MATERNAL EXPOSURE TO PROSTAGLANDIN E2 AFFECTS HIPPOCAMPAL SYNAPTIC PLASTICITY IN MICE OFFSPRING – A LINK TO AUTISM SPECTRUM DISORDER.

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ABSTRACT

Prostaglandin E2 (PGE2) is a lipid signaling molecule involved in early healthy brain development. Exposure to environmental risk factors such as air pollutants, infections, and drugs such as acetaminophen during early pregnancy have shown to impact PGE2 levels and have all been linked to Autism Spectrum Disorders (ASDs). Our previous studies show that maternal exposure to PGE2 and the lack of the PGE2 producing enzyme Cyclooxygenase-2 (COX2) results in sex-specific abnormal dendritic morphology within the cerebellum and the hippocampus as well as ASD-like behaviors including motor deficits and anxiety in mice offspring. In this study, I investigated sex-dependent effects of prenatal PGE2 exposure on hippocampal electrophysiology in the C57bl/6 mice offspring at postnatal day 90-100. I measured Schaffer collateral long-term potentiation (LTP), paired-pulse facilitation (PPF), input/output (I/O) responses, the expression of glutamate receptor components NMDAR subunit 2A, AMPA subunit GluR1, beta-actin, and morphological characteristics such as primary dendrite length and cell soma size of pyramidal neurons in the hippocampus. I found that PGE2 exposure decreased LTP in males and I/O responses in females at higher stimulation intensities with no effect on PPF. PGE2 also increased the expression of NMDAR2A in males with no effect on GluR1 or β-actin. However, PGE2 did not affect pyramidal cell morphology. Overall, our data suggests that prenatal PGE2 exposure disrupts innate sex differences by reducing LTP maintenance in males, while impairing basal synaptic strength in females. I propose a model that PGE2-dependent upregulation of NMDAR2A observed in male offspring may reflect a

neurotoxicity effect of PGE2 mediated by glutamate, subsequently leading to neuronal death, which could explain the corresponding decrease in LTP. In summary, this study adds further evidence that abnormal maternal PGE2 levels known to be influenced by many environmental risk factors may affect hippocampal function and contribute to specific deficits in ASDs in a sex-dependent manner.

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AA – Arachidonic acid

aCSF – artificial cerebrospinal fluid

ADDM – Autism and Developmental Disabilities Monitoring

ADHD – Attention deficit hyperactivity disorder

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

ASD – Autism spectrum disorders

BPA – Bisphenol A

CA – Cornu ammonis

CaMKII – calcium/calmodulin-dependent protein kinase II

CF – Control female (saline-exposed female)

CM – Control male (saline-exposed male)

COX1 – cyclooxygenase-1

COX2 – cyclooxygenase-2

COX2-KI – cyclooxygenase-2 knockin

DHA – docosahexanoic acid

dmPGE2 – 16, 16 dimethyl prostaglandin E2

EP – E Prostanoid

EPA – eicosapentaenoic acid

fEPSP – field excitatory post-synaptic potential

FXS – Fragile X Syndrome

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GI – Gastrointestinal

G11 – Gestational day 11

HFS – high frequency stimulation

HRP – Horseradish peroxidase

IL-6 – interleukin-6

I/O – Input/Output

LTD – Long-term depression

LTP – Long-term potentiation

MDA – malondialdehyde

mPFC – medial prefrontal cortex

Mpges-1 – microsomal Prostaglandin E synthase-1

NLGN – Neuroligin

NMDAR – *N*-methyl-D-aspartate receptor

NRXN – Neurexin

PBS - Phosphate-buffered saline

PFA – paraformaldehyde

PGE2 – Prostaglandin E2

PGE2F – PGE2-exposed female

PGE2M – PGE2-exposed male

PI-3K – phosphatidylinositol-3 kinase

PKA – Protein Kinase A

PKC – Protein Kinase C

PLA2 – phospholipase A2

$PM_{2.5}$ – particulate matter 2.5

PN – Post-natal

PPF – paired-pulse facilitation

PTEN – phosphatase and tensin homolog

PUFA – Poly-unsaturated fatty acid

RIPA – radio-immunoprecipitation assay

SC – Schaffer Collateral

SDS-PAGE – Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

TBS-T – Tris Buffer Saline–Triton

VPA – Valproic acid

Wnt – Wingless

CHAPTER 1: INTRODUCTION

1.1 Autism Spectrum Disorder

Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by deficits in three prime domains – social interaction, communication (expressive and receptive language), as well as repetitive and restricted behavior (American Psychiatric Association, 2014; Centers for Disease Control and Prevention [CDC], 2018). ASD can be diagnosed before a child reaches one year of age, however, symptoms become more prominent by 2-3 years of age (American Psychiatric Association, 2014; CDC, 2018). There is a large sex bias in ASD, where males are 4 times more likely to be diagnosed than females (American Psychiatric Association, 2014; CDC, 2018). Various genetic and environmental risk factors have been associated with ASDs. For example, having a twin or sibling with ASD (Le Couteur et al., 1996), parents getting pregnant at an older age (American Psychiatric Association, 2014; CDC, 2018), having genetic conditions such as Fragile X Syndrome (FXS) (Bregman et al., 1988), exposure to metals such as lead (London & Etzel, 2000) and mercury (Mutter et al., 2005) and consuming medications during pregnancy such as thalidomide and valproic acid (Williams et al., 2001). Studies have also reported various neurological and non-neurological conditions such as attention deficit hyperactivity disorder (ADHD), depression, anxiety (CDC, 2018), epilepsy, sleep disorders and gastrointestinal (GI) disorders (Bauman, 2010) to occur comorbidly with ASD. Apart from the main symptoms mentioned above, individuals with ASD also face other challenges such as deficits in cognition, learning, language skills and atypical behavioral patterns such as hyperactivity and impulsivity (CDC, 2018). Although there is no treatment for ASD, a range of individualized methods such as pharmacological or behavioral interventions are often

implemented to improve specific symptoms (CDC, 2018). The most alarming aspect of ASD in the recent years has been its increasing prevalence rates. According to the data collected by the Autism and Developmental Disabilities Monitoring (ADDM) Network on 8-year-old children living in 11 communities across the United States from the year 2000 to 2020, there has been a drastic increase in incidence rates of ASD (CDC, 2020). In the year 2000, the prevalence of ASD was 6.7 per 1000 8-year-old children (**Figure 1**). By the year 2010, the prevalence almost doubled to an average of 14.7 in 1000 children. The prevalence subsequently almost doubled again to 27.6 per 1000 children or 1 in 36 children by the year 2020.

Several environmental and genetic factors have been linked to the etiology of ASD. Both pre- and perinatal factors such as maternal medication, maternal infection, immune dysfunction, and exposure to various drugs and environmental toxins have been shown to increase the risk of ASD in individuals (Chaste & Leboyer, 2012). Additionally, several genes have also been implicated in ASD. Specifically, synaptic genes including neuroligins (NLGN3) which are postsynaptic cell adhesion molecules, postsynaptic scaffolding proteins (SHANK3), and neurexins (NRXN1) which are presynaptic cell adhesion molecules have been reported to be mutated in ASD individuals (Almandil et al., 2019). More recently, studies focusing on environmental risk factors of ASD, such as drug exposure, have been on the rise (Gardener et al., 2009). Specifically, recent studies have suggested a link between ASD and exposure to environmental risk factors during pregnancy that affect signaling of specific biolipids in the brain called prostaglandins (discussed in more detail below) (Tamiji & Crawford, 2011; Wong & Crawford, 2014). The increasing financial burden, mental stress, and reduction in quality of life of families of children with autism makes it more compelling to study the etiology of this disorder.

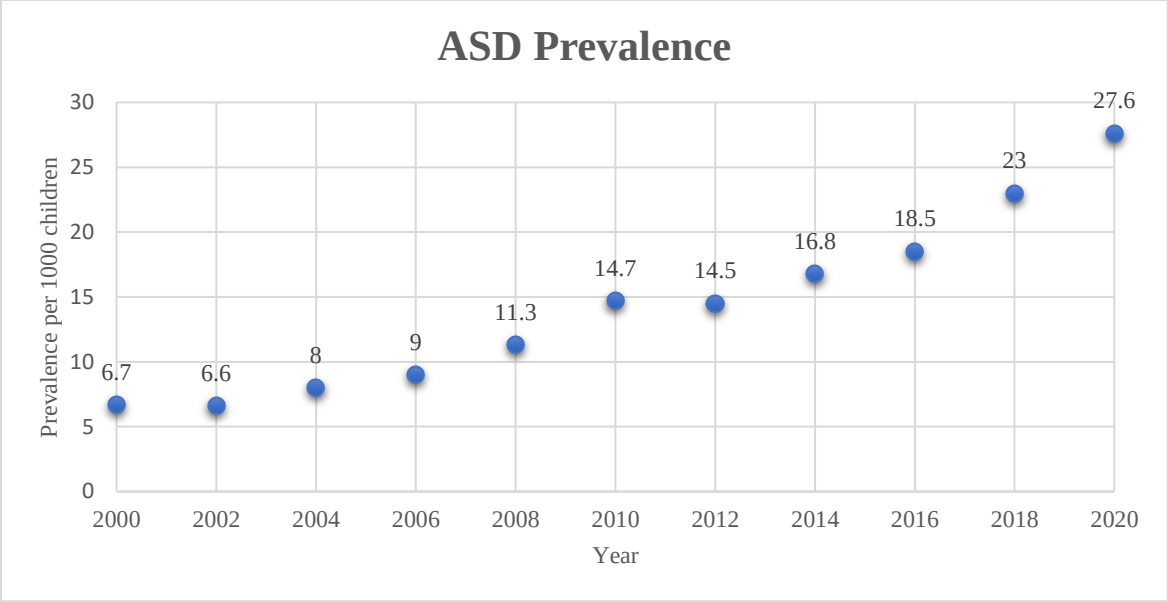


Figure 1: Prevalence of ASD per 1000 8-year-old children across 11 sites in the United States.
Data was obtained from CDC's ADDM Network (CDC, 2020).

1.2 Lipid Signaling in the Healthy Brain

Lipids, an essential component of the cell membrane, are important for healthy development of the brain. Lipids constitute approximately 60% of the dry mass of the adult human brain, of which 35% accounts for poly-unsaturated fatty acids (PUFAs). PUFAs are involved in critical processes such as synaptic plasticity, neuronal survival, inflammatory responses, neurogenesis, synaptogenesis, myelin sheath formation, cognition (Bazinet & Layé, 2014), maintenance of membrane fluidity, signaling, enzyme and receptor activity (Yehuda et al., 1997). Arachidonic acid (AA) and docosahexanoic acid (DHA) are major PUFAs synthesized via the metabolism of linoleic acid from the omega-6 family and alpha-linolenic acid from the omega-3 family of fatty acids respectively. AA is released from the phospholipid membrane by the enzymatic activity of phospholipase A2 (PLA2) and is further converted to a major bioactive lipid mediator known as Prostaglandin E2 (PGE2) by the enzymes cyclooxygenase-1 (COX1) or cyclooxygenase-2 (COX2) (Sang & Chen, 2006) (**Figure 2**). PGE2 exerts its diverse physiological responses by binding on the four G-protein coupled E-prostanoid receptors EP1, EP2, EP3 and EP4 (Burks et al., 2007). PGE2 is involved in crucial processes including neuroprotection (McCullough et al., 2004), neuroinflammatory responses (Shi et al., 2010), synaptic transmission (Marty et al., 2008), neuronal development, long-term potentiation, spatial learning (Tassoni et al., 2008), cell maturation (Kubo et al., 2004) and cell survival (Andreasson, 2010).

Although both COX1 and COX2 are responsible for synthesizing PGE2, there are key differences in their function and location. COX-1 is constitutively expressed in peripheral tissues and the brain (Kim et al., 2017), mainly by microglia, however, its expression can be upregulated in response to brain injury (Schwab et al., 2002) and in pathological conditions (Hoozemans et al., 2001). In contrast, COX-2 expression is induced in peripheral tissues primarily in response to

inflammatory stimuli but is constitutively expressed in the brain primarily in neurons, specifically in post-synaptic dendritic spines of excitatory neurons, hence playing a significant role in neurotransmission and synaptic plasticity (Choi et al., 2009; Kaufmann et al., 1996; López & Ballaz, 2020; Yamagata et al., 1993; Yang & Chen, 2008). Normal functioning of the COX2/PGE2 pathway is crucial for healthy brain development as these pathways are involved in chief functions including synaptic plasticity, cognition (Sang & Chen, 2006), development (Saint-Dizier et al., 2011), cell migration, speed of migration, cell division (Wong et al., 2014), and dendritic spine formation (Burks et al., 2007).

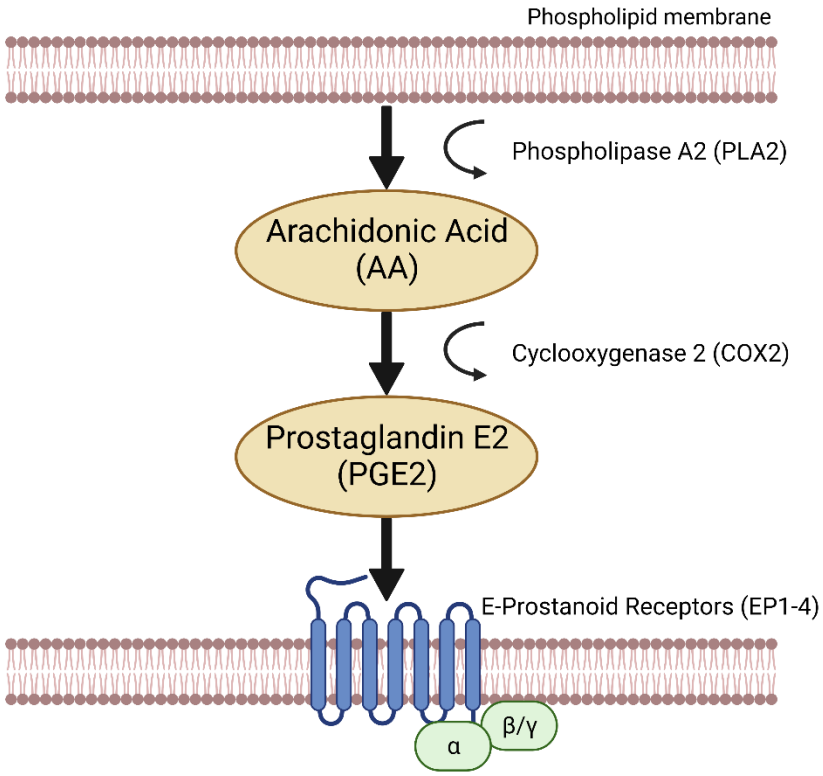


Figure 2: The production of PGE2. Arachidonic acid is released from the phospholipid bilayer by the enzymatic activity of phospholipase A2. AA is then converted to PGE2 by COX2. PGE2 binds to EP receptors which leads to further downstream signaling cascades. Figure created using BioRender.com.

1.3 Abnormal Lipid Metabolism in ASD

Abnormalities in the COX2/PGE2 pathways have been linked to pathologies of the nervous system such as ASD (Kalkman, 2012; Okerlund & Cheyette, 2011; Tamiji & Crawford, 2011; C. Wong & Crawford, 2014). Clinical and epidemiological studies have shown that abnormal levels of PGE2 due to genetic causes and environmental risk factors are associated with neurodevelopmental disorders like ASD (Wong & Crawford, 2014). For example, elevated levels of PGE2 (Brigandi et al., 2015; El-Ansary & Al-Ayadhi, 2012), COX2 and microsomal Prostaglandin E synthase-1 (Mpges-1) – enzymes involved in the production of PGE2, – have been reported in the plasma of ASD patients (Qasem et al., 2018). In the same study, ASD patients had decreased levels of PGE2 receptor EP2 compared to healthy controls. Furthermore, polymorphism of the COX2 encoding gene *Ptgs2* has also been linked to ASD related behaviors (Yoo et al., 2008). Other studies have also provided evidence showing an increase in lipid peroxidation biomarkers such as malondialdehyde (MDA) in ASD patients compared to controls (Meguid et al., 2011; Rossignol & Frye, 2012). Additionally, increased levels of PLA2 and decreased membrane fluidity or increased membrane rigidity have been linked to ASD as well (Bell et al., 2004; Chauhan & Chauhan, 2006; Helena Fávero de Souza Tostes et al., 2013). Furthermore, studies conducted in ASD patients have shown an increase in PUFAs DHA and eicosapentaenoic acid (EPA) and a decrease in AA in red blood cells and plasma (Bell et al., 2004; Brigandi et al., 2015; El-Ansary et al., 2011; Vancassel et al., 2001). In contrast, a decrease in DHA levels were found in ASD subjects in other studies (Brigandi et al., 2015; Vancassel et al., 2001). In summary, these lipid mediators, especially COX2 and PGE2, have been suggested to be important biomarkers for ASD (Bjørklund et al., 2018; El-Ansary & Al-Ayadhi, 2012; Qasem et al., 2018; Shen et al., 2020).

1.4 Environmental Risk Factors of ASD

Many environmental factors shown to influence the level of PGE2 have been linked to the etiology of ASD. Specifically, exposure to drugs such as valproic acid, acetaminophen, misoprostol and thalidomide, toxic heavy metals such as lead, mercury and arsenic, pesticides, inflammatory factors including cytokines, and dietary imbalances in vitamin D and folic acid have been associated with abnormal lipid metabolism and ASD (Ashmawi & Hammoda, 2022). Interestingly, exposure to these risk factors during critical time periods in development both pre- and postnatally are known to pose a greater risk of developing ASD. Specifically, prenatal and early postnatal periods of neurodevelopment are known to be more vulnerable to environmental insults (Dietert et al., 2011). Some of these environmental risk factors relevant to the COX2/PGE2 pathway are discussed below.

1.4.1 Drug Exposure

Valproic acid

Exposure to various drugs during a critical time in early pregnancy has been associated with a high risk of ASD prevalence in the offspring in both humans and animal models. Valproic acid (VPA) is an anti-epileptic drug used against seizures, bipolar disorders, and migraines. Children born to epileptic mothers who have taken VPA have been diagnosed by fetal valproate syndrome, a condition phenotypically similar to ASD (Ardinger et al., 1988; Mawer et al., 2002; Moore et al., 2000). Hence, the VPA exposed rodent model has been extensively studied to understand the neurobiology of ASD in humans. Studies have provided evidence of major congenital malformations (Dean et al., 2002; Tomson et al., 2019), intellectual disabilities and delayed childhood milestones in children exposed to VPA prenatally (Daugaard et al., 2020).

Furthermore, several studies on human participants have provided evidence for increased risk of ASD in children prenatally exposed to VPA (Bromley et al., 2013; Christensen et al., 2013; Moore et al., 2000; Rasalam et al., 2005). Developmental issues often linked to ASD were found in children prenatally exposed to VPA, including speech and language delay, behavior problems, joint laxity (Dean et al., 2002), poor verbal and non-verbal cognitive abilities, social skills (Meador et al., 2011), mild motor delay and learning difficulties (Rasalam et al., 2005).

To this end, animal models of autism have been well established through studies conducted on rodents exposed to VPA pre- and postnatally. Interestingly, prenatal exposure to VPA has also shown to affect levels of the COX-2 encoding *Ptgs2* gene in the cerebral cortex of young-adult male rats (Gąssowska-Dobrowolska et al., 2020). Rats exposed to VPA have shown ASD-like behaviors such as anxiety, impaired emotional learning (Banerjee et al., 2014), hyperactivity and repetitive behavior, delayed motor maturation, and reduced exploratory and social behavior which are consistent with human autistic behavior (Schneider & Przewłocki, 2005; Yang et al., 2016). VPA exposure in rats have also been linked to developmental retardation, regression and intrusive behaviors associated with abnormal cerebellar and hippocampal functioning (Wagner et al., 2006). Moreover, VPA exposure has been linked to the suppression of neurotrophin production which subsequently leads to apoptotic neurodegeneration in the developing brain (Bittigau et al., 2002). Additionally, other molecular abnormalities including increased dendritic spine density, decreased expression of phosphatase and tensin homolog (PTEN), a gene implicated in ASD, in the hippocampus and the cortex, reduced number of cells in the Cornu ammonis (CA1) region of the hippocampus (Yang et al., 2016), reduction in the number of purkinje cells in the cerebellum (Ingram et al., 2000), and reduced

COX1, COX2 and PGE2 levels in the brain were also found in rodents exposed to VPA (Bosetti et al., 2003).

Acetaminophen

Acetaminophen is a common over-the-counter analgesic used to treat fever and pain. This drug has been widely used by pregnant females and recent research has provided evidence for increased risk of ASD in children exposed to acetaminophen *in utero* (Avella-Garcia et al., 2016; Liew et al., 2014, 2016). Interestingly, acetaminophen is known to inhibit the synthesis of PGE2 (Dean et al., 2012; Parker et al., 2017). Prenatal acetaminophen exposure has been linked to higher number of autism spectrum symptoms in males, decreased attention function, and higher risk of hyperactivity and impulsivity in both genders (Avella-Garcia et al., 2016), hyperkinetic symptoms (Liew et al., 2016), and behavior problems (Liew et al., 2014). An ecological study showed a strong positive correlation between prenatal and early postnatal acetaminophen exposure and ASD prevalence (Bauer & Kriebel, 2013). In another case-control parental survey study, administration of acetaminophen after MMR vaccination in children aged 1-5 years was associated with ASD (Schultz et al., 2008). Furthermore, a study conducted on Norwegian children whose mothers' used acetaminophen at a critical time during pregnancy, revealed deficits in motor development and communication (Brandlistuen et al., 2013). It is also suggested that acetaminophen is an endocrine disruptor that can cross the placenta, induce neurotoxicity, oxidative stress and cell death which can ultimately affect neurodevelopment (Liew et al., 2016; Parker et al., 2017). Additionally, pre- and postnatal exposure to acetaminophen has also shown to affect prefrontal cortex gene expression in mice (Baker et al., 2023) and reduce pyramidal cell

layer thickness in the CA region of rats respectively (Albo Hussin & Al-Allaf, 2022). Ultimately, all these findings suggest a strong link between acetaminophen exposure and ASD.

Misoprostol

Misoprostol is a drug analogous to Prostaglandin E, used to treat ulcers and misused by pregnant mothers to induce labor and terminate pregnancies (Dodd & Crowther, 2010). Misoprostol can bind to Prostaglandin E receptors EP1-4, regulate calcium levels in the cell and subsequently affect gene expression (Breyer, 2001) (**Figure 3**). Several studies have reported an increased risk of ASD and congenital malformations associated with exposure to misoprostol. A case study conducted in Brazil reported the diagnosis of Mobius syndrome in children born to mothers who misused misoprostol during early stages of their pregnancy (Pottie, 1999). In another study conducted in northeastern Brazil, ASD-like symptoms were reported in children prenatally exposed to misoprostol (Bandim et al., 2003). Our lab has shown that exposure to misoprostol and PGE2 can affect calcium levels in growth cones and decrease the number and length of neurites during differentiation (Tamiji & Crawford, 2010).

Overall, pre- and postnatal exposure to certain drugs during a critical time in neurodevelopment can lead to molecular abnormalities, altering lipid signaling in the brain, which can ultimately manifest into behavioral deficits associated with ASD.

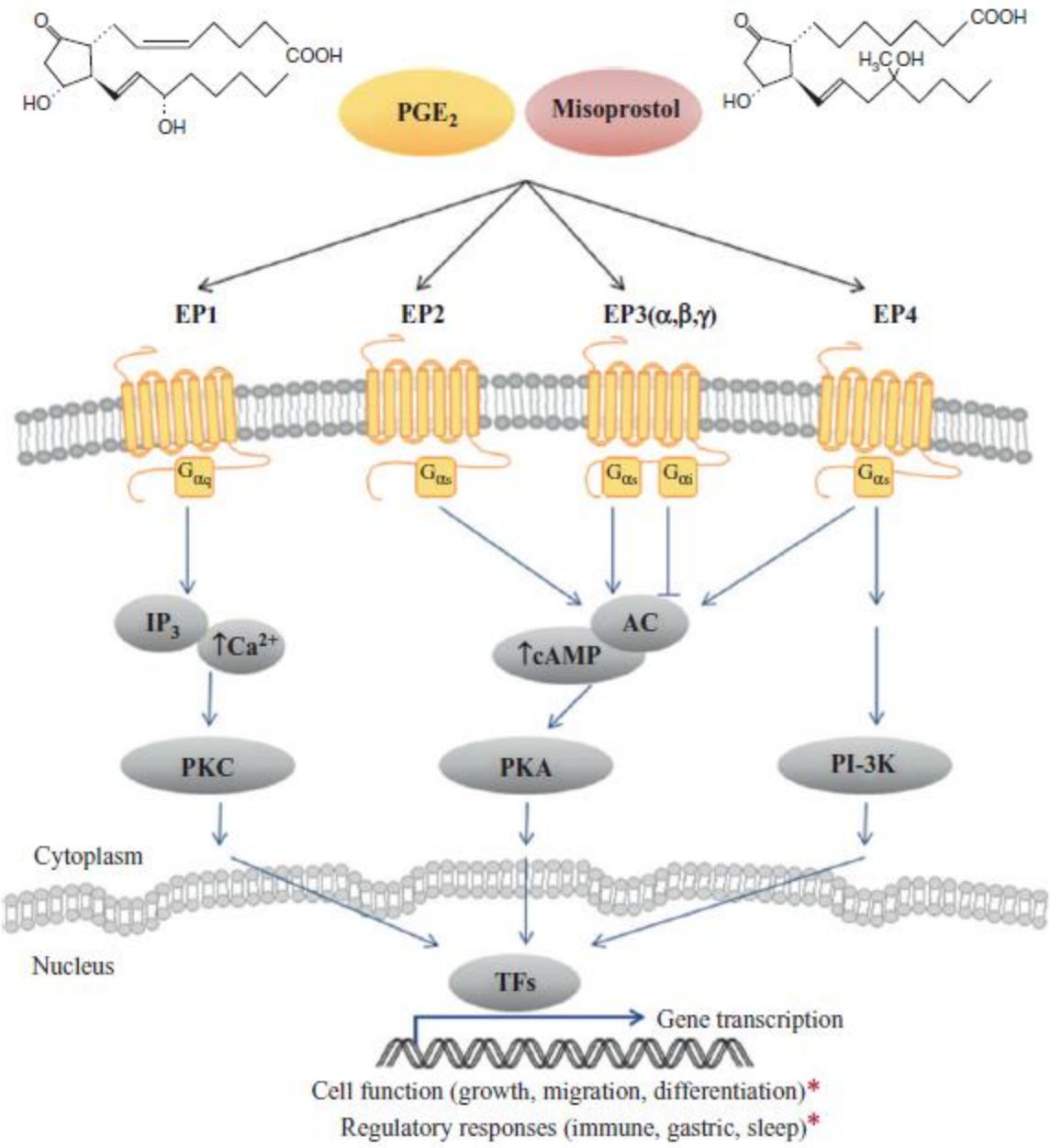


Figure 3: The interaction between misoprostol and PGE2 signaling. Misoprostol is a PGE2 analog that can bind to the PGE2 receptors EP1-4 and regulate gene transcription via the activation of protein kinase C (PKC), protein kinase A (PKA) and phosphatidylinositol-3 kinase (PI-3K). The asterisks indicate abnormalities linked to ASD. Figure and copyright obtained from (Wong & Crawford, 2014)**.**

1.4.2 Maternal Injection and Immune Dysfunction

Several studies have recently emerged, indicating a link between maternal infection and inflammation during pregnancy and the risk of ASD in their offspring (Jiang et al., 2016). A study conducted in Denmark reported the development of ASD in children whose mothers suffered viral infections such as influenza during the first trimester of pregnancy (Atladóttir et al., 2010). Additionally, mothers having 2 or more infections, urinary tract infection being the most common, were at a higher risk of having children with ASD (Zerbo et al., 2016). Maternal influenza infection, prolonged episodes of fever and use of antibiotics during pregnancy were associated with autism (Atladóttir et al., 2012). Animal studies have also supported findings from human studies, for example, mice born to influenza infected mothers also showed behavioral deficits reflective of hyper-anxiety and impaired social behavior indicating altered fetal brain development (Shi et al., 2003).

Post-mortem studies on the brains of ASD individuals have shown an increase in proinflammatory cytokines such as interleukin-6 (IL-6) in the cerebrospinal fluid (CSF), and the activation of microglia and astroglia in the cerebellum, which is suggested to be part of the neuroinflammatory response (Vargas et al., 2005). High levels of tumor necrosis factor-alpha, suggested to be a possible marker for inflammatory damage, was detected in the CSF of children with ASD (Chez et al., 2007). Another study reported an increase in microglial cell density in the dorsolateral prefrontal cortex of individuals with ASD (Morgan et al., 2010). Elevated levels of chemokines in the plasma of ASD children were also associated with behavioral deficits, impaired communication, and aberrant behaviors (Ashwood et al., 2011).

Overall, maternal immune activation and dysfunction during pregnancy has shown to be strongly associated with the development of ASD in the offspring.

1.4.3 Heavy Metal Exposure and Air Pollution

Heavy metals are toxic to the brain and can alter fetal brain development, consequently resulting in impairments in cognition, neurodevelopment, and behavior (Gorini et al., 2014). Studies have provided evidence suggesting a link between exposure to metals such as lead, mercury and cadmium, and the development of ASD (Gorini et al., 2014). Higher levels of blood mercury levels were reported in ASD subjects compared to controls (Geier et al., 2010). Increased levels of lead and mercury were found in the nail and hair samples of children with ASD compared to healthy children which can result in detrimental effects on cognition and development (Lakshmi Priya & Geetha, 2011). In a study conducted in Oman, higher levels of heavy metals including lead, aluminum, silicon, molybdenum, vanadium, chromium, cadmium, cobalt, nickel, boron, and barium were found in the hair samples of ASD diagnosed children compared to controls (Al-Farsi et al., 2013). Exposure to cadmium and lead *in utero* was associated with lower intelligence scores, poor motor, and perceptual functioning in children (Bonithon-Kopp et al., 1986; Kippler et al., 2012). Postnatal exposure to lead was associated with behavior problems in 8-year-old school children (Bellinger et al., 1994). Deficits in motor speed (Debes et al., 2006), language, attention and memory were reported in children prenatally exposed to methylmercury (Grandjean et al., 1997).

Exposure to various air pollutants both pre- and postnatally have been suggested as a possible risk factor for ASD. Perinatal exposure to hazardous air pollutants such as methylene chloride, quinoline and styrene was linked to a higher risk of ASD in 8-year-old children (Kalkbrenner et al., 2010). Prenatal exposure to air pollutants including nitric oxide (Pagalan et al., 2019), lead, benzene, toluene, xylene, formaldehyde, and chlorinated solvents were suggested to increase the risk of ASD in children (Von Ehrenstein et al., 2014). Air pollutants

including diesel, manganese and methylene chloride was associated with ASD (Roberts et al., 2013). Pre- and early postnatal exposure to traffic related air pollutants such as nitrogen dioxide, particulate matter 2.5 ($PM_{2.5}$) and PM_{10} was also linked to ASD (Volk et al., 2013). Another study reported an increased risk of ASD in children exposed to air pollutants including ozone, carbon monoxide, nitrogen dioxide, sulfur dioxide and PM_{10} (Jung et al., 2013). Ultimately, pollutants and heavy metals can alter neurodevelopment and lead to behavioral deficits seen ASD (Frye et al., 2021; Von Ehrenstein et al., 2014).

1.5 Current Research Evidence for Abnormal COX2/PGE2 Signaling and its Link to ASD from our Lab.

Based on previous studies, our lab developed 2 potential models for ASD – the genetic COX-2 deficient (COX2-KI) model and the PGE2-exposed model. Studies conducted on mice and neuronal stem cells have shown that COX-2 deficiency and PGE2 exposure modifies the expression of several genes involved in the Wingless (Wnt) pathway (Rai-Bhogal, Ahmad, et al., 2018; Rai-Bhogal, Wong, et al., 2018; Wong et al., 2014, 2016, 2019). Interestingly, these Wnt genes have been linked to critical processes in the brain such as neuronal proliferation, migration, and differentiation during early embryonic stages and some of them have also been associated with neurodevelopmental disorders including ASD (Rai-Bhogal, Wong, et al., 2018; Wong et al., 2014). Studies in our lab have also shown abnormal neuronal and dendritic morphology including looping, arborization, cell soma size, branch length and spine morphology in the cerebellum and the hippocampus of the COX-2 and PGE2-exposed mice (Kissoondoyal et al., 2021; Kissoondoyal et al., Unpublished; Iyer et al., Unpublished). Axonal looping, neurite extension and neurite retraction have also been shown in PGE2 treated NE4C and Neuro-2a cells

(Kissoondoyal & Crawford, 2021; Tamiji & Crawford, 2010). Abnormalities in dendritic morphology have been attributed to abnormal expression of proteins involved in cytoskeletal architecture (Kissoondoyal et al., 2021; Kissoondoyal & Crawford, 2021; Iyer et al., Unpublished). Furthermore, behavioral studies also strengthened the association of abnormal COX2/PGE2 signaling with ASD as studies showed ASD-like symptoms such as anxiety, motor impairments, repetitive behavior, and deficits in social interaction (Wong et al., 2019; Kissoondoyal et al., Unpublished). Most importantly, majority of these genetic, morphological and behavioral abnormalities were sex specific, where males were affected more than females, and/or developmental stage specific indicating that COX2/PGE2 signaling affects the brain in a sex and stage dependent manner (Kissoondoyal et al., 2021; Rai-Bhogal, Ahmad, et al., 2018; Rai-Bhogal, Wong, et al., 2018; Wong et al., 2019; Iyer et al., Unpublished).

1.6 Functional and Anatomical Abnormalities in the Hippocampus Linked to ASD

As mentioned earlier, ASD is behaviorally characterized by anxiety, repetitive behavior and social deficits, with many implicated brain regions such as the cerebellum, amygdala, hippocampus and prefrontal cortex, contributing to these complex behavioral impairments. Recent research has suggested that the hippocampus may be involved in some of these functions, particularly social behavior, and its abnormal development may lead to manifestation of these symptoms in ASDs (Banker et al., 2021). Moreover, ASD studies have also demonstrated memory impairments, which is a function closely associated with the hippocampus (Cooper et al., 2017; Williams et al., 2005). The role of hippocampus in the etiology of ASDs has long been overlooked, therefore, it is important to study this brain region in the context of ASD. The hippocampus, like most other brain regions, is involved in several functions that are critical for

development. Although it is primarily associated with learning and memory, emerging studies have suggested its involvement in other functions including spatial navigation, emotional behavior (Anand & Dhikav, 2012), and social interactions (Banker et al., 2021; Immordino-Yang & Singh, 2013; Rubin et al., 2014; Trinkler et al., 2009). Specifically, researchers have highlighted memory and social interaction as notable functions of the hippocampus that have been implicated in ASD (Banker et al., 2021). The Schaffer collaterals, which are pyramidal neuron axon projections from the CA3 to the CA1 are known to play a critical role in memory (Anand & Dhikav, 2012). Additionally, the dorsal hippocampus is known to be involved in cognitive functions such as learning and memory, whereas the ventral hippocampus is more so associated with emotional behavior such as anxiety (Bannerman et al., 2004).

Several animal and human studies have reported hippocampus-related behavioral deficits in ASD. Research has provided evidence for hippocampal dependent memory impairments in ASD. However, social deficits in ASD have not been directly linked to the hippocampus. Regardless, hippocampal lesion studies have provided evidence for abnormalities in social behavior (Sams-Dodd et al., 1997; Uekita & Okanoya, 2011). ASD subjects have demonstrated a reduction in memory retrieval success and hippocampal connectivity after a memory task relative to control participants (Cooper et al., 2017). Impairments in spatial memory, memory for faces and social interaction scenes were also reported in ASD individuals (Williams et al., 2005). Deficits in social behavior and spatial memory have been reported in animal models of ASD as well (Etherton et al., 2011; Jaramillo et al., 2017; Sowers et al., 2013).

In addition to functional deficits mentioned above, several studies have reported various anatomical and molecular abnormalities in the hippocampus of human ASD subjects as well as animal models. Specifically, abnormalities in shape, volume and neuronal morphology have been

reported in several ASD studies (Aylward et al., 1999; Dager et al., 2007; Edalatmanesh et al., 2013; Schumann et al., 2004). Interestingly, contradictory results have been reported across studies that compared hippocampal volume in ASD to neurotypical individuals. In one study, computational mapping of the hippocampus revealed a decrease in volume of the right medial posterior hippocampus in individuals with ASD after controlling for total brain size but no difference in total hippocampal volume (Nicolson et al., 2006). In a magnetic resonance imaging study, hippocampal volume of ASD children was higher relative to controls (Schumann et al., 2004). Additionally, an increase in the left hippocampal volume was reported in adolescents with autism (Groen et al., 2010). Contrastingly, a reduction in hippocampal volume after correcting for total brain volume was observed in adolescents with autism (Aylward et al., 1999). A Golgi-Cox analysis conducted on the brains of children with autism revealed a reduction in the size of the neuronal perikaryon in CA4 neurons and decreased branching in the CA1 and CA4 pyramidal neurons (Raymond et al., 1995). Furthermore, a study conducted on the VPA-exposed rat model of ASD provided evidence for increased cell density in the CA1, CA2, CA3, dentate gyrus and the subiculum region of the hippocampus (Edalatmanesh et al., 2013). Reduced neuronal size and increased cell packing density were also observed in the hippocampus of an individual with autism (Bauman & Kemper, 1985).

1.7 Hippocampal Synaptic Plasticity and ASD

Synaptic plasticity includes several steps involving the pre- and/or post-synaptic neuronal membranes such as synaptic transmission, ion flow, receptor activity, and several other downstream signaling cascades that result in gene expression and ultimately physiological responses. As mentioned previously, both human subjects and rodent models of ASD have

exhibited impairments in memory (Cooper et al., 2017; Etherton et al., 2011; Jaramillo et al., 2017; Sowers et al., 2013; D. L. Williams et al., 2005). Long-term potentiation (LTP) is the neural mechanism underlying learning and memory which has been impaired in several rodent models of ASD (Blundell et al., 2010; Bozdagi et al., 2010; Jaramillo et al., 2016, 2017; Kouser et al., 2013; Won et al., 2012; Yang et al., 2012). Hence, in this study, we wanted to examine whether abnormal PGE2 signaling would affect hippocampal electrophysiology, specifically LTP.

1.7.1 Long-Term Potentiation

LTP is an activity-dependent long-lasting enhancement in synaptic strength which has been identified in several neural circuits of different brain regions including the hippocampus, cerebellum, cortex, and the amygdala (Lynch, 2004). However, the most extensively studied is the *N*-methyl-D-aspartate receptor (NMDAR) dependent LTP observed in the Schaffer collateral pathway (CA3-CA1) of the hippocampus (Citri & Malenka, 2008). This involves high frequency stimulation (HFS), typically 100Hz in 1s, of excitatory pyramidal neuronal projections from the CA3 to the CA1 region of the hippocampus. The presynaptic CA3 neuron, upon activation, releases glutamate which binds to the glutamate receptors α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and NMDA located on the post-synaptic membrane in the CA3 region of the hippocampus (Citri & Malenka, 2008) (**Figure 4**). This allows the passage of Ca^{2+} which results in the induction of LTP shown by the large outward current in a typical field excitatory post-synaptic potential (fEPSP) (Citri & Malenka, 2008). Ca^{2+} is an important second messenger that activates further downstream signaling cascades which consequently results in

insertion of new receptors, synaptogenesis, and ultimately synaptic plasticity (Citri & Malenka, 2008; Kawamoto et al., 2012).

Many studies have suggested the involvement of COX2/PGE2 signaling in synaptic transmission and plasticity. COX-2 inhibition has shown to decrease LTP induction in the perforant pathway (Chen et al., 2002). COX-2 expression is also known to be upregulated by high frequency stimulation associated with LTP induction in the perforant pathway (Yamagata et al., 1993). Additionally, PGE2 has also been suggested to serve as a retrograde messenger via a presynaptic EP2 receptor-PKA pathway during synaptic transmission (Sang et al., 2005). The importance of endogenous PGE2 in regulating membrane excitability and synaptic transmission has been demonstrated in studies (Chen & Bazan, 2005). Similarly, exogenous administration of PGE2 has shown to reverse the reduction of LTP induced by COX-2 inhibition suggesting the importance of PGE2 in synaptic plasticity (Chen et al., 2002). Interestingly, COX2 inhibition has been associated with abnormal cognitive behavior involving the hippocampus including memory acquisition, consolidation, and retention (Rall et al., 2003; Sharifzadeh et al., 2006; Shaw et al., 2003; Teather et al., 2002). Many studies have reported LTP abnormalities in ASD. Studies conducted on various animal models of ASD such as the Shank3 mutant and VPA exposed rodents have exhibited abnormal LTP, AMPA and NMDA receptor subunit expression and aberrant hippocampal dependent learning and memory behavior related to ASD. Shank3 mutant mice have shown a reduction in Schaffer collateral LTP, AMPA subunit GluR1 expression as well as learning and memory behavior (Bozdagi et al., 2010; Kouser et al., 2013; Wang et al., 2011; Yang et al., 2012). Studies have also reported a reduction in LTP in the hippocampal CA1, mossy fibers, dentate gyrus and medial prefrontal cortex (mPFC) of rodents exposed to VPA (Chang et al., 2010; Lee et al., 1996; Martin & Manzoni, 2014; West et al., 2014). In contrast,

prenatal exposure to VPA has also shown enhancements in LTP, NMDAR2A, and GluR1 expressions in the PFC (Liu et al., 2021). Diminished dentate gyrus LTP and NMDA-mediated neurotransmission in FXS and diminished Schaffer collateral LTP and impaired memory in Rett Syndrome were also reported in studies (Moretti et al., 2006; Yun & Trommer, 2011). Other rodent models of ASD have shown contradicting results where some reported enhancements in LTP, AMPA receptor mediated neurotransmission, and NMDAR2B expression while others reported LTP reductions (Etherton et al., 2011; George et al., 2022; Wang et al., 2018), potentially contributing to the heterogeneity in ASD symptomatology.

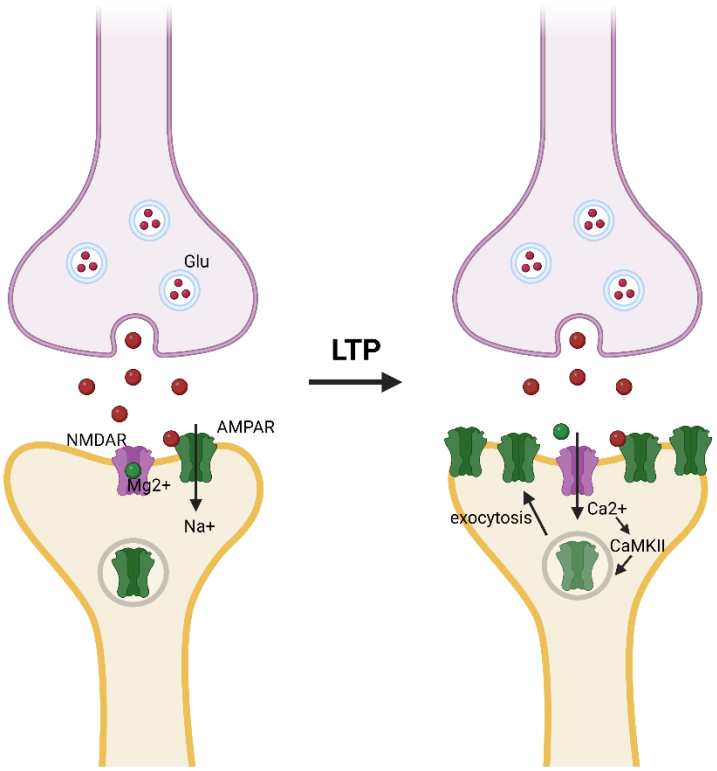


Figure 4: Synaptic transmission and receptor trafficking during NMDA-dependent LTP at the hippocampal Schaffer collateral pathway. Glutamate released from the presynaptic terminal binds to the AMPA receptors allowing the passage of Na^{+}. LTP induced by high frequency stimulation releases the Mg^{2+} block in NMDA receptors thus allowing Ca^{2+} to enter the postsynaptic terminal. Ca^{2+} leads to the insertion of more AMPA receptors in the membrane through exocytosis. This process is mediated by kinases such as calcium/calmodulin-dependent protein kinase II (CaMKII). Figure created using BioRender.com.

1.7.2 Paired-Pulse Facilitation and Input/Output Responses

PPF is a measure of short-term activity dependent presynaptic plasticity (Santschi & Stanton, 2003). When a presynaptic neuron is stimulated twice in brief succession, typically within hundreds of milliseconds, the residual Ca^{2+} in the presynaptic neuron from the first stimulus adds to the Ca^{2+} flow from the second stimulus (Santschi & Stanton, 2003). This 'facilitation' of neurotransmitter release by the residual Ca^{2+} from the first stimulus is seen as a large increase in the slope and amplitude of the second fEPSP relative to the first (Santschi & Stanton, 2003). PPF is measured as the ratio of the slope of the second fEPSP to the slope of the first fEPSP known as the paired-pulse ratio (PPR) (Santschi & Stanton, 2003). PPF is known to be a purely presynaptic mechanism (Grover, 1998) and hence independent of the NMDA receptor activity (Bliss & Collingridge, 1993). PPR has an inverse relationship with release probability, meaning if PPR is high, presynaptic release probability is low (Dobrunz & Stevens, 1997).

While some studies in animal models of ASD such as the Shank3 mutants have reported no differences in PPF (George et al., 2022; Kouser et al., 2013; Wang et al., 2011), other Shank3 and Rett syndrome models, a condition that falls on the ASD spectrum, have shown a decrease in PPF (Bozdagi et al., 2010; Moretti et al., 2006), suggesting low presynaptic release probability. Interestingly, PGE2 has shown to reduce PPF and hence increase presynaptic release probability of glutamate at both the Schaffer collateral and perforant path synapses of hippocampal slices (Sang et al., 2005).

I/O curves are a measure of basal synaptic strength or transmission which is achieved by recording fEPSPs in response to increasing stimulation intensities. Inconsistent findings in I/O responses have been reported in studies where an increase, decrease or no differences have been

found in pathological models relative to healthy controls. Some animal models of ASD such as the VPA-exposed rats have shown no change in I/O responses relative to controls in the mPFC and hippocampal CA1 region (Martin & Manzoni, 2014; Wang et al., 2011; Wang et al., 2018). Other studies have reported a reduction in I/O response and thereby basal synaptic strength in the Schaffer collateral pathway in ASD (Bozdagi et al., 2010; Kouser et al., 2013; MYang et al., 2012). Animal models of ASD such as the Neuroligin-3 mutant, and Rett syndrome such as the Mecp2 mutant have also exhibited enhanced basal synaptic transmission in the CA1 region (Etherton et al., 2011; George et al., 2022; Moretti et al., 2006).

Overall, studies on animal models of ASD reveal a spectrum of abnormalities in synaptic plasticity and function, owing to the diverse behavioral manifestations observed in ASD patients.

1.7.3 Pyramidal Cell Morphology

Pyramidal cells in the CA3 and CA1 region of the hippocampus are key players in hippocampal synaptic plasticity (Marr, 1971). Pyramidal neurons have a characteristic pyramidal shaped large cell body and are abundant in number in the hippocampus (Marr, 1971). They are particularly important in the context of synaptic plasticity associated with learning and memory because these cells are involved in LTP (Spruston, 2008). Pyramidal cells in the hippocampus are known to establish connections between various brain regions by receiving inputs from associative neocortical areas, processing the information, and sending outputs to multiple brain regions including the amygdala, mPFC and the medial entorhinal cortex (Cenquizca & Swanson, 2007). This process is thought to be involved various cognitive functions including memory (Fanselow & Poulos, 2005). Therefore, understanding the morphology of pyramidal neurons is critical as it gives us information about the structural organization and connectivity of the

hippocampus. Specifically, researchers have investigated morphological features such as size, length and shape of dendritic arbors and cell soma of hippocampal neurons in various pathological conditions. More importantly, morphology of neuronal dendrites and spines have been studied extensively as these are the structures that make synaptic contacts, receive inputs from other neurons, and process and transmit those signals (Martínez-Cerdeño, 2017). Additionally, the complex nature of these structures allows for the integration of signals from other cells such as interneurons and glial cells which is important for healthy functioning of the nervous system (Martínez-Cerdeño, 2017).

Studies have provided evidence for abnormal dendritic and spine morphology in the hippocampal pyramidal neurons as well as other neurons in various brain regions of ASD subjects. Postmortem studies conducted on human subjects with autism have shown evidence for reduction in spine density in cortical pyramidal neurons (Williams et al., 1980) and reduced cell soma size and dendritic branching in hippocampal pyramidal neurons suggesting a curtailment of maturation in those subjects rather than a malformation (Raymond et al., 1995). In VPA-exposed rats, a well established rodent model of ASD, increased dendritic arborization in the apical dendrites of pyramidal neurons in the motor cortex as well as reduced cell soma size in the hippocampal CA1 pyramidal neurons was observed relative to the controls (Hajisoltani et al., 2019; Snow et al., 2008). VPA-exposed rats have also shown a reduction in dendritic branching, spine density and branch length in the mPFC (Raza et al., 2015). In addition to these findings, our lab has provided evidence for abnormal dendritic morphology in the cerebellum of the COX-2 Knockin (KI) and hippocampus of PGE2-exposed mice including dendrite thickness, arborization, looping, branch length, cell soma size, and spine morphology with some of them being sex dependent effects (Kissoondoyal et al., 2021). These abnormalities have been linked to

the abnormal expression of proteins involved in cytoskeletal architecture including spinophilin and β-actin (Kissoondoyal et al., 2021; Kissoondoyal & Crawford, 2021). Additionally, abnormalities in neurite length and axonal looping were also observed in neuronal stem cells in response to increased PGE2 and misoprostol (Kissoondoyal & Crawford, 2021; Tamiji & Crawford, 2010). Although several ASD studies have largely focused on spine morphology of pyramidal neurons in various parts of the brain, not much is known about dendritic morphology of hippocampal pyramidal neurons.

CHAPTER 2: OBJECTIVES AND HYPOTHESIS

2.1 Overall Objectives and Hypothesis

Overall, this thesis aims to investigate whether abnormal COX2/PGE2 signaling affects changes in hippocampal electrophysiology, protein expression and neuronal morphology and whether these changes are sex-dependent. Previous studies conducted in our lab in mouse offspring exposed to PGE2 during pregnancy (increased PGE2 levels) or lacking the PGE2 producing enzyme (COX2-Knockin/KI; decreased PGE2 levels) have demonstrated sex-dependent abnormalities in dendritic morphology including branching, looping, dendrite length, cell soma size, spine morphology, abnormal expression level of developmental genes and cytoskeletal protein in the cerebellum and hippocampus and ASD-like behavior (Kissoondoyal et al., 2021; Wong et al., 2019, Kissoondoyal et al., Unpublished, Iyer et al., Unpublished). In this study, I will further examine the effects of maternal PGE2 exposure on hippocampal short- and long-term synaptic plasticity, the expression of synaptic proteins, and morphology of pyramidal neurons in male and female offspring.

Recent findings from our lab have shown that PGE2 affects dendritic morphology in cells across the hippocampus including increases in dendritic looping, arborization and primary branch length as well as decreased cell soma size (Iyer et al., Unpublished). This study stems from these findings. I hypothesize that maternal exposure to PGE2 during a critical time in pregnancy at gestational day 11: 1) affects neuronal electrophysiology within the hippocampus, 2) downregulates expression level of proteins involved in synaptic plasticity and cytoskeletal architecture and 3) contributes to changes in dendritic morphology of pyramidal neurons. We also expect to see sex-dependent differences in all these measures.

2.2 Experimental Model System

The experiments in this study will be conducted on the male and female offspring born to mothers injected with PGE2 on G11 (see methods section for details). In brief, pregnant C57BL/6J mice were subjected to a one-time subcutaneous injection of 0.25µg/g of 16, 16 dimethyl prostaglandin E2 (dmPGE2). dmPGE2 is a stable analog of PGE2 and has a slower metabolic rate, thus remaining active for a longer duration when compared to PGE2 (Steffenrud, 1980). The control group received a subcutaneous injection of the saline solution. The injections were administered on G11 because neurogenesis in various parts of the mouse brain typically begins at this critical time point (Rodier, 1980). Additionally, G11 is equivalent to the critical time period during which pregnant women misused the drug misoprostol to terminate their pregnancies which resulted in babies born with Mobius syndrome and ASD-like behavior (Bandim et al., 2003; Pottie, 1999).

2.3 Aim 1: Investigating Hippocampal Electrophysiology

2.3.1 Rational

Many studies have previously reported abnormalities in LTP, I/O curves and PPF in animal models of ASD. Specifically, the Shank3 mutant and VPA exposed rodent models have shown a reduction in LTP, PPF and I/O responses (Bozdagi et al., 2010; Kouser et al., 2013; Yang et al., 2012). Additionally, the importance of the COX-2/PGE2 signaling in synaptic transmission and plasticity has also been demonstrated in many studies (Andreasson et al., 2001; Chen et al., 2002; Chen & Bazan, 2005; Sang et al., 2005; Yang et al., 2008). For example, COX-2 inhibitors have shown to significantly reduce post-synaptic membrane excitability and hippocampal LTP induction in mice (Chen et al., 2002). A graduate student in our lab has

recently shown sex-specific abnormal dendrite and dendritic spine morphology within cells across the hippocampus including arborization, branch length, branch order, looping and cell soma size (Iyer et al., Unpublished). Therefore, this study will further investigate if these morphological changes observed may influence hippocampal electrophysiology and whether they are manifested differently in male and female offspring.

2.3.2 Objectives and Hypothesis

The main objective of this aim was to examine the effect of prenatal PGE2 exposure on hippocampal Schaffer collateral LTP, I/O responses and PPF in male and female mice offspring at postnatal day 90-100 (PN90-PN100). Based on our recent dendritic morphology findings, **I hypothesized** that maternal PGE2 exposure will contribute to sex-dependent changes in LTP, I/O and PPF in the offspring. Specifically, I predicted that all the electrophysiological measurements will be lower in the PGE2-exposed offspring compared to the saline-exposed wildtype controls.

2.3.3 Methodology

Male and female mice exposed to PGE2 (PGE2M and PGE2F) and saline (CM AND CF) were sacrificed between PN90 and PN100 (see methods for details). In short, the hippocampi were extracted and sliced to 400 microns. The slices were placed in an interface chamber and bathed in oxygenated artificial cerebrospinal fluid (aCSF). The LTP protocol was conducted by stimulating the CA3 region of the hippocampus and recording field excitatory post-synaptic potentials (fEPSPs) from the CA1 region. Baseline activity was recorded for 20mins followed by high frequency stimulation (HFS) at 100Hz X 1sec. Post-HFS fEPSP was recorded for 80mins.

For the I/O protocol, slices were stimulated at 20, 30, 40, 50, 60, 70, 80, 90, 100 and 110µA, and for the PPF protocol, the slices were stimulated twice in brief succession at 50, 100, 150 and 200ms.

2.4 Aim 2: Investigating the Expression of Proteins Involved in Hippocampal Synaptic Plasticity

2.4.1 Rational

Previous studies in our lab have attributed abnormal dendritic morphology in the cerebellum to abnormal protein expression. Specifically, COX2-KI and PGE2-exposed mice have shown differences in the expression of β-actin and spinophilin, proteins involved in cytoskeletal architecture and highly enriched in dendritic spines in the cerebellum (Kissoondoyal et al., 2021; Kissoondoyal et al., Unpublished). AMPA, NMDA receptors are also involved in synaptic transmission and plasticity (Citri & Malenka, 2008). Specifically, the NMDA receptor subunit GluN2A or NMDAR2A is involved in LTP (Dalton et al., 2012). Studies have also reported that the AMPA receptor subunit GluR1 is involved in LTP while subunit GluR2 is involved in long term depression (LTD) (Din et al., 2010). These receptors have shown to be implicated in various animal models of ASD (Bozdagi et al., 2010; Kim et al., 2016; Rinaldi et al., 2007). In this study, I aim to quantify the level of β-actin, GluR1 and GluN2A in individual biological replicates from the PGE2 exposed male and female mice hippocampi.

2.4.2 Objectives and Hypothesis

The main objective of this aim was to examine the effect of prenatal PGE2 exposure on the expression of critical proteins involved in hippocampal synaptic plasticity, namely, β-actin,

GluR1 and GluN2A, at PN90. Based on my LTP results and previous findings from our hippocampal dendritic morphology study, **I hypothesized** that maternal exposure to PGE2 will downregulate β-actin, GluR1 and GluN2A in the hippocampus of mice offspring, specifically in males.

2.4.3 Methodology

The hippocampi were extracted from different biological replicates of saline- and PGE2-exposed males and females from 3 different litters. Protein samples were extracted from these tissues via homogenization and the concentrations were determined using the Pierce BCA assay kit. The control samples were pooled while the PGE2 male and female samples were individually tested using western blot (see methods section for details).

2.5 Aim 3: Investigating Hippocampal Pyramidal Cell Morphology

2.5.1 Rational

Our lab has shown previously that a decrease in PGE2 (COX2-KI) and increase in PGE2 (PGE2-exposure) can result in abnormal dendrite and dendritic spine morphology throughout the cerebellum and the hippocampus (Kissoondoyal et al., 2021; Kissoondoyal et al., Unpublished; Iyer et al., Unpublished). However, dendritic morphology of pyramidal neurons specifically has not been quantified. Pyramidal neurons play a significant role in hippocampal LTP and learning and memory (Spruston, 2008). Information about pyramidal neuron morphology can give us insight into its development, firing properties and connectivity pattern with other brain regions. In this study, I will quantify dendrite length and cell soma size of hippocampal pyramidal neurons.

2.5.2 Objectives and Hypothesis

The objective of this aim is to investigate the effect of prenatal PGE2 exposure on morphological characteristics of pyramidal neurons such as dendrite length and cell soma size. **I hypothesize** that PGE2 exposure will contribute to sex-dependent differences in dendrite length and cell soma size at PN90. Specifically, I predict that dendrite length will increase, and cell soma size will decrease in the PGE2-exposed offspring.

2.5.3 Methodology

Brains of mice offspring exposed to PGE2 and saline were extracted at PN90 and stained using a well-established method in our lab called the Golgi-Cox staining (Kissoondoyal et al., 2021; Zaqout & Kaindl, 2016). The brains were then sliced to a thickness of 100µm and imaged under the 20X objective of the confocal microscope using bright field microscopy. The slices were tiled and z-stacked to obtain the full area and depth of the hippocampus. Dendritic morphology was analyzed using imageJ.

CHAPTER 3: METHODOLOGY

3.1 Animal Breeding and Injections

Male and female mice of the C57BL/6J strain were obtained from the Jackson Laboratory and housed at the York University animal facility. Mice were housed at room temperature on a 12h/12h light/dark cycle with access to ad libitum water and standard mouse chow. Males and females were paired and housed together for breeding overnight, and the females were checked each morning for a vaginal plug. The day the plug was found in the female was determined to be gestational day 0 (G0), following which the females were housed separately from their male partners for the rest of their pregnancy. On G11, the females were weighed to ensure they were carrying, before they received a one-time subcutaneous injection of 0.25µg/g of 16, 16 dimethyl prostaglandin E2 (dmPGE2), diluted in physiological saline (Sodium Chloride 0.9%) sterile solution, to a final volume of 200µl. The pups born to these females were the PGE2-exposed males (PGE2M) and females (PGE2F). dmPGE2 is a stable analog of PGE2 and has a slower metabolic rate, thus remaining active for a longer time when compared to PGE2 (Steffenrud, 1980). The control group received 200µl of the saline solution and the pups born to these females were the saline-exposed control males (CM) and females (CF). The injections were administered on G11 because neurogenesis in various parts of the mouse brain typically occurs during a critical time window between G11 and G17 (Rodier, 1980). Additionally, it is this critical time period during which pregnant females used to consume misoprostol to terminate their pregnancies which resulted in a high risk of ASD and Mobius syndrome incidence in the offspring (Bandim et al., 2003; Pottie, 1999). On post-natal day 28 (PN28), male and female mice offspring were weaned and housed separately for further use in experiments. All

procedures, experiments and protocols were conducted in accordance with the ethical guidelines set by York University's Animal Care Committee and were approved by the Research Ethics Board of York University.

3.2 Mouse Hippocampal Slice Preparation for Electrophysiology Experiments

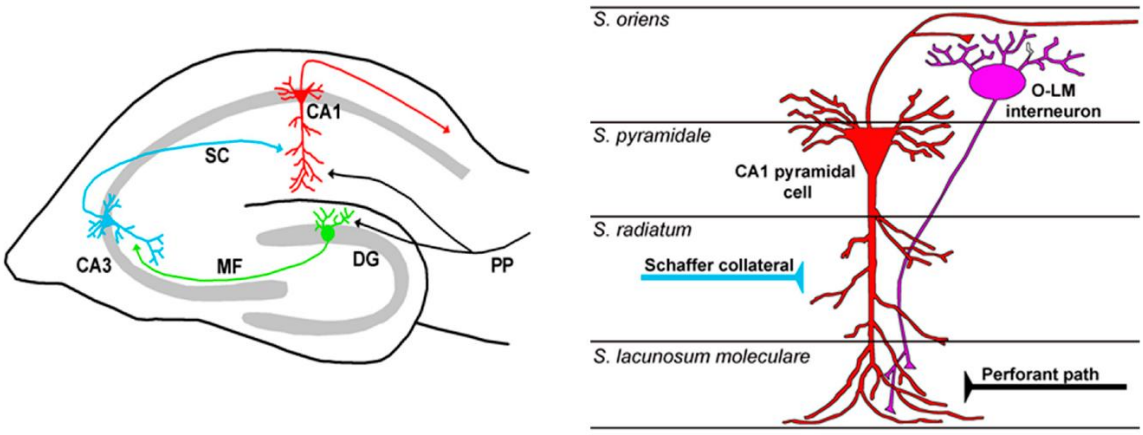
PN90 mice were sacrificed by cervical dislocation followed by decapitation. This method was chosen over chemical anesthesia in order to avoid any potential effects of the anesthetic on electrophysiological recordings. The brains were then extracted and rapidly cooled in ice-cold artificial cerebrospinal fluid (aCSF) **(Table 1)** bubbled with carbogen (95% O2, 5% CO2) for 30-45 seconds. The hippocampi were then removed from both hemispheres and sliced to a width of 400 microns using a manual tissue slicer. 4 slices from each end of the hippocampus were obtained in order to separate the dorsal and ventral slices for experiments. The slices were then transferred to an interface chamber that was maintained at 30°C and supplied with fresh oxygenated aCSF via an IV line at 1-2 ml/min. The slices were left to recover for 1.5 hours in this interface chamber before starting experiments. All electrophysiology experiments were conducted using the Axon Instruments recording system.

Table 1: Constituents of the artificial cerebrospinal fluid. The pH was adjusted to 7.35 by bubbling the solution with carbogen.

Component	**Amount for 2L ddH20**
Sodium Chloride	14.5g
Potassium Chloride	656mg
Magnesium Sulfate	2600µl (1mM)
Sodium Phosphate monobasic	240mg
Sodium Bicarbonate	4.42g
Calcium Chloride	5000µl (1mM)
D-Glucose	3.6g

3.3 Long-Term Potentiation

Field excitatory postsynaptic potentials (fEPSPs) were induced by stimulating the Schaffer collaterals in the CA3 region and recording the responses from the stratum radiatum in the CA1 region (**Figure 5**). A stimulating electrode made of nichrome wire was placed on the CA3 region and a recording electrode made of sliver wire coated with chloride placed in a glass capillary pulled using a micropipette puller (Sutter Instruments) with a resistance of 1-2 mΩ was placed on the CA1 region (**Figure 5**)**.** Slices were stimulated at increasing intensities to obtain the maximal response. 40% of the maximum amplitude was determined as the baseline response (Blundell et al., 2010). Baseline recordings were obtained for 20 mins (1 fEPSP per min). Following baseline recordings, LTP was induced by a high frequency stimulation (HFS, 1 second x 100 Hz). fEPSPs were then recorded for 80 mins post-HFS. A total of 20 PN90 mice were used. This includes 5 saline-exposed males, 5 saline-exposed females, 6 PGE2-exposed males, and 4 PGE2-exposed females from at least 3 separate litters. For all electrophysiology experiments, the sample size (n) was the number of hippocampal slices taken from animals from at least 3 different litters.



(a) (b)

Figure 5: Neural circuitry of the hippocampus. (a) The trisynaptic pathway of the hippocampus. Pyramidal neuron projections from the CA3 to the CA1 (indicated in blue) is the Schaffer collateral pathway stimulated during hippocampal NMDA-dependent LTP. The stimulating electrode is placed on the CA3 and the fEPSP responses are recorded from the CA1. (b) Schematic drawing of the Schaffer collaterals synapsing on the CA1 pyramidal neuron, with its cell body located in the stratum pyramidale. Figure obtained from (Cammalleri et al., 2019).

3.4 Input/Output Responses and Paired-Pulse Facilitation

For I/O curves, slices were stimulated with increasing stimulation intensities and the fEPSPs were recorded. The stimulation intensities used were 20, 30, 40, 50, 60, 70, 80, 90, 100, and 110µA. This protocol was not performed using baseline activity since the stimulation intensities were being manipulated. A total of 17 PN90 mice were used. This includes 5 saline-exposed males, 4 saline-exposed females, 4 PGE2-exposed males, and 4 PGE2-exposed females from at least 3 separate litters.

For PPF, field excitatory postsynaptic potentials (fEPSPs) were recorded in response to 2 rapid stimulations in brief successions. 2 fEPSPs were induced during PPF protocols. The interstimulus intervals used for this protocol were 50, 100, 150 and 200ms. PPF protocols were also conducted at baseline activity (40% of the maximum response). A total of 14 PN90 mice were used. This includes 3 saline-exposed males, 4 saline-exposed females, 3 PGE2-exposed males, and 4 PGE2-exposed females from at least 3 separate litters.

3.5 Western Blot

The Polytron power homogenizer was used to homogenize each hippocampal sample at PN90 in 300µl of 1X radio-immunoprecipitation assay (RIPA, ab156034) lysis buffer and 1% protease inhibitor cocktail (1:100). The samples were then placed on a shaker at 4°C for 2 hours, followed by centrifugation at 12,000 rpm at 4°C for 20 minutes. The supernatant was collected, and the protein concentrations were measured using the Pierce BCA protein assay kit. To examine differences in protein expression between individual PGE2-exposed male and female mouse, western blots were performed on 3 biological replicates from separate litters. The saline exposed control samples were pooled from 3 age-matched animals from separate litters for both

males and females separately. 15µg of protein for each sample was loaded on a 10% SDS-PAGE gel along with the Laemmli loading buffer. Three technical replicates of the western blot were performed. Once the proteins separated through the gel, they were transferred to a 0.2µm nitrocellulose membrane using cold transfer buffer for one hour at 100V, followed by four washes in 1X TBS-T (Tris Buffer Saline 0.01% Tween 20) for a total of 20 minutes. The membranes were blocked using 5% milk diluted in 1X TBS-T at room temperature for 1 hour. The membranes were then incubated with primary antibodies diluted in blocking buffer (milk in TBS-T) at 4°C overnight on a shaker. The primary antibodies were mouse monoclonal anti-GAPDH (1:20,000, ab8245), rabbit monoclonal anti-GluR1 (AMPAR) (1:2500, ab183797), mouse monoclonal anti-beta actin (1:10,000, ab6276) and mouse monoclonal anti-NMDAR2A (GluN2A) (1:2500, ab133265). The membranes were washed in 1X TBS-T four times for 5 minutes each the next morning and then incubated with horse radish peroxidase (HRP)-tagged secondary antibodies diluted in blocking buffer for one hour at room temperature on a shaker. The secondary antibodies used were goat anti-rabbit (1:10,000, ab6721) and goat anti-mouse (1:10,000, ab6789). The membranes were washed in 1X TBS-T again four times for 5 minutes each and then imaged with the help of the ChemiDoc XRS+ System. The bands were quantified using ImageJ. Protein band intensities were normalized to GAPDH, and a fold change was measured for each protein expression relative to the saline-exposed males i.e., protein expression in saline exposed male was set as 1.

3.6 Golgi-Cox Staining

Golgi-Cox staining is frequently used for dendritic morphology analysis. The Golgi-Cox solution and the tissue protectant solution were prepared before sacrificing the animals as per the

protocol established by Zaqout and Kaindl (Zaqout & Kaindl, 2016). The Golgi-Cox solution consists of potassium chloride, potassium dichromate and mercuric chloride in ddH20 (5% w/v). The tissue protectant solution consists of 30% w/v sucrose, 20% w/v ethylene glycol, and 1% polyvinylpyrrolidone in ddH20. The whole brain samples were extracted from a total of 14 PN90 mice, including 3 saline-exposed males, 3 saline-exposed females, 4 PGE2-exposed males, and 4 PGE2-exposed females from at least 3 separate litters. After extraction, the brains were washed in 1X PBS, hemisected, and immediately fixed in 4% paraformaldehyde (PFA) solution for 24 hours. The brains were transferred into a fresh PFA solution the next day for another 24 hours. Subsequently, the brains were immersed in 5ml of the Golgi-Cox solution and left in the dark for 24 hours at room temperature. The next day, samples were immersed in fresh Golgi-Cox solution and left in the dark at room temperature for 5 days. The samples were then moved to the tissue protectant solution and left in the dark at 4°C. After 24 hours, samples were transferred to a fresh tissue protectant solution and left in the dark at 4°C for another 7 days before they were sent to the Pathology Research Program Laboratory at the University Health Network in Toronto. The brains were sliced sagittally to a thickness of 100µm using a cryostat and the stain was developed using a 3:1 ammonia to H20 solution and 5% w/v sodium thiosulfate in H20. The slices were then serially dehydrated using 70%, 95% and 100% ethanol, dipped in xylene and then sealed with a cover slip.

3.7 Confocal Microscopy

The stained slices were imaged on the Carl Zeiss Observer Spinning Disk confocal microscope using brightfield microscopy at the Advanced Light and Electron Microscopy services at York University (Observer Z1 spinning disk confocal microscope). The hippocampus

was imaged under the 20X objective, tiled, and z-stacked every ~1µm (~60 stacks on average). Finally, the tiles were fused, and the z-stacks were compressed into a 2D jpeg image during image processing.

3.8 Image Analysis

Pyramidal neurons were selected based on the location of neuronal cell body in the cell body layer (Marr, 1971). Primary branch length and cell soma area was quantified using the open-source software Fiji ImageJ. The segmented line tool was used to trace the primary branches and measure the length. For each neuron, all primary branches were traced, and the average of all the primary branch lengths were calculated. The analysis included 10 neurons from the saline-exposed males, 11 neurons from the saline-exposed females, 9 neurons from the PGE2-exposed males, and 10 neurons from the PGE2-exposed females. The cell soma area was measured using the wand tracing tool on imageJ which outlined the cell soma. The analysis included 10 neurons from the saline-exposed males, 9 neurons from the saline-exposed females, 9 neurons from the PGE2-exposed males, and 10 neurons from the PGE2-exposed females.

3.9 Statistics

Data collection and image analyses were conducted in a randomized order and blinded to the experimental group. ImageJ (Western blot and Golgi-Cox analysis) and Clampfit 11.2 (electrophysiology) were used to analyze images and collect data. We used linear mixed effect modelling to analyze all data as it allows to take confounding variables, main effects and interactions into consideration. Variables of interest such as treatment condition (PGE2 vs saline) and sex (male vs female) were assigned as fixed effects. Their main effects and interactions were

analyzed. Confounding variables including litter, slice, technical replicate, sample ID, mouse ID, and hippocampal side (dorsal vs ventral) were assigned as random effects and included in the baseline model M0. All comparisons were made to M0 and the model of best fit was determined using the Akaike Information criteria (AIC) and p-values. Pairwise comparisons were then conducted to examine differences between groups. p-values less than 0.05 were considered statistically significant.

CHAPTER 4: RESULTS

4.1 Hippocampal Electrophysiology

4.1.1 Long-Term Potentiation

Studies conducted on animal models of ASD such as the Shank3, Shank2 mutant and VPA exposed rodents have exhibited impaired hippocampal LTP (Bozdagi et al., 2010; Jaramillo et al., 2016, 2017; Kouser et al., 2013; Won et al., 2012; Yang et al., 2012). Additionally, many studies have highlighted the importance of normal COX2/PGE2 signaling in synaptic transmission and plasticity (Andreasson et al., 2001; Chen et al., 2002; Chen & Bazan, 2005; Sang et al., 2005; Yang et al., 2008). Our lab has also previously reported dysregulation of genes associated with synaptic transmission in COX2-KI mice such as *CaMK2b* and *NRXN1* (Rai-Bhogal, Ahmad, et al., 2018). In this study, we investigated sex-dependent effects of prenatal PGE2 exposure on hippocampal electrophysiology by stimulating the pyramidal neuron axon projections in the CA3 region and recording field excitatory post-synaptic potentials (fEPSPs) from the CA1 region (**Figure 6A**). Slopes of the fEPSPs were extracted using the software Clampfit 11.2 (**Figure 6B**). After averaging the 20 slopes from the baseline recordings, each slope from the baseline as well as the post-high frequency stimulation (HFS) recordings was divided by the average baseline slope. For each fEPSP, the percent of the average baseline slope was plotted against the time in minutes (**Figure 6C**).

We assigned treatment condition (saline vs PGE2), sex and time interval (minutes) and their interactions as fixed effects while litter, slice, and hippocampal side (dorsal vs ventral) were assigned as random effects to account for any potential biases. The baseline model (M0) included litter, slice and hippocampal side as random effects. Model 1 (M1) included condition,

sex and time interval as fixed effects and Model 2 (M2) included condition and time interval as fixed effects (see methods). All comparisons were made to the baseline model M0. The analysis includes data from 6 hippocampal slices (n=6) per experimental group.

The model of best fit included condition, sex and time as a factor (AIC = 25221, $p < 2.2e-16$). Within this model, there was a significant interaction between condition, sex and time interval ($t(2376) = 4.937$, $p = 8.48e-07$). Hence, we conducted pairwise comparisons at each minute to examine the differences in LTP between the saline- and PGE2- exposed males and females.

Sex differences in the saline- and PGE2-exposed mice offspring

Sex differences in LTP were examined in the saline-exposed and PGE2-exposed mice offspring. Comparisons within the saline-exposed mice offspring revealed a significant difference between the males and females where the saline-exposed females had a lower potentiation compared to their male counterparts throughout the entire post-HFS period ($p < 0.05$). However, comparisons within the PGE2-exposed mice showed no significant differences between the males and females at any time post-HFS ($p > 0.05$) indicating an innate loss of sex difference due to PGE2 exposure.

Treatment condition differences in males and females

Next, we examined differences between the treatment groups in the male and female mice offspring. We found no significant differences between the PGE2 and control males at 21-70 mins or at 91-100 mins ($p > 0.05$). However, PGE2-exposed males showed lower potentiation

than the control males at 71-90 mins ($p < 0.05$). No significant differences in LTP were found between the PGE2 and control females at any time post-HFS ($p > 0.05$).

Overall, we found an innate sex difference in LTP for the control mice where males had a higher response compared to females at every time interval post-HFS. However, PGE2 exposure had a sex-specific effect where it decreased LTP in males only.

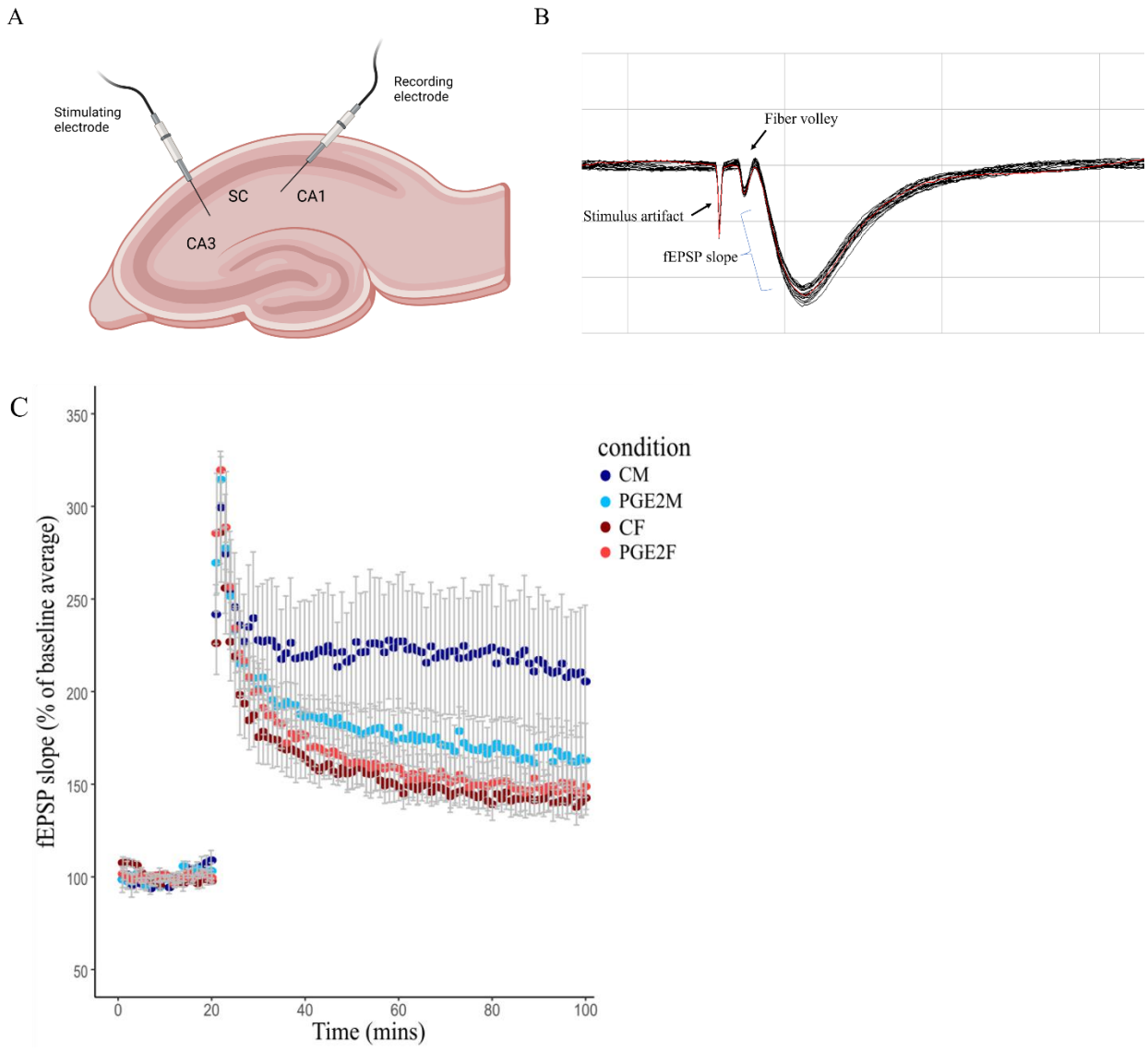


Figure 6: Schaffer collateral LTP in the PGE2 and control males and females. A. Placement of the stimulating and recording electrode on the hippocampal slice. The stimulating electrode is placed on the axons of pyramidal neurons in the CA3 region and the recording electrode is placed on the stratum radiatum of the CA1 region. SC = Schaffer Collateral. Figure created using BioRender.com. **B.** fEPSP recording obtained from the CA1 region. The stimulus artifact indicates that the slice is being stimulated, the fiber volley indicates presynaptic activity, and the large EPSP indicates Ca^{2+} flow into the post-synaptic terminal. **C.** LTP graph showing fEPSP slope calculated as the percent of the baseline average plotted against time. Data is presented as the mean ± SEM. n=6 slices per experimental group. Figure 6A was created using BioRender.com

4.1.2 Input/Output Responses

Our aim was also to investigate whether prenatal exposure to PGE2 would affect input-output responses and thereby basal synaptic strength in a sex-dependent manner. fEPSP responses were recorded for the stimulation intensities 20, 30, 40, 50, 60, 70, 80, 90, 100 and 110µA and the slopes were extracted using Clampfit 11.2 (**Figure 7A**). The slopes of each fEPSP were plotted against their corresponding stimulation intensity (**Figure 7B**). We assigned treatment condition, sex, and stimulation intensity as fixed effects while litter, slice and animal ID were assigned as random effects to account for potential biases. The model of best fit included treatment condition, sex and stimulation intensity as determining factors (AIC = 674.28, p = 1.818e-05). Within this model, there was a significant interaction between treatment condition, sex, and stimulation intensity (t(359.999) = 5.123, p = 4.9e-07). Hence, we conducted pairwise comparisons at each stimulation intensity to examine the differences between the saline- and PGE2- exposed males and females (**Table 2**). The analysis includes data from 10 hippocampal slices in each group (N=10).

Sex differences in input-output responses

Sex differences in input-output responses were examined at each stimulation intensity in the saline-exposed mice offspring. Comparisons within the control mice offspring revealed no significant difference between the males and females at any of the stimulation intensities: 20µA (t(23.2) = 0.092, p = 0.927, CM = 0.270, CF = 0.358), 30µA (t(23.2) = 0.160, p = 0.874, CM = 0.505, CF = 0.624), 40µA (t(23.2) = 0.325, p = 0.749, CM = 0.738, CF = 0.929), 50µA (t(23.2) = 0.443, p = 0.661, CM = 0.906, CF = 1.150), 60µA (t(23.2) = 0.594, p = 0.558, CM = 1.089, CF = 1.401), 70µA (t(23.2) = 0.733, p = 0.471, CM = 1.241, CF = 1.615), 80µA (t(23.2) = 1.269, p

= 0.217, CM = 1.320, CF = 1.933), 90µA (t(23.2) = 1.428, p = 0.167, CM = 1.411, CF = 2.094), 100µA (t(23.2) = 1.607, p = 0.121, CM = 1.472, CF = 2.235), and 110µA (t(23.2) = 1.638, p = 0.115, CM = 1.572, CF = 2.349).

Comparisons within the PGE2-exposed mice offspring also revealed no significant difference between the males and females at any of the stimulation intensities: 20µA (t(21.2) = -0.032, p = 0.975, PGE2M = 0.082, PGE2F = 0.119), 30µA (t(21.2) = -0.198, p = 0.845, PGE2M = 0.333, PGE2F = 0.297), 40µA (t(21.2) = -0.518, p = 0.610, PGE2M = 0.633, PGE2F = 0.453), 50µA (t(21.2) = -0.723, p = 0.477, PGE2M = 0.865, PGE2F = 0.593), 60µA (t(21.2) = -0.926, p = 0.364, PGE2M = 1.059, PGE2F = 0.696), 70µA (t(21.2) = -1.067, p = 0.298, PGE2M = 1.205, PGE2F = 0.780), 80µA (t(21.2) = -1.205, p = 0.242, PGE2M = 1.363, PGE2F = 0.876), 90µA (t(21.2) = -1.385, p = 0.180, PGE2M = 1.526, PGE2F = 0.958), 100µA (t(21.2) = -1.508, p = 0.146, PGE2M = 1.643, PGE2F = 1.020), and 110µA (t(21.2) = -1.479, p = 0.154, PGE2M = 1.739, PGE2F = 1.129).

Overall, we did not observe any significant sex differences within the control or the PGE2-exposed offspring.

Treatment condition differences in input-output responses

Treatment condition differences were examined at each stimulation intensity in the male and female mice offspring. Comparisons within the males revealed no significant difference between the PGE2-exposed and control offspring at any of the stimulation intensities: 20µA (t(23.2) = -0.471, p = 0.642, CM = 0.270, PGE2M = 0.0816), 30µA (t(23.2) = -0.432, p = 0.669, CM = 0.505, PGE2M = 0.333), 40µA (t(23.2) = -0.282, p = 0.780, CM = 0.738, PGE2M = 0.633), 50µA (t(23.2) = -0.138, p = 0.892, CM = 0.9056, PGE2M = 0.865), 60µA (t(23.2) = -

0.115, p = 0.909, CM = 1.089, PGE2M = 1.059), 70µA (t(23.2) = -0.127, p = 0.900, CM = 1.241, PGE2M = 1.205), 80µA (t(23.2) = 0.049, p = 0.961, CM = 1.320, PGE2M = 1.363), 90µA (t(23.2) = 0.210, p = 0.835, CM = 1.411, PGE2M = 1.526), 100µA (t(23.2) = 0.336, p = 0.740, CM = 1.472, PGE2M = 1.643), and 110µA (t(23.2) = 0.329, p = 0.745, CM = 1.572, PGE2M = 1.739).

Comparisons within the females revealed no significant difference between the PGE2-exposed and control offspring at lower stimulation intensities (20µA-70µA): 20µA (t(21.2) = -0.593, p = 0.559, CF = 0.358, PGE2F = 0.119), 30µA (t(21.2) = -0.788, p = 0.439, CF = 0.624, PGE2F = 0.297), 40µA (t(21.2) = -1.121, p = 0.275, CF = 0.929, PGE2F = 0.453), 50µA (t(21.2) = -1.302, p = 0.207, CF = 1.150, PGE2F = 0.593), 60µA (t(21.2) = -1.633, p = 0.117, CF = 1.401, PGE2F = 0.696), and 70µA (t(21.2) = -1.924, p = 0.068, CF = 1.615, PGE2F = 0.780). However, PGE2-exposed females showed significantly reduced input-output responses compared to their saline counterparts at higher stimulation intensities (80µA-110µA): 80µA (t(21.2) = -2.420, p = 0.024, CF = 1.933, PGE2F = 0.876), 90µA (t(21.2) = -2.598, p = 0.017, CF = 2.094, PGE2F = 0.958), 100µA (t(21.2) = -2.774, p = 0.011, CF = 2.235, PGE2F = 1.020), and 110µA (t(21.2) = -2.783, p = 0.011, CF = 2.349, PGE2F = 1.129) (**Figure 7B**).

Overall, we found that PGE2 exposure had a sex-specific effect where females had a reduction in I/O responses at higher stimulation intensities compared to their saline counterparts (**Table 2**).

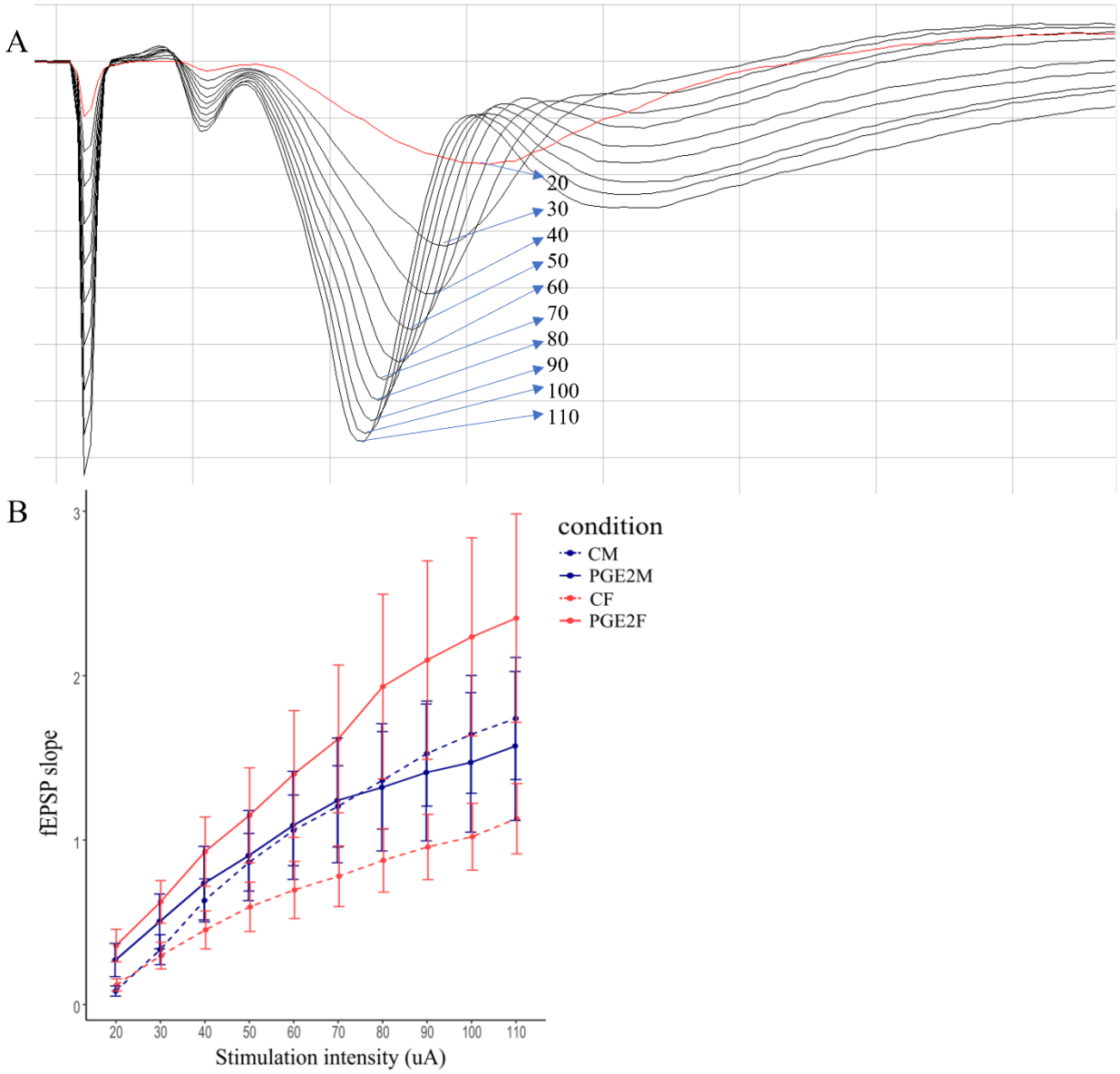


Figure 7: Input/Output responses in the mouse hippocampus of PGE2-exposed and control mice. A. I/O curves recorded from the mouse hippocampus. fEPSP slopes were extracted from these recordings using Clampfit 11.2. **B**. Graph showing I/O responses of male and female control and PGE2 mice at increasing stimulation intensities. Data is presented as mean ± SEM. n=10 slices per experimental group.

Table 2: Summary of sex- and treatment condition differences in I/O responses.
Sex differences in control and PGE2 mice are shown in comparison to males. Treatment condition differences in males and females are shown in comparison to controls. – indicate no significant difference, ↑ indicate an increase and ↓ indicate a decrease in the measured response.

Stimulation intensity (µA)	**Sex differences** *(compared to male)*		**PGE2 exposure** *(compared to controls)*	
	Control Female	**PGE2 Female**	**PGE2 Male**	**PGE2 Female**
20	–	–	–	–
30	–	–	–	–
40	–	–	–	–
50	–	–	–	–
60	–	–	–	–
70	–	–	–	–
80	–	–	–	↓
90	–	–	–	↓
100	–	–	–	↓
110	–	–	–	↓

4.1.3 Paired-Pulse Facilitation

In this study, we aimed to investigate whether prenatal exposure to PGE2 would affect paired-pulse facilitation and thereby short-term activity dependent presynaptic plasticity. We stimulated the CA3 region of the hippocampus twice in rapid succession with inter-pulse intervals of 200ms, 150ms, 100ms and 50ms. For each inter-pulse interval, the paired-pulse ratio, which is the slope of the second evoked fEPSP divided by the slope of the first evoked fEPSP (**Figure 8A**) was averaged across four recordings from the same hippocampal slice for a total of 5 slices per experimental group. The paired-pulse ratio was then plotted against the corresponding inter-pulse intervals (**Figure 8B**). We assigned treatment condition, sex, hippocampal side and inter-pulse interval as fixed effects and compared this to the baseline model which included litter, hippocampal slice and animal ID as random effects. The model of best fit included treatment condition and inter-pulse interval as determining factors (AIC = -66.671, p = 1.083e-11). Within this model, there was no significant interaction between treatment condition and inter-pulse interval (t(1.200e+02) = -0.388, p = 0.699). We conducted pairwise comparisons at each inter-pulse interval to examine differences between the saline- and PGE2-exposed mice offspring. Since the model of best-fit excluded sex and hippocampal side as a factor, the 2 groups included in this analysis are saline-exposed mice and PGE2-exposed mice with 20 hippocampal slices (10 male slices and 10 female slices) per group.

Treatment condition differences

Treatment condition differences in PPF responses were examined at each inter-pulse interval. Comparisons between the PGE2-exposed and control mice offspring revealed no significant differences in PPF at any of the inter-pulse intervals: 50ms (t(60.2) = -1.042, p =

0.302, control = 1.358, PGE2 = 1.451), 100ms (t(60.2) = -0.644, p = 0.522, control = 1.190, PGE2 = 1.287), 150ms (t(60.2) = -0.349, p = 0.729, control = 1.149, PGE2 = 1.204) and 200ms (t(60.2) = -0.835, p = 0.407, control = 1.098, PGE2 = 1.201). Overall, we observed no effect of PGE2 on PPF.

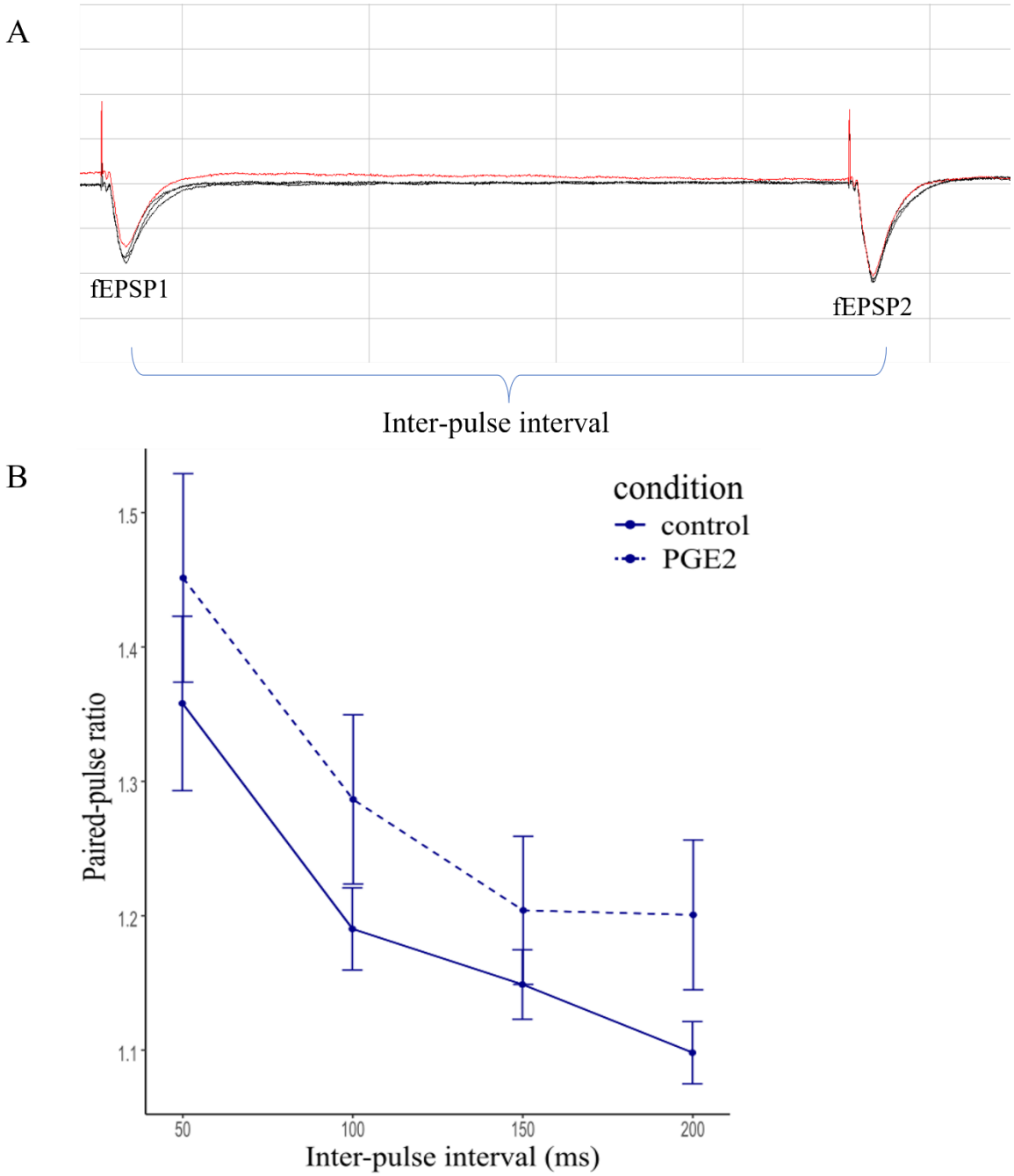


Figure 8: Paired-pulse facilitation in the hippocampus of control and PGE2 mice. A. fEPSP recordings obtained in response to rapid stimulation of the CA3 region with brief inter-pulse intervals. **B.** Graph showing paired-pulse ratio (slope of fEPSP 2 / slope of fEPSP 1) plotted against different inter-pulse intervals. Each point represents averages of four consecutive trials recorded. Data is presented as mean ± SEM. n=20 slices per experimental group.

4.2 Protein Expression in the Hippocampus

The above data demonstrated a sex-specific effect of PGE2-exposure on hippocampal LTP and I/O responses. We further investigated whether these responses may be influenced by the expression level of hippocampal proteins involved in synaptic plasticity including two post-synaptic glutamate receptors AMPAR subunit GluR1, NMDAR subunit GluN2A or NR2A and cytoskeletal architecture protein β-actin (**Figure 9A**). These proteins have been implicated in ASD and have been shown to be abnormally expressed in various rodent models of autism (Bozdagi et al., 2010; K. C. Kim et al., 2016; Rinaldi et al., 2007). Studies in our lab have also shown sex-specific changes in β-actin expression in the cerebellum of the COX2-KI and PGE2-exposed mice offspring (Kissoondoyal et al., 2021; Kissoondoyal et al., Unpublished).

We examined the effect of sex and treatment condition on the expression of β-actin, AMPA and NMDAR2A at PN90. Model 0 (M0) included technical replicate and the protein sample ID as random effects while Model 1 (M1) included treatment condition and sex as fixed effects. In Model 2 (M2), we included treatment condition as a fixed effect while excluding sex as a factor. Both M1 and M2 were compared to the baseline model M0.

4.2.1 AMPA

For AMPA, neither M1 (AIC = -1.506, $p = 0.649$) nor M2 (AIC = -4.643, $p = 0.880$) was a representative model. However, we conducted pairwise comparisons on M1 and plotted the fold change of AMPA against the 4 experimental groups in order to see the mean expression for each group (**Figure 9B**). There was no significant interaction between the treatment condition and sex ($t(21) = -0.887$, $p = 0.385$). Additionally, the main effect of treatment condition was also not significant ($t(21) = -0.151$, $p = 0.881$).

Sex differences in the saline- and PGE2-exposed mice offspring

Sex differences in AMPA expression were examined in the PGE2-exposed and control mice offspring. Comparisons within the control mice offspring revealed no significant differences between the males and females ($t(21) = -0.922$, $p = 0.367$, CM = 1.000, CF = 1.119). Comparisons within the PGE2-exposed mice offspring also revealed no significant differences between the males and females ($t(21) = 0.176$, $p = 0.862$, PGE2M = 1.055, PGE2F = 1.041) (**Figure 9B)**.

Treatment condition differences in males and females

Treatment condition differences in AMPA expression were examined in the male and female mice offspring. Comparison within the males revealed no significant difference between the PGE2-exposed and control mice ($t(21) = -0.518$, $p = 0.610$, CM = 1.000, PGE2M = 1.055) (**Figure 9A,B**). Comparison within the females also revealed no significant difference between the PGE2-exposed and control mice ($t(21) = 0.736$, $p = 0.470$, CF = 1.119, PGE2F = 1.041).

Overall, we found no difference in AMPA expression between males and females or between the saline- and PGE2-exposed mice offspring at PN90.

4.2.2 NMDAR2A

For NMDAR2A, M1 which included treatment condition and sex as fixed effects was chosen as the model of best fit (AIC = 53.004, $p = 0.019$). Within this model, we found a significant interaction between treatment condition and sex ($t(7.212) = -3.745$, $p = 0.007$).

Sex differences in the saline- and PGE2-exposed mice offspring

Sex differences in NMDAR2A expression were examined in the PGE2-exposed and control mice offspring. Comparisons within the control mice offspring revealed a significant difference, where the females showed higher NMDAR2A expression compared to males ($t(7.2) = -3.003$, $p = 0.019$, CM = 1.000, CF = 2.575) (**Figure 9A,C**). However, comparison within the PGE2-exposed mice offspring revealed no significant difference between the males and females ($t(7.2) = 2.290$. $p = 0.055$, PGE2M = 2.388, PGE2F = 1.694) indicating a loss of innate sex difference in NMDAR2A expression due to PGE2 exposure.

Treatment condition differences in males and females

Treatment condition differences in NMDAR2A expression were examined in the male and female mice offspring. Comparison within the males revealed a significant difference, where the PGE2-exposed mice showed higher NMDAR2A expression compared to the control mice ($t(7.2) = -3.240$, $p = 0.014$, CM = 1.000, PGE2M = 2.388). However, comparison within the females revealed no significant difference between the PGE2-exposed and control mice ($t(7.2) = 2.056$, $p = 0.078$, CF = 2.575, PGE2F = 1.694).

Overall, at PN90, we found an innate sex difference where the control females had higher NMDAR2A expression compared to their male counterparts. Interestingly, PGE2 exposure increased NMDAR2A expression specifically in males (**Figure 9A,C**).

4.2.3 β-actin

For β-actin, neither M1 (AIC = -23.813, $p = 0.422$) nor M2 (AIC = -26.090, $p = 0.845$) was a representative model. However, we conducted pairwise comparisons on M1 and plotted

the fold change of β-actin against the 4 experimental groups in order to see the mean expression for each group (**Figure 9D**). There was no significant interaction between treatment condition and sex ($t(21) = 1.244$, $p = 0.227$). Additionally, the main effect of treatment condition was also not significant ($t(21) = -0.195$, $p = 0.847$).

Sex differences in the saline- and PGE2-exposed mice offspring

Sex differences in β-actin expression were examined in the PGE2-exposed and control mice offspring. Comparisons within the control mice offspring revealed no significant differences between the males and females ($t(21) = 0.828$, $p = 0.417$, CM = 1.000, CF = 0.930). Comparisons within the PGE2-exposed mice offspring also revealed no significant differences between the males and females ($t(21) = -1.054$, $p = 0.304$, PGE2M = 0.929, PGE2F = 0.981) (**Figure 9D**).

Treatment condition differences in males and females

Treatment condition differences in β-actin expression were examined in the male and female mice offspring. Comparison within the males revealed no significant difference between the PGE2-exposed and control mice ($t(21) = 1.023$, $p = 0.318$, CM = 1.000, PGE2M = 0.929). Comparison within the females also revealed no significant difference between the PGE2-exposed and control mice ($t(21) = -0.736$, $p = 0.470$, CF = 0.930, PGE2F = 0.981).

Overall, we found no difference in β-actin expression between males and females or between the PGE2-exposed and control mice offspring at PN90 (**Figure 9A,D**).

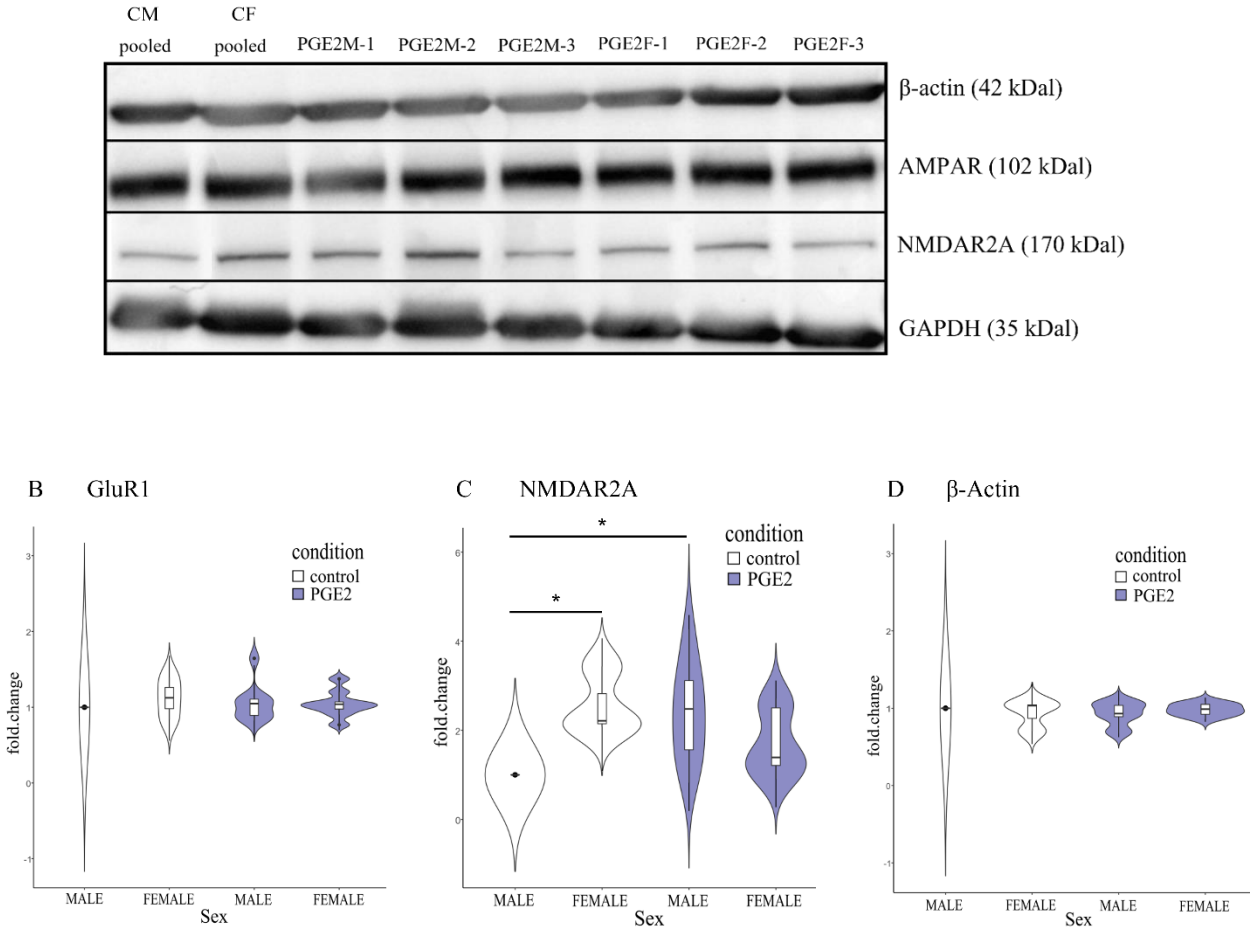


Figure 9: Protein expression of AMPAR (GluR1), NMDAR2A (GluN2A) and β-actin in the hippocampus at PN90. A. Western blots of pooled control samples and individual PGE2 samples of male and female mice obtained from 3 biological replicates from different litters (n=3). **B**. Violin plot of AMPAR expression. **C**. Violin plot of NMDAR expression. **D**. Violin plot of β-actin expression. All proteins were standardized using GAPDH (control). The fold change was then calculated relative to CM (CM=1). Data is presented as mean ± IQR. 3 technical replicates were performed.

4.3 Pyramidal Cell Morphology in the Hippocampus

Studies in our lab have provided evidence for abnormal dendritic morphology in the cerebellum and hippocampus of COX2-KI and PGE2-exposed mice (Kissoondoyal et al., 2021; Kissoondoyal et al., Unpublished; Iyer et al., Unpublished). Recent data from our lab examined the morphology of cells across the hippocampus at PN30 and PN90. The data has shown that PGE2 affected primary branch length, dendritic arborization, cell soma size and dendritic looping in cells across the hippocampus in a sex- and developmental stage-dependent manner. More specifically, PGE2 increased dendritic arborization in males and females at PN30 and males at PN90, but decreased arborization in females at PN90 (**Appendix A, Table 3**). PGE2 also increased primary branch length in males at PN30 only while decreasing cell soma size in males and females at PN90. Additionally, PGE2 exposure has also shown to increase the odds of observing dendritic looping in these neurons. Furthermore, PGE2 disrupted the innate increase in spine density observed across development (Iyer et al., Unpublished). This study aimed to further quantify neuronal morphology, specifically of pyramidal cells, which are key players in hippocampal synaptic plasticity (Spruston, 2008). We quantified primary branch length and cell soma size using the open software imageJ (**Figure 10A, 11A**).

In this study, our aim was to examine the effect of sex and treatment condition on the morphological characteristics of hippocampal pyramidal neurons. Specifically, we investigated changes in primary dendrite length and cell soma size at PN90. Model 0 (M0) included litter and animal ID as random effects while Model 1 (M1) included treatment condition and sex as fixed effects. In Model 2 (M2), we included treatment condition as a fixed effect while excluding sex as a factor. Both M1 and M2 were compared to the baseline model M0.

4.3.1 Primary Dendrite Length

Sex differences in the saline- and PGE2-exposed mice offspring

For primary dendrite length, neither M1 (AIC = 295.37, p = 0.533) nor M2 (AIC = 292.63, p = 0.111) was a representative model. There was no significant interaction between the treatment condition and sex (t(40) = -0.105, p = 0.917). Additionally, the main effect of treatment condition was also not significant (t(40) = -1.116, p = 0.271).

Sex differences in primary dendrite length were examined in the saline- and PGE2-exposed mice offspring. Comparisons within the saline-exposed mice offspring revealed no significant differences between the males and females (t(40) = 0.753, p = 0.456, CM = 29.621, CF = 26.939). Comparisons within the PGE2-exposed mice offspring also revealed no significant differences between the males and females (t(40) = 0.861, p = 0.394, PGE2M = 25.442, PGE2F = 22.216) (**Figure 10B**).

Treatment condition differences in males and females

Treatment condition differences in primary dendrite length were examined in the male and female mice offspring. Comparison within the males revealed no significant difference between the saline- and PGE2-exposed mice (t(40) = 1.116, p = 0.271, CM = 29.621, PGE2M = 25.442). Comparison within the females also revealed no significant difference between the saline- and PGE2-exposed mice (t(40) = 1.326, p = 0.192, CF = 26.939, PGE2F = 22.216).

Overall, we found no difference in primary dendrite length of hippocampal pyramidal neurons between males and females or between the saline- and PGE2-exposed mice offspring at PN90 (**Figure 10B**).

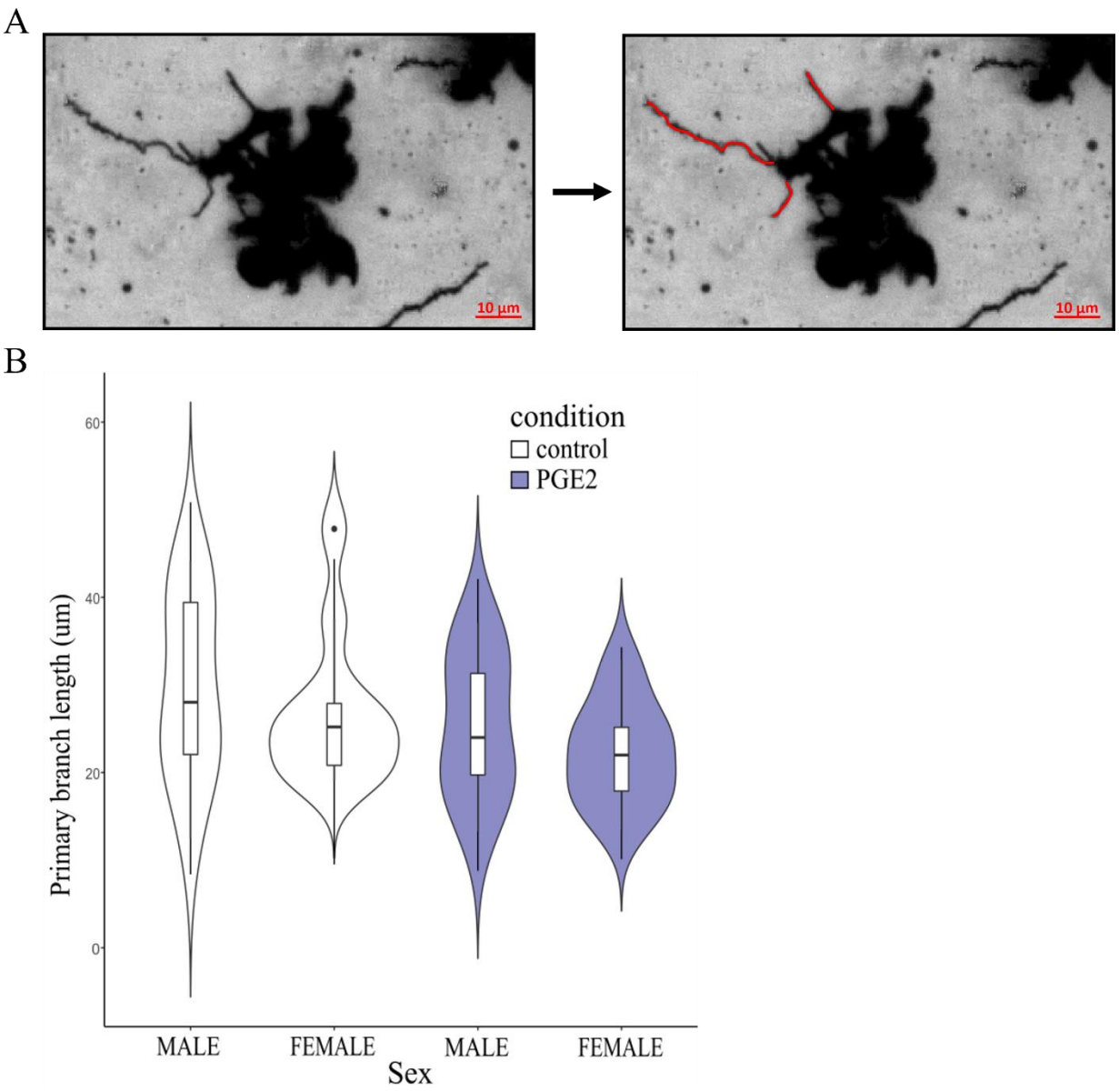


Figure 10: Primary branch length of pyramidal neurons in male and female control and PGE2-exposed mice. A. Primary dendrites were traced from the surface of the cell soma using imageJ **B.** Violin plot showing primary dendrite length in male and female control and PGE2 mice. Data is presented as mean ± IQR. n = 10 neurons for CM, 11 for CF, 9 for PGE2M and 10 for PGE2F.

4.3.2 Cell Soma Size

Sex differences in the saline- and PGE2-exposed mice offspring

For cell soma size, neither M1 (AIC = 542.50, p = 0.990) nor M2 (AIC = 539.48, p = 0.266) was a representative model. There was no significant interaction between the treatment condition and sex (t(11.265) = 0.771, p = 0.456). Additionally, the main effect of treatment condition was also not significant (t(12.794) = 0.298, p = 0.770).

Sex differences in cell soma size were examined in the saline- and PGE2-exposed mice offspring. Comparisons within the saline-exposed mice offspring revealed no significant differences between the males and females (t(9.3) = 0.151, p = 0.883, CM = 656.438, CF = 613.640). Comparisons within the PGE2-exposed mice offspring also revealed no significant differences between the males and females (t(14.7) = -0.997, p = 0.335, PGE2M = 688.316, PGE2F = 817.032) (**Figure 11B)**.

Treatment condition differences in males and females

Treatment condition differences in cell soma size were examined in the male and female mice offspring. Comparison within the males revealed no significant difference between the saline- and PGE2-exposed mice (t(12.8) = -0.298, p = 0.770, CM = 656.438, PGE2M = 688.316). Comparison within the females also revealed no significant difference between the saline- and PGE2-exposed mice (t(10.1) = -1.331, p = 0.212, CF = 613.640, PGE2F = 817.032).

Overall, we found no difference in cell soma size of hippocampal pyramidal neurons between males and females or between the saline- and PGE2-exposed mice offspring at PN90 (**Figure 11B**).

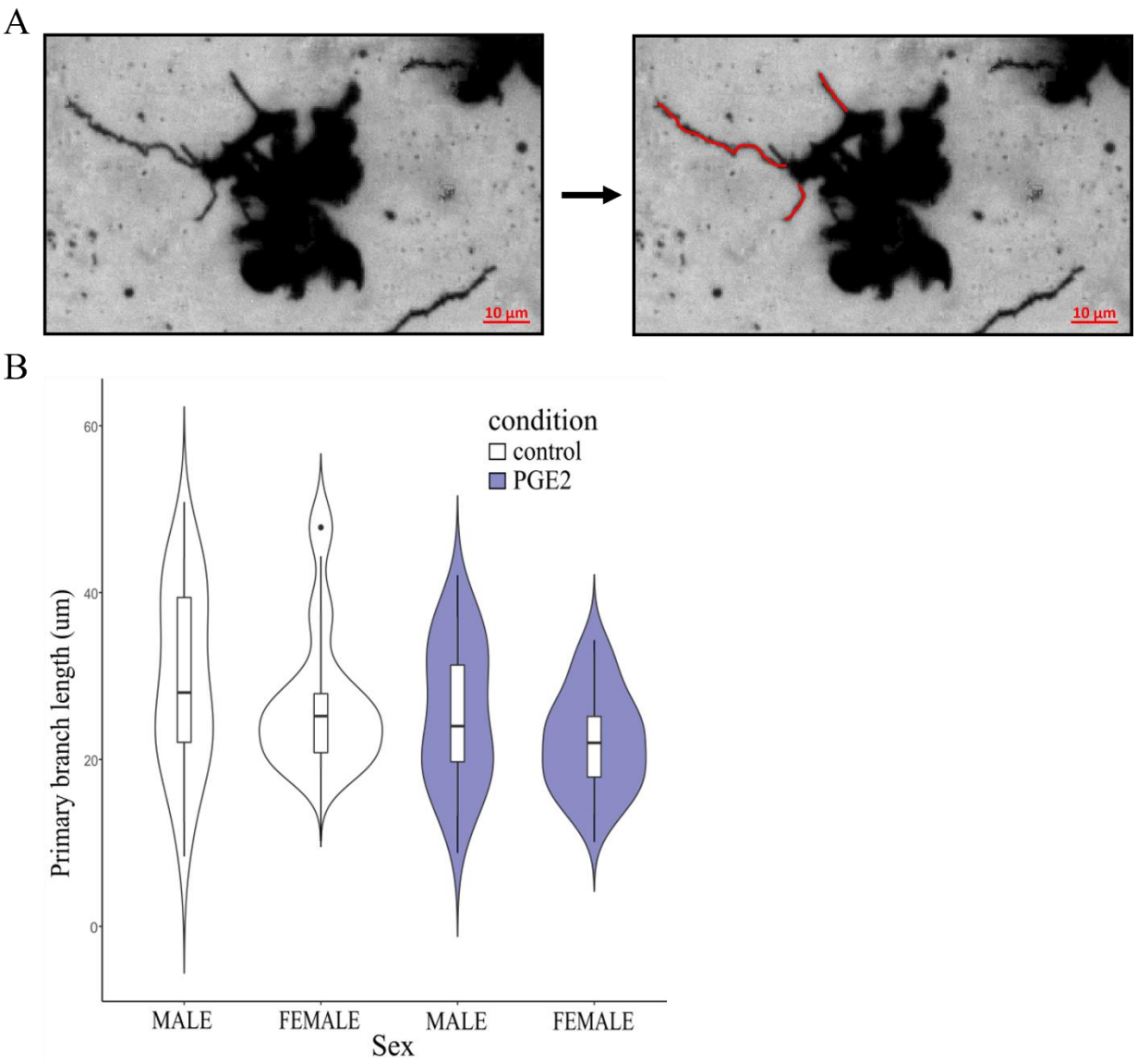


Figure 11: Cell soma size of pyramidal neurons in male and female control and PGE2-exposed mice. A. Cell bodies of neurons were traced using imageJ **B.** Violin plot showing cell soma size in male and female control and PGE2 mice. Data is presented as mean ± IQR. n = 10 neurons for CM, 9 for CF, 9 for PGE2M and 10 for PGE2F.

CHAPTER 5: DISCUSSION

5.1 Overview

The prevalence of ASD has been increasing at an alarming rate in the past two decades (CDC, 2020). Research has shown that various environmental risk factors such as prenatal exposure to drugs including misoprostol and acetaminophen, pollutants and toxins, maternal infection and inflammation can increase the risk of ASD (Ashmawi & Hammoda, 2022). These factors have been found to influence the level of PGE2, an important biolipid involved in healthy neurodevelopment (Tamiji & Crawford, 2010, 2011; Wong & Crawford, 2014). Our lab has developed mice models to study how increased (PGE2-exposed offspring) and reduced PGE2 levels (COX2-KI mice) can influence molecular changes in the brain and what the resulting behavioral consequences are later in life. Although various brains regions including the amygdala, cerebellum and prefrontal cortex has been implicated in ASD, the contribution of hippocampal deficits to the etiology of ASD is less understood especially in the wake of increasing evidence supporting memory impairments in individuals affected by the disorder (Weston, 2019).

My research study is an extension of a project recently conducted by another student in the lab showing sex-specific changes in branching, dendrite length, cell soma size and dendritic looping in neurons across the hippocampus in offspring exposed to PGE2 (Iyer et al., Unpublished). In this study, I further examined a sex-specific effect of PGE2 on hippocampal electrophysiology, dendritic morphology of pyramidal cells specifically and expression of synaptic proteins at PN90. I showed that PGE2 had a male-specific effect on LTP and a female-specific effect on I/O responses (Aim 1), as well as male-specific effect on NMDAR2A

expression level (Aim 2). However, we observed no differences in pyramidal cell dendrite length or cell soma size (Aim 3). We speculate that PGE2 may exert an effect on other cell types within the hippocampus as observed in (Iyer et al., Unpublished) and consequently influence the function of pyramidal cells (see limitations section). In the section below, I discuss the complementing findings of my research projects in the context of our recent data within the hippocampus and existing literature.

5.2 The Effect of PGE2 on Hippocampal Electrophysiology

5.2.1 Long-Term Potentiation

Sex differences in LTP

Hippocampal LTP is the underlying neural mechanism of learning and memory which has been impaired in various pathological conditions. In this study, we observed sexual dimorphism in LTP in control animals, where control males exhibited enhanced LTP relative to the control females throughout the entire post-HFS period (**Figure 6C**). We did not observe any significant difference between PGE2-exposed males and females suggesting a loss of innate sex difference in LTP responses. Our findings within the control animals are in line with other studies that investigated sexual dimorphism in hippocampal perforant path-dentate gyrus, schaffer collateral, and temporoammonic-CA1 LTP in rats (Maren, 1995; Maren et al., 1994; Monfort et al., 2015; Qi et al., 2016; Safari et al., 2021; Yang et al., 2004). These studies have also reported a corresponding difference in hippocampal learning and memory behavior where males performed better in the Morris Water Maze task, radial arm maze, and acquisition of contextual fear conditioning (Joseph et al., 1978; Maren et al., 1994; Qi et al., 2016; Roof, 1993; Safari et al., 2021). Researchers have attributed this sex difference to higher levels of

allopregnanolone, a progesterone metabolite, in adult female rats relative to males (Paul & Purdy, 1992), that has the capacity to enhance GABA receptor mediated inhibition (Shen et al., 2000; Smith et al., 1987) which may have caused the sex difference in LTP observed in our study. Overall, our results are in line with findings from other studies, however, further experiments need to be conducted to investigate receptor activity during LTP in these hippocampal slices.

Effect of PGE2 on LTP

As mentioned above there was no sex difference between PGE2-exposed male and female offspring, indicating that the effect can be male or female specific. When we compared these responses to the corresponding age-matched controls (CM and CF), we observed a decrease in LTP in response to PGE2 exposure in the male offspring only (**Figure 6C**). This data shows for the first time that maternal exposure to external PGE2 has a sex-specific effect on LTP. Previous studies in rodent models of ASD, such as the Shank3 mutants have investigated changes in the hippocampus of males only (Bozdagi et al., 2010) or in males and females combined (Jaramillo et al., 2016). Similar to our findings, rats prenatally exposed to bisphenol A (BPA), a potential model of ASD, showed impaired spatial learning in males but not females (Thongkorn et al., 2021). Several ASD rodent models including the Shank3, Shank2 and the Neuroligin-1 mutants also have exhibited impaired Schaffer collateral LTP compared to their controls (Blundell et al., 2010; Bozdagi et al., 2010; Jaramillo et al., 2016, 2017; Kouser et al., 2013; Won et al., 2012; Yang et al., 2012). The resulting effect of abnormal PGE2 signaling on long-term synaptic plasticity and hippocampus-dependent learning and memory still needs to be investigated. The studies mentioned above have also demonstrated deficits in social behavior and

spatial learning and memory in these animal models which have been previously linked to ASD (Blundell et al., 2010; Bozdagi et al., 2010; Jaramillo et al., 2016, 2017; Kouser et al., 2013; Won et al., 2012). Interestingly, other studies have also suggested a link between changes in PGE2 levels and aromatase activity, an enzyme that converts testosterone to estrogen, which may potentially offer some insight into sex bias observed in our data (Simpson, 2000). Intracerebellar injection of PGE2 during the second postnatal week has shown to enhance aromatase activity and estradiol production, subsequently reducing dendritic spines and dendritic tree size of rat Purkinje cells (Dean, Wright, et al., 2012). Additionally, aromatase inhibition has shown to impair LTP in female (Vierk et al., 2012) and male rodents (Grassi et al., 2011). These findings suggest that abnormal levels of PGE2 during prenatal development can influence aromatase activity and estradiol production and in turn influence neuronal morphology and synaptic plasticity in the brain. Hence the impairment in LTP observed in our PGE2-exposed males may be attributed to sex-specific long-term effects of aromatase-estradiol dysregulation caused by increased PGE2, to the extent where males exhibited LTP similar to females (**Figure 6C**). This connection still needs to be investigated.

5.2.2 Input/Output Responses

Sex differences I/O responses

In the Schaffer collateral synapses, we observed no difference in AMPAR mediated I/O responses between the CM and CF or between PGE2-exposed males and females (**Figure 7B**). I/O curves are a measure of basal synaptic transmission or synaptic strength under normal conditions and is known to be mediated by AMPA receptors. Findings from one study conducted on male and female rats revealed similar results where no changes in Schaffer collateral I/O

responses were observed between the sexes (Warren et al., 1995). Our western blot results also reveal a lack of difference in AMPAR expression between the control and PGE2-exposed males and females which may be attributed to the observations here. Although not many studies have investigated sex differences in I/O responses, one study has found increased I/O responses in the temporoammonic – CA1 synapses of males relative to females (Qi et al., 2016). These findings suggest that sex differences in I/O responses may be specific to the hippocampal region.

PGE2 on I/O responses

Next, we examined the effect of PGE2 exposure on AMPAR mediated I/O responses in the male and female offspring compared to the age-matched controls. We observed sex-specific effect of PGE2 where PGE2 females had lower I/O responses compared to their control counterparts at higher stimulation intensities (80-110µA) (**Figure 7B**), indicating that PGE2 exposure decreased basal synaptic strength or transmission in females. Many studies in ASD rodent models such as the Shank3, Shank2, Cntnap2 and Neuroligin-1 mutants have demonstrated no difference in I/O responses in the Schaffer collateral synapses relative to controls (Blundell et al., 2010; Jaramillo et al., 2016, 2017; Jurgensen & Castillo, 2015; Won et al., 2012). Other studies have shown a decrease in Schaffer collateral I/O responses in Shank3 ASD rodents, similar to what we observed in our PGE2-exposed females (Bozdagi et al., 2010; Kouser et al., 2013; Lee et al., 2015; Yang et al., 2012). However, these studies have not looked at sex differences and were conducted on younger animals.

Our study provides novel evidence that PGE2 impairs I/O responses in a sex-specific manner. The sex-specific trend observed due to PGE2 exposure may also be due to increased glutamatergic transmission in females relative to males as was observed in the mPFC of WT

adult C57BL/6J mice (Knouse et al., 2022). However, this difference in glutamate levels between our control males and females would need to be investigated in future experiments. Additionally, we did not observe changes in AMPA expression corresponding to the changes observed in I/O responses. Our lab has previously demonstrated developmental stage specific effects of PGE2 exposure on dendritic morphology (Iyer et al., Unpublished). For example, PGE2 exposure has shown to affect dendritic arborization, primary branch length and cell soma size differently in younger (PN30) and older mice (PN90) (Iyer et al., Unpublished). It may be possible that changes in receptor protein expression may also depend on the developmental stage, the effects of which may persist into adulthood. Further research would need to be conducted in order to test this possibility.

5.2.3 Paired-Pulse Facilitation

PGE2 on PPF

PPF is a measure of short-term activity dependent pre-synaptic plasticity. In this study, we wanted to investigate whether prenatal exposure to PGE2 would affect presynaptic activity in the hippocampus. In the Schaffer collateral synapses, we observed no changes in PPF in response to PGE2 exposure as compared to the control animals. Almost all of the ASD rodent studies have demonstrated similar results where Shank3, Shank2, Cntnap2 and Neuroligin-1 mutants exhibited PPF similar to their control counterparts (Blundell et al., 2010; Jaramillo et al., 2016, 2017; Jurgensen & Castillo, 2015; Kouser et al., 2013; Won et al., 2012), indicating that presynaptic release probability is not affected in autism. Our findings suggest that prenatal PGE2 exposure likely does not affect short-term activity-dependent presynaptic plasticity.

5.3 The Effect of PGE2 on Hippocampal Protein Expression

5.3.1 AMPAR and NMDAR2A

AMPA and NMDA receptors carry out significant roles in synaptic transmission. Specifically, these receptors are involved in the hippocampal Schaffer collateral NMDA-dependent LTP, where glutamate binds to them and allows for the entry of Ca^{2+} into the post-synaptic neuron, ultimately resulting in the insertion of new AMPA receptors (Citri & Malenka, 2008). This cellular mechanism is known to underlie learning and memory (Hölscher, 1999). In this study, we showed that there was no sex difference in AMPAR expression level in healthy controls and that prenatal exposure to PGE2 also has no effect on AMPAR expression level in males and females. However, there was an innate sex difference in NMDAR2A expression where control females exhibited higher levels compared to control males. Additionally, PGE2 exposure increased NMDAR2A expression in males only compared to their control counterparts.

Many ASD studies have provided evidence for the dysregulation of AMPA and NMDA receptors in the brain. For example, NMDAR2A expression was found to be reduced in the striatum of Shank3 mutant ASD mice (Peça et al., 2011). Rats prenatally exposed to VPA have shown enhanced levels of NMDAR2A in the neocortex associated with enhanced LTP (Rinaldi et al., 2007). Multiple animal models of ASD have also exhibited reduced NMDA/AMPA ratio in various brain regions (Blundell et al., 2010; Won et al., 2012). Ultimately, both upregulation and downregulation of AMPAR and NMDAR2A have been observed in other animal models of ASD such as the Shank3 mutants, VPA exposed and Neuroligin-3 mutant rodents (Bozdagi et al., 2010; Choi et al., 2016; Etherton et al., 2011; Kim et al., 2014; Rinaldi et al., 2007).

Our results reveal no change in AMPAR expression levels in response to PGE2 exposure. This is in line with the findings from studies conducted on other ASD models. For example, in

VPA exposed rats, Neuroligin-3 mutant and Shank3 mutant mice, similar results were observed in the neocortex, hippocampus and striatum of relatively much younger and older animals respectively where no change was observed in AMPAR expression levels (Etherton et al., 2011; Peça et al., 2011; Rinaldi et al., 2007). Interestingly, administration of PGE2 into the preoptic area was found to induce masculinization of behaviors in females which was mediated by AMPA receptors (Wright & McCarthy, 2009). Additionally, PGE2 was also shown to increase AMPAR phosphorylation and membrane insertion in the preoptic area of rats (Lenz et al., 2011). However, PGE2 was administered soon after birth and the tests were conducted within hours of PGE2 administration. Our study was conducted on prenatally exposed PGE2 mice that were mature (PN90). Previously, our lab has shown that PGE2 contributes to developmental stage-specific changes in dendritic morphology. Hence, further studies need to be conducted to examine whether AMPAR expression is altered early in development.

In this study, we also observed a sex-specific change in NMDAR2A expression in the PGE2-exposed mice offspring. Specifically, in males, PGE2 exposure increased NMDAR2A levels in the hippocampus. Although most ASD rodent models such as the Shank3 and Shank2 mutants have shown a reduction in NMDAR levels associated with a reduction in LTP and ASD-like behaviors (Jaramillo et al., 2017; Won et al., 2012), some studies have shown the opposite effect where enhanced NMDAR levels, LTP and spatial memory have been reported in animal models of ASD including the VPA exposed and Neuroligin-3 mutants (Etherton et al., 2011; Rinaldi et al., 2007). In either case, the direction of change in LTP and NMDAR expression is usually the same, where we see either an increase in both NMDAR expression and LTP or a decrease in both. However, in our study, we saw an increase in NMDAR expression level corresponding to a decrease in LTP responses in PGE2-exposed male offspring. Similar sex

differences were observed in a study conducted on VPA-exposed rats, where NMDAR2A expression was found to be increased in VPA-exposed males during post-natal weeks 2 and 4 but only post-natal week 2 in females (Kim et al., 2016). Researchers suggested that this maybe due to the switch in expression from NMDAR2B, a subunit expressed early in life, to NMDAR2A during later post-natal stages (Kim et al., 2016). NMDAR2B is known to induce longer excitatory post-synaptic potentials thus increasing synaptic efficacy and memory compared to NMDAR2A subunits (Monyer et al., 1994; Newcomer et al., 2000). This decrease in NMDAR2B subunits with age have been associated with the age-related cognitive decline in memory (Newcomer et al., 2000). These findings and our results suggest that such age- and sex-dependent changes may start during the early weeks of post-natal development and persist into adulthood.

Additionally, there might be an interesting link between the PGE2 signaling pathway and the activation of NMDA receptors. Studies have shown that COX2 expression in neurons is enhanced by synaptic NMDARs (Stark & Bazan, 2011). Pharmacological inhibition of NMDAR2A in cultured mice cortical neurons have shown to reduce COX2 and PGE2 levels (Rajagopal et al., 2019). Moreover, activation of NMDA receptors was reported to enhance PLA2 activity, the first enzyme in the PGE2 synthesis pathway (Kim et al., 1995). Interestingly, increased PLA2 activity has been reported in children with ASD (Bell et al., 2004; Helena Fávero de Souza Tostes et al., 2013). PGE2 is also known to enhance glutamatergic neurotransmission by increasing presynaptic glutamate release probability (Sang et al., 2005; Yang et al., 2008). However, it is important to note that excessive glutamate release can result in over-excitation and subsequently lead to cell death, injury or synaptic dysfunction (Yang & Chen, 2008). Therefore, we propose a model for the effect of PGE2 on hippocampal function in

which, increased PGE2 levels (perhaps due to maternal exposure to various environmental risk factors) upregulate NMDA receptor expression via a feedback mechanism (**Figure 12**), thus impairing LTP in a male-specific manner (the feedback mechanism in our proposed model is described below). These findings may suggest that increased NMDA expression may alter COX2/PGE2 signaling and vice-versa, however, further studies would need to investigate this relationship.

Our results also reveal an innate sex difference where NMDAR2A expression was higher in control females relative to control males. This innate sex difference was disrupted by PGE2 exposure. Studies examining basal expression of glutamate receptors in males and females are scarce. However, one study reported lower NMDAR expression in the hippocampus of female rats compared to males (Palomero-Gallagher et al., 2003). However, this study did not investigate changes in the expression level of specific subunits of the NMDA receptor. Our study is the first to provide evidence for differential expression of NMDAR2A in control males and females. Interestingly, sex hormones have been suggested to play a role in synaptic transmission. A study conducted on ovariectomized female rats treated with estradiol provided evidence for increased NMDA sensitivity but not AMPA in the hippocampus, suggesting that females might naturally have increased NMDA activity or expression due to their hormones (Woolley et al., 1997). This study provides novel evidence that basal NMDAR2A level is likely higher in the female hippocampus relative to males. Overall, these findings suggest that NMDAR expression may be specific to the sex and the type of receptor subunit.

5.3.2 β-Actin

β-actin is one of 2 distinct isoforms of the actin cytoskeleton involved in neurodevelopment (Cheever & Ervasti, 2013). It is known to play critical roles in axon guidance, migration and synaptogenesis (Ayala et al., 2007; Cheever & Ervasti, 2013). In this study, we found no difference in β-actin expression in biological replicates of PGE2-exposed mice hippocampi. However, our lab has previously associated impairments in dendritic morphology in the cerebellum of the COX2-KI and the PGE2-exposed mice to a decrease in β-actin expression at an earlier developmental stage (PN25, PN30) (Kissoondoyal et al., 2021). It is important to note that our study was conducted on PN90 mice during which the hippocampus is mature, while the previous study was conducted on PN30 mice during which the hippocampus is still developing. Our results suggest that changes in β-actin expression levels due to PGE2 exposure may be specific to the brain region as well as developmental stage. Moreover, PGE2 has shown to affect hippocampal dendritic morphology in a developmental stage-dependent manner (Iyer et al., Unpublished). Hence, further studies need to focus on examining protein expression in mice at an earlier developmental stage.

5.4 The Effect of PGE2 on Hippocampal Pyramidal Cell Morphology

5.4.1 Primary Dendrite Length

In the hippocampus, we saw that primary dendrite length of pyramidal neurons was not affected by PGE2 or sex at PN90 (**Figure 10B**). However, our lab has recently shown an innate sex difference in primary dendrite length of cells across the hippocampus where PGE2-exposed females had increased branch length compared to males at PN30 (Iyer et al., Unpublished). Additionally, in the same study, PGE2 had a sex-specific effect on primary branch length where

PGE2-exposed males had increased length relative to control males (Iyer et al., Unpublished). However, my study shows no change in primary dendrite length of pyramidal neurons at PN90. Testing earlier developmental stages would provide a better representation of the effect of PGE2 on branch length in the pyramidal neurons. The developmental stage appears to have an effect where there was an overall reduction in dendrite length across development from PN30 to PN90 for each of the groups (Iyer et al., Unpublished). Our study specifically focused on pyramidal neurons in the CA3-CA1 region at PN90. Interestingly, it is known that hippocampal pyramidal neurons are highly vulnerable to injury in pathological conditions (Medvedeva et al., 2017). Our results showing no change in dendrite length might also be a consequence of dendritic pruning and refining mechanisms that occur over development as evidenced in hippocampal pyramidal neurons and cerebellar granule cells (Dailey & Smith, 1996; Dhar et al., 2018). Dendrites are highly dynamic structures that exhibit an initial growth spurt followed by stabilization and then retraction later in development (Puram & Bonni, 2013). Further research would need to be conducted at an earlier developmental stage in order to confirm the effects of PGE2 on pyramidal cell morphology.

5.4.2 Cell Soma Size

In the hippocampus, we found that cell soma size of pyramidal neurons was not affected by PGE2 or sex at PN90 (**Figure 11B**). We have recently shown that PGE2 exposure had no effect on cell soma size of cells across the hippocampus at PN30, but decreased cell soma size at PN90 in both males and females (Iyer et al., Unpublished). Many ASD and Rett's syndrome rodent models such as the bisphenol A (BPA) exposed and *Mecp2* mutants as well as human studies have demonstrated a reduction in neuronal soma size in various parts of the brain such as

the hippocampus, cerebrum, cerebellum, thalamus, amygdala, cortical and subcortical regions (Bauman et al., 1995; Chen et al., 2001; Goffin et al., 2012; Thongkorn et al., 2021; Wegiel et al., 2014). As mentioned previously, the highly vulnerable nature of pyramidal neurons might be the reason for our observations. These findings suggest that the effect of various environmental risk factors on neuronal cell soma size may depend on the brain region, cell type and developmental stage. Future studies will need to focus on quantifying pyramidal cell soma size at a younger developmental stage to further elucidate this.

5.5 Implications of Abnormal Hippocampal Plasticity in ASD

In this study, we observed sex-dependent changes in hippocampal LTP, I/O responses and NMDAR2A expression in the PGE2-exposed mice offspring which can potentially lead to memory impairments. The contribution of hippocampal deficits to the etiology of ASD has been long overlooked despite the fact that many ASD studies have reported impairments in LTP and memory behavior. Hippocampal abnormalities have also been linked to deficits in social behavior (Banker et al., 2021). Findings from a recent study conducted by a previous graduate student in our lab also provided evidence for abnormal hippocampal neuronal morphology in the same mouse model (Iyer et al., Unpublished). Ultimately, deficits in all these factors, namely, synaptic function, protein expression and neuronal morphology in the hippocampus can result in abnormal hippocampal synaptic plasticity and consequently lead to memory impairments. Interestingly, the onset of ASD symptoms is known to temporally coincide with synaptic development and maturation. Hence, exposure to environmental insults during this critical time in development interferes with normal synaptic development thus increasing the risk of ASD (Guang et al., 2018; Nisar et al., 2022).

In this study, we examined the effect of PGE2 exposure on hippocampal Schaffer collateral LTP at PN90. The high frequency stimulation used to induce LTP is known to mimic the theta rhythm that naturally occurs in the hippocampus during exploratory behavior (Diamond et al., 1988). Hippocampal LTP is known to be the neural basis that underlies learning and memory, particularly cued spatial memory (Lynch, 2004). Our results revealed a sex-specific effect of PGE2 on LTP which can be attributed to the sex-specific effect of PGE2 on NMDAR2A expression in the hippocampus. However, the LTP findings could also be attributed to various other factors that need to be further investigated such as the expression of second messenger molecules including Ca^{2+}, CAMKII, PKA, glutamate receptors as well as glutamate levels in the brain (Nisar et al., 2022). Although we measured NMDAR2A levels in the hippocampus, a more accurate way of linking LTP to NMDAR activity would be to measure the receptor activity and expression levels in the same animals that were subjected to the HFS during LTP. Metabotropic glutamate receptors (mGluRs), specifically mGluR1 and mGluR5 are the most studied in autism (Nisar et al., 2022). Studies have reported increased levels of mGluR5 in the vermis region of the cerebellum and the superior frontal cortex of children with autism (Fatemi et al., 2011; Fatemi & Folsom, 2011). Group 1 mGluRs are particularly associated with NMDA-dependent LTP, which was shown to be impaired in our study in the PGE2-exposed males. The deletion or inhibition of these Group 1 mGluRs has been associated with poor performance in memory-related tasks such as the Morris water maze task (Xu et al., 2009). Glutamate transporter genes such as *SLC1A1* have also been implicated in ASD where it has been linked to anxiety and repetitive behaviors in children with autism (Gadow et al., 2010).

As mentioned previously, both increased and decreased expression of NMDAR subunits have been reported in ASD. In our study, we observed an increase in NMDAR2A expression

corresponding to a decrease in LTP in the PGE2-exposed mice. This can be attributed to the hyper-glutamatergic hypothesis of autism where increased levels of glutamate were reported in the serum of adult individuals with autism (Shinohe et al., 2006). Higher glutamate levels are known to occur as a result of reduced levels of the enzyme glutamate acid decarboxylase and consequently a reduction in GABA (Fatemi et al., 2002). Higher glutamate levels could be a possible reason for the higher expression of its receptor NMDA observed in this study. Elevated levels of glutamate can also lead to other problems such as excitotoxicity and oxidative stress which has also been implicated in ASD (El-Ansary, 2016). Stimulating glutamate receptors allow the flow of Ca^{2+} into the cell which can result in the activation of inducible nitric oxide and the phosphorylation of PKC (Nisar et al., 2022). However, higher levels of nitric oxide can lead to an increase in free radicals and reactive oxygen species which can damage the cell (Nisar et al., 2022). Besides, PKC can also induce PLA2, which can further produce pro-inflammatory molecules (Babu et al., 1994). These can lead to toxic reactions in the cell ultimately causing cell death (Nisar et al., 2022). Abnormal levels of GABA (inhibitory neurotransmitter) and glutamate (excitatory neurotransmitter) can also cause an excitatory/inhibitory imbalance which is also detrimental to normal synaptic functioning and has been implicated in ASD (Lee et al., 2017). Studies have also reported higher levels of GluR1 in the cerebellum of individuals with ASD (Purcell et al., 2001). Glutamate also plays a significant role in neurodevelopment and synaptogenesis suggesting that moderate levels of glutamate and its receptors are important in the formation of brain cytoarchitecture (Johnston, 1995).

CaMKII, an important downstream enzyme involved in LTP, has also been associated with ASD. Our lab has provided evidence for the dysregulation of the *Camk2b* gene in the COX2-KO mice during the embryonic stages (Rai-Bhogal, Ahmad, et al., 2018). Similarly,

PKA, another second messenger involved in synaptic transmission has also shown to be downregulated in the frontal cortex of individuals with autism (Ji et al., 2011). Interestingly, PGE2 is also known to regulate synaptic transmission via the EP2 receptor-PKA pathway (Sang et al., 2005). Ultimately, the effect of PGE2 on LTP and NMDAR2A could have been mediated by any of these second messengers discussed above, further research needs to be conducted to investigate the activity and/or expression of these proteins to fully understand the pathway involved in synaptic dysfunction.

5.6 Proposed Model/Pathway Mechanism

Our study demonstrated that PGE2 exposure increased NMDAR2A expression and decreased LTP in males while decreasing I/O responses specifically in females only. Based on these findings and previous literature, we propose a novel mechanism for the interaction between the COX2/PGE2 signaling pathway and NMDA regulated synaptic transmission within the hippocampus (**Figure 12**). Various studies have linked NMDA receptor activity to increased COX-2 production subsequently leading to neurotoxicity and cell death (Hewett et al., 2000, 2006; Stark & Bazan, 2011; Strauss & Marini, 2002). This neurotoxicity and cell death is known to be mediated by PGE2 and glutamate induced neuroinflammation and excitotoxicity (Hewett et al., 2000; Stark & Bazan, 2011). As mentioned previously, PGE2 has shown to enhance glutamatergic neurotransmission by increasing presynaptic glutamate release probability (Sang et al., 2005; Yang et al., 2008). Based on this evidence, we propose that PGE2 exposure may have resulted in an increase in glutamatergic neurotransmission due to PGE2-induced NMDAR expression mediated by E prostanoid (EP) receptors via PKA (Sang et al., 2005). Abnormally increased amounts of NMDAR expression levels as observed in our study may result in

overexcitation induced by glutamate causing neuronal death. This neurotoxicity induced cell death could explain the decrease in LTP observed in PGE2-exposed males in our study. Moreover, findings from other studies demonstrated an increase in COX-2 and PGE2 levels in response to NMDA exposure resulted in excitotoxicity (Hewett et al., 2000; Stark & Bazan, 2011). Hence, we propose that the NMDA receptor expression – COX2/PGE2 signaling pathway interaction might be regulated through a feedback mechanism (as shown by the red dotted line) (**Figure 12**) and play an important role during prenatal development. However, various environmental risk factors known to influence PGE2 levels prenatally (Tamiji & Crawford, 2011; Wong & Crawford, 2014) may impair the homeostasis between these pathways and contribute to abnormal excitation of neurons leading to neurotoxicity. Further investigations of these mechanisms are required to fully elucidate this.

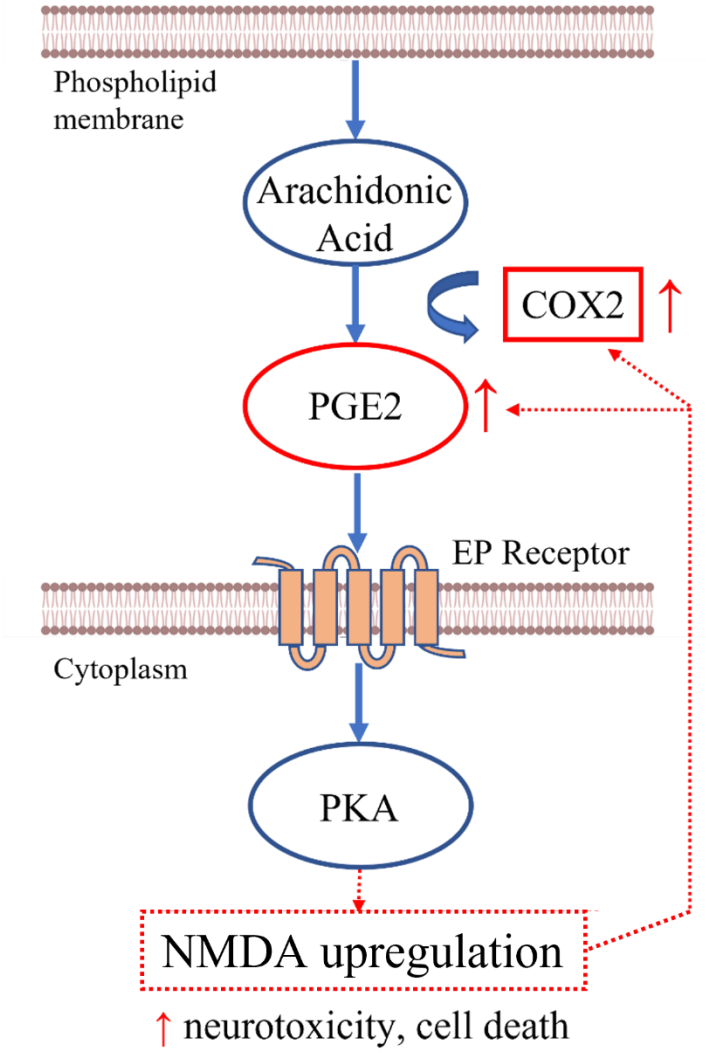


Figure 12: Proposed model of the COX2/PGE2 signaling pathway affecting NMDA expression. Abnormal COX2/PGE2 signaling may lead to increased NMDA gene transcription in the brain via the PGE2-EP receptor-PKA pathway. Alternatively, exposure to abnormal levels of NMDA may increase the production of COX2 and PGE2, subsequently leading to neurotoxicity and cell death. The red dotted lines indicate a potential feedback mechanism.

5.7 Limitations and Future Studies

In Aim 1 of this study, I examined the effect of PGE2 exposure on hippocampal electrophysiology at PN90. However, the hippocampus has different regions, mainly the dorsal and ventral hippocampus which has distinct functions including the involvement in cognition and emotion, respectively. These functional regions could be affected differently by PGE2 exposure, and our study did not take this into account due to the lower sample size. Hence, future studies should investigate the distinct effects of PGE2 on the dorsal and ventral hippocampus. Additionally, our lab has previously shown that dendritic morphology was affected in a developmental stage-dependent manner. Therefore, in order to get a developmental perspective, it is important to investigate the effect of PGE2 on hippocampal electrophysiology at an earlier developmental stage, perhaps PN30, which is the maturation timepoint of the hippocampus. In Aim 2 of our study, we measure hippocampal protein expression in control and PGE2 hippocampal protein samples from 3 mice only. Future experiments should focus on conducting western blots on larger sample size from control and PGE2-exposed samples. As mentioned previously, stage-specific effects also need to be examined in younger mice. Additionally, a more accurate way would be to examine receptor activity by blocking one type of receptor and measuring the contribution of the other type of receptor using patch clamp recording and quantifying the AMPA/NMDA ratio. In Aim 3 of this study, we examined the morphology of pyramidal neurons at PN90. However, the pyramidal neurons were selected based on their location due to the lack of a characteristic pyramidal structure. This may have been due to an overall pruning in the older mice. Hence, future experiments would need to focus on characterizing neuronal morphology at a younger stage when the neurons still have their characteristic pyramidal shape. Additionally, more morphological characteristics such as

branching, dendritic looping and most importantly spine density and morphology need to be quantified in order to give us a better insight into how synaptic dysfunction may have occurred. Additionally, the hippocampus has different regions (CA3, CA1, dentate gyrus etc.) and cell types (granule cells, basket cells etc.) that have different functions and may be affected differently by PGE2. Hence, it is important to characterize and quantify these using techniques such as primary cell culture. Lastly, behavioral experiments such as Morris water maze will be conducted by a current graduate student in our lab to test whether these morphological, molecular and synaptic deficits will manifest into spatial memory impairments.

5.8 Conclusion

This study examined the role of prenatal PGE2 exposure on hippocampal electrophysiology, protein expression and neuronal morphology. Overall, we found that PGE2 contributed to changes in hippocampal electrophysiology and protein expression in mice offspring at PN90. In Aim 1, we conducted LTP, I/O and PPF experiments and observed a male specific effect on LTP and a female-specific effect on I/O responses. In Aim 2, we conducted western blots to measure the expression of AMPAR, NMDAR2A and β-actin in the hippocampus. We observed a male-specific effect of PGE2 on NMDAR2A expression suggesting that the LTP impairments observed in our study may be due to the abnormal NMDAR2A expression. NMDA receptors are critical in regulating LTP, and studies have suggested a strong link between COX2/PGE2 signaling and NMDAR activity (Lacroix et al., 2015; Rajagopal et al., 2019; Stark & Bazan, 2011). Impairments in LTP and NMDA receptor expression could lead to memory deficits which needs to be investigated in future studies. Overall, our findings provide convincing evidence suggesting that abnormal COX2/PGE2

signaling can affect the physiology of cells within the hippocampus, likely contributing to the pathophysiology of ASD.

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APPENDIX A

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Figure 2: The production of PGE2.

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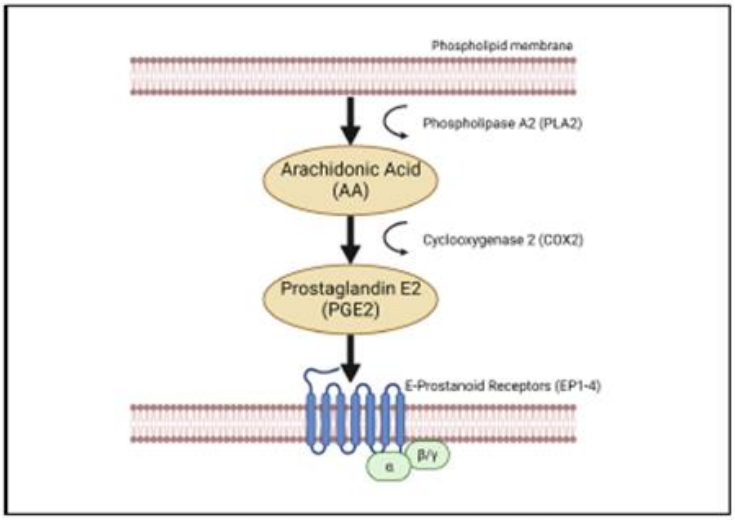

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Figure 3: The interaction between misoprostol and PGE2 signaling. (Wong & Crawford, 2014).

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Figure 4: Synaptic transmission and receptor trafficking during NMDA-dependent LTP at the hippocampal Schaffer collateral pathway.

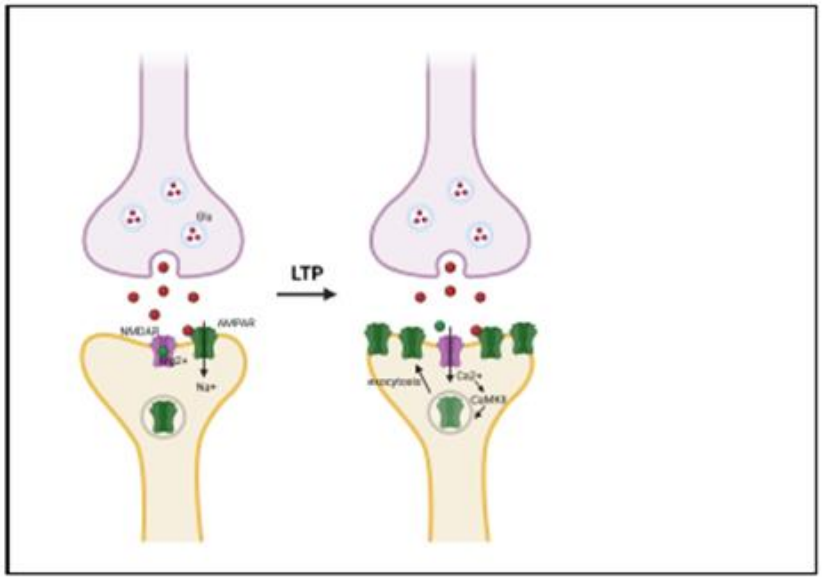

Figure 6A: Schaffer collateral LTP in the PGE2 and control males and females.

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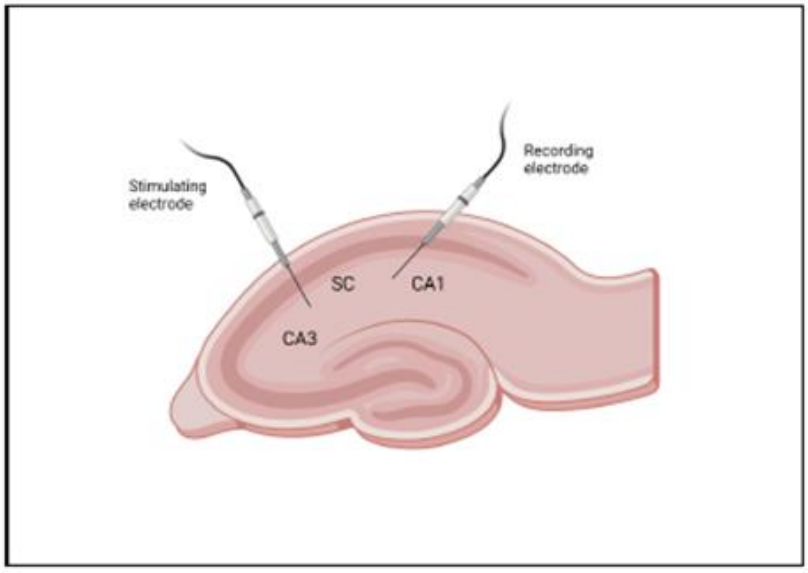

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Table 3: The effect of prenatal exposure to PGE2 on male (M) and female (F) mice offspring hippocampal neuronal morphology at PN30 and PN90. (N.S. = Non-Significant, ↑ = increased, ↓ = decreased). Data obtained from (Iyer et al., Unpublished).

Factor	**PN30**	**PN90**
Dendritic arborization	M: ↑ at 60 – 100 µm F: ↑ at 60 – 100 µm	M: ↑ at 20 µm F: ↓ at 20 – 40 µm
Primary branch length	M: ↑	N.S
Cell soma size	M & F: N.S	M & F: ↓
Dendritic looping	M & F: ↑	