflect differences in regulatory mechanisms, such as enzyme expression patterns involved in SPM biosynthesis [45]. Further investigation is needed to elucidate how these enzymatic processes specific to SPM biosynthesis are regulated during critical periods of brain development.

DHA status has been linked to health outcomes in conditions involving disrupter neurodevelopment, inflammation, and neurodegeneration, such as preterm birth and Alzheimer's disease [6]. This study suggests that DHA's cognitive benefits may arise through SPM-mediated neuroinflammation resolution and support of synaptic plasticity [49]. SPMs have been shown to act via specific G-protein-coupled receptors on immune cells, initiating signaling cascades that resolve inflammation while preserving immune function [46, 49], creating an optimal environment for neural development and function. The role of SPMs in mediating inflammatory and neurodegenerative conditions is emerging as a critical area of interest [9-11]. In the context of preterm birth, DHA status has been linked to reduced risk and improved neonatal outcomes [50]. Specifically, average maternal DHA levels were found to be significantly lower in patients with preterm births [51], and pregnant women with plasma DHA concentrations below 2.0-2.5% are considered to be at a greater risk of preterm birth [52]. Furthermore, in a cohort of preterm newborns, higher DHA levels in the first few weeks of life were associated with improved microstructural brain development and corresponding improved developmental scores in a follow-up analysis [53]. Evidence suggests that SPMs may play a protective role by mitigating inflammation in the placenta and fetal tissues [18, 47, 54], a pathology characteristic of preterm birth [15]. The placenta is a modulator that can influence both the maternal and fetal immune

systems, and this has been marked by strong inflammatory responses initiated by placental infections leading to heightened levels of pro-inflammatory cytokines that precede preterm labor [55]. SPM levels have been found to be relatively high in placental tissue, with protectin levels increasing near term, suggesting a role in maintaining inflammatory balance and preventing preterm birth [18].

Similarly, DHA status has been widely studied in Alzheimer's disease, with reduced brain DHA levels linked to increased neuroinflammation and cognitive decline, as reviewed by Devassy et al. [56]. Omega-3 PUFA supplementation in patients with mild cognitive impairment increased RvD1 levels, enhanced amyloid beta plaque phagocytosis, and improved cognitive test scores [57]. Patients with Alzheimer's disease exhibited significantly reduced levels of PD1 and Maresin1 in the hippocampus [22, 58], as well as RvD1 in the cerebrospinal fluid, with lower SPM levels in the cerebrospinal fluid correlating with decreased mini-mental state examination scores, a marker of cognitive function [22]. Interestingly, PD1, Maresin1, and RvD1 administration have shown neuroprotective effects by boosting microglial phagocytosis of amyloid-beta plaques, improving neuronal survival, and shifting microglial activity from pro-inflammatory to anti-inflammatory [10]. The regional specificity of SPM action observed in this study provides new insights into how DHA and its metabolites may support cognitive resilience in Alzheimer's disease.

The strong correlation between prefrontal SPM levels and activation in cerebellar and sensorimotor networks suggests these metabolites may contribute to inter-regional communication or neuronal connectivity. This study builds on existing research by linking functional network maturation to cognitive development [59]. Specifically, the cerebellar network, traditionally associated with motor control, is increasingly recognized for its role in

cognitive and emotional processing [60]. The cerebellar network activation correlated with SPM levels may reflect broader neurodevelopmental benefits mediated by these metabolites. Similarly, the sensorimotor network, essential for integrating sensory input with motor responses [61], could rely on SPMs for optimal connectivity and function during early development. These results emphasize the potential for DHA-derived metabolites to promote the structural and functional integration of key brain networks critical for higher-order cognitive processes.

These findings highlight the potential of this novel class of metabolites to mediate neurodevelopmental programming by resolving inflammation and enhancing neuronal connectivity. Further research is needed to investigate their impact on cognition later in life and their translational relevance to human health, particularly in at-risk groups such as preterm infants and those with a predisposition to neurodevelopmental disorders. This study underscores the critical role of maternal nutrition in shaping offspring brain health and provides a foundation for targeted interventions utilizing DHA and its bioactive metabolites.

AUTHOR CONTRIBUTIONS

Stephanie Dubrof: Writing – original draft, Visualization, Investigation, Formal analysis. **Jillien G. Zukaitis:** Investigation. **Ishfaqe Ahmed:** Investigation. **Wenwu Sun:** Investigation. **Qun Zhao:** Methodology, Supervision. **Hea Jin Park:** Writing – review & editing, Methodology, Conceptualization, Supervision, Funding acquisition.

Co-authors have agreed for this work to be included in this dissertation.

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Table 3.1. Diet composition of the sow basal diet during gestation and lactation for sows supplemented with or without docosahexaenoic acid

Ingredients (g/kg)	Gestation Diet¹	Lactation Diet¹
Corn	535.4	389.6
Soybean Meal	32.3	172.3
Distillers Dried Grains with Solubles	400.0	400.0
Fat		4.6
Dicalcium Phosphate	2.8	
Limestone	17.4	21.6
Salt	3.5	3.5
Vitamin pre-mix²	2.5	2.5
Trace Mineral pre-mix³	1.5	1.5
Sow Vitamin pre-mix⁴	2.5	2.5
L-Lysine HCl	2.1	2.0
Total	1000.0	1000.0
Calculated Analysis		
Crude Protein, %	17.4	22.4
Metabolizable Energy, kcal/kg	3330	3300
Crude Fiber, %	4.18	5.10
Ether Extract, %	6.23	6.14
Lysine, %	0.70	1.10
TSAA, %	0.68	0.86
Threonine, %	0.64	0.92
Tryptophan, %	0.15	0.25
Ca, %	0.79	0.90
Total P, %	0.52	0.57
Available P, %	0.29	0.40

¹Basal diet was supplemented daily with 75 mg/kg BW/day DHA or 75 mg/kg BW/day safflower oil in the DHA group and CON group, respectively. ²Supplied per kg of premix: vitamin A 4400 IU; vitamin D 660,000 IU; vitamin E 17,600 IU; vitamin K 1760 IU; riboflavin 3960 mg; niacin 22,000 mg; vitamin B12 17,600 µg. ³Supplied per kg of premix: iron 110,000 mg; copper 11,000 mg; manganese 26,400 mg; zinc 110,000 mg; iodine 198 mg; selenium 198 mg. ⁴Supplied per kg of premix: biotin, 88 mg; choline, 220.5 g; folic acid, 661.5 mg; pyridoxine, 1.98g; vitamin E8,882 IU.

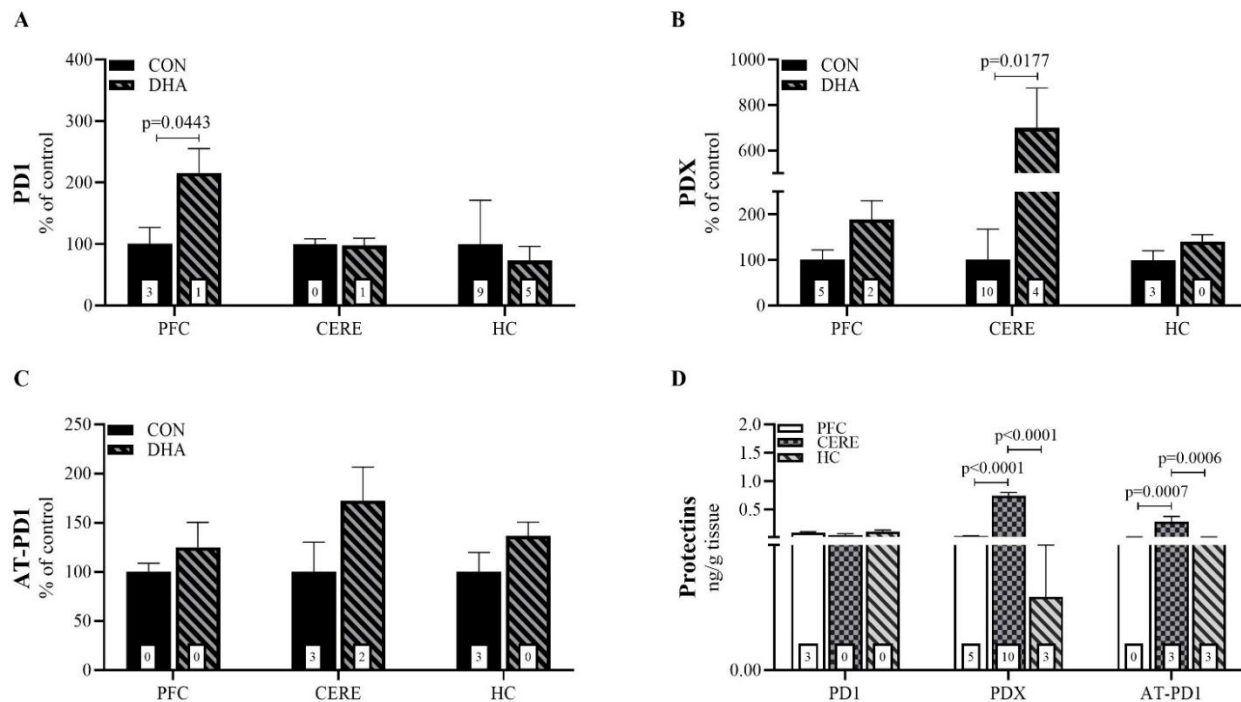


Figure 3.1. Protectin levels in the prefrontal cortex, cerebellum, and hippocampus of weaning piglets from sows fed a diet with (n=12) or without (n=12) docosahexaenoic acid. Shown as percent change relative to control: levels of PD1 (A), levels of PDX (B), levels of AT-PD1 (C). Brain regional differences of PD1, PDX, and AT-PD1 in CON (n=12) piglets (D). Number in box indicates the number of samples below the level of detection (0.0001 ng/g tissue). CON: control; DHA: docosahexaenoic acid; PD1: protectin D1; PDX: protectin DX; AT-PD1: aspirin triggered protectin D1; PFC: prefrontal cortex; CERE: cerebellum; HC: hippocampus.

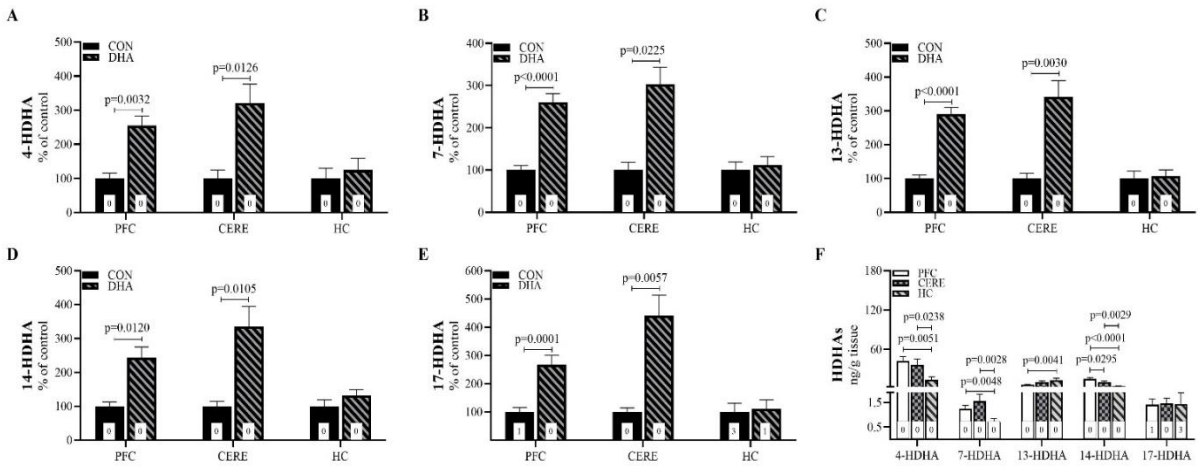


Figure 3.2. Hydroxydocosahexaenoic acid levels in the prefrontal cortex, cerebellum, and hippocampus of weaning piglets from sows fed a diet with (n=12) or without docosahexaenoic acid (n=12). Shown as percent change relative to control: levels of 4-HDHA (A), levels of 7-HDHA (B), levels of 13-HDHA (C), levels of 14-HDHA (D), levels of 17-HDHA (E). Brain regional differences of 4-, 7-, 13-, 14-, and 17-HDHA in CON (n=12) piglets (F). Number in box indicates the number of samples below the level of detection (0.0001 ng/g tissue). CON: control; DHA: docosahexaenoic acid; HDHA: hydroxydocosahexaenoic acid; PFC: prefrontal cortex; CERE: cerebellum; HC: hippocampus.

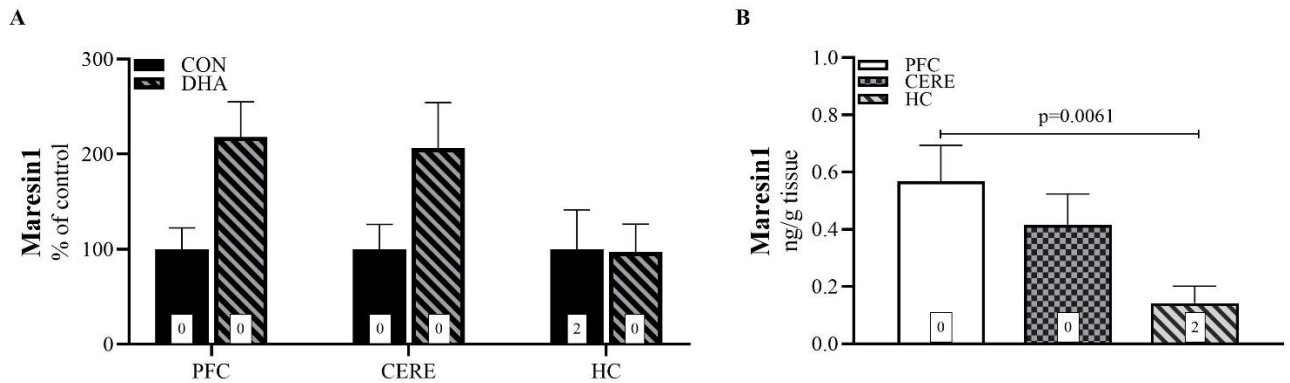


Figure 3.3. Maresin levels in the prefrontal cortex, cerebellum, and hippocampus of weaning piglets from sows fed a diet with (n=12) or without docosahexaenoic acid (n=12). Maresin levels shown as percent change relative to control (**A**). Brain regional differences of Maresin in CON (n=12) piglets (**B**). Number in box indicates the number of samples below the level of detection (0.0001 ng/g tissue). CON: control; DHA: docosahexaenoic acid; PFC: prefrontal cortex; CERE: cerebellum; HC: hippocampus.

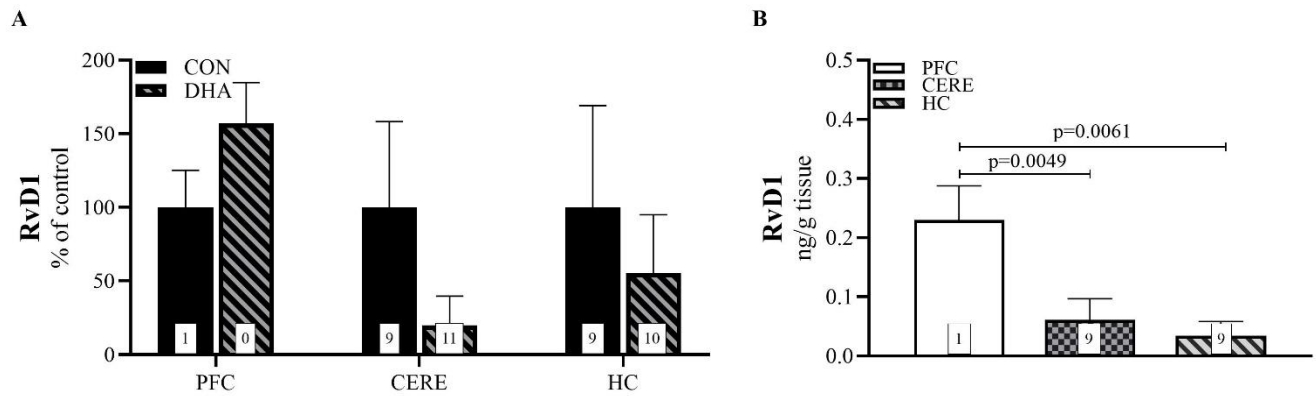


Figure 3.4. D-series Resolvin 1 levels in the prefrontal cortex, cerebellum, and hippocampus of weaning piglets from sows fed a diet with (n=12) or without docosahexaenoic acid (n=12). RvD1 levels are shown as percent change relative to control (**A**). Brain regional differences of RvD1 in CON (n=12) piglets (**B**). Number in box indicates the number of samples below the level of detection (0.0001 ng/g tissue). CON: control; DHA: docosahexaenoic acid; RvD1: D-series resolvin 1; PFC: prefrontal cortex; CERE: cerebellum; HC: hippocampus.

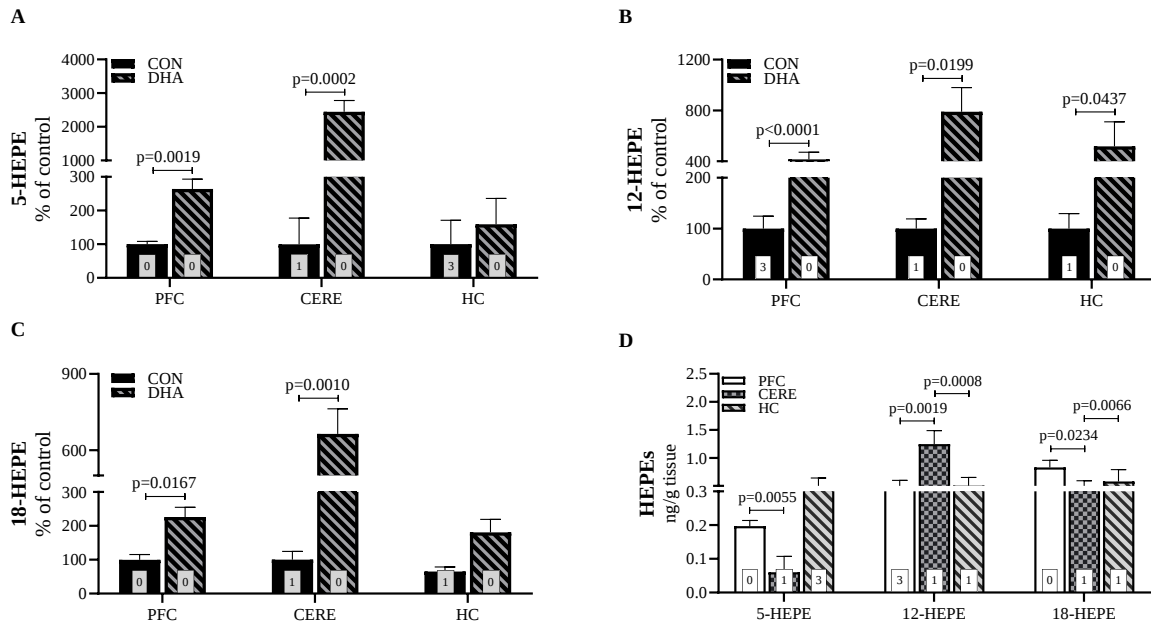


Figure 3.5. Hydroxyeicosapentaenoic acid levels in the prefrontal cortex, cerebellum, and hippocampus of weaning piglets from sows fed a diet with (n=12) or without docosahexaenoic acid (n=12). Shown as percent change relative to control: 5-HEPE (A), 12-HEPE (B), 18-HEPE (C). Brain regional differences of 5-, 12-, and 18-HEPE in CON (n=12) piglets (D). Number in box indicates the number of samples below the level of detection (0.0001 ng/g tissue). CON: control; DHA: docosahexaenoic acid; HEPE: hydroxyeicosapentaenoic acid; PFC: prefrontal cortex; CERE: cerebellum; HC: hippocampus.

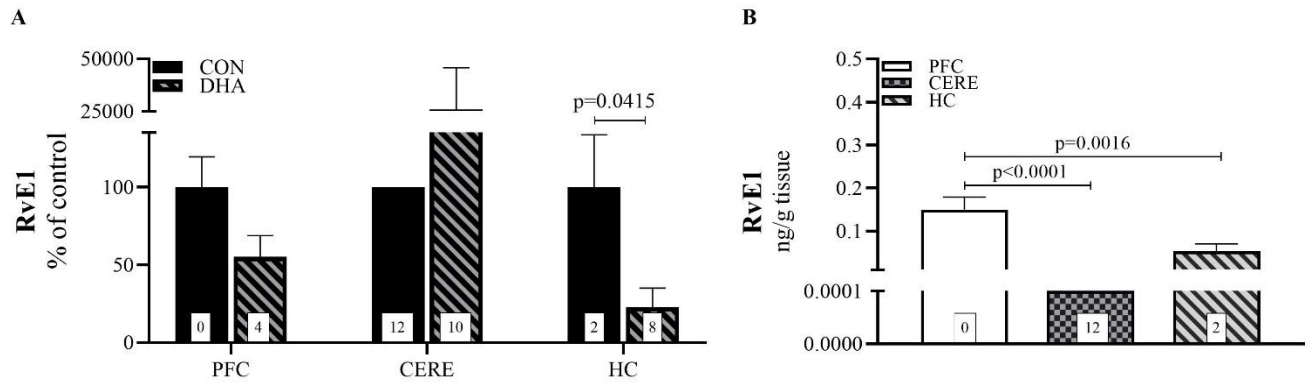


Figure 3.6. E-series Resolvin 1 levels in the prefrontal cortex, cerebellum, and hippocampus of piglets from sows fed a diet with (n=12) or without docosahexaenoic acid (n=12). RvE1 levels are shown as percent change relative to control (**A**). Brain regional differences of RvE1 in CON (n=12) piglets (**B**). Number in box indicates the number of samples below the level of detection (0.0001 ng/g tissue). CON: control; DHA: docosahexaenoic acid; RvE1: E-series resolvin 1; PFC: prefrontal cortex; CERE: cerebellum; HC: hippocampus.

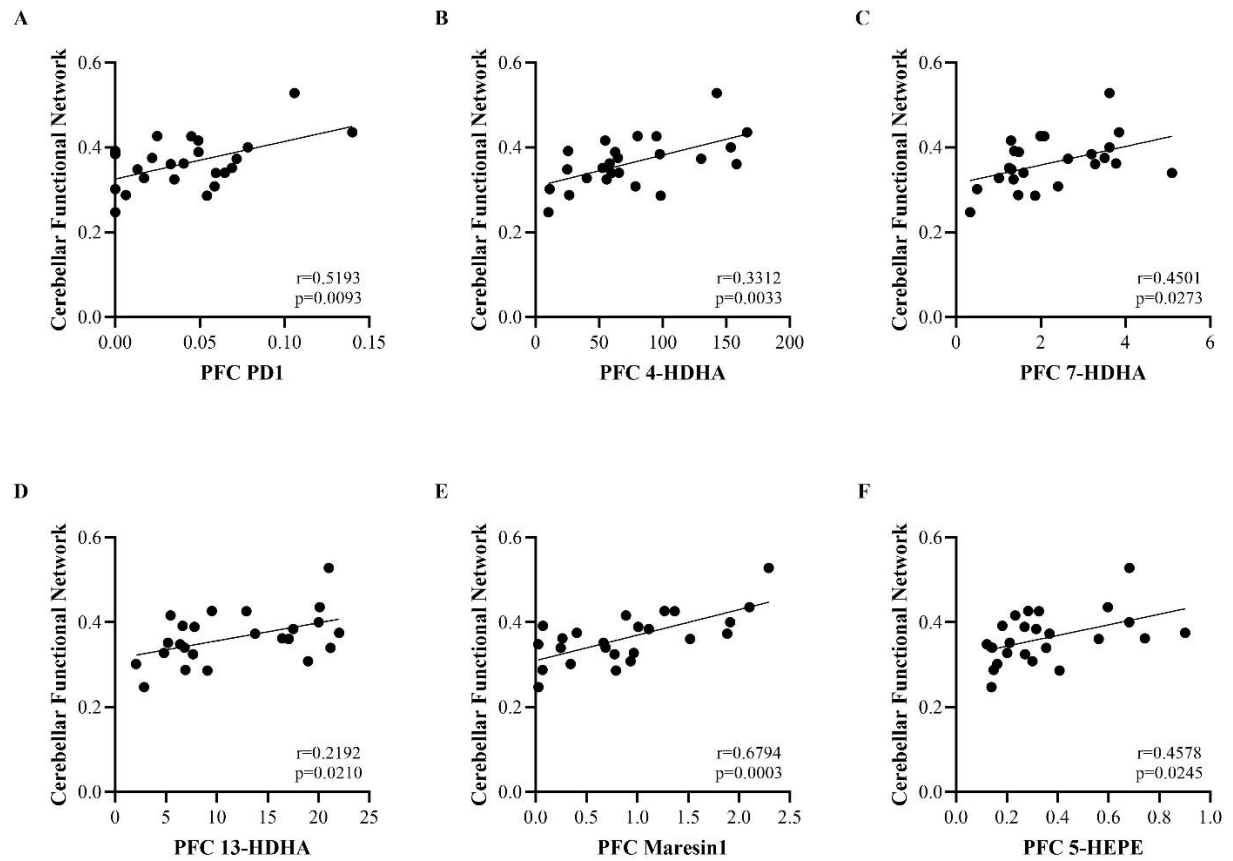


Figure 3.7. Correlation of SPMs with activation of brain functional networks of piglets at weaning. PFC PD1 (A), PFC 4-HDHA (B), PFC 7-HDHA (C), PFC 13-HDHA (D), PFC Maresin1 (E), PFC 5-HEPE (F) correlated with cerebellar functional network activation. PFC: prefrontal cortex; PD1: protectin D1; HDHA: hydroxydocosaheaxanoic acid.

CHAPTER 4

MATERNAL SUPPLEMENTATION OF EGG YOLK MODULATES BRAIN FUNCTIONAL ORGANIZATION AND FUNCTIONAL OUTCOMES OF OFFSPRING ¹

¹ Dubrof S, Zukaitis J, et al. Maternal supplementation of egg yolk modulates brain functional organization and functional outcomes of offspring. 2024. *Nutrition Research*. 131:147-158.

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ABSTRACT

Maternal nutrition during the perinatal stage is critical to offspring brain development. Egg yolks are a balanced and nutrient-dense food that are rich in bioactive components crucial to optimal neurodevelopment early in life. Egg consumption is often recommended to pregnant women to enhance both maternal and fetal health. We hypothesized that maternal intake of egg yolk from late gestation and throughout lactation would enhance functional organization and cognitive developmental outcomes in offspring using a pig model. Sows were fed a control diet (n=6) or a diet containing egg yolks (n=5, 350 mg egg yolk powder/kg BW/day, equivalent to ~3 eggs/day for humans) from late gestation through lactation. At weaning, piglet offspring (n=2/sow, total n=22) underwent structural magnetic resonance imaging (MRI) and resting-state functional MRI (rs-fMRI). Piglets underwent novel object recognition (NOR) testing to assess hippocampal-dependent learning and memory. fMRI results demonstrated that egg yolk significantly increased functional activation in the executive network ($p=0.0343$) and cerebellar network ($p=0.0253$) in piglets when compared to control. DTI analysis showed that perinatal intake of egg yolks significantly increased white matter fiber length in the hippocampus ($p=0.0363$) and cerebellum ($p=0.0287$) in piglet offspring compared to control piglets. Furthermore, piglets from egg yolk-fed sows spent significantly more proportional frequency exploring the novel object than the familiar object in NOR testing ($p=0.0370$). The findings from this study support egg yolk-altered activation of specific brain networks may be associated with functional cognitive outcomes in weaning piglets.

INTRODUCTION

The perinatal period is a critical phase of brain development to ensure healthy brain function [1]. Maternal diet and supplement intake can greatly influence neurocognitive development during this crucial period of life, as optimal nutrition status has been highlighted as having a major impact on brain function throughout all stages of life [2-4]. Diminished prenatal nutrition may negatively impact essential neurodevelopmental processes such as neurogenesis and synaptic plasticity, therefore, a well-balanced and adequate maternal diet during the perinatal period is vital to proper brain development [5, 6].

Eggs are a nutrient rich food often recommended for consumption during the perinatal period to benefit both fetal and maternal health [7]. Eggs serve as a proficient source of several essential macronutrients and bioactive compounds, such as fatty acids and proteins, that are vital to infant brain development and cognitive outcomes [7-10]. Consumption of eggs by children has been shown to reduce stunted growth, which is a marker that is highly attributed to diminished cognitive outcomes [11]. Specifically, consumption of the egg yolk has been observed to result in increased short-term learning and memory scores when compared to egg white consumption alone in children, overall supporting a higher cognitive performance [12]. Evidence of the beneficial effects of specific egg yolk nutrients, such as choline and lutein, have on infant neurodevelopmental outcomes following maternal intake has been compelling and extensively studied [13, 14]. Investigation into perinatally supplementing egg yolk as a whole food, however, has been limited.

The pig is suggested as a more robust translational animal for investigating the effects of maternal nutritional programming in offspring neurodevelopment, as compared to rodents, due to several physiological and anatomical similarities they share with humans [15-17]. Most notably, while rodents have a lissencephalic brain, both pigs and humans have a gyrencephalic brain, which

have a direct effect on brain activation and function and is well attributed to increased neurological intricacy [18-20]. Both the swine and human brain are comprised of more than 60% white matter, while the rodent brain is composed of approximately 12% or less [21, 22]. The pig and human experience similar brain growth spurts and maturation patterns, with the human brain growing the fastest at the last trimester of pregnancy until end of lactation and the pig brain growing the fastest from about 50 days prior to farrowing to about 40 days after birth [23, 24]. Previous longitudinal magnetic resonance imaging (MRI) studies quantified neonatal pig brain volumes to investigate brain growth and showed that 95% of all brain growth had occurred by the age of 21-23 weeks, which is similar and translatable to that of human neonates [25, 26]. These key similarities in early brain development between humans and pigs make the pig an excellent model to study the effects of egg yolk on perinatal brain development.

In this novel study, we aim to investigate how perinatal supplementation of egg yolk can modulate neurodevelopment by influencing brain structure, functional activation, and early cognition in offspring, as evaluated by structural MRI, resting state-fMRI (rs-fMRI), and cognitive behavior testing. We hypothesized that maternal intake of egg yolk from late gestation and throughout lactation would alter and potentially enhance brain structural and functional development and cognition in offspring. The findings of this study provide novel insights into the critical role of perinatal nutrient intake throughout this crucial period of early pediatric brain development.

METHODS

Animal Handling and Study Design

A cross-bred commercial line of healthy Landrace sows (n=11) were artificially inseminated at the University of Georgia Swine Unit and delivered to the Large Animal Research Unit animal facility at approximately day 60-65 of gestation. Sows were assigned to treatment group

accounting for parity and body weight. Sows were fed a treatment diet with (EGG, n=5) or without egg yolk powder (CON, n=6) from day 70 of gestation and throughout lactation. All sows were fed a basal diet throughout gestation and lactation that met the metabolic requirements of maternal health and maximized fetal growth as according to the National Research Council's recommendations for the nutrient requirements of swine (**Table 1**) [27]. Animal numbers for this study were determined by a power analysis. The basal diet fed to sows in the EGG group was supplemented with 350 mg of dried egg yolk powder (NestFresh, Fullerton, CA) per kg of body weight each day, which is equivalent to approximately 3 large eggs per day for an adult human. This dose was chosen based on previous evidence that about three egg yolks per day can improve cognitive function while not causing adverse health outcomes [11, 28-30]. At birth, 1 male and 1 female piglet nearest the average birth weight from each litter were chosen to include in the study (CON, n=12; EGG, n=10). At weaning, approximately postnatal day (PND) 20-21, piglets underwent MRI and behavior testing. This study was conducted in accordance with the University of Georgia Institutional Animal Care and Use Committee guidelines (Animal Use Protocol: A2021 01-026).

MRI Acquisition and Analysis

Acquisition

At weaning (PND 20-21), piglets (CON, n=12; EGG, n =10) underwent structural MRI, including diffusion tensor imaging (DTI) to acquire detailed information pertaining to white matter fibers, and rs-fMRI as previously described [31-33] using a 3.0 Tesla General Electric HDx scanner and a quadrature 8-channel knee coil at the University of Georgia's Bioimaging Research Center [34]. Briefly, piglets were sedated with propofol and maintained under mild anesthesia with 1.5% isoflurane throughout the scan. The data acquisition sequence consisted of:

- 1) 3D fast spoiled gradient echo sequence (repetition time = 5.5 seconds, echo time = 2.1 ms, flip

angle = 9°, field-of-view = 12.8 x 12.8 x 6.4 cm, slice thickness = 1 mm, a reconstruction matrix size of 256 x 256 x 112, axial slice plane and an acquisition time of 10 minutes and 57 seconds; 2) a spin-echo echo-planar imaging (EPI) sequence (TR = 15.5 s, TE = min-full, FOV = 12.8 × 12.8 × 6.4 cm, acquired matrix = 64 × 64 × 32, and 30 diffusion weighted images using b = 1000 s/mm²) was used for DTI acquisition; 3) gradient echo-planar imaging sequence: repetition time = 3 seconds, echo time = 30 ms, flip angle = 80°, field-of-view = 12.8 x 12.8 x 6.2 cm, a matrix size of 96x96x32, coronal slice plane, for a total of 305 volumes for rs-fMRI [34].

Anatomical Analysis

Each piglet brain image was coregistered with a previously standardized pig brain atlas [35] using MATLAB's (The MathWorks, Inc., Natick, MA, USA) Statistical Parameter Mapping 12 (SPM12) toolbox. Percentage volumes for 19 brain regions were calculated using MATLAB.

DTI Analysis

To remove motion and susceptibility artifacts, all DTI datasets were minimally preprocessed using FMRIB Software Library (FSL, v6.0) followed by manual segmentation with 3D Slicer from non-brain tissues and skull. Mapping of the white matter tracts, or tractography, was implemented utilizing the Tensor Toolkit in MedInria for tensor estimation and tensor tractography analysis. DTI datasets for each piglet subject were individually coregistered with a three-dimensional digital segmented pig brain atlas [33] through MATLAB's (The MathWorks, Inc., Natick, MA, USA) SPM12 toolbox to subsequently obtain mean diffusivity (MD), fractional anisotropy (FA), and fiber length (FL) in targeted brain structures.

Functional Activation Analysis

Rs-fMRI data was preprocessed and analyzed as previously described [33, 34, 36]. Briefly, all rs-fMRI data was converted from digital imaging and communications in medicine (DICOM)

format to neuroimaging informatics technology initiative (NIfTI) format utilizing the MRICron software package (v1.0.20190902). SPM12 toolbox based on MATLAB (The MathWorks, Inc., Natick, MA, USA) was used to apply head motion corrections, to apply slice timing corrections, and to register individual subject data to the reference template subject [36]. The non-brain signals were removed from the data by applying a binary mask that was created using 3D Slicer (4.11.20210226). The baseline functional activation maps (independent components, ICs) were estimated by running an independent component analysis on control group only (group ICA, gICA) for subsequent group comparison. The baseline functional activation maps were regressed in each individual via FSL's dual regression command followed by spatial normalization to a higher resolution space/anatomical space (image dimensions of 129x129x57 and voxel size 1x1x1 mm³). Spatial Pearson correlation values were computed for eight resting state networks (RSNs) including the executive control network (ECN), cerebellar network (CERE), visual network (VIS), sensorimotor network (SMN), auditory network (AUD), default mode network (DMN), salience network (SMN), and basal ganglia network (BGN) for each subject.

Novel Object Recognition Test

The novel object recognition (NOR) test was conducted to evaluate memory retention, which has effectively been carried out in pigs previously [31, 32, 37, 38]. Piglets took part in behavior testing at weaning (PND 20-21) (CON, n=12; EGG, n=10). Testing occurred in a 2.7 x 2.7 m² arena. The NOR test is made up of two test trials, including a sample trial to rule out any innate preference to location in the arena and a test trial. The sample trial consisted of a ten minute session for the piglet to explore two identical objects attached in the center of the open arena. The piglet was then removed from the arena for a 10-minute interphase, where one of the objects was replaced with a novel object. The subsequent test trial was an additional ten minutes in the

arena for the piglet to explore the novel and the familiar object. The amount of time the piglet spent exploring each of the objects was measured using EthoVision software (Noldus, Wageningen, the Netherlands). The proportional time was calculated as the ratio of time exploring the novel object to the total time exploring both objects, and the proportional frequency was calculated as the ratio of visits to the novel object to the total number of visits to both objects. Piglets were handled daily from birth to the day of the behavior test to habituate them to human interaction and being temporarily away from the litter mates.

Statistical Analyses

Data expressed as mean $\pm$ S.E. was analyzed using RStudio (Version 2022.07.2+576, R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism (Version 9.00, GraphPad Software, Inc.; San Diego, CA, USA). Treatment effects were analyzed using a linear mixed-effect model to control for sex as a fixed effect and for maternal as a random effect. NOR behavior data was analyzed using a linear mixed-effect model with logit transformation to test for differences in time spent with each object. Significance of the treatment effect was determined at a significance level of 0.05 based on t-test statistics.

RESULTS

Food intake was similar amongst sows

Daily sow feed intake was measured throughout the study to ensure that potential outcomes were not due to differences in feed intake. Sows were fed 2.04 kg of basal feed per day during gestation, and sows consumed similar amounts of feed during lactation per day (16.60 ± 0.32 kg and 16.24 ± 0.33 kg, CON and EGG, respectively, $p > 0.05$)

Maternal egg yolk intake showed limited effects on anatomical brain volumes in offspring

Key brain structures, such as the hippocampus and thalamus, were measured for volumetric changes utilizing structural MRI in weaning piglets from both CON and EGG sows (**Table 2**).

Given that these are healthy piglets, profound differences in brain structure volumes were not expected following maternal dietary intervention. However, it was observed that EGG piglets had a decreased left cortex volume, increased right cortex volume, and increased left hippocampal volume when compared to CON piglets. While there were some changes in brain structural volumes, the clinical significance of these findings is unclear.

Perinatal egg yolk intake altered brain functional network activity in offspring

Functional activation maps in eight resting state networks (RSNs) were examined to determine the influence of perinatal egg supplementation in the offspring brain. Figure 1A shows representative visualizations of the maximum activation maps of eight resting state networks in piglet brains. Maternal egg yolk supplementation during late gestation and lactation resulted in a significant hyper functional activity, with a 15.63% increase ($p=0.0343$) in activation in the executive control network when compared to the control (**Figure 1B**).

Furthermore, maternal egg yolk intake caused a significant increase ($p=0.0253$) in cerebellar functional activation by 4.95% in piglet offspring when compared to the control (**Figure 1B**).

These results demonstrate that maternal egg yolk consumption may enhance the performance of brain networks associated with high-level cognitive processes and voluntary motor function in offspring. In other RSN's of the brain, no significant ($p>0.05$) change (hyper or hypo) in terms of functional activation was determined (**Figure 1B**).

Perinatal egg yolk intake increased fiber length in the hippocampus and cerebellum in offspring

DTI allows for white matter tract-specific analysis of fiber length and integrity, which are vital for optimal information processing and neuronal communication. The hippocampus is a structure critical to memory formation, the cerebellum contributes to increased cognitive

function, and the thalamus is crucial for distributing sensory information to the appropriate cortical regions. Perinatal egg yolk supplementation was found to significantly increase fiber length in the hippocampus and the cerebellum (16.83 ± 1.40 , and 13.61 ± 1.20 , respectively) of offspring when compared to control (**Figure 2C and 2F**), suggesting that perinatal egg supplementation may enhance white matter structural integrity in the hippocampus and cerebellum in developing piglets. Perinatal egg yolk intake did not influence fiber length within the thalamus ($p > 0.05$) (**Figure 2I**). Egg yolk supplementation did not alter mean diffusivity (**Figure 2A, 2D, and 2G**) or fractional anisotropy (**Figure 2B, 2E, and 2H**) in hippocampus, cerebellum, or thalamus ($p > 0.05$).

White matter structural fiber length was associated with functional activation in piglet brains

To further investigate changes in offspring neurodevelopment, we evaluated the association of fractional anisotropy and white matter structural fiber length with functional activation. Interestingly, we observed that hippocampal fractional anisotropy was positively correlated with functional activation of the visual network, while cerebellar fractional anisotropy was negatively associated with visual network activation (**Figure 3**). In addition, cerebellar fiber length was negatively correlated with auditory network functional activation (**Figure 3**). These findings suggest that brain structural development and integrity may be associated with functional network activation early in neurodevelopment.

Perinatal egg yolk intake increased proportional frequency in the NOR test in piglets

Piglets showed no preference in exploring the two identical objects in the sample test ($p > 0.05$), which indicated that there was no preference in location in the behavior arena. In the NOR test, CON piglets spent similar proportional frequency (**Figure 4A**) and proportional time (**Figure 4B**) engaging with the familiar and novel objects ($p > 0.05$). Interestingly, EGG piglets

spent significantly more proportional frequency (**Figure 4C**) exploring the novel object than with the familiar object, although proportional time (**Figure 4D**) was statistically similar ($p>0.05$).

Proportional time and frequency in the NOR was associated with default mode network activation

To assess the association between the NOR outcomes of proportional time and frequency and functional activation, we assessed the potential for correlations between these NOR metrics and rs-fMRI network activity. Functional activation of the default mode network was found to be negatively associated with both proportional time and frequency spent with the novel object in NOR testing (**Figure 5A and 5B**, both p -value <0.05), suggesting that development of the functional network activation may be associated with observed cognitive outcomes early in life.

DISCUSSION

In this study, we demonstrated that maternal supplementation of egg yolk from late gestation throughout lactation led to enhanced functional activation in the executive and cerebellar networks and lengthened white matter fiber length in the hippocampus and cerebellum in the piglet brain. These changes in brain organization were accompanied by cognitive changes in the NOR test that may be associated with hippocampal-dependent performance. These findings add credence to the importance of maternal nutrition status during this critical period of neurodevelopment and how it can impact brain functional organization, white matter fiber length, and functional outcomes in offspring.

The perinatal stage is a period of rapid and dynamic brain development that is foundational for lifelong memory, learning and processing functions, and emotional regulation [39, 40]. During this amenable time, the brain undergoes periods of structural refinement and neural network formation that are largely associated with maternal nutrition status [41, 42]. Maternal

nutrition characterized by low macronutrient and micronutrient intake during pregnancy and breastfeeding has been associated with increased risk of neurodevelopmental disorders, cognitive impairments, and behavioral challenges in children, suggesting an unsupplemented maternal diet may be inadequate for the required nutrients for optimal fetal health and neurodevelopment during this critical time [43, 44]. Supplementation of both macronutrients, such as polyunsaturated fatty acids, and micronutrients, such as choline, is evidenced to have a favorable effect on neurodevelopment [4, 45]. In our study, we used a sow and piglet dyad model to supplement a nutrient-dense whole food, the egg yolk, which likely enhanced maternal nutrition status, thus aiding in establishing indicators of brain health and functional outcomes that were observed in offspring.

We found that maternal supplementation of a human dose equivalent to three egg yolks per day led to changes in neurodevelopmental markers in piglet offspring, as illustrated through MRI analysis and behavior testing. Lutter et al. [7] have provided considerable evidence indicating that maternal consumption of eggs could potentially improve birth outcomes, childhood nutrition status, and brain development due to eggs being a major source of protein, fatty acids, and other bioactive compounds shown to play a role in early neurodevelopment. Previously, childhood consumption of eggs as a whole decreased the incidence of stunted growth and development in children, which is an indicator strongly correlated with diminished cognitive development [11, 46]. Specifically, the egg yolk contains the bulk of the lipids, vitamins, and minerals that comprise the egg's nutritional value [47]. Choline is in high demand during the perinatal period, as it is essential for neuronal membrane formation, neurotransmission, and brain development in offspring [13, 47, 48]. The egg yolk is a considerable source of choline, providing 680 mg per 100 g of egg yolk [47]. Studies using a rodent model have repeatedly highlighted several markers

of enhanced offspring memory and overall cognitive function following maternal supplementation of choline during the perinatal period [49-52]. Egg yolk also contains a significant amount of lutein, approximately 640 ug per 100 g of egg yolk, a carotenoid that is the most abundant and preferentially accumulates in the developing brain [53, 54]. Lutein comprises about 50% of the total carotenoid composition in human breast milk, implicating its possible role in infant neurodevelopment [55]. Maternal supplementation of lutein during lactation resulted in a significant increase of lutein concentration in breast milk, maternal plasma, and infant plasma [14]. Interestingly, post-mortem metabolomic analyses on human infant brain tissue in regions integral to memory and learning revealed that lutein concentration is positively associated with amino acid neurotransmitters in the infant hippocampus and occipital cortex [56]. Moreover, lutein was positively associated with the antioxidant homocarnosine in the infant hippocampus and frontal cortex, providing evidence as to how lutein may influence early learning and memory [56]. Consequently, it is possible that the NOR test outcomes associated with heightened cognitive function observed in this study may be due to the choline or lutein content present in egg yolk.

In addition, egg yolk is a rich source of essential fatty acids, including linoleic acid, which is a fatty acid that cannot be synthesized and thus must be obtained through diet [47, 57]. Linoleic acid is of particular importance as it is an essential precursor to arachidonic acid, a major long-chain polyunsaturated fatty acid in the brain, therefore, integral in neurodevelopment [58, 59]. Notably, the egg yolk provides 15.9 grams of protein per 100 grams of egg yolk, making it a considerable source of protein for the maternal diet [47]. Protein is essential for the fundamental formation and repair of tissues and for the production of enzymes, hormones, and antibodies [60]. Further research is warranted to fully understand how choline, lutein, protein, and the other

nutrients in egg yolk may mechanistically work together to ultimately have an impact on the brain of the developing infant.

Optimal white matter growth in the developing brain during the perinatal period is critical, as white matter consists of myelinated axons that facilitate the rapid communication between different brain regions [61, 62]. Proper white matter development in the cerebellum is essential for the transmission of signals between different regions, ensuring efficient motor control and cognitive functioning [63]. White matter integrity and growth in the hippocampus are vital for the integration of information and communication between the hippocampus and other brain regions, supporting memory consolidation and emotional processing [64]. Although present in relatively smaller quantities in egg yolk, iron and DHA are nutrients that have been observed to be involved in white matter integrity [65-68]. In our study, DTI analysis revealed enhanced white matter fiber length in the cerebellum and hippocampus of weaning offspring following perinatal egg yolk supplementation, which may be attributed to the iron and DHA found in egg yolk [7, 65-68].

Using rs-fMRI analysis, we observed that maternal supplementation of egg yolk enhanced functional activation in the cerebellar network and the executive network in the brain of offspring. Recent findings investigating the relationship between functional activation and perinatal nutrition suggest a significant association between protein and lipid intake in the first postnatal month with functional activation at preschool age [69]. Furthermore, a study using a translational macaque model found that a supplemented carotenoid-enriched formula, including lutein, was associated with functional activation outcomes in infant macaques [70]. Our findings of enhanced functional activation in offspring following maternal egg yolk supplementation may be, in part, due to egg yolk's rich protein, lipid, and lutein content.

In this study, we observed an association of hippocampal and cerebellar fractional anisotropy and cerebellar fiber length with functional activation in the visual and auditory networks. Perinatal nutritional status and nutrient intake has been noted for its association with both infant white matter development and establishing functional activation in the brain [71-74]. Evidence has suggested that maternal adiposity is correlated with changes in resting-state functional activation and is negatively associated with white matter development in the newborn offspring's brain [72-74]. Additionally, total white matter volume was lower in children born to mothers with low intake of omega-3 fatty acid during pregnancy, and a recent neuroimaging study found that low DHA levels during perinatal development resulted in impaired functional activation in some cortical networks in young adult monkeys [71, 75]. These previous findings and the findings from this current study support the idea that brain structural organization and activation may be interrelated and likely influenced by maternal nutritional status and specific nutrient intake.

In conclusion, the findings from this study suggest an association of perinatal egg yolk intake with brain developmental outcomes in piglet offspring. This data offers preclinical evidence that maternal supplementation of egg yolk may enhance white matter tracts, modulate brain functional organization, and lead to improved cognitive outcomes in weaning offspring. The insights from this translational research will aid in bridging the gap of further understanding the role of maternal nutrient intake and optimal nutritional status throughout gestation and lactation on the developmental trajectory in the brain of offspring.

AUTHOR CONTRIBUTIONS

Stephanie Dubrof: Writing – original draft, Investigation, Formal analysis. **Jillien G. Zukaitis:** Investigation, Formal analysis. **Ishfaque Ahmed:** Formal analysis. **Wenwu Sun:** Formal analysis. **Kelly M. Scheulin:** Methodology, Formal analysis. **Xi Fang:** Investigation. **Julie Jeon:** Investigation. **Franklin D. West:** Writing – review & editing, Project administration, Methodology. **Qun Zhao:** Writing – review & editing, Methodology, Formal analysis. **Hea Jin Park:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Co-authors have agreed for this work to be included in this dissertation.

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Table 4.1. Diet composition of the sow basal diet during gestation and lactation for sows supplemented with or without egg yolk powder

Ingredients (gram per kilogram of feed)	Gestation Diet¹	Lactation Diet¹
Corn	689.95	664.55
Soybean Meal	65.00	280.00
Distillers Dried Grains with Solubles	200.00	0.00
Fat	10.00	20.00
Dicalcium Phosphate	1.50	6.20
Limestone	18.50	15.20
Salt	3.50	3.50
Vitamin pre-mix ²	2.50	2.50
Trace Mineral pre-mix ³	1.50	1.50
Sow Vitamin pre-mix ⁴	5.00	5.00
L-Lysine HCl	2.30	1.30
Phytase	0.25	0.25
Total (g)	1000.00	1000.00
<i>Calculated Analysis</i>		
Crude Protein, %	17.40	22.40
Metabolizable Energy, kcal/kg	3330.00	3300.00
Crude Fiber, %	4.18	5.10
Ether Extract, %	6.23	6.14
Lysine, %	0.70	1.10
TSAA, %	0.68	0.86
Threonine, %	0.64	0.92
Tryptophan, %	0.15	0.25
Ca, %	0.79	0.90
Total P, %	0.52	0.57
Available P, %	0.29	0.40

¹Basal diet was fed daily to CON (n=6) group. Basal diet was supplemented daily with 350 mg/kg BW/day egg yolk powder to the EGG (n=5) group. ²Supplied per kg of premix: vitamin A 4400 IU; vitamin D 660,000 IU; vitamin E 17,600 IU; vitamin K 1760 IU; riboflavin 3960 mg; niacin 22,000 mg; vitamin B12 17,600 µg. ³Supplied per kg of premix: iron 110,000 mg; copper 11,000 mg; manganese 26,400 mg; zinc 110,000 mg; iodine 198 mg; selenium 198 mg. ⁴Supplied per kg of premix: biotin, 88 mg; choline, 220.5 g; folic acid, 661.5 mg; pyridoxine, 1.98g; vitamin E 8,882 IU. TSAA: total sulfur amino acids; Ca: calcium; P: phosphorous.

Table 4.2. Percentage volumes of anatomical brain regions in piglets from sows supplemented with or without egg yolk powder

Brain Region	CON	EGG	p-Value
Caudate	0.8603 ± 0.0004	0.8600 ± 0.0007	0.6107
Cerebellum	11.3890 ± 0.0012	11.3870 ± 0.0014	0.2360
Left Cortex	31.2630 ± 0.0012	31.2590 ± 0.0017	0.0446
Right Cortex	31.1420 ± 0.0007	31.1460 ± 0.0015	0.0320
Lateral Ventricle	1.0117 ± 0.0008	1.0122 ± 0.0009	0.7273
Third Ventricle	0.1065 ± 0.0004	0.1063 ± 0.0003	0.8082
Cerebral Aquaduct	0.0734 ± 0.0003	0.0737 ± 0.0002	0.3665
Fourth Ventricle	0.0996 ± 0.0002	0.0994 ± 0.0002	0.5243
Left Hippocampus	0.9141 ± 0.0006	0.9160 ± 0.0007	0.0493
Right Hippocampus	0.9242 ± 0.0005	0.9235 ± 0.0007	0.4129
Medulla	3.3614 ± 0.0009	3.3612 ± 0.0011	0.8929
Midbrain	3.4119 ± 0.0013	3.4114 ± 0.0009	0.7866
Pons and Globus Pallidus	2.1559 ± 0.0007	2.1573 ± 0.0005	0.1223
Putamen	0.7310 ± 0.0006	0.7297 ± 0.0007	0.1504
Hypothalamus	0.5014 ± 0.0004	0.5012 ± 0.0005	0.7304
Thalamus	2.7686 ± 0.0007	2.7671 ± 0.0007	0.1461
Olfactory Bulb	4.6325 ± 0.0010	4.6330 ± 0.0007	0.7056
Corpus Callosum	0.7752 ± 0.0009	0.7760 ± 0.0010	0.5782
Internal Capsule	2.8777 ± 0.0011	2.8800 ± 0.0021	0.3114
Total Voxels	411294 ± 16.8750	411319 ± 15.8330	0.3032

Percentages of volumes of brain regions in weaning piglet offspring from sows fed a CON (n=12) or EGG (n=10) diet. Data is expressed as the mean ± S.E.M. A linear mixed-effect model was used to control for sex (fixed) and maternal (random) effects.

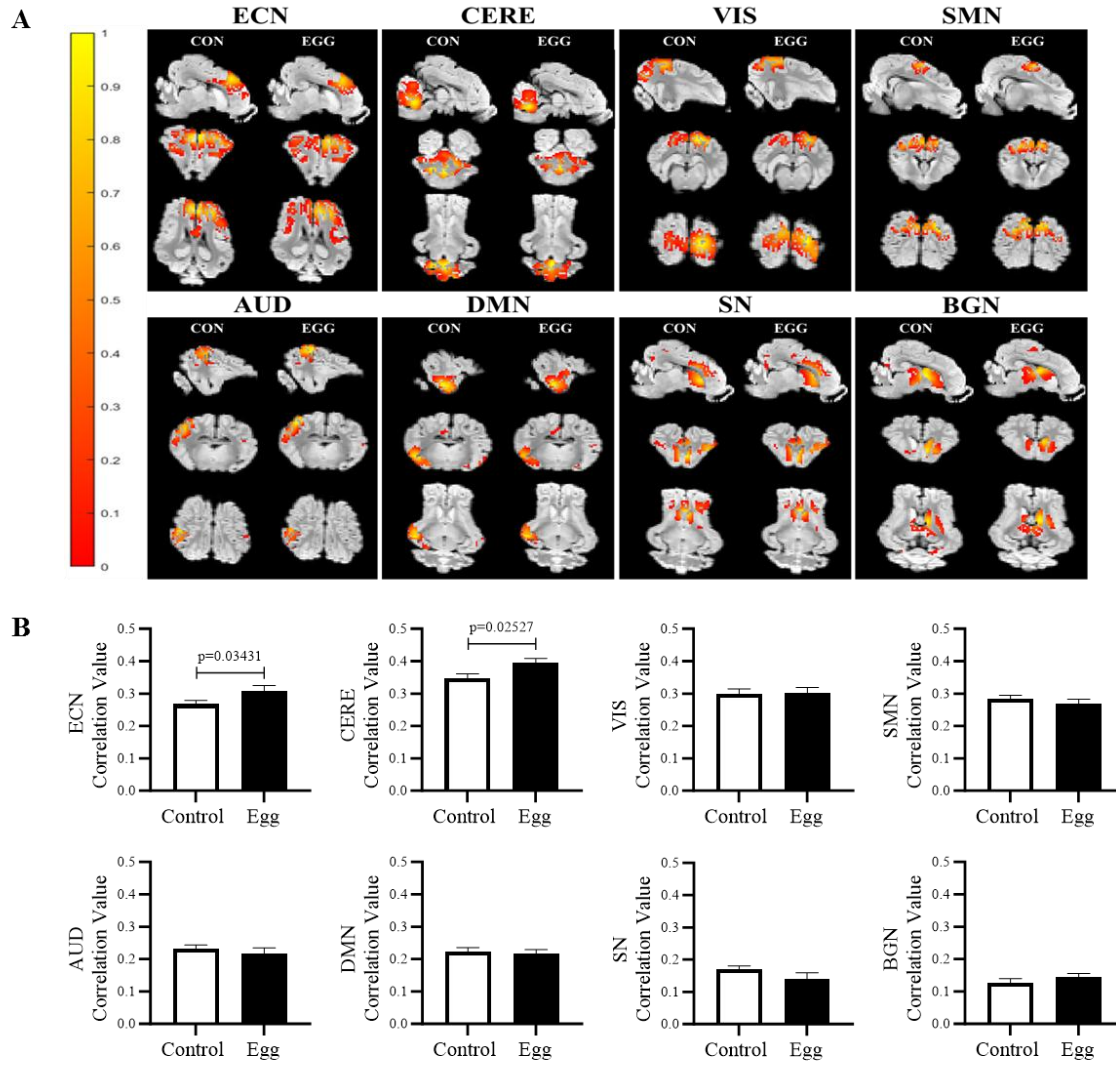


Figure 4.1. Perinatal egg yolk intake altered brain functional network activation in piglet offspring. Visualization of sagittal (top), coronal (middle), and axial (bottom) images of maximum activation maps within eight resting state networks, including ECN, CERE, VIS, SMN, AUD, DMN, SN, and BGN in piglets born to sows fed a CON (n=12) or EGG (n=10) diet. Yellow patterns are resting-state network atlases and orange patterns are the activations within the region (A). Analysis of functional activation from rs-fMRI scans of eight resting state networks in weaning piglet offspring from sows fed a CON (n=12) or EGG (n=10) diet. Graphs display correlation coefficients of the activation maps of piglet offspring (B). A linear mixed-effect model was used to control for sex (fixed) and maternal (random) effects. ECN: executive control network; CERE: cerebellar network; VIS: visual network; SMN: sensorimotor network; AUD: auditory network; DMN: default mode network; SN: salience network (SN); BGN: basal ganglia network. p values greater than 0.05 are not shown.

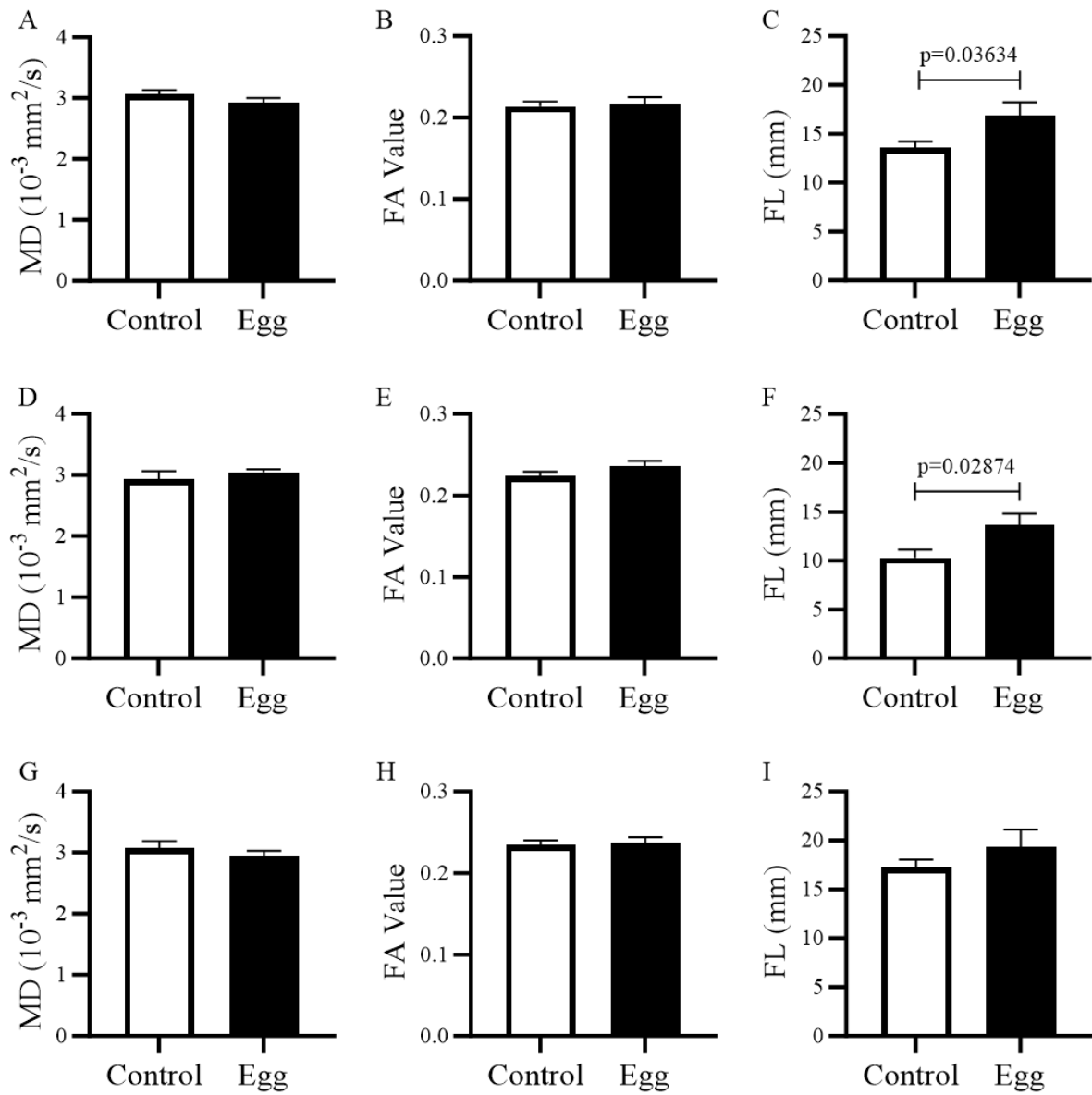


Figure 4.2. Perinatal egg yolk intake increased white matter fiber length in the hippocampus and cerebellum in piglet offspring as shown through diffusion tensor imaging analysis. Data shows DTI MRI analysis for CON (n=12) and EGG (n=10) piglets. Hippocampal mean diffusivity (A), fractional anisotropy (B), and fiber length (C). Cerebellar mean diffusivity (D), fractional anisotropy (E), and fiber length (F). Thalamic mean diffusivity (G), fractional anisotropy (H), and fiber length (I). Data is expressed as the means $\pm$ S.E.M. A linear mixed-effect model was used to control for sex (fixed) and maternal (random) effects. MD: mean diffusivity; FA: fractional anisotropy; FL: fiber length. p values greater than 0.05 are not shown.

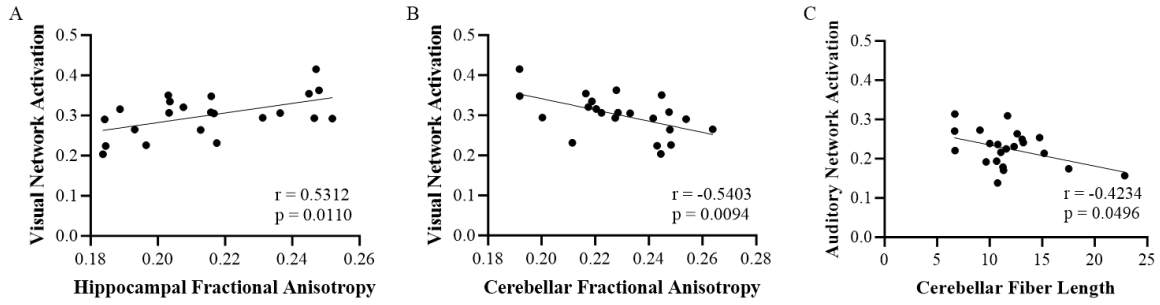


Figure 4.3. Hippocampal and cerebellar diffusion tensor imaging parameters correlated with visual and auditory functional activation in weaning piglet offspring. Data shows correlation results for all piglets (n=22). Hippocampal fractional anisotropy correlated with visual network functional activation (A), cerebellar fractional anisotropy correlated with visual network functional activation (B), and cerebellar fiber length correlated with auditory network functional activation (C). r = Pearson's correlation coefficients; p values less than 0.05 are shown.

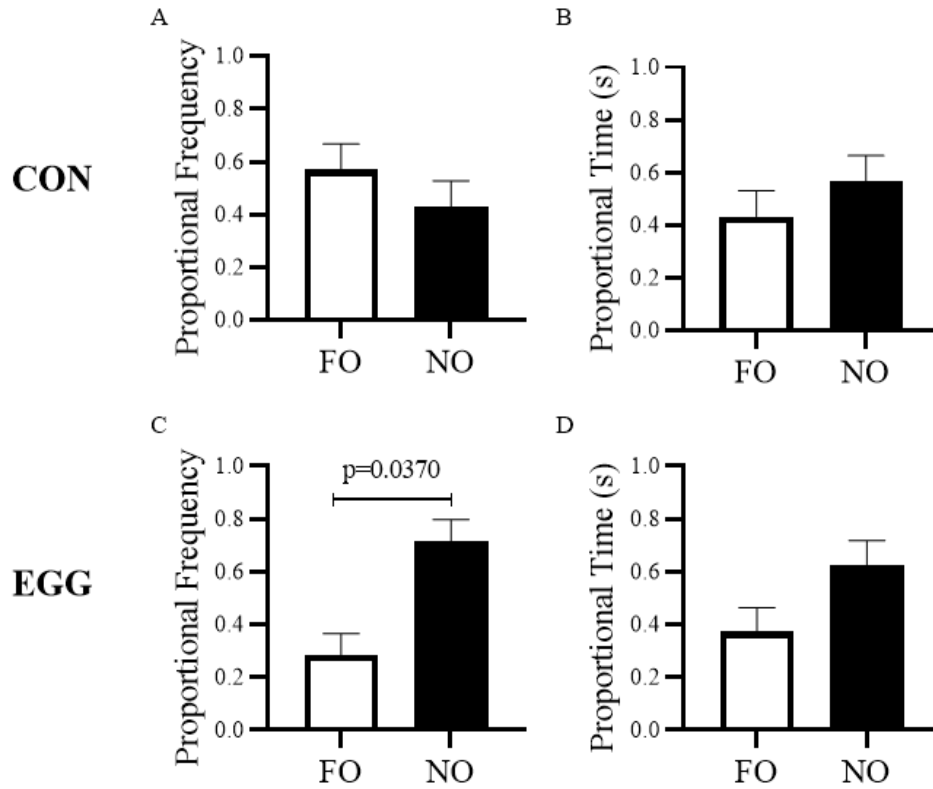


Figure 4.4. Perinatal egg yolk intake increased proportional frequency in novel object recognition testing in piglets. Proportional frequency (A) and proportional time (B) CON (n=12) piglets spent with each object. Proportional frequency (C) and proportional time (D) EGG (n=10) piglets spent with each object. Data was analyzed using a linear mixed-effect model with logit transformation to test for differences in time spent with each object. FO: familiar object; NO: novel object.

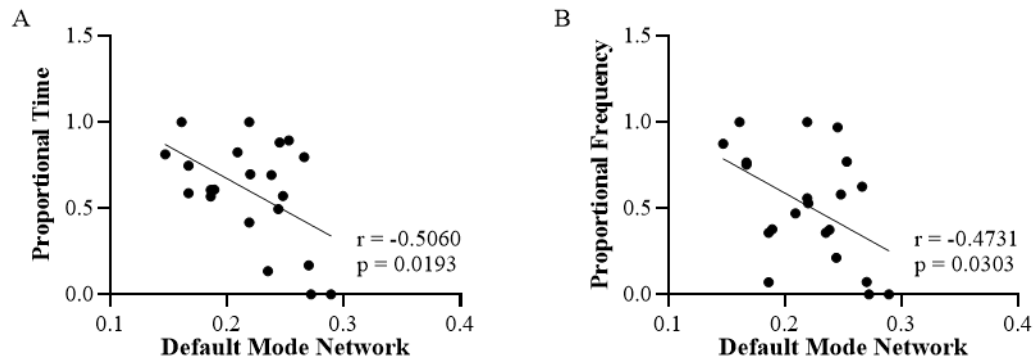


Figure 4.5. Functional outcomes in weaning piglet offspring of novel object recognition tests correlated with brain functional network activation. Data shows correlation results for all piglets ($n=22$). Default mode network activation correlated with proportional time with novel object (**A**) and default mode network activation correlated with proportional frequency with novel object (**B**). r = Pearson's correlation coefficients; p values less than 0.05 are shown.

CHAPTER 5

CATALASE ACTIVITY IN THE BRAIN IS ASSOCIATED WITH RECOVERY FROM BRAIN INJURY IN A PIGLET MODEL OF TRAUMATIC BRAIN INJURY ¹

¹ Dubrof S, Schantz S, et al. Catalase activity in the brain is associated with recovery from brain injury in a piglet model of traumatic brain injury. To be submitted to *Brain Sciences*.

ABSTRACT

Traumatic brain injury (TBI) is a global leading cause of disability and death with millions of new cases added each year. Oxidative stress significantly exacerbates the primary TBI injury leading to increased levels of intracerebral cell death, tissue loss, and long-term functional deficits in surviving patients. Catalase and superoxide dismutase (SOD) mitigate oxidative stress and play a critical role in dampening injury severity. This study examines the neuroprotective effects of the novel antioxidant alpha lipoic acid-based therapeutic, CMX-2043, on antioxidant enzymes in a preclinical TBI model via various drug administration routes. Piglets (n=28) underwent cortical controlled impact to induce moderate-severe TBI and were assigned to placebo (n=10), subcutaneous CMX-2043 (SQ, 10 mg/kg; n=9), or intravenous CMX-2043 (IV, 9 mg/kg; n=9) treatment groups. Treatments began 1 hour after TBI induction and continued for 5 days. MRI was performed throughout the study period to evaluate brain recovery. Blood was collected at 1, 7, and 42 days post-TBI, and liver and brain tissues were collected at 42 days post-TBI to measure catalase and SOD activity. CMX-2043 IV-treated piglets showed 46.3% higher hepatic catalase activity than placebo (p=0.0038), while the SQ group did not show significant changes in hepatic catalase activity compared to placebo. In the brain, SQ-treated piglets had significantly higher catalase activity than both IV (p=0.0163) and placebo (p=0.0003) groups (45.8340 ± 3.0855 , 36.4822 ± 1.5558 , 31.6524 ± 1.3129 nmol/min/mg protein for SQ, IV, and placebo, respectively), while IV-treated piglets did not show significant changes compared to placebo. IV-treated piglets did exhibit 39.3% higher brain SOD activity than placebo (p=0.0148), while the SQ group did not show a significant change. CMX-2043 treatment did not alter plasma antioxidant enzyme activity during the study period. Importantly, within CMX-2043 treated TBI groups, piglets with significantly decreased lesion volumes, midline shift, and combined swelling

and atrophy had better brain recovery, determined by MRI on day 1, 7, and 42 days post-injury TBI, exhibited higher brain catalase activity at 42 days post-injury TBI regardless of administration route, suggesting a link between improved recovery and sustained local catalase activity. This study highlights the impact of administration route on tissue-specific antioxidant responses, with IV administration enhancing liver catalase and brain SOD activity, while SQ administration primarily elevated brain catalase activity. In addition, this study shows an association between increased brain catalase activity and decreased TBI brain lesioning, midline shift, and combined swelling and atrophy, thus emphasizing the role of antioxidant defenses in neuroprotection post-injury.

INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of disability and death worldwide with millions of new cases added each year [1]. TBI tissue damage is a combined result of a complex injury and pathophysiology including the primary and secondary injury pathways [2-5]. The primary injury is typically due to a mechanical insult such as a jolt or blow to the head leading to brain lesioning, hemorrhage, swelling, and atrophy [2,3]. This primary injury is quickly followed by the activation of a secondary injury cascade that includes oxidative stress, excitotoxicity, and inflammation, resulting in increased neuropathology [1,5,6]. Specifically, oxidative stress occurs as a result of a loss of equilibrium between free radical formation and the antioxidant system leading to a buildup of free radicals such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) [6,7]. Moreover, the brain is more susceptible to oxidative damage due to a high amount of peroxidizable fatty acid content [6,8]. In response to injury, however, the brain can initiate the transcription of genes encoded for endogenous antioxidant systems, such as catalase and superoxide dismutase (SOD), that can terminate free radical reactions before there is widespread permanent damage [9,10]. The persistence of oxidative stress with higher levels of catalase and SOD in the brain correlates with neural damage and functional impairment, highlighting the potential for these antioxidant enzymes to serve as biomarkers for assessing injury severity and therapeutic efficacy [1,5,11].

Catalase and SOD are key components of the brain's physiological antioxidant defense system, playing essential roles in neutralizing reactive oxygen species (ROS) and mitigating oxidative damage [12]. Together, these enzymes regulate redox balance and safeguard cells from oxidative injury, which is especially vital in the brain due to its high metabolic activity and lipid-

rich composition, making it particularly vulnerable to oxidative damage [12-14]. Dysregulation of catalase and SOD activity has been implicated in various neurodegenerative diseases and injury models, including TBI, where excessive ROS production exacerbates secondary injury [6,15]. Understanding the regulation of these antioxidant enzymes in response to injury and therapeutic interventions is essential for developing targeted neuroprotective strategies.

CMX-2043 is a novel alpha-lipoic acid (ALA) based therapeutic compound that has neuroprotective, metabolic, and antioxidative properties that may limit TBI-induced tissue damage and long-term functional deficits [16]. ALA is a metabolic antioxidant proven to be a potent protector of neuronal cells from oxidative stress in in vitro and in vivo animal and human studies [16-19]. However, CMX-2043 has demonstrated superior antioxidant potential than ALA in preclinical studies, showing enhanced efficacy in protecting cardiac cells from ischemia-reperfusion injury [16]. In this context, the antioxidant CMX-2043 is a promising candidate for mitigating TBI-associated oxidative damage.

Route of administration can affect drug bioavailability, distribution, and metabolism, ultimately influencing its capacity to reach target tissues [20,21]. Intravenous delivery ensures rapid systemic distribution, potentially leading to higher concentrations in circulation [22], whereas subcutaneous administration may result in slower absorption and prolonged release [23,24]. Examining how these differences may influence antioxidant enzyme activity and recovery outcomes is essential for optimizing treatment strategies for TBI [21]. Therefore, this study examines both subcutaneous and intravenous administration of CMX-2043 to compare therapeutic efficacy.

While previous research has shown that CMX-2043 enhances antioxidant capacity in vitro [16], its effects on enzymatic antioxidant activity within the brain remain unexplored. This study

examines the effects of CMX-2043 administered both subcutaneously and intravenously on antioxidant enzyme activity in a preclinical pig TBI model and explores its potential role in neuroprotection by assessing the relationship between oxidative stress markers and MRI based injury and recovery metrics. The translational pig model employed in this study closely mimics the human brain in anatomy and physiology, increasing the clinical relevance and overall translation of these findings [25-27].

METHODS

Animal handling and study design

Six week old Yorkshire crossbred piglets (n=28) were used for this study. Piglets were allowed ad libitum access to a standard pig diet. At 6 weeks of age, piglets were randomly assigned to experimental groups: saline (placebo, 40 mg/kg; n=10), subcutaneous administration of CMX-2043 (SQ, 10 mg/kg; n=9), or intravenous administration of CMX-2043 (IV, 9 mg/kg; n=9). SQ piglets were given CMX-2043 beginning 1 hour post-TBI and continued thereafter every 8 hours for a total period of 5 days. IV piglets were given CMX-2043 beginning 1 hour post-TBI and continued every 12 hours for a total period of 5 days. The inclusion of both subcutaneous and intravenous administration allowed for the evaluation of potential differences in drug efficacy, which may influence treatment outcomes. Piglets underwent MRI analysis at 1, 7, and 42 days post-TBI using methods as previously described [28,29]. Briefly, multiplanar MRI sequences, including T2 Weighted (T2W), were acquired to assess lesion volume, hemispheric swelling, and atrophy [28,29]. Using T2W sequences, trained and blinded analysts manually identified regions of interest (ROIs), and OsiriX software calculated ipsilateral and contralateral hemisphere volumes (cm³), lesion volumes (cm³), and midline shift (MLS) by measuring the deviation from the ideal midline (mm) [28,29]. This study was conducted in accordance with the University of Georgia

Institutional Animal Care and Use Committee guidelines (Animal Use Protocol: A2022 08-005-Y1-A0).

Controlled cortical impact of traumatic brain injury

The TBI induction procedure was carried out using previously established methods [25,26,28-30], including controlled cortical impact to induce a moderate-severe TBI. Briefly, a 4 cm left-sided incision was made to expose the skull. A 20 mm craniectomy was performed over the motor cortex, and TBI was induced using a controlled cortical impact device with a 15 mm blunt impactor tip (4 m/s velocity, 9 mm depression depth, 400 ms dwell time).

Plasma, liver, and brain tissue collection

Blood samples were collected from all piglets via jugular vein access into EDTA tubes to isolate plasma. Blood was collected at baseline (pre-TBI), immediately prior to treatment administration on day 1 post-TBI, and again at 7 and 42 days post-TBI. Plasma samples were flash frozen in liquid nitrogen and stored in -80°C until analysis.

At 42 days post-TBI, all piglets were sacrificed, and liver and brain tissues were collected. Liver and brain tissue samples were immediately flash frozen in liquid nitrogen and stored in -80°C until analysis.

Measurement of catalase and superoxide dismutase activity in plasma and tissue

Catalase and SOD activity in plasma, liver, and brain tissue were assessed to evaluate antioxidant defense in piglets. Tissue samples from the liver and brain were homogenized in potassium phosphate buffer for the catalase assay and in HEPES buffer for the SOD assay. Catalase and SOD activity were measured using the catalase assay kit (Cayman, Ann Arbor, MI, USA) and SOD assay kit (Cayman, Ann Arbor, MI, USA), following the manufacturer's instructions. Protein content of the homogenates was quantified using the Pierce BCA protein

assay (Thermo Fisher Scientific, Waltham, MA, USA), and catalase and SOD activity were normalized to protein content.

Statistical analysis

Treatment and time effects were analyzed using one-way ANOVA. Data is expressed as mean $\pm$ S.E.M. Pearson correlations were performed to determine relationships between brain catalase and SOD activity with MRI measurements that were obtained following TBI, including midline shift (mm), lesion volume (cm³), and swelling/atrophy (cm³). To further investigate the relationship between antioxidant activity and brain preservation and recovery, MRI measurements of CMX-2043-treated piglets were stratified into top and bottom half groupings and analyzed in relation to catalase activity, allowing for a focused assessment of antioxidant levels and their potential link to MRI outcomes. An unpaired t-test was used to assess for differences in catalase activity in the brain in treated piglets based on these MRI-defined recovery groups. All analyses were done using GraphPad Prism (Version 10.1.0, GraphPad Software, Inc.; San Diego, CA, USA).

RESULTS

Antioxidant enzyme activity in plasma changed over time following TBI

Catalase and SOD activity was measured in plasma collected from piglets at baseline (pre-TBI) and on days 1, 7, and 42 following TBI (**Figure 1**). Catalase exhibited temporal changes following TBI, with an initial increase followed by a decline over time in all three groups (**Figure 1A**). The area under the curve (AUC) analysis for catalase activity (**Figure 1B**) revealed no significant differences between the treatment groups, suggesting that CMX-2043 administration did not significantly alter overall catalase activity in plasma. SOD activity (**Figure 1C**) also showed an early (1 and 7 days) increase post-TBI and a decrease at 42 days but

still remained elevated relative to baseline. However, the AUC analysis for SOD activity (**Figure 1D**) again showed no significant differences between treatment groups, further reinforcing that the treatment did not significantly alter overall SOD activity in plasma.

Despite the lack of significant treatment effect, there was a significant effect of time on antioxidant enzymatic activity in plasma following TBI. This indicates that while treatment did not directly influence plasma antioxidant activity, the temporal changes observed may reflect the natural physiological response associated with limiting TBI injury and promoting recovery.

Administration route of CMX-2043 differentially altered antioxidant status in the liver and brain following TBI

Catalase and SOD activities were assessed in both the liver and brain to evaluate tissue-specific antioxidant responses following TBI (**Figure 2**). CMX-2043 increased hepatic catalase activity, as well as catalase and SOD activity in the brain. Interestingly, administration route of CMX-2043 influenced catalase and SOD activity differently in brain and liver tissues. Specifically, IV piglets exhibited 46.3% higher catalase activity in the liver than placebo (5.7900 ± 0.4518 $\mu\text{mol}/\text{min}/\text{mg}$ protein and 3.6126 ± 0.2608 $\mu\text{mol}/\text{min}/\text{mg}$ protein for IV and placebo, respectively; $p=0.0038$), while the SQ group was not statistically different (4.6410 ± 0.4894 $\mu\text{mol}/\text{min}/\text{mg}$ protein; $p>0.05$) (**Figure 2A**). SOD activity in the liver did not differ significantly between SQ, IV, and placebo treatment groups (83.6760 ± 5.2844 , 71.5300 ± 4.1467 , 77.4373 ± 7.0645 U/mL/mg protein, respectively; $p>0.05$).

Administration route of CMX-2043 also differentially affected catalase and SOD activity in the brain (**Figure 2B**). SQ-treated piglets had significantly higher brain catalase activity than both IV ($p=0.0163$) and placebo ($p=0.0003$) groups (45.8340 ± 3.0855 , 36.4822 ± 1.5558 , 31.6524 ± 1.3129 nmol/min/mg protein for SQ, IV, and placebo, respectively). IV piglets' brain

catalase activity was not different from placebo ($p>0.05$). However, brain SOD activity in IV piglets was 39.3% higher than placebo piglets (0.8522 ± 0.0875 and 0.5722 ± 0.0475 U/mL/mg protein for IV and placebo, respectively; $p=0.0148$), while the SQ group did not show a significant difference relative to placebo (0.7330 ± 0.0521 U/mL/mg protein; $p>0.05$). These findings demonstrate that CMX-2043 enhances antioxidant enzyme activity in a tissue and delivery dependent manner, as IV administration increased catalase activity in the liver and SOD activity in the brain, while SQ administration primarily elevated brain catalase activity.

Additionally, catalase and SOD activity in the brain was notably lower than activity levels in the liver (**Figure 2**), thus supporting previous literature that characterized antioxidant activities in the mouse brain and liver, where brain antioxidant activity was substantially lower than liver antioxidant activity [31]. This difference highlights tissue-specific enzymatic activity and suggests that the liver may play a larger role in systemic antioxidant defense following TBI. Moreover, the brain's heightened vulnerability to oxidative damage is partly due to its relatively weak antioxidant defense systems, which is consistent with the lower antioxidant activity levels observed [1,11].

Catalase activity in the brain is associated with decreased brain tissue damage following TBI

To evaluate the relationship between antioxidant status and tissue damage within the brain post-TBI, catalase and SOD activity values were assessed against MRI measurements in piglets to identify correlations (**Table 1**). It was observed that catalase activity in the brain was negatively correlated with multiple MRI measurements, including 1 day midline shift ($r=-0.4557$, $p=0.0148$), 7 day midline shift ($r=-0.6241$, $p=0.0004$), and 42 day midline shift ($r=-0.5448$, $p=0.0033$). There was a trending negative correlation between catalase activity in the brain with 1 day ($r=-0.3433$, $p=0.0860$) and 7 day ($r=-0.3419$, $p=0.0749$) lesion volume, as well as a

significant negative correlation with 42 day lesion volume ($r=-0.5770$, $p=0.0016$). A significant negative correlation between catalase activity and 1 day swelling/atrophy was also observed ($r=-0.4084$, $p=0.0383$).

To further investigate this, an analysis of only piglets treated with CMX-2043 ($n=19$) was conducted to substantiate if there was a relationship between catalase activity and MRI markers of TBI injury. Piglets were stratified into two groups based on midline shift, lesion volume, and swelling/atrophy severity using MRI measures, creating "low" and "high" groups for each parameter (**Figure 3**). The catalase activity was then compared between low and high groups within a given parameter allowing for a more focused assessment of catalase activity across varying degrees of injury. The results indicated that among CMX-2043 treated piglets, those with improved MRI-assessed markers of brain injury also exhibited higher catalase activity in the brain, independent of administration route. Specifically, catalase activity in the brain trended higher in the low 1 day midline shift group (44.8512 ± 2.1394 nmol/min/mg protein) compared to the high group (37.5749 ± 3.2969 nmol/min/mg protein; $p=0.0760$) (**Figure 3A**). Catalase activity in the brain was significantly higher in the low 7 day midline shift group (46.5803 ± 2.6927 nmol/min/mg protein) versus the high group (35.6537 ± 1.7765 nmol/min/mg protein; $p=0.0042$) (**Figure 3B**). Similarly, catalase activity in the brain was higher in the low 7 day lesion volume group (43.7768 ± 2.2649 nmol/min/mg protein) relative to the high group (36.7293 ± 2.3542 nmol/min/mg protein; $p=0.0465$) (**Figure 3C**), as well as in the low 42 day swelling/atrophy group (44.2151 ± 2.8407 nmol/min/mg protein) compared to the high group (36.2910 ± 1.3349 nmol/min/mg protein; $p=0.0225$) (**Figure 3D**). These findings demonstrate that within CMX-2043 treated piglets, higher catalase activity in the brain suggested reduced TBI-induced neural injury based on MRI biomarkers. Importantly, this relationship was

independent of the route of administration (SQ or IV), suggesting that catalase brain activity may serve as a potential marker of recovery following treatment. Furthermore, catalase activity in the brain at 42 days post-TBI, which is also long after the final treatment, remained a predictor of recovery across the multiple MRI time points. These results also emphasize the variability in antioxidant responses and the need for further investigation to establish a causal link between antioxidant enzyme activity and brain recovery post-TBI.

DISCUSSION

CMX-2043 treatment significantly increased hepatic catalase activity, as well as brain catalase and SOD activity in a piglet TBI model. Additionally, the effects of CMX-2043 on antioxidant enzyme activity in tissue varied depending on SQ or IV administration, influencing catalase and SOD activity differently in the liver and brain. Notably, our findings showed that higher catalase activity in the brain at 42 days post-TBI was related to improved MRI-based biomarkers of brain injury in CMX-2043 treated piglets, regardless of administration route. CMX-2043 treatment did not affect plasma antioxidant enzyme activities, although TBI itself altered these systemic markers throughout the study period. This is the first study to show that CMX-2043 treatment leads to increased levels of catalase and SOD brain activity in a post-TBI large animal model and that increased levels of brain catalase is associated with decreased brain injury. This highlights the potential of CMX-2043 to serve as a therapeutic agent for enhancing brain antioxidant defenses and promoting recovery after injury.

Catalase is a key endogenous antioxidant enzyme that helps remove ROS and mitigate oxidative damage following TBI [12]. Physiologically, superoxide anion is rapidly and efficiently converted into $\text{H}_2\text{O}_2 + \text{O}_2$ by the enzyme SOD, and H_2O_2 is then detoxified into $\text{O}_2 + \text{H}_2\text{O}$ mainly by glutathione peroxidase and, partly, by catalase [13,14]. This enzymatic process involving

catalase and SOD is essential for mitigating the detrimental effects of oxidative damage [14] and promoting recovery [15] after TBI. Maintaining the proper function and activity levels of these antioxidant enzymes is therefore crucial for facilitating the brain's recovery [1,3,6]. This study demonstrates that higher brain catalase activity correlates with improved MRI measures, suggesting a potential link between antioxidant enzyme activity and TBI recovery. While this association was independent of administration route, SQ administration may offer logistical advantages in acute settings, as it can be administered more rapidly and without the need for trained personnel, unlike IV treatment [52]. Earlier intervention is vital given the rapid onset of secondary injury following TBI. Although direct measurement of brain catalase requires invasive, post-mortem tissue collection, these findings highlight the critical role of antioxidant defenses in neuroprotection and the importance of oxidative stress regulation in recovery [1,3,32]. To address this limitation, non-invasive biomarkers that reflect similar oxidative stress responses should be explored. Emerging techniques such as magnetic resonance spectroscopy (MRS) offer a promising approach for assessing oxidative stress-related metabolites in the brain, enabling evaluation at earlier stages of recovery [33,34].

Antioxidants, often supplemented through diet [35], can diminish the potency of various oxidants [12,13,36]. Although endogenous antioxidants can mitigate free radical damage, elevated free radicals can overwhelm the body's natural defenses, thus supplementing the brain's antioxidant capacity may help prevent and alleviate oxidative damage during injury [35,36]. Studies have shown that alpha-lipoic acid, the analog of CMX-2043, can increase the activity of the antioxidant enzymes catalase and SOD in various models of diseases linked to oxidative stress, such as kidney and heart conditions [19,37,38]. In the liver of experimental hyperoxaluric rats, administration of ALA was found to enhance catalase activity and decrease peroxidative

levels [39]. Similarly, ALA supplementation significantly increased serum SOD activity in patients undergoing hemodialysis [37], and markedly restored SOD activity in both the serum and renal cortex of diabetic rats [38]. CMX-2043 is composed of ALA covalently linked to a dipeptide adduct molecule [16]. In an investigation evaluating the antioxidant capacity of CMX-2043, ALA, and the dipeptide adduct individually, CMX-2043 demonstrated superior efficacy in scavenging peroxyl radicals, as measured by the oxygen radical absorbance capacity assay [16]. Notably, the dipeptide adduct had minimal antioxidant activity [16]. These findings suggest that the structural combination of ALA with the dipeptide is essential for enhancing the overall antioxidant potential of CMX-2043 [16]. The current study demonstrated that CMX-2043 treatment increased hepatic catalase activity and increased catalase and SOD activity in the brain, reinforcing the potential of the novel antioxidant molecule of CMX-2043 to restore antioxidant enzyme function under oxidative stress conditions.

Our findings also indicated that elevated catalase activity in the brain was related to improved MRI markers of TBI injury in piglets treated with CMX-2043. Prior research has shown that enhanced activity of endogenous antioxidants, such as SOD and glutathione peroxidase, correlate with reduced oxidative damage and improved neuroprotection in both preclinical and clinical settings [12,40,41]. Despite this association, there remains no established approach for the use of antioxidants in the post-injury period to alleviate the effects of TBI [35]. Many studies have assessed the efficacy of other antioxidants to reduce TBI-associated oxidative damage in animal models and in a limited number of clinical trials as reviewed by Di Pietro et al. [35]. For example, vitamin E is a powerful antioxidant, as it can aggressively scavenge reactive oxygen species [42]. In a rodent model of TBI, Wu et al. found that vitamin E supplementation following TBI significantly normalized levels of oxidative markers, including SOD [42].

Administration of quercetin, another antioxidant, in a rodent TBI-model reduced cognitive deficit and increased catalase and SOD antioxidant activity in the hippocampus [43]. In a rat model of TBI assessing the effects of the antioxidant hydroxysafflor yellow A, it was observed that hydroxysafflor yellow A reduced markers of oxidative stress as observed through increased activity of both catalase and SOD in the brain [44]. These results fall in line with what was observed in the current study, as we also observed changes in enzymatic activity in the liver and brain in response to antioxidant administration. While the findings from this study contribute to the growing body of evidence, further work is needed to establish a rigorous protocol for antioxidant administration in the acute phase post-TBI.

The route of administration of CMX-2043 had distinct effects on antioxidant enzyme activity in the liver and brain. When administered SQ, a drug may be more readily absorbed and metabolized in the subcutaneous adipose tissue before reaching the liver and brain [20,24,45]. This could result in lower levels of the active drug reaching the intended tissue compared to IV administration, which would allow the drug to more directly access these organs due to increased bioavailability [20,46]. Studies have shown that differences in pharmacokinetics can impact drug bioavailability and distribution, ultimately influencing its therapeutic efficacy in different organs [47,48]. For instance, in a rodent study, IV administration of ALA resulted in higher peak plasma levels compared to oral intake, though both routes shared comparable elimination half-lives [49]. Another rodent study revealed that IV administration of CMX-2043 showed rapid clearance of the drug, exhibiting a half-life in rats of about 10 minutes [50]. Conversely, subcutaneous (SQ) administration involves ALA absorption through the interstitial tissues, leading to a slower onset and prolonged presence in circulation. In California sea lions, a single SQ dose of 10 or 20 mg/kg ALA peaked within 20 to 30 minutes, with a half-life of 40 and 32 minutes for 10 and 20

mg/kg doses, respectively [51]. Our results specifically indicated IV administration enhanced catalase activity in the liver and SOD activity in the brain, while SQ administration increased catalase activity in the brain, suggesting the administration route can differentially impact the ability of antioxidant therapies to modulate enzymatic activity in key tissues.

Though CMX-2043 treatment did not alter catalase or SOD activity in the plasma, a significant overall time difference was observed in the antioxidant activity of the plasma following TBI. In a cohort of patients with subarachnoid hemorrhage, SOD concentrations in plasma increased from day 1 to day 7 [53]. This falls in line with the observations in our study where there is an apparent increase in SOD activity on day 7 before it begins to return to normal levels. Among ROS, the superoxide anion is the first to be produced after TBI by cerebral cells via multiple mechanisms, but mainly through the malfunctioning of the mitochondrial electronic transport chain [54]. This may explain why plasma SOD activity on day 1 was 83% higher than baseline, while plasma catalase activity increased by only 35% from baseline on the same day following TBI.

In conclusion, this study demonstrates a dynamic relationship between brain catalase activity and improved MRI markers of injury following TBI. Moreover, CMX-2043 treatment increased brain catalase levels, which was associated with reduced lesion volume, midline shift, and swelling/atrophy. These findings reinforce the critical role of antioxidant defenses in neuroprotection and post-injury repair. Importantly, CMX-2043 treatment enhanced antioxidant enzyme activity in the liver and brain, with its effects varying by administration route, thus highlighting the importance of delivery method in optimizing therapeutic efficacy for TBI recovery. Future research should focus on elucidating the causal pathways linking antioxidant responses to structural and functional recovery, exploring the integration of enzymatic

biomarkers with imaging modalities, and leveraging advanced analytics to enhance prognostic and therapeutic strategies. By addressing these gaps, we can advance our understanding of TBI pathology and develop more targeted interventions to improve outcomes.

AUTHOR CONTRIBUTIONS

Stephanie T. Dubrof: Methodology, Formal analysis, Investigation, Data curation, Writing - original draft preparation. **Sarah L. Schantz:** Formal analysis, Investigation, Data curation.

Taylor H. LePage, Sydney E. Sneed, Savannah R. Cheek: Formal analysis. **Holly A. Kinder,**

Kylee J. Duberstein: Methodology, Writing – review & editing. **Erin E. Kaiser:** Formal analysis, Methodology, Validation, Writing – review & editing, Project administration,

Supervision, Funding acquisition. **Franklin D. West:** Methodology, Validation, Writing – review & editing, Project administration, Supervision, Funding acquisition. **Hea Jin Park:**

Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Co-authors have agreed for this work to be included in this dissertation.

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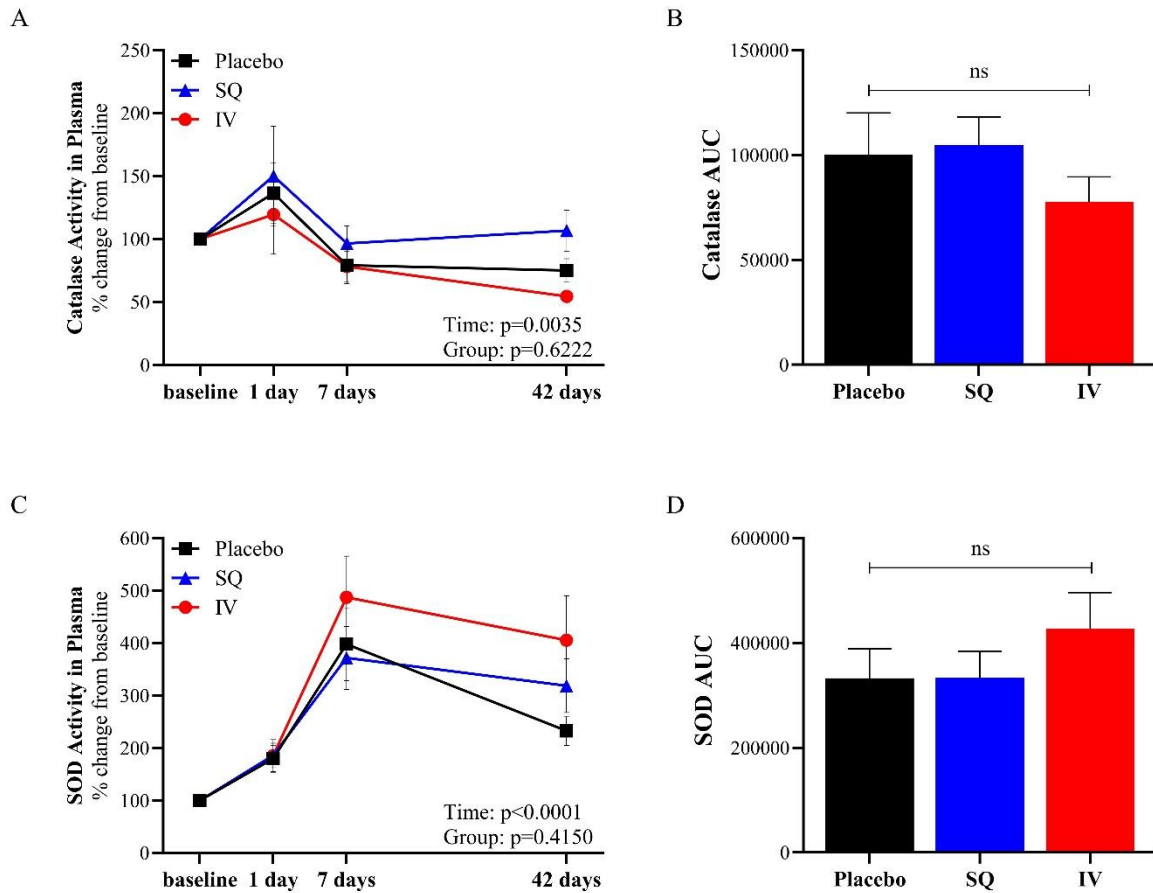


Figure 5.1. Antioxidant enzymatic activity in plasma from piglets following TBI induction. Catalase activity in plasma (A), AUC calculated from catalase activity in plasma from baseline to 42 days post-TBI (B), SOD activity in plasma (C), and AUC calculated from SOD activity in plasma from baseline to 42 days post-TBI (D). AUC: area under the curve; SOD: superoxide dismutase. SQ: subcutaneous treatment group; IV: intravenous treatment group.

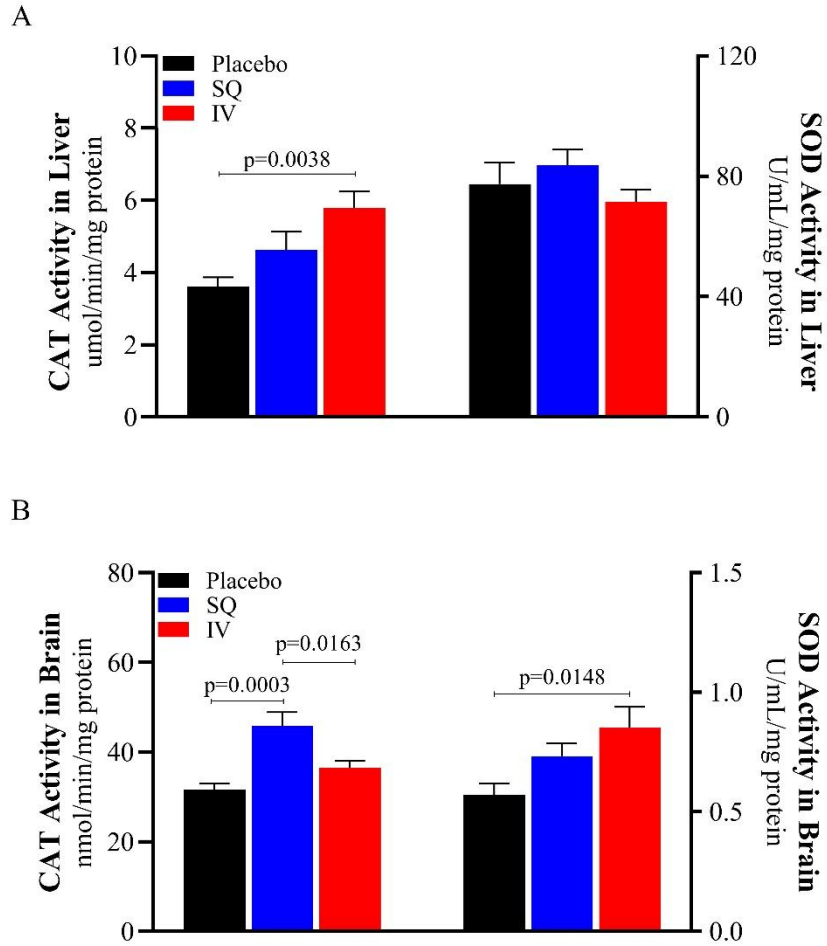


Figure 5.2. Antioxidant enzymatic activity in liver and brain from piglets at 42 days after traumatic brain injury. Catalase and SOD activity in liver (**A**), catalase and SOD activity in brain (**B**). CAT: catalase; SOD: superoxide dismutase; SQ: subcutaneous treatment group; IV: intravenous treatment group.

Table 5.1. Correlations of MRI measurements with catalase activity in the brain 42 days after traumatic brain injury. Pearson's correlations between MRI measurements of midline shift, lesion volume, and swelling/atrophy at 1, 7, and 42 days post-TBI and catalase activity in the brain of all piglets (n=28). r=Pearson's correlation coefficient.

MRI Measurement	r	p-value
1 Day Midline Shift	-0.4557	0.0148
7 Days Midline Shift	-0.6241	0.0004
42 Days Midline Shift	-0.5448	0.0033
1 Day Lesion Volume	-0.3433	0.0860
7 Days Lesion Volume	-0.3419	0.0749
42 Days Lesion Volume	-0.5770	0.0016
1 Day Swelling/Atrophy	-0.4084	0.0383
42 Days Swelling/Atrophy	0.1686	0.4005

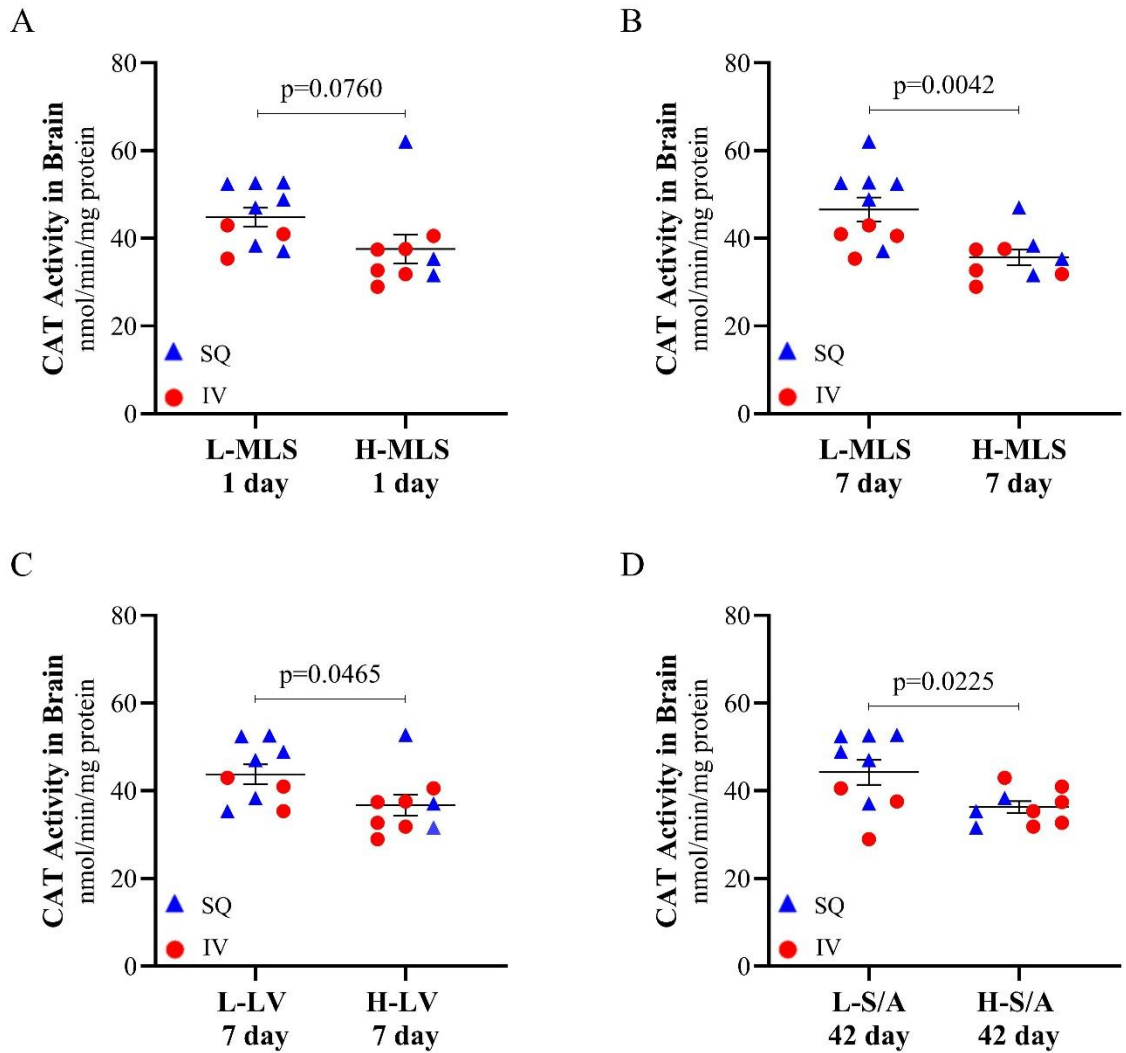


Figure 5.3. Catalase activity at 42 days post-traumatic brain injury in the brain of CMX-2043-treated piglets stratified by MRI-defined injury severity groups. Catalase activity is stratified by low and high 1 day midline shift (**A**), low and high 7 day midline shift (**B**), low and high 7 day lesion volume (**C**), and low and high 42 day swelling/atrophy (**D**) in piglets receiving CMX-2043 treatment (n=19). CAT: catalase; L-: low group; H-: high group; MLS: midline shift; LV: lesion volume; S/A: swelling/atrophy; SQ: subcutaneous treatment group; IV: intravenous treatment group.

CHAPTER 6

SUMMARY & CONCLUSIONS

The overarching objective of this dissertation was to investigate the role of nutritional and therapeutic interventions in brain development and brain health. This dissertation aimed to 1) examine how perinatal supplementation of docosahexaenoic acid (DHA) impacts specialized pro-resolving lipid mediators (SPMs) in offspring brains, 2) assess the functional outcomes associated with perinatal egg yolk supplementation on offspring brain activity, and 3) evaluate the effects of an antioxidant therapeutic drug on oxidative stress markers and neuroprotection in a traumatic brain injury (TBI) model.

In Chapter 3, perinatal DHA supplementation significantly elevated SPM levels in the offspring brain, particularly within regions associated with cognitive function, such as the prefrontal cortex and cerebellum. Notably, increased prefrontal cortex SPM levels correlated with enhanced functional activation in the cerebellar network, revealing a potential mechanism through which maternal DHA intake may shape early cognitive processes in offspring. These findings reinforce the significance of maternal dietary DHA intake for modulating neurodevelopmental pathways that underpin early brain connectivity and function, highlighting a promising mechanism for DHA in optimizing cognitive outcomes through its anti-inflammatory and pro-resolving properties. Collectively, this research provides essential preclinical support for maternal DHA supplementation as a nutritional intervention to promote cognitive outcomes, potentially guiding dietary recommendations aimed at optimizing brain developing during pregnancy and lactation.

In the second study presented in Chapter 4, maternal egg supplementation significantly enhanced brain functional organization and cognitive performance in offspring, as demonstrated by increased activation in the executive and cerebellar networks compared to controls. Additionally, piglets from egg yolk-supplemented sows exhibited enhanced hippocampal-dependent learning and memory. These findings underscore the critical role of maternal diet quality, particularly the consumption of nutrient-rich whole foods like egg yolk, which provide essential nutrients such as choline and lutein to support optimal neurodevelopment and cognitive function in offspring. Overall, this study provides critical preclinical support for maternal egg yolk supplementation as a nutritional strategy to enhance offspring cognitive development, highlighting its potential importance in promoting lifelong brain health.

In Chapter 5, findings revealed significant tissue-specific differences in antioxidant responses within the liver and brain, which were distinctly influenced based on the administration route of CMX-2043. Specifically, intravenous administration effectively increased liver catalase and brain superoxide dismutase (SOD) activity, while subcutaneous administration predominantly increased brain catalase activity. Importantly, the elevated brain catalase activity was associated with reduced brain lesion volume, decreased midline shift, and diminished brown swelling and atrophy. These findings highlight the essential role of antioxidant defenses in protecting neural tissue. Collectively, this study provides evidence supporting targeted antioxidant therapy as a promising approach for improving neuroprotection and recovery outcomes following TBI in humans, potentially shaping clinical strategies for managing brain injury.

In conclusion, this dissertation demonstrates that nutritional and therapeutic interventions have profound potential to shape the trajectory of brain development, cognitive performance, and recovery following neurological injury. By identifying specific nutritional components, such as

DHA and nutrient-rich whole foods like egg yolk, along with therapeutic strategies that leverage antioxidant defenses, these studies provide critical evidence to inform dietary and clinical recommendations. The clear mechanistic insights and translational findings presented here underscore the necessity of prioritizing maternal nutritional status and targeted antioxidant therapies to enhance cognitive resilience, mitigate neurological damage, and ultimately promote optimal lifelong brain health.