NEUROBIOLOGY OF LIPID SIGNALLING IN THE DEVELOPING BRAIN: LINK TO AUTISM SPECTRUM DISORDERS

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ABSTRACT

Autism spectrum disorders (ASDs) are neurodevelopmental conditions diagnosed by atypical behaviours in social interaction and communication, along with stereotyped, restricted and repetitive behaviours. The exact cause of ASDs is unclear but is likely from a combination of genetic and environmental influences. Various clinical studies have identified an association between irregular lipid signalling and ASDs.

Lipids are major components of brain cells and serve as a supply for signalling molecules such as prostaglandin E2 (PGE_2). Cyclooxygenase-2 (COX-2) is the key enzyme responsible for PGE_2 production in the brain. The COX-2/PGE_2 signalling pathway is essential for development and maintenance of healthy neural functions. Genetic defects or exposure to various environmental agents—such as infections or drugs—can disrupt the levels of PGE_2.

However, investigation into the molecular mechanisms by which disrupted COX-2/PGE_2 signalling or irregular PGE_2 levels might affect the development of the nervous system and contribute to the pathogenesis of ASDs is sparse. Addressing this gap in knowledge was the main purpose of this dissertation. We found that PGE_2 interacts with the key developmental Wnt signalling pathway *in vitro* by affecting neuroectodermal stem cell motility and proliferation (Study 1) and by promoting their differentiation into neurons (Study 2). In addition, prenatal PGE_2 exposure disrupted cell density and increased migration in ASD-implicated areas of the mouse brain (Study 3). Decreased PGE_2 (via COX-2-deficiency) or increased PGE_2 in mice also led to abnormal microglial density and morphology (Study 4). Lastly, behavioural outcomes related to ASDs were quantified in COX-2-deficient and PGE_2-exposed mice (Study 5 and 6).

Our findings support epidemiological and clinical reports implicating the COX-2/PGE_2 pathway in ASDs. We provide novel evidence that disturbances in the COX-2/PGE_2 pathway

results in aberrant neurodevelopment, including molecular, cellular, and behavioural differences analogous to those described in ASDs. Importantly, this array of research studies is one of the first to investigate mechanisms related to ASDs in both males and females and at various developmental stages, which is greatly underrepresented in the current literature. Altogether, this dissertation exposes the COX-2/PGE_2 pathway as an autism candidate pathway and offers important insight into the complex, unknown etiology of ASDs.

DEDICATION

This collection of works is dedicated to four important people in my life:

My grandmother, *mama*: although our time together did not feel long enough, you showed me and taught me so much about love, passion, and perseverance. No matter what life threw at you, you tackled it with such grace and impressive grit. These fundamentals have become essential through this journey of life and academics.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
Ach	Acetylcholine
ALA	Omega-3 α-linolenic acid
ANOVA	Analysis of variance
ASDs	Autism spectrum disorders
ADHD	Attention deficit-hyperactivity disorder
BBB	Blood-brain barrier
BPA	Bisphenol A
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CldU	5-Chloro-2′-deoxyuridine
CNS	Central nervous system
COX-2	Cyclooxygenase-2
$COX\text{-}2^{-}$	Cyclooxygenase-2 knockin
DHA	Docosahexaenoic acid
E1, E11, E16	Embryonic day 1, 11, 16
EEDCs	Estrogen-like endocrine disrupting chemicals
EFA	Essential fatty acids
EP	E-prostanoid
EPA	Eicosapentaenoic acid
FADS	Fatty acid desaturase
FXS	Fragile X syndrome
FZD	Frizzled

GABA	Gamma-aminobutyric acid
GSH	Glutathione
GSK-3β	Glycogen synthase kinase 3 beta
H89	H89 dihydrochloride hydrate
HAHs	Halogenated aromatic hydrocarbons
IdU	Iododeoxyuridine
KI	Knockin
LA	Omega-6 linoleic acid
MEM	Minimal essential media
MTs	Monozygotic twins
NE-4C	Neuroectodermal
NSAIDs	Nonsteroidal anti-inflammatory drugs
OCPs	Organochlorine pesticides
OPPs	Organophophate pesticides
P8	Postnatal day 8
PBDEs	Polybrominated dyphenyl esters
PBS	Phosphate-buffered saline
PCBs	Polychlorinated bisphenyls
PFA	Paraformaldehye
PGE_2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PLA2	Phospholipase A2
PPs	Pyrethrins and pyrethroids
PUFA	Polyunsaturated fatty acids

PVC	Polyvinyl chloride
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
RQ	Relative quantification
SFM	Serum-free media
SLOS	Smith-Lemli-Opitz syndrome
TXA_2	Thromboxane A2
Wnt	Wingless-related MMTV integration site
WntA	Wnt agonist
WORT	Wortmannin
WT	Wild-type

CHAPTER 1.

1.1. General Introduction

Lipids serve as important building blocks in the brain and are essential for the production of crucial developmental signalling molecules such as prostaglandin E2 (PGE_2). Endogenous production of PGE_2 in the brain is predominately controlled through the enzymatic activity of cyclooxygenase-2 (COX-2), which expression is both constitutive and inducible in the nervous system (Kirkby et al., 2016). Insufficient dietary supplementation, genetic defects, or influence of various environmental agents such as exposure to infections, pesticides, air pollution, or specific drugs have all been shown to cause abnormal lipid metabolism and disrupt the levels of PGE_2 (Wong et al., 2015, Wong and Crawford, 2014). These same factors have also been associated with Autism Spectrum Disorders (ASDs). For example, clinical studies have revealed that prenatal exposure to the drug misoprostol, an analogue to PGE_2, during the first trimester of pregnancy may lead to neurodevelopmental aberrations, including Mobius sequence and ASDs (Miller et al., 2005, Bandim et al., 2003, Schuler et al., 1999, Costa, 1998, Gonzalez et al., 1998). Polymorphisms of the gene that encodes COX-2 called *Ptgs2* have been associated with ASDs and related core symptoms including abnormalities in communication, social interaction, and overactivity (Yoo et al., 2008). Despite clinical and epidemiological evidence that the COX-2/PGE_2 signalling pathway may have etiological significance to the pathogenesis of ASDs (see Chapter 2 for review of literature), basic research surrounding the function of PGE_2 in the developing brain and its potential implications in ASDs are sparse. The studies presented in this dissertation provide a range of molecular, cellular, and behavioural research conducted in cell and mice model systems, revealing mechanisms by which abnormalities of the COX-2/PGE_2

pathway can influence brain development and result in pathologies that may contribute to the cause of ASDs.

1.2. Objectives and Hypothesis

The studies of this dissertation in its entirety aim to address the overarching objectives:

To discover the molecular mechanisms by which abnormal signalling of lipid mediators, specifically prostaglandin E2 (PGE_2), may affect brain development and contribute to ASDs-related pathologies. Another important objective was to describe sex differences in mouse studies, which are often overlooked although critical for understanding disorders such as ASDs.

Overall Hypothesis: Abnormalities to the COX-2/PGE_2 lipid signalling pathway disrupts normal brain development that leads to molecular characteristics and behaviours associated with ASDs in a sex-dependent manner.

Specific objectives of the current dissertation were:

Using an *in vitro* cell model (Study 1 and 2):

1) To study the effects of PGE_2 exposure on the proliferation and migration of early neuroectodermal (NE-4C) stem cells.
2) To determine if an elevated PGE_2 level influences the differentiation of NE-4C stem cells.
3) To investigate the possible interaction between the PGE_2 pathway and a major developmental pathway of the nervous system called the Wnt signalling pathway.

Using an *in vivo* mouse model (Study 3 thourgh 6):

4) To verify *in vitro* findings and study the consequences of elevated maternal PGE_2 levels on cell proliferation and neuronal migration in the developing brain of offspring mice.

5) To describe if altered COX-2/PGE_2 signalling disrupts microglial density and morphology in the developing brain.

6) To examine the behavioural outcomes of COX-2-deficient and PGE_2-exposed mice.

1.3. Dissertation Layout

The general introduction (Chapter 1) is followed by a literature review (Chapter 2) on lipid signalling with a focus on major lipid metabolite PGE_2 and its significance in ASDs. To address the objectives of my dissertation, six studies were conducted and are presented in manuscript style in the subsequent chapters. The first two studies utilized NE-4C stem cells as an *in vitro* model to study the fundamental roles of PGE_2 on neuronal cell function. The next four studies were completed *in vivo* to examine the sex-dependent molecular, cellular, and behavioural consequences of altered COX-2/PGE_2 levels during early brain development.

The first study (Chapter 3) examined the possible interaction of PGE_2 and Wnt signalling, as well as the resulting effects on cell motility and proliferation. The second study (Chapter 4) focused on the effects of increased PGE_2 in neuronal differentiation. The third study (Chapter 5) explored the consequence of PGE_2 exposure on neuronal cell density and neocortical migration in the mouse brain. The fourth study (Chapter 6) investigated how elevated PGE_2 (in a PGE_2-injected model) and decreased PGE_2 (in a COX-2-deficient model) levels effect microglial density and morphology. Lastly, the fifth and sixth studies (Chapter 7 and 8) described the behavioural outcomes of PGE_2 exposure and COX-2-deficiency in mouse offspring.

The closing chapter of this dissertation (Chapter 9) provides an overview of the key novel findings across all studies and our contributions to the existing literature on ASDs. This chapter will also discuss the influence of abnormal COX-2/PGE_2 signalling on neurogenesis during brain

development, the convergence of the COX-2/PGE_2 and Wnt signalling pathways, and its distinct role in the male and female brain at various developmental ages. Our findings confirm what has been suggested in various clinical studies by providing new scientific evidence for the link between the COX-2/PGE_2 pathway and the etiology of ASDs.

1.4. Overview of Experimental Models

Over 90% of the mouse and human genomes are analogous (Waterston et al., 2002) indicating that the mouse model can serve as a valuable model for studying human processes such as early development. The studies within this dissertation utilize murine cell (Study 1 and 2) and animal (Study 2, 3, 4, 5, and 6) models.

In vitro model for Study 1 and 2: The complete gestational period of a mouse is around 20 days (Xu et al., 2010, Lanman and Seidman, 1977). At the 8-9th day of gestation, pluripotent stem cells specialize into tissue-specific neural stem cells, which are destined to become neuronal and glial cells. Neuroectodermal (NE-4C) stem cells originate from prenatal day 9 of the mouse embryo (Schlett and Madarasz, 1997). They undergo *in vitro* migration, aggregation, neurosphere formation, and differentiation similar to processes that occur in the developing brain (Kelava and Lancaster, 2016) and thus serve as an appropriate model to investigate the effect of abnormal PGE_2 signalling on neuronal function.

In vivo model for Study 2, 3, 4, 5, and 6: To study the molecular, cellular, and behavioural effects of altered PGE_2 signalling in the animal model, mice offspring prenatally exposed to PGE_2 or deficient in the PGE_2-producing enzyme, COX-2, were examined.

In the PGE_2-exposed mouse model, C57bl/6 pregnant mice were subcutaneously injected with 16, 16-dimethyl prostaglandin E2 ($dmPGE_2$) on embryonic day 11 (E11). A subcutaneous injection was administered to the mother to mimic an increase in PGE_2 levels in the maternal environment that would result from exposure to infections or inflammation (Sugimoto and Narumiya, 2007). $dmPGE_2$ is a stable analog of PGE_2 that is often used *in vivo* (Cook et al., 2016, Cruz Duarte et al., 2012, Ohno et al., 1978). It also better mimics the increase of PGE_2 that may arise due to factors that have been associated with ASDs, including inflammation, infections, and oxidative stress. E11 was chosen as the day of $dmPGE_2$ administration for two reasons. First, the onset of neurogenesis in the mouse brain occurs at this time point (Zhang and Jiao, 2015). Second, this period corresponds to the time reported in human studies in which misoprostol, a PGE_2 analog, was misused for the termination of pregnancy resulting in the manifestation of Moebius syndrome and autism symptoms (Bos-Thompson et al., 2008, Miller et al., 2005, Bandim et al., 2003, Schuler et al., 1999, Costa, 1998, Gonzalez et al., 1998, Pastuszak et al., 1998). The PGE_2-exposed mouse model was used in Study 2, 3, 4, and 6.

COX-2-deficient mice exhibit a decrease in the level of PGE_2 in the brain (Bosetti et al., 2004). $COX\text{-}2^-$ knockin (KI) mice, also known as B6.129S6(FVB)-*Ptgs2*$^{tm1.1Fun}$/J mice, were investigated. $COX\text{-}2^-$ KI mice are a genetic mouse model for selective COX-2 inactivation. They were made by a targeted point mutation of the *Ptgs2* gene, specifically, a substitution of the Y385F amino acid. This mutation leads to the complete inhibition of COX-2 activity (and its production of PGE_2) but downstream peroxidase activity remains unaltered (Yu et al., 2006). $COX\text{-}2^-$ KI founder mice were backcrossed for at least 5 generations to wild-type 129S6/SvEvTac mice to generate the COX-2-deficient mouse model used in Study 4 and 5.

Handling and behavioural testing of all mouse subjects were conducted by the same female researcher to avoid increased stress levels in rodents reported when handling is done by male researchers (Sorge et al., 2014).

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CHAPTER 2.

Literature Review: Lipid Signalling in Autism Spectrum Disorders

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Components of this review have been published in a book chapter with the publisher, ***Springer Reference,*** and in an invited review in the journal, ***European Journal of Neuroscience***. All writings presented in this section were composed by Christine T. Wong and edited by Dr. Dorota A. Crawford. Copyright permissions can be found in Appendix B.

The research in our lab stems from the clinical studies presented in the two published manuscripts. Our main objective was to review the environmental and genetic risk factors that affect lipid signalling and result in Autism Spectrum Disorders (ASDs). The important role of lipids in the brain is summarized, followed by an introduction to the signalling pathway of a major lipid-derived molecule, prostaglandin E2 (PGE_2). The current literature on the connection between the PGE_2 signalling pathway and ASDs will then be presented, including topics such as genetic abnormalities, oxidative stress, immunological factors, and exposure to common environmental agents. This chapter will provide an overview of key findings and concepts related to the field of research in this dissertation.

2.1. Introduction to Autism Spectrum Disorders

Autism is a neurodevelopmental disorder that is categorized under the umbrella term Autism Spectrum Disorders (ASDs), which also includes Asperger's and Fragile X syndrome. Over recent years, there has been a dramatic increase in the prevalence of ASDs in children. Autism is behaviourally defined by a combination of qualitative impairments in social interaction, reciprocal communication, and abnormalities in restrictive and stereotyped patterns of behaviours that typically emerge at 3 years of age (Pelphrey et al., 2014, Folstein and Rosen-Sheidley, 2001). However, increasing lines of evidence suggest that differences between children with autism and typically developing children are already present at the time of birth (Nelson et al., 2001). The Centers for Disease Control and Prevention reported that 1 in 88 children had an ASD in 2008 (CDC, 2012) and in 2010 the prevalence increased to 1 in 68 children (CDC, 2014). Furthermore, school-aged boys were more than four times as likely to have an ASD compared to their female counterparts (Blumberg et al., 2013). Although some argue that the increased prevalence is the result of changes in diagnostic criteria, this cannot fully explain the observed increases (Hertz-Picciotto and Delwiche, 2009). ASDs are heterogeneous disorders, etiologically and phenotypically, and it is well established that the etiology of ASDs involves the complex interaction of genetic composition and exposure to environmental factors (Kim and Leventhal, 2015, Banerjee et al., 2014, Hall and Kelley, 2014, Rossignol et al., 2014, Tordjman et al., 2014, Meek et al., 2013, Herbert, 2010, Muhle et al., 2004). Since genes do not evolve very rapidly in evolution, influence of environmental factors might contribute to the developmental differences in ASDs through modifications in gene expression.

2.1.2 Development of ASDs

Emerging literature suggests the pathogenesis of autism may occur as early as prenatal development (Croen et al., 2011, Atladottir et al., 2010, Brown et al., 2008, Arndt et al., 2005). The critical period of brain development occurs prenatally and during this period, gross changes are occurring throughout the body, particularly in the nervous system. Neural stem cells proliferate, migrate and differentiate into specialized neurons and astrocytes (Kornblum, 2007). The rapid changes in the nervous system involve carefully guided coordination, accomplished by tightly regulated signalling molecules (Vaccarino et al., 2001), including PGE_2 and Wnt. For these reasons, the critical period is particularly sensitive to environmental insults that might influence the level of these important molecules. Major congenital anomalies, such as neural tube defects and mental retardation, could occur as a result of exposure to harmful environmental agents during the first trimester of pregnancy (Moore and Persaud, 1998).

2.1.3. Genetic and Environmental Contributions to ASDs

Family and twin studies have provided support for a strong genetic component in the etiology of ASDs. The first twin study examining autism was in 1977 and provided breakthrough evidence on the involvement of genetics in the disorder (Folstein and Rutter, 1977). It reported that a much higher concordance was seen in monozygotic twins compared to dizygotic twins, and it has since been verified in several studies (Hu et al., 2006, Bailey et al., 1995). Moreover, the risk for siblings of individuals with autism to be affected or to display milder autistic behaviours is higher than the average population (Constantino et al., 2010). Further support for a genetic contribution to ASDs comes from a study that suggests relatives may be at a higher risk of being affected by difficulties related to autistic behaviours (Bailey et al., 1998). There has

been no single gene associated with autism, but instead it is likely that multiple genes are involved (Muhle et al., 2004). Candidate genes have been identified through various techniques such as genome screening (Risch et al., 1999), homozygous haplotype mapping (Casey et al., 2012) and using various *in vivo* and *in vitro* models (Kumar et al., 2011, Meehan et al., 2011). Genetic variations could be inherited or de novo and these genetic differences as well as possible deviations in gene expression could be promoted by environmental factors. Human studies have provided evidence for candidate genes, while animal studies performed *in vivo* and *in vitro* have enabled further biological characterization of these genes.

Although twin studies have provided evidence for the heritability of ASDs, the most recent evidence suggests that in monozygotic twins (MTs) that share the same genetic material, the concordance rates range from 43-88% (Ronald and Hoekstra, 2014, Hallmayer et al., 2011, Lichtenstein et al., 2010, Stilp et al., 2010, Rosenberg et al., 2009). Additionally, MTs that are diagnosed with ASDs often display different subsets of autism symptoms (Mitchell et al., 2009, Belmonte and Carper, 2006, Kates et al., 2004, Kates et al., 1998). Furthermore, the concordance rates for dizygotic twins are around double that of non-twin siblings, suggesting that the uterine and maternal environment likely contributes to autism concordance rates (Bohm et al., 2013). This suggests that investigations beyond heritable genetic differences should be taken to uncover the etiologies of ASDs. Various studies on ASDs using animal models and human samples have shown significant differences in gene expression during pre- and postnatal brain development (Gupta et al., 2014, Bhogal et al., 2013, Garbett et al., 2008). Gene expression studies in individuals with ASDs have revealed dysregulation of particular pathways, including those involved with the immune response, cell communication and motility, and neuronal differentiation (Gupta et al., 2014, Garbett et al., 2008). Given that the expression of genes as a

result of gene-environment interactions determine phenotype outcomes (Kanherkar et al., 2014), exposure to environmental risk factors during vulnerable developmental periods may alter gene expression and contribute to the phenotypes of ASDs. The exponential rise in the prevalence of ASDs and the increasing evidence for the contribution of environmental factors in the etiology of these disorders has prompted urgency in determining the potential exogenous causes and underlying mechanisms involved (Landrigan et al., 2012). Environmental factors such as increased oxidative stress (which could be due to infections and inflammation or exposure to toxins), dietary imbalances of omega-3 and -6, and use of the drug misoprostol (prostaglandin type E analogue) can interfere with the homeostasis of lipid metabolism (Tamiji and Crawford, 2010). Lipids are particularly important to brain development and will be reviewed in Chapter 2.2.

The development of the human brain and nervous system is extraordinarily complex, involving time-sensitive events that are impacted by an ongoing interplay of genetic and environmental factors. Human brain development begins in the 3rd week of gestation and continues after birth through to adolescence, and arguably into adulthood (Stiles and Jernigan, 2010). Normal prenatal development of the brain—including events like cell fate specification and axon guidance—requires highly specific signalling from key biological pathways (Charron and Tessier-Lavigne, 2005). These pathways carefully regulate the expression of genes, which can be turned on or off during different stages of development and expressed in specific concentration gradients (Charron and Tessier-Lavigne, 2005). The environment, endogenous signals found within the brain and exogenous agents originating outside the body, can influence gene expression during development (Andersen, 2003). Exposure to exogenous insults can perturb the normal developmental trajectory during the critical prenatal and perinatal period

(Andersen, 2003). The foundations for brain formation are being established during this period, making it particularly susceptible to harmful environmental agents that may occur through maternal exposure (Tannahill et al., 2005, Moore and Persaud, 1998).

Since the human brain contains high lipid content, healthy development of the brain relies on the supply and function of these macromolecules (Calderon and Kim, 2004). Fatty acids are the simplest form of lipids that serve as the building blocks for more complex lipids such as phospholipids, cholesterol, and vitamin E. A sufficient supply and balance of fatty acids is integral to cell membrane integrity, which is an indicator for healthy development, maintenance, and function of the nervous system (Lawrence, 2010). Environmental factors such as diet, increased levels of oxidative stress, and exposure to infections and inflammation can lead to altered lipid metabolism (Wong and Crawford, 2014, Tamiji and Crawford, 2010, Adibhatla and Hatcher, 2008). Lipid mediators such as prostaglandin E2 (PGE_2) are key molecules important in the development and function of the human brain (Carlson, 2009, Innis, 2007, Uauy and Dangous, 2006).

2.2. Introduction to Lipids

Fatty acids are the simplest type of lipids that serve as building blocks for more complex lipids such as phospholipids, cholesterol, and vitamin E, which are integral to cell membranes (Fig. 2-1). Abnormal metabolism and imbalances of these three complex lipids have all been associated with autism spectrum disorders (ASDs) and will be outlined in following sections. Fatty acids can be saturated, monounsaturated, or polyunsaturated; the latter will be elaborated upon due to its link with ASDs. Essential fatty acids (EFAs), also called polyunsaturated fatty

acids (PUFAs), play an important role in maintaining the structural and functional integrity of the central nervous system (CNS). They serve as major components of neural cell membrane phospholipids and are necessary for the modulation of ion channels, enzymes, and receptor activity (Boland et al., 2009, Guizy et al., 2008). PUFAs must be obtained through the diet since the ability to synthesize them is limited. The two major types of PUFAs are omega-6 linoleic acid (LA; 18:2n-6) and omega-3 α-linolenic acid (ALA; 18:3n-3), which are converted to arachidonic acid (AA, 20:4n-6) and to eicosapentaenoic acid (EPA, 20:5n-3) or docosahexaenoic acid (DHA, 22:6n-3), respectively (Haag, 2003) (Figs. 2-1 and 2-2).

One of the most commonly derived PUFAs from cell membranes is AA. Through the action of phospholipase A_2 (PLA_2), an enzyme important for cell membrane maintenance, AA is cleaved from membrane phospholipids and further metabolized to potent bioactive messengers including prostaglandins, thromboxanes, leukotrienes, lipoxins, and cannabinoids. The metabolic products of AA are collectively known as eicosanoids.

2.2.1. *The Importance of Lipids in the Brain*

Compared to all organs other than adipose tissue, the human brain has the highest percentage of lipids by mass, with approximately 60 % of its dry mass being lipids – specifically phospholipids and PUFAs (Lawrence, 2010). AA and DHA/EPA are the most abundant PUFAs in the brain (Sastry, 1985), and a sufficient supply of and balance between omega-6 and omega-3 fatty acids are important for plasma membrane integrity, which is tightly integrated with healthy development, maintenance, and function of the nervous system (Lawrence, 2010). An extensive body of research has been devoted to determining the role of fatty acids in the nervous system.

Rapid brain growth begins during prenatal fetal development and continues postnatally for 2 years following birth. The majority of brain growth occurs during the fetal stage, and at birth, a newborn's brain is already 70 % the size of the adult brain (Clandinin et al., 1994). There is a high PUFA demand during the brain growth spurt (Lauritzen et al., 2001), and during this period, the omega-6 and omega-3 fatty acid content in grey and white matter increases significantly (Martinez, 1992). The importance of PUFAs in fetal development is emphasized by the high lipid content found in breast milk, as well as the increasing proportions of PUFAs in the phospholipids when comparing levels found in the maternal blood to the placenta and to the fetal brain (Crawford et al., 1981). This process ensures that the high concentrations of long-chain PUFAs necessary for the developing brain can be acquired (Crawford et al., 1989).

Healthy development of the human brain relies greatly on the functions of lipids (Calderon and Kim, 2004). For example, AA and DHA are important for retinal development and vision (Hoffman et al., 2009), neuroplasticity and signal transduction (Wu et al., 2008), inflammation (Ferrucci et al., 2006), learning and memory (Su, 2010), and sleep (Ladesich et al., 2011). The final structure and breakdown of phospholipids (AA and DHA precursors) represent a unique interaction between genetic and environmental factors. Proper brain functioning relies on the supply of dietary PUFAs and the release of their metabolites from membrane phospholipids through the activity of enzymes such as PLA_2 (Yehuda et al., 1999) (Fig. 2-3). Therefore, alterations in phospholipid metabolism may affect normal functions of the CNS. Anomalies in lipid concentrations due to insufficient dietary intake or aberrant lipid regulation may lead to irregular lipid profiles and signalling and could be highly detrimental to the developing brain. Recent literature provides sufficient evidence that dysregulation of the lipid metabolic pathway may be an underlying biological component involved in the pathophysiology

of ASDs. Abnormalities at multiple sites of this pathway have been associated with ASDs and will be further discussed in proceeding sections.

2.3. Lipid Imbalances in ASDs

2.3.1. *Essential Fatty Acid Profiles in ASDs*

Current literature suggests that imbalances in the lipid profiles of omega-6 (i.e., AA) and omega-3 (i.e., DHA and EPA) fatty acids may be an underlying mechanism to the manifestation of autistic-like behaviours. Specifically, several studies have reported altered AA, DHA, and EPA levels in patients with autism. Investigation of blood samples revealed that children with autism have a higher AA: DHA ratio (Meguid et al., 2008) and a higher AA: EPA ratio (Bell et al., 2010) compared to healthy controls. Other studies have reported significantly lower than normal levels of AA and DHA in the blood plasma of individuals with autism (Wiest et al., 2009, Vancassel et al., 2001). Another study revealed an increase in plasma DHA levels in high-functioning males with autism (Sliwinski et al., 2006). Though the causes of atypical PUFA levels have yet to be fully understood at the molecular level, potential reasons include defects in the enzymes that convert dietary PUFAs into longer and highly unsaturated derivatives, inadequacy in the integration of PUFAs into membrane phospholipids, or deficient dietary intake of PUFA precursors (Vancassel et al., 2001). Moreover, aberrant activity of fatty acid desaturase-1 and fatty acid desaturase-2 (FADS1 and FADS2), the enzymes that catalyze the breakdown of LA and ALA into AA and DHA, can lead to irregular lipid profiles (Morales et al., 2011) and may be involved in lipid imbalances reported in autism (Figs. 2-1 and 2-3). Notably, the chromosomal locations of FADS1 and FADS2 are in close proximity to an autism-candidate

linkage peak on chromosome 11q22 (Ma et al., 2007, Craddock and Lendon, 1999). The presence of altered lipid composition and its potential contribution to the behavioural outcomes of ASDs have been conveyed in animal studies using rodent models, which revealed that altered brain phospholipid composition induced by environmental agents, including enteric bacteria and diet, can lead to the appearance of autism-like behaviour (Thomas et al., 2010, Shultz et al., 2009). Altogether, these studies provide evidence that irregular lipid profiles are a common characteristic of ASDs.

2.3.2. *Insufficient Dietary Fatty Acids and Autism*

The availability of fatty acids for brain development can be heavily impacted by the maternal diet during the prenatal stage and by the infant's diet (i.e., breast milk or formula) in the postnatal stage. Since abnormal lipid profiles have commonly been characterized in individuals with ASDs, the ability to alter lipid concentrations through dietary means is encouraging. Healthy infants fed with PUFA-supplemented milk formula showed improvements in infant cognitive scores compared to infants on a no-PUFA formula, suggesting that inadequate intake of dietary PUFAs during early development may be associated with altered cognition (Willatts et al., 1998). Considering that higher problem-solving scores in infancy have been associated with higher childhood IQ scores, PUFA supplementation may modify the development of childhood intelligence. There is also a significantly higher likelihood for infants to develop autism if they were not breastfed or not supplemented with PUFAs (Schultz et al., 2006), and administration of PUFA supplementation has been shown to restore normal fatty acid profiles and improve behavioural outcomes in children with autism. For example, when diet was supplemented with

fish oils high in omega fatty acids for 6 months, children with autism or Asperger's syndrome exhibited reduced omega-6:omega-3 and AA: EPA ratios compared to supplementation-naïve autistic controls (Bell et al., 2010). Another study found that supplementation of omega-6 and omega-3 fatty acids in children with autism led to elevated levels of fatty acids in the blood, decreased AA: DHA ratio, and improvements in several autism-affected behavioural domains such as eye contact, concentration, language development, and motor skills (Meguid et al., 2008). Another group revealed that stereotypy and hyperactivity associated with autism were mitigated with omega-3 fatty acid supplementation (Amminger et al., 2007). Such supplementation also led to improvements in basic language and learning skills in children with autism (Patrick and Salik, 2005).

A case study on a child with autism and associated high levels of anxiety and agitation showed the elimination of irregular fatty acid levels and the complete resolution of anxiety and agitation after EPA supplementation was administered (Johnson and Hollander, 2003). A better quality of life was also reported in the study. Additionally, these improvements in fatty acid levels and behaviour were stable at the 8-month follow-up. In contrast, a study investigating young adults (18–40 years old) with autism revealed that omega-3 fatty acid supplementation did not lead to significant improvements in behaviour (Politi et al., 2008), which suggests that there may be a specific time window for the potential beneficial effects of supplementation.

Furthermore, cholesterols are lipids that are important components of cell membranes (Fig. 2-1) involved in the induction of normal motor and mental capacity (Gilbert, 2003). Cholesterol is needed for proper embryonic and fetal development as well. Specifically, it is necessary for the Sonic Hedgehog signal transduction pathways, which are crucial for the induction of brain formation, neural patterning, mood regulation, and cerebral development

(Gilbert, 2003). Cholesterol affects the availability of essential lipid molecules, and as such, its insufficient consumption by the mother could lead to unhealthy metabolic deficiencies that may contribute to the development of autism in the offspring (Tallberg et al., 2011). Imbalances in cholesterol levels have also been associated with ASDs (Tierney et al., 2006). Prevailing evidence for this stems from studies on Smith-Lemli-Opitz Syndrome (SLOS), which is a genetic condition of impaired cholesterol biosynthesis (Bukelis et al., 2007, Sikora et al., 2006). Cholesterol treatment in individuals with SLOS resulted in a decrease in autism-related behaviours, infections, and symptoms of irritability and hyperactivity, as well as improvements in physical growth, sleep, and social interactions (Aneja and Tierney, 2008). It is interesting to note that cholesterol supplementation also improved other behaviours such as aggression, self-injury, temper outbursts, and trichotillomania (Aneja and Tierney, 2008).

Taken together, the existing literature indicates that dietary imbalances in lipids and fatty acids may influence the development of autism-related behavioural outcomes. However, the molecular mechanisms of lipid dysregulation in ASDs still have to be determined. This would aid in establishing recommended dietary supplementation for individuals with ASDs.

2.4. Association of Prostaglandin Metabolic Pathway with ASDs

While PUFAs such as AA or DHA are crucial for membrane structure and function, their metabolites, such as bioactive prostanoids, are very important for the normal functioning of the brain (Tassoni et al., 2008). Upon the release of AA from membrane phospholipids via the action of PLA_2, cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2) converts AA into an unstable intermediate, PGG_2, which is then metabolized to the prostanoid precursor, PGH_2 (Fig.

2-3). Prostaglandin (PG) or thromboxane synthases convert PGH_2 to active lipid signalling messengers including prostanoids, PGs (PGE_2, PGF_2a, PGD_2, PGI_2), and thromboxane A2 (TXA_2) (Tamiji and Crawford, 2010). Prostanoids can elicit cellular effects by binding to their respective G-protein-coupled receptors called E-prostanoid (EP), FP, DP, IP, and TP receptors, respectively (Breyer et al., 2001) (Fig. 2-3).

2.4.1. *Phospholipase A2*

Phospholipases are enzymatic molecules that break down phospholipids, which are precursors of important lipid mediators such as PGs. Phospholipase A_2 (PLA_2) is of particular interest since it is an upstream regulator of a wide range of physiologic functions. PLA_2 selectively hydrolyzes the sn-2 fatty acid ester bond of phospholipids to produce free fatty acids such as AA (Murakami and Kudo, 2002). PLA_2 activation and the subsequent release of AA and its metabolites can be stimulated by various neurotransmitters such as glutamate (Marin et al., 1997). Alternatively, the presence of other stimuli, such as cytokines activated during inflammation, can cause the release of AA and DHA from phospholipid membranes (Farooqui et al., 2007).

The PLA_2 protein family has been associated with autism. Specifically, clinical studies have reported significantly higher concentrations of PLA_2 in the red blood cells of individuals with autism and Asperger's syndrome compared to healthy controls (Bell et al., 2004) (Fig. 2-3). The increased activity of PLA_2 in patients with ASDs could interfere with the proper maintenance of membrane phospholipids, and this may contribute to their abnormal PUFA profiles. Current literature provides support for a link between aberrant PLA_2 enzyme activity or

concentrations and ASDs, reinforcing the hypothesis that abnormal lipid metabolism may play a role in the pathophysiology of these disorders.

2.4.2. *COX Enzymes*

The enzymes cyclooxygenases-1 and -2 (COX-1, -2) catalyze the reaction of oxygen with PUFAs, producing bioactive lipid substances, such as prostaglandins (PGs), from AA (Sang and Chen, 2006). Collectively, these lipid metabolites are known as eicosanoids, each of which is formed by a different sequence of enzyme- catalyzed reactions. Both COX enzymes assist in the production of autoregulatory and homeostatic prostanoids, which may be released during inflammation. The enzymatic activity of COX can be inhibited by nonsteroidal anti-inflammatory drugs, consequently inhibiting the biosynthesis of PGs (Vane, 1971).

COX-1, the constitutive form, is expressed in nearly all human tissues including the brain, lung, kidney, and stomach (O'Neill and Ford-Hutchinson, 1993). Although most commonly known as a mediator of housekeeping functions, new evidence shows that COX-1 may also be important in developmental and inflammatory functions (Loftin et al., 2002). For example, COX-1 activity may alter progenitor cell proliferation and hippocampal neurogenesis (Russo et al., 2011), neuroinflammation (Shukuri et al., 2011), as well as parturition (Gross et al., 1998). COX-2, the inducible form, is heavily involved with the inflammatory response. It is normally found in low levels, but various stimuli such as growth factors (Hinz and Brune, 2002), inflammation due to injury or infection (Ricciotti and FitzGerald, 2011), synaptic activity (Yamagata et al., 1993), and oxidative stress (Kwiecien et al., 2012) can trigger its up-regulation.

Interestingly, COX-2 plays important roles in the CNS, gastrointestinal tract, and pain perception (Hinz and Brune, 2002) via the production of downstream signalling metabolites such as PGE_2. Abnormalities in the CNS, GI, and nociception have been reported in cases of ASDs (Gorrindo et al., 2012, Kohane et al., 2012, Dubois et al., 2010) increasing lines of evidence demonstrates that these abnormalities are associated with alterations in the COX-2/PGE_2 pathway (Fig. 2-3). The activity and production of COX-2 are induced by cytokines and proinflammatory agents, and irregular immune responses have been characterized in patients with ASDs (Ashwood et al., 2006). Additionally, COX-2 is a key contributor to neurodevelopment. COX-2 is richly expressed in the dendrites of neurons (Yamagata et al., 1993). However, its distribution pattern is anomalous in the brain tissue of individuals with Rett syndrome, a form of ASD (Kaufmann et al., 1997). Furthermore, ASDs have been associated with a polymorphism in *PTGS2*, the gene encoding for the COX-2 protein (Yoo et al., 2008).

2.4.3. *Prostaglandin E_2 during Development*

PUFAs are essential structural components of cell membranes, while eicosanoids are crucial metabolites required for the normal functioning of the brain (Tassoni et al., 2008). PGE_2 is an eicosanoid that is generated from the metabolism of PGH_2 by the enzymatic action of PG synthase. PGE_2 diffuses rapidly through membranes, exerting its signalling effects by binding to E-prostanoid receptors (EP1-4).

EP receptors are G-protein-coupled receptors and are expressed differentially across varying tissue types (Sugimoto and Narumiya, 2007). PGE_2 is an important lipid mediator that typically acts within the local microenvironment, providing autocrine and paracrine stimulation

to a number of signalling pathways in the nervous system (Legler et al., 2010) and regulating the function of many immune cell types, thus mediating the inflammatory response in the brain (Zhang and Rivest, 2001). Evidence shows that it is also involved in dendritic spine formation (Burks et al., 2007), synaptic plasticity (Koch et al., 2010), pain transmission (Harvey et al., 2004), and cell survival (Jiang et al., 2010) or death (Jonakait and Ni, 2009). In early prenatal development, there is an increase in the number of COX-2 and PG synthase transcripts (Saint-Dizier et al., 2011), as well as elevated levels of EP receptor transcripts (Tamiji and Crawford, 2010), suggesting that PGE_2 is involved in early development. The PGE_2 pathway is summarized in Fig. 2-4.

Clinical studies have revealed a connection between misuse of the drug misoprostol during the first trimester of pregnancy and neurodevelopmental aberrations, including Mobius sequence and ASDs (Bandim et al., 2003) (Fig. 2-3). Misoprostol is an analogue of prostaglandin type E, which has been proven to bind and activate EP receptors activating the PGE_2 pathway (Tamiji and Crawford, 2010) (Fig. 2-4). It was initially used to treat stomach ulcers and has since been used also to induce labour and to terminate pregnancy (Lin et al., 2011). During the early stages of pregnancy (5–6 weeks after fertilization), the embryo is the most vulnerable to misoprostol exposure (Genest et al., 1999). Exposure to this drug during early embryonic development may alter the PGE_2 signalling pathway and have toxic effects on the developing nervous system. Misoprostol and PGE_2 can increase the intracellular level and fluctuation amplitude of calcium in neuronal growth cones, as well as reduce the number and length of neurite extensions through the activation of EP receptors (Tamiji and Crawford, 2010, Tamiji and Crawford, 2010). Dysfunctional calcium regulation has been previously suggested in the etiology of ASDs (Krey and Dolmetsch, 2007). When considering these studies together, they

demonstrate that abnormalities in the PGE_2 pathway through misoprostol exposure could lead to neurotoxic effects on the development and communication of neurons.

The importance of the PGE_2 signalling pathway during early development is further established through the significantly increased expression of four EP receptors transcripts – EP1, EP2, EP3b, and EP4 – during early neurogenesis (embryonic day 11–15) in the mouse (Tamiji and Crawford, 2010). It is intriguing that this embryonic period marks the development of many brain structures (Rice and Barone, 2000) including the cerebellum, amygdala, hippocampus, and caudate nucleus of the basal ganglia, which have been reported to be abnormal in individuals with autism (Amaral et al., 2008). However, it is still unclear how COX-2/PGE_2 signalling may directly affect the development of these brain areas.

There is growing evidence supporting an interaction between the PGE_2 and the Wnt (*wingless*) pathways (Evans, 2009). PGE_2-dependent signalling may converge with the Wnt pathway at the level of β-catenin through EP1-4 receptors, including the association of the $G_{\alpha s}$ subunit with axin, the stimulation of the cAMP/PKA pathway, or the phosphorylation of GSK-3β by PI-3K (Buchanan and DuBois, 2006) (Fig. 2-5). Such an interaction is of particular interest since Wnts are morphogens necessary for the formation of a healthy nervous system (Ciani and Salinas, 2005). They are signalling molecules that act through Frizzled (FZD) transmembrane receptors and are vital to embryonic development, participating in the determination of cell fates through activating transcription of various target genes (Buechling and Boutros, 2011). Cross talk and joint regulation between these pathways have been reported previously in various non-neuronal cells. For example, in osteocytes, PGE_2 interacts with β-catenin to modulate bone mass and density (Genetos et al., 2011), as well as apoptosis following induced stress (Kitase et al., 2010). The COX-2/PGE_2 signalling pathway is highly stimulated in

gastric cancer cells, which results in significantly increased levels of PGE_2 and subsequent activation of Wnt (Oshima et al., 2009). In these cells, PGE_2 and Wnt signalling cooperated to cause dysplastic growth and development (Oshima and Oshima, 2010), while a dose-dependent effect of PGE_2 on Wnt activation in bone and prostate cancer cells has been reported (Liu et al., 2010). Furthermore, in colon cancer cells, PGE_2 induces a decrease in β-catenin phosphorylation, which increases the activation of TCF/LEF transcription factors and up-regulates certain genes, resulting in increased proliferation (Castellone et al., 2005). Wnt and PGE_2 also collaborate to promote the survival and proliferation of zebra fish hematopoietic stem cells (Goessling et al., 2009). Through this model, it was also demonstrated that PGE_2 and Wnt are important in the regulation of vertebrate regeneration and recovery.

Although the interaction between these two pathways in the nervous system still remains elusive, our current studies have shown that PGE_2 can modulate the expression of Wnt-target genes and change the proliferation and migration behaviour of neuroectodermal (NE-4C) stem cells (Wong et al., 2016, Wong et al., 2014). Wnt signalling proteins are also essential for neuronal precursor proliferation (Zhou et al., 2006), radial migration of cortical neurons (Zhou et al., 2004), and synapse formation (Sahores and Salinas, 2011), all of which are cellular events driven by calcium signalling. Therefore, calcium fluctuations induced by PGE_2 or misoprostol as demonstrated by Tamiji and Crawford (2010) may interfere with the Wnt signalling pathway, influencing its various functions. For example, calcium signalling triggered by neuronal activity mediates the CREB-dependent transcription of *Wnt-2* as well as the production and secretion of its corresponding protein. *Wnt-2*, one of many wingless genes, contributes to proper dendritic outgrowth and branching (Wayman et al., 2006). Notably, this gene is located in the chromosome region 7q31-33, which has been linked to autism, and its polymorphisms have been

associated with speech delay inherent in ASDs (Lin et al., 2012, Lennon et al., 2007). Moreover, Wnt signalling participates in the formation of the cerebellum (Hall et al., 2000), hippocampus (Galceran et al., 1999), and amygdala (Abu-Khalil et al., 2004). As mentioned earlier, structural deviations in these brain areas have been implicated in autism. Misguided neuronal migration resulting in misplaced neurons, especially Purkinje cells, has also been connected to autism (Wegiel et al., 2010). Given that Wnts are crucial regulators of cell fate specification, cell proliferation, and cell migration during development (Buechling and Boutros, 2011), PGE_2-dependent dysregulation of Wnt signalling could result in brain abnormalities and contribute to autism. These lines of evidence validate the increasing interest in investigating the molecular mechanisms underlying the interaction between the COX-2/PGE_2 and Wnt signalling pathways and its link to autism.

2.5. Oxidative Stress and Lipid Peroxidation in ASDs

Under normal conditions, there is a dynamic equilibrium between the production of reactive oxygen species (ROS) and the rate at which they are neutralized by antioxidants. Oxidative stress occurs when there is an imbalance in this equilibrium and the level of ROS produced by a cell exceeds its antioxidant capacity. ROS such as oxygen ions, free radicals, and peroxides are highly reactive molecules that can damage the cell by targeting lipids, key building blocks of cells (Choe, 2010). Elevated levels of free radicals and reduced antioxidant defense mechanisms are the prime initiators of oxidative stress (Sies, 1997). The activity of ROS can lead to altered cell function due to abnormal lipid metabolism affecting proliferation and differentiation during neurodevelopment and can even cause cell death (Filomeni and Ciriolo,

2006). The PUFAs from membrane phospholipids are the primary targets of ROS because they contain many double bonds, making them particularly vulnerable to lipid peroxidation, which is the oxidative breakdown of lipids (Adibhatla and Hatcher, 2008). This results in increased metabolism of AA and activates the PGE_2 signalling pathway (Pepicelli et al., 2005). A number of neurological disorders, such as autism, schizophrenia, and Alzheimer's, have been associated with dysregulation of lipid metabolism and increased levels of oxidative stress. A combination of genetic and environmental factors influencing lipid peroxidation may contribute to the etiology of autism (Deth et al., 2008).

An imbalance between the oxidative and antioxidative stress systems due to increased oxidative damage and decreased antioxidant neutralization has been reported in individuals with autism (Ghanizadeh et al., 2012). The brain is particularly vulnerable to oxidative stress and consequent lipid peroxidation because lipids are the primary component of the human brain (Adibhatla and Hatcher, 2008), and because the brain uses a great amount of oxygen, generating potentially elevated levels of harmful reactive by-products. The high oxygen requirement of the brain is exemplified by the fact that although the human brain is approximately 2 % of an individual's total body mass, it uses around 20 % of the available body oxygen (Shulman et al., 2004). The brain also has limited antioxidant defense mechanisms because neurons cannot produce glutathione (GSH), one of the most important antioxidants for the free radical neutralization of environmental toxins (Shulman et al., 2004). As the majority of the energy consumed in the brain is used by neurons, they are the first cells to be affected by reduced antioxidant levels and thus in the most danger. Infants who are in a sensitive period of development are more vulnerable than adults to oxidative stress because they have naturally lower levels of GSH (Ono et al., 2001), which may suggest greater risk of oxidative stress.

Children with autism have been reported to have lower levels of GSH and a deficiency in GSH precursors, methionine, and cysteine, which indicate their increased susceptibility to oxidative stress and the associated risk of developing brain disorders (Al-Gadani et al., 2009, James et al., 2004). The diminished antioxidant capacity could amplify oxidative damage to the cells. Several studies have also discovered genetic variances (allele deletions and polymorphisms) that directly impact methionine and GSH metabolism in individuals with autism (Ming et al., 2010, Williams et al., 2007, Buyske et al., 2006), hinting at the influence of genetics in the abnormalities of lipid peroxidation observed in ASDs. In addition to GSH, aberrations in a number of other antioxidants have been reported in several studies. Protein antioxidants, ceruloplasmin, and transferrin levels were significantly reduced, and lipid peroxidation was significantly elevated in the plasma samples of children with autism compared to controls (Chauhan et al., 2004).

Current literature also provides support for the connection between elevated oxidative damage and lipid/prostanoid metabolites in cases of autism. Two protein adducts, carboxyethyl pyrrole (CEP) and iso[4]levuglandin (iso[4]LG)E_2 (Fig. 2-1), are uniquely formed through the peroxidation of DHA- and AA-containing lipids, respectively, and are markers of oxidative damage. Significantly increased fluorescent immunoreactivity of these compounds was detected in the cortical tissue of subjects with ASD compared to healthy controls (Evans et al., 2008). Moreover, a distinct expression pattern specific to the autistic brain was also visualized – significant staining was found in white and grey matter. Increased levels of lipid peroxidation markers 2, 3-dinor-thromboxane (TXB_2) and 6-ketoprostaglandin PGF_1a (6-keto-PGF_1a), which are metabolites of TXA_2 and PGI_2, respectively (Fig. 2-3), were quantified in urine samples of children with autism (Yao et al., 2006). Likewise, elevated levels of 8-isoprostane-F_2a(8-iso-

PGF_2a) and isoprostane F_2a- VI (iPF_2a-VI), by-products of PGF_2a peroxidation via the oxidation of AA (Fig. 2-3), have been measured in the urine of children with autism compared to healthy controls (Yao et al., 2006). A significantly higher concentration of iPF_2a was also reported in plasma samples of children with autism (Mostafa et al., 2010) and patients with Rett syndrome (De Felice et al., 2009). Under normal conditions, the accumulation of F_2-isoprostanes induces platelet aggregation and vasoconstriction (Pratico et al., 2001). However, when the accumulation becomes abnormal, as evident in the blood and urine samples from individuals with autism, it may contribute to the altered platelet reactivity and vascular irregularities reported in these patients (Yao et al., 2006).

There has also been mounting evidence that links abnormalities in vitamin E and the pathology of ASDs. Vitamin E, a lipid and a powerful antioxidant capable of protecting cells and tissues from oxidative damage, is important in the regulation of lipid peroxidation. It is a key component of cell membranes (Fig. 2-1) that is also stored in fat and liver for future mobilization during elevated levels of oxidative stress (Lawrence, 2010). Deficient levels of vitamin E have been reported in blood samples of children with autism (Al-Gadani et al., 2009). Supplementation of PUFAs and vitamin E led to dramatic improvements in a number of areas including speech, imitation, coordination, eye contact, behaviour, sensory issues, and the development of pain sensation in 97 % of 187 ASD-affected children (Morris and Agin, 2009). When the supplementation was halted, speech and coordination regressed in some children, suggesting that abnormal fatty acid metabolism may contribute to the manifestation of these autism-associated traits.

In summary, the reported findings provide additional evidence for irregular lipid biogenesis in ASDs. Current publications reveal altered antioxidant capacity and increased

oxidative stress markers in individuals with autism. Lipids are the prime targets for oxidative stress, suggesting that abnormal lipid signalling may be associated with the development and/or expression of symptomatic deficits in affected individuals.

2.6. Immunological Factors Contributing to Lipid Dysregulation

Immunological events associated with infections in early fetal life through alterations in the vasculature and maternal-fetal immune responses may play a role in the initiation of neurological disorders such as autism, epilepsy, and schizophrenia (Stolp et al., 2012, Atladottir et al., 2010, Brown and Derkits, 2010). Immune dysfunctions and insults during sensitive periods of brain development could predispose the developing nervous system to potential failures in subsequent cell migration, target selection, and synapse maturation (Harvey and Boksa, 2012). During embryonic and fetal life, the immune system is still immature. This may be an adaptive measure to safeguard the fetus from premature maternal immune rejection, which could lead to fetal damage or even death (France de La Cochetiere et al., 2007). Paradoxically, this beneficial adaption also increases the risk for the fetus to acquire an infection.

Although autism is a disorder that primarily affects brain function, many cases have shown that other systems, such as the immune system, are also affected. Clinical studies and case reports have revealed the potential contribution that viral infections and aberrant immune responses have towards the pathology of ASDs (Libbey et al., 2005). Additionally, women who develop a fever while pregnant may be more than twice as likely to have a child with an ASD or some other form of developmental delay (Zerbo et al., 2013). Research by various scientists utilizing animal models has demonstrated that certain pre- and postnatal infections can induce

gene alterations in the brain, morphological changes in cells and brain regions, immunological deviations in offspring, and irregular behaviours analogous to those characterized in ASDs (Shi et al., 2009, Fatemi et al., 2008, Ponzio et al., 2007, Fatemi et al., 2005, Shi et al., 2003, Fatemi et al., 2002).

Although the direct molecular mechanisms by which infections and inflammation may lead to the pathological outcomes of autism through the COX-2/PGE_2 lipid signalling pathway remain to be fully elucidated, existing literature supplies indirect evidence of their possible roles. Eicosanoids derived from omega-6 and omega-3 PUFAs are central regulators of the immune and inflammatory responses (Wall et al. 2010). Specifically, AA (omega-6 metabolite) has proinflammatory and immunoactive properties, while EPA and DHA (omega-3 metabolites) have anti-inflammatory roles. Cyclooxygenase-derived mediators, such as the lipid signalling molecule PGE_2, are significantly elevated during infections and the inflammatory response (Fitzgerald et al., 2012), especially if the viral or bacterial exposure occurs during pregnancy (Christiaens et al., 2008). PGE_2, the most abundantly produced eicosanoid derived from AA, can modulate the sensitivity of sensory nerves and functions as a mediator to induce fever (Lawrence, 2010). Heightened PGE_2-related inflammatory responses during early development may produce developmental defects in the nervous system. The inflammatory response in the brain can be mediated by PGE_2 since it regulates the function of many immune cell types (Zhang and Rivest, 2001). It promotes the production of cytokines (Legler et al., 2010), which are compounds made and secreted by leukocytes at the site of inflammation. Cytokines, along with other products of immune activation, have widespread effects on neuronal pathways, which may contribute to common features of ASDs (Ashwood and Van de Water, 2004). They also represent the primary mediators of communication between the brain and the immune system.

Cytokines act locally as signalling molecules between immune cells, but they may also enter the bloodstream and target remote structures including the brain (Rothwell and Hopkins, 1995). The activation of endothelial cells associated with the blood brain barrier is responsible for the subsequent release of second messengers such as prostaglandins that act on specific brain targets (de Vries et al., 1996). Cytokines are also involved in many aspects of CNS function and development as well. They are of particular importance during early neural development, including the induction of the neuroepithelium (Gaulden and Reiter, 2008). They are also critical modulators of neuronal and glial migration (Zhu et al., 2002), differentiation (Soleymaninejadian et al., 2012), and selective pruning or programmed cell death to acquire proper neural connections (Sedel et al., 2004). Taken altogether, studies demonstrate that infections and the resultant inflammatory responses could disturb several processes involved in brain development and potentially be in part responsible for the abnormalities of autism. Additional studies are necessary to establish a direct link between the effects of immunological factors and the COX-2/PGE_2 pathway, and how consequent alterations are manifested in the molecular and behavioural outcomes of ASDs.

2.7. Exposure to Common Environmental Factors with ASDs

Exposure to various exogenous risk factors during prenatal and perinatal development can disrupt important neurodevelopmental processes, such as the patterning and growth of the brain, by altering normal gene expression and cell function (Jurewicz et al., 2013, Braw-Tal, 2010, Grandjean and Landrigan, 2006, Weiss, 2000). The maternal environment can have direct consequences on the developing embryo and fetus. It is known that molecules found in the maternal system, such as lipids, can be passed into the developing embryo or fetus during

pregnancy and to the growing infant through breast milk following birth (Lassek and Gaulin, 2006). Exposure to natural and manmade chemicals occurs on a daily basis through the air, soil, foods, water, and consumer products. Transmission of these chemicals into the body can occur via inhalation, ingestion, or contact with skin and it has been reported that accumulations of chemicals can be found in organs (Johnson-Restrepo et al., 2005); predominately in fatty tissues (De Saeger S. et al., 2005). A greater buildup of toxins in the mother could increase the likelihood of exposure to the developing fetus or child. Goldman and Koduru reported that approximately 85,000 chemicals were manufactured in the United States (U.S.) in year 2000 and with each following year, about 2,000-3,000 new chemicals are reviewed by the U.S. Environmental Protection Agency (Goldman and Koduru, 2000). In this year, roughly 2,800 chemicals were used in high volumes with over one million pounds produced annually. They also state that nearly 80% of these chemicals lacked screening for developmental toxicity and almost half that were found in consumer products had no test data for developmental toxicity. Moreover, a study by the Environmental Working Group in the U.S. found that 287 of the 413 toxic substances tested were present in the umbilical cord of newborns (Houlihan, 2005). These chemicals include heavy metals, numerous pesticides, and estrogen-like endocrine disrupting chemicals (EEDCs). Alarmingly, 157 of these chemicals were found to affect the brain and nervous system and are related to developmental defects (Houlihan, 2005). This striking information gives precedence to the investigation of potentially harmful chemicals—including air pollutants, pesticides, and toxins in consumer products—that could impact the developing baby.

Below we describe how these environmental risk factors are capable of directly affecting the PGE_2 signalling pathway during early brain development. Moreover, we reveal how air

pollutants, pesticides, and toxins in consumer products may indirectly affect the PGE_2 signalling pathway by elevating levels of inflammation, increasing levels of oxidative stress, and acting as EEDCs that can affect PGE_2 signalling. We also discuss how these exogenous chemicals have been associated with increased risk for ASDs.

2.7.1. Air Pollution and Heavy Metals

Exposure to air pollution has been shown to induce increased levels of inflammation and oxidative stress in the brains of children, adolescents and adults (Calderon-Garciduenas et al., 2014, Moller et al., 2014, Calderon-Garciduenas et al., 2007). Moreover, sustained exposure could lead to DNA damage and pathologies in the brain (Calderon-Garciduenas et al., 2014, Moller et al., 2014, Calderon-Garciduenas et al., 2007). In turn, inflammation is highly associated with increased levels of PGE_2 (Nakanishi and Rosenberg, 2013, Andreasson, 2010). PGE_2 is the most abundant prostaglandin and can induce fever (Lawrence, 2010) and promote the production of cytokines (Legler et al., 2010). Immune activation and the production of cytokines can cause disturbances in the development of neuronal pathways that have been associated with ASDs (Ashwood and Van de Water, 2004). Increased levels of oxidative stress can cause lipid peroxidation of cell membranes, including membranes of endothelial cells in the blood-brain barrier (BBB), which leads to the subsequent release of second messengers like prostaglandins (de Vries et al., 1997, de Vries et al., 1996). In fact, exposure to air pollution particles and common air pollutant, sulfur dioxide (SO_2), have been found to elevate PGE_2 levels in macrophages (Schneider et al., 2005), lung fibroblasts (Alfaro-Moreno et al., 2002), and neurons (Sang et al., 2011).

Toxic air pollutants can arise from human activity—such as vehicles, factories, and household cleaning solvents—and from natural activity including volcano eruptions. Toxic air pollutants are fine particles that can be found in diesel exhaust, tobacco smoke, and industrial emission. They include organic compounds like styrene, and metals like mercury, lead, and cadmium. Metals are of concern because they stay in the body for prolonged periods of time (Wang and Du, 2013, Suwazono et al., 2009). For example, the biological half-life of cadmium in humans is between 13-24 years (Wang and Du, 2013, Suwazono et al., 2009). They are especially dangerous to the developing brain because upon inhalation and entry into the circulation, many metals can translocate across various tissues including the BBB or can result in increased systemic levels of inflammation and oxidative stress that can also be measured in the brain (Jomova and Valko, 2011, Lopez et al., 2006, Peters et al., 2006, Valko et al., 2005). Elevation in inflammation (Nakanishi and Rosenberg, 2013, Andreasson, 2010) and oxidative stress (de Vries et al., 1997, de Vries et al., 1996) can also lead to abnormal PGE_2 production.

A number of extensive case-control studies completed in recent years across the U.S. investigated the possible association of exposure to toxicants in the air and the risk of neurodevelopmental disorders such as ASDs. Each child's residential area was compared to the exposure of surrounding air pollutants which included metals, particulates, and volatile organic compounds. They report that exposure to metals (antimony, arsenic, cadmium, chromium, lead, mercury, manganese, nickel) (Roberts et al., 2013, Palmer et al., 2009, Roberts et al., 2007, Windham et al., 2006), diesel particulates (Volk et al., 2014, Roberts et al., 2013, Windham et al., 2006), methylene chloride (Roberts et al., 2013, Kalkbrenner et al., 2010, Windham et al., 2006), vinyl chloride (Windham et al., 2006), styrene (Kalkbrenner et al., 2010), and trichloroethylene (Windham et al., 2006) are associated with increased risk of ASDs.

Additionally, reviews from the U.S. Environmental Protection Agency have reported that each of these pollutants has been demonstrated to have adverse effects on the developing fetus in clinical and animals studies (Environmental Protection Agency, 2013). Interestingly, stronger associations were observed in boys compared to girls for most air pollutants, indicating a sex-specific interaction similar to that found in autism (Roberts et al., 2013).

Porphyrin levels in the urine are often used as a biomarker for heavy metal toxicity from air pollution, including mercury and lead. Numerous independent studies have found that children with ASDs have significantly elevated levels of urinary porphyrins, which are indicative of greater symptom severity (Kern et al., 2011, Kern et al., 2010, Youn et al., 2010, Geier et al., 2009, Austin and Shandley, 2008, Geier and Geier, 2007, Geier and Geier, 2006). Although measuring porphyrins levels might not be a valid diagnostic tool for ASDs on its own (Shandley et al., 2014), it may help identify a subgroup of ASDs subjects (Heyer et al., 2012).

Individuals having a genetic variant in the promoter region (*rs1858830* "C" allele) of the MET receptor tyrosine kinase gene and who are exposed to high levels of air pollutants were at a greater risk of ASDs (Volk et al., 2014). A polymorphism in the delta-aminolevulinic acid dehydratase (ALAD), which is associated with heavy metal toxicity and elevated levels of oxidative stress, has also been associated with autism (Rose S. et al., 2008). This suggests that individuals with ASDs may have a decreased ability to eliminate heavy metals from the body due to a genetic etiology (Kern et al., 2007). Furthermore, individuals with ASDs have impairments in detoxification and have lower levels of antioxidants, such as glutathione-s-transferase and vitamin E (Alabdali et al., 2014). This may cause these individuals to be more susceptible to the accumulation of toxic metals such as mercury and lead. Altogether, many

studies report that perinatal exposure to air pollutants, in combination with genetic susceptibility, may increase risk for ASDs.

2.7.2. Pesticides

Pesticides are chemical agents that are distributed widely throughout our environment for two common uses: to eradicate or discourage the involvement of pests and to protect plants in agriculture. Exposure to pesticides is of great concern since they are capable of passing the placental barrier and the BBB through specific transporters, such as the OAT3 transporter, which normally transport PGE_2. Furthermore, many pesticides can act as EEDCs, estrogen-like compounds that can result in hormonal disturbances (Kojima et al., 2004, Soto et al., 1994). In rodent models, exposure to estrogen compounds have been shown to induce PGE_2 production during development causing permanent changes, such as masculinization of the brain and sexual behaviour (Amateau and McCarthy, 2004). Given that pesticides are EEDCs that are capable of crossing protective barriers, it is probable that pesticides may directly or indirectly disrupt the PGE_2 lipid signalling pathway during development. Moreover, many pesticides have been found to induce oxidative stress (Abdollahi et al., 2004), which can lead to abnormal increases of PGE_2 levels that may disturb sensitive periods of neurodevelopment.

The general population, including pregnant women, is exposed to these types of compounds from a wide variety of sources including household products, food with pesticide residue or their metabolites, and air from areas where agricultural or urban spraying has occurred (Shelton et al., 2012). Maternal exposure to various pesticides during pregnancy has been associated with adverse effects in cognitive development in children (Bouchard et al., 2011) and increased risk of ASDs (Shelton et al., 2012, Roberts et al., 2007). Numerous pesticides have

been shown to disrupt critical neurodevelopmental signalling pathways—such as the γ-aminobutyric acid (GABA) and acetylcholine (ACh) pathways—and important hormones, including thyroid hormones (reviewed in (Shelton et al., 2012)). Interestingly, dysregulation of GABA, ACh, and thyroid hormone signalling have been associated with ASDs (Khan et al., 2014, Coghlan et al., 2012, Deutsch et al., 2010). Moreover, pesticides can increase levels of oxidative stress and the production of reactive oxygen species (Franco et al., 2009). This leads to a decline in mitochondrial function (Cui et al., 2012), which has been associated with ASDs (Rossignol and Frye, 2014). Considering that pesticides have the potential to impair neurodevelopment, we review the three most widely used pesticides: organochlorine pesticides (OCPs), organophosphate pesticides (OPPs), and pyrethrins and pyrethroids (PPs).

OCPs are a group of pesticides that was used widely across the globe for agricultural purposes. Due to its low biodegradability, toxicity, and incorporation into food webs, the use of these pesticides has been banned in many countries, including the U.S. and Canada. However, their levels remain persistent in the environment and continue to pose a risk to human health (Crinnion, 2009, Brun et al., 2008). In fact, a recent study conducted in the U.S. found that the presence of OCPs were detected in all of the pregnant women tested (Woodruff et al., 2011). In addition to their lasting presence in the environment, OCPS are able to cross the intestinal barrier, BBB, and skin barrier (Escuder-Gilabert et al., 2009). Examples of OCPs include dichlorodiphenyltrichloroethane (DDT), endosulfan, and dicofol. Many OCPs are EEDCs, which mimic endogenous estrogen that can affect calcium signalling (Wozniak et al., 2005) and PGE_2 signalling (Amateau and McCarthy, 2004). Moreover, in a large scale case-control study on children, prenatal exposure to OCPs during the first trimester was reported to increase the risk of children developing ASDs (Roberts et al., 2007). Another study identified two critical periods of

vulnerability for exposure to OCPs that were associated with ASDs: from 1 month prior to conception to 5 months post conception, and approximately 2 months to 8 months after birth (Roberts and English, 2013).

OPPs are another group of pesticides that were originally manufactured to replace numerous banned OCPs. Examples of commonly used OPPs include chlorpyrifos, dichlorvos, and malathion. OPPs act on the enzyme acetylcholinesterase and inhibit its function, causing nerve damage to unwanted pests (Costa, 2006). Unfortunately, OPPs also pose a potential risk to human health. Children whose mothers reside near application sites of OPPs during gestation were at a greater risk for ASDs (Shelton et al., 2014). Furthermore, a prospective cohort study by Rauh and colleagues found that higher concentrations of chlorpyrifos in the umbilical cord plasma was associated with a greater likelihood to develop symptoms of pervasive developmental disorder by 3 years of age (Rauh et al., 2011). Another investigation by the same group utilized magnetic resonance imaging to show that children with increased concentrations of chlorpyrifos had structural changes in brain areas associated with attention, social cognition, and receptive language processing (Rauh et al., 2012). Exposure to OPPs, including those which are commonly used to deter mosquitos and fruit flies, has been shown to induce oxidative stress, mitochondrial dysfunction, and cytotoxicity to neurons and liver cells (Moore et al., 2010, Kaur et al., 2007). As mentioned earlier, oxidative stress and mitochondrial dysfunction has been reported in ASDs cases (Rossignol and Frye, 2014). Additionally, the effects of OPPs, such as impaired cognition and altered neurochemistry, have been reported to be more severe in males than females (Levin et al., 2010), comparable again to the trend found with ASDs. Similar to the findings regarding heavy metals, there have been various reports about the possibility of genetic susceptibility to OPP toxicity (reviewed in (Costa, 2006)). This greater susceptibility would

decrease the ability to excrete OPPs and their metabolites (Costa, 2006). A study by Pasca and associates found that children with ASDs may be affected more harshly by OPPs due to relatively less active paroxonases; the enzymes responsible for metabolizing OPPs (Pasca et al., 2006).

A third group of pesticides are the naturally-derived pyrethrins and synthetically-adapted pyrethroids. Pyrethrins and pyrethroids (PPs) have been reported by the U.S. Environmental Protection Agency to be found in over 3,500 products in the U.S. (Environmental Protection Agency, 2013). Substantial increases in the use of PPs have been observed since 2000, when household use of OPPs was banned in the U.S. (Williams et al., 2008). 88% of women surveyed between 2000 and 2008, reported the use of PPs during their pregnancy, with 55% reporting high-exposure use including professional pesticide application of PPs (Williams et al., 2008). Even though PPs are short-lived, their metabolites have been found in over 75% of American children and adults, and 80% in adolescence (Barr et al., 2010). This suggests that utilization of PPs in daily living likely occurs in the majority of Americans. PPs and their metabolites could have toxic effects on humans since they can alter calcium signalling, induce oxidative stress, and affect voltage-sensitive sodium channels (Soderlund, 2012, Shafer et al., 2005). PPs have also been shown to cause neurotoxic developmental effects. For example, permethrin is a pyrethroid that is commonly found in treatment creams against lice and is also used as an agricultural pesticide. Studies show that permethrin increases oxidative stress leading to immunotoxic effects and neural apoptosis (Shi et al., 2011, Gabbianelli et al., 2009). Cyfluthrin is another example of a common household pyrethroid. It was found to modulate the production and signalling of interleukin-6 and interferon-γ (Mense et al., 2006), cytokines associated with ASDs (Li et al., 2009). Furthermore, the authors highlighted its potential to disrupt brain development (Mense et

al., 2006). In addition, maternal proximity to agricultural sites (that use PPs) just prior to conception or during the third trimester was associated with an increased risk for both ASDs and developmental delay (Shelton et al., 2014).

Taken together, these studies reveal that all three major groups of pesticides (OPPs, OCPs, and PPs) can lead to neurodevelopmental disturbances, including increased risk for developing ASDs.

2.7.3. Consumer Products

The daily use of consumer products potentially containing chemicals that accumulate within fatty tissues due to their lipophilic nature has become a concern for the human population. Many of these chemicals in consumer products have been shown to cross into the body and bloodstream through the skin, as well as through the protective barriers between a pregnant mother and fetus. Recent literature presented here reveals examples of chemicals that elicit physiological changes including aberrant fluctuations and dysregulation of PGE_2 signalling, hormone activity, and calcium function that may lead to developmental abnormalities. Examples of some consumer products that may contain harmful chemicals include lubricants, fire retardants, plastic containers, flooring and building materials, lotions, cosmetics, and fragrances. Persistent use of such products could potentially lead to absorption and bioaccumulation of certain compounds in the body.

Halogenated aromatic hydrocarbons (HAHs) are toxic compounds that are resistant to degradation and have been found in consumer products. Polychlorinated bisphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are two examples of HAHs that were used in lubricants (for industrial pipelines, cables, scientific equipment, etc.) and flame retardants (compounds

added to wood and manufactured materials such as plastics and textiles), respectively (Goines and Ashwood, 2013). Despite being banned in many industrial countries after it was discovered that they can cause serious adverse health effects on both wildlife and humans, PCBs and PBDEs still remain present in human tissues and breast milk (Daniels et al., 2010, Johnson-Restrepo et al., 2005). Exposure to PCBs and PBDEs has been shown to increase levels of PGE_2 in uterine and placental cells leading to proinflammatory responses (Peltier et al., 2012, Wrobel et al., 2010, Wang et al., 2008). Interestingly, the immune systems of children with ASDs react uniquely to PBDEs compared to typically developing children: peripheral blood cell samples of ASDs subjects displayed an increased cytokine response compared to control subjects indicating an overactive immune system in ASDs (Ashwood et al., 2009). This is important since the tight connection existing between the development of the immune system and central nervous system suggests that aberrations in immune responses may contribute to neurobehavioural disorders (Goines and Ashwood, 2013, Hertz-Picciotto et al., 2008). This is in line with other studies, which have found that exposure to PCBs and PBDEs can disrupt normal neuronal development (Kimura-Kuroda et al., 2007) and result in behavioural deficits observed with ASDs in the human population (Eskenazi et al., 2013) and animal model (Jolous-Jamshidi et al., 2010), such as social impairments . Additionally, maternal exposure to PBDEs in rats has been associated with hormonal disruptions as well as cognitive and behavioural abnormalities in the offspring (Kodavanti et al., 2010). PCBs and PBDEs have also been found to cause disruptive effects on the endocrine system (Lema et al., 2008, Morse et al., 1993) and cause dysfunction of calcium homeostasis (Wayman et al., 2012, Pessah et al., 2010); a potential marker for neurodevelopmental disorders like autism (Wayman et al., 2012).

Phthalate esters, also referred to as phthalates for short, are synthetic compounds that have been used as plasticizers for a variety of consumer products such as polyvinyl chloride (PVC) flooring material and building materials, children's toys, plastic containers, and personal care products (e.g. cosmetics, lotions, and fragrances) (Crinnion, 2010, Witorsch and Thomas, 2010). Phthalates found in food packaging, plastic containers, polluted soil, and polluted water can contaminate our foods and beverages; this is concerning because ingesting trace amounts of phthalates may have health consequences (Serrano et al., 2014, Schecter et al., 2013). In fact, phthalates are widely present in foods from the U.S. with high concentrations found in poultry, pork, cooking oils, and cream-based dairy products (Serrano et al., 2014, Schecter et al., 2013). Although, phthalates are short-lived chemicals that do not bio-accumulate and are rapidly excreted from the body (Heudorf et al., 2007), they still present a potential risk to human health since they are EEDCs. As EEDCs, they mimic endogenous estrogen by activating estrogen receptors and by acting as an antagonist to androgen receptors (Sharpe, 2008, Takeuchi et al., 2005). Moreover, exposure to phthalates has been shown to disrupt the levels of prostaglandins, including PGE_2, in uterine and amniotic cells (Wang et al., 2010, Pavan et al., 2001). It has been found through retrospective case and clinical studies that phthalate exposure is linked to behavioural abnormalities and developmental disorders including ADHD and ASDs (Testa et al., 2012, Miodovnik et al., 2011, Engel et al., 2010, Larsson et al., 2009). A study conducted in Sweden investigating potential harmful indoor environmental factors found that if PVC flooring was present in the parents' or children's room, which is a source of airborne phthalates, the child was at an increased risk of developing ASDs compared to wood flooring (Larsson et al., 2009). Prenatal exposure to phthalates during the third trimester of pregnancy (determined by urine samples) has been associated with adverse effects on childhood behaviour and executive

functioning, with behavioural outcomes commonly found in children with ADHD (Engel et al., 2010). Since phthalates are expelled quickly from the body, detection of phthalates in the urine indicates that daily phthalate exposure is likely occurring. Another study also measuring the urine samples of pregnant women in their third trimester found that phthalate exposure was associated with deficits in social behaviour, communication, social awareness, and social cognition (Miodovnik et al., 2011). In a study examining the phthalate levels in children, a significant increase in the urinary concentrations of phthalate metabolites were detected in children with ASDs compared to control children (Testa et al., 2012). Strikingly, the authors of this study were able to identify ASDs subjects with 91.1% specificity through the measurement of phthalate metabolite, 5-oxo-MEHP. In summary, these studies provide evidence that prenatal and postnatal exposure to phthalates has been associated with behavioural differences and developmental disorders like ASDs.

Bisphenol A (BPA) has been used to make epoxy resins and polycarbonate plastics, both of which are used in many household products including reusable plastic food containers, the internal lining of tin cans, food packaging materials, and cash register receipts (Biedermann et al., 2010, vom Saal and Hughes, 2005). BPA can enter the body through the skin, eating or drinking contaminated sources, hand-to-mouth contact, and manufacturer workplace exposure (CDC, 2013, Biedermann et al., 2010). Leaching of BPA molecules from consumer product sources has been shown to be increased when washing polycarbonate plastics and heating BPA-containing containers to sterilize foods (Howdeshell et al., 2003, Kang et al., 2003). Furthermore, it has been shown that BPA can leach from landfills into surrounding ecosystems, affecting drinking and bathing water (Coors et al., 2003). In a national health and nutrition examination study conducted by the Centre for Disease Control and Prevention in the U.S. in

2003-2004, nearly all individuals tested had BPA in the urine, suggesting widespread BPA exposure (CDC, 2013). Similarly, a study conducted by Statistics Canada in 2009-2011 found that BPA was detected in the urine of 95% of Canadians between the ages of 3 to 79 years old, with the highest levels of BPA measured in children between the ages of 3 to 5 and 6 to 11 years old (Statistics-Canada, 2013). Because BPA has been found to be rapidly metabolized (Volkel et al., 2002), this suggests that human exposure occurs in a continuous manner most likely from multiple sources. BPA has been found in various human body fluids, such as fetal serum and full-term amniotic fluid (Ikezuki et al., 2002), indicating that BPA has the ability to pass through the placenta that acts as a maternal-fetal protective barrier.

Exposure to BPA has been shown to alter the human uterine microenvironment by disrupting PGE_2 production in the endometrium and corpus luteum, which could disturb embryonic and fetal development (Mannelli et al., 2014, Romani et al., 2013). BPA is also known as a common EEDC (vom Saal and Hughes, 2005) that can exert its toxic affects at low human-relevant doses (Welshons et al., 2003). Studies completed on mouse and rat models found that prenatal and perinatal exposure to BPA can affect the offspring by up-regulating the immune response (Yoshino et al., 2004), altering social behaviours and expression of estrogen receptors (Wolstenholme et al., 2012), increasing hyperactive behaviour (Ishido et al., 2004), impairing neural pathways involving fear and learning (Negishi et al., 2004), decreasing levels of sonic hedgehog (Shh), a crucial developmental signalling molecules, and affecting dopaminergic neuron development (Miyagawa et al., 2007), and changing levels of DNA methyltransferases in the cortex, suggesting the possibility of epigenetic and transgenerational effects (Kundakovic et al., 2013). A clinical prospective study found that gestational BPA exposure was associated with deficits in behavioural and emotional regulation in children at 3 years of age (Braun et al., 2011).

Postnatal exposure to BPA in animal model studies reveal that exposure led to elevated levels of reactive oxygen species and lipid peroxidation and decreased levels of antioxidant enzymes (Chitra et al., 2003), higher estrogen receptor levels in the brain (Aloisi et al., 2001), and increased calcium signalling in hippocampal neurons (Tanabe et al., 2006). To summarize, prenatal and postnatal BPA exposure was found to be toxic by altering gene expression, disrupting immunological and neural pathways, and altering behaviours later in life.

Cosmetics are another group of consumer products that possesses chemicals that may be harmful to human health. For example, cosmetic eyelash growth products often include ingredients bimatoprost or dechloro ethylcloprostenolamide, which are prostaglandin analogues that can activate prostaglandin receptors (Alm et al., 2008, Choy and Lin, 2008, Toris et al., 2008), and thus are capable of disrupting normal prostaglandin signalling. Furthermore, siloxanes and parabens are chemicals that are often found in cosmetics that have been contested as substances that put human health at risk. Siloxanes (cyclic and linear) are used in cosmetics as spreading agents (Nair and Cosmetic Ingredients Review Expert, 2003). They are of concern since they can accumulate in fatty tissues and are EEDCs that are able to elicit estrogenic activity (He et al., 2003, Luu and Hutter, 2001, McKim et al., 2001) and indirectly disrupt PGE_2 levels (Amateau and McCarthy, 2004). Additionally, they may cause adverse effects on the nervous system by disrupting normal dopamine neurotransmission (Alexeeff, 2007). Parabens are largely used as antimicrobial preservatives in products such as cosmetics, pharmaceuticals, and on foods (Crinnion, 2010) and can also increase estrogenic activity (Darbre and Harvey, 2008). As mentioned earlier, elevated estrogenic activity can disturb regular PGE_2 signalling during development. Taken altogether, a variety of chemicals that can be found in the environment and in consumer products are capable of bio-accumulating in human tissue and are capable of

altering the signalling of important developmental pathways, such as the lipid mediator PGE_2 signalling pathway (Fig. 2-6). In addition to the chemicals mentioned above, Health Canada has published a science-based document, an "Ingredient Hotlist" containing a list of prohibited and restricted substances for use in cosmetics due to their hazardous properties (Government of Canada, 2014). Given that these chemicals are considered dangerous to the health of an adult, they could produce profound disturbances on the developing brain, which starts prenatally and continues into adolescence.

2.8. Literature Review Summary

In closing, lipids and lipid signalling pathways, such as PGE_2, are crucial in the development of the brain. There are several lines of research that provide evidence supporting the involvement of abnormal lipid metabolism in individuals with ASDs. Lipid signalling participates in the development of the nervous system, oxidative balance, immune function, as well as gastrointestinal repair and sleep behaviour consistent with reported medical complications in some children with autism. The COX-2/PGE_2 signalling pathway can become defective due to genetic aberrations or environmental conditions, such as exposure to drugs, infections, or common environmental agents and toxic chemicals. There is evidence linking the impairment of this pathway at various steps and the development of ASDs. Collectively, the available literature suggests that lipid signalling may have a crucial impact during pre- and postnatal periods. Anomalous changes along this pathway can be detrimental to the development of the nervous system and may contribute to the pathogenesis and symptomatic manifestations of autism. Considering that ASDs are complex neurodevelopment disorders characterized by a wide range of behaviours, dysregulation at different points of the lipid signalling pathways discussed

herein might provide some explanation to the diverse behavioural outcomes of ASDs. Current research gives compelling evidence that lipid irregularities may be a potential underlying cause of autism. However, additional investigation into the various genetic and environmental factors involved with lipid signalling in autism is essential for understanding the molecular mechanisms of the disorder and may aid in the development of novel diagnostic and therapeutic tools as well as preventative strategies for ASDs.

2.9. Figures

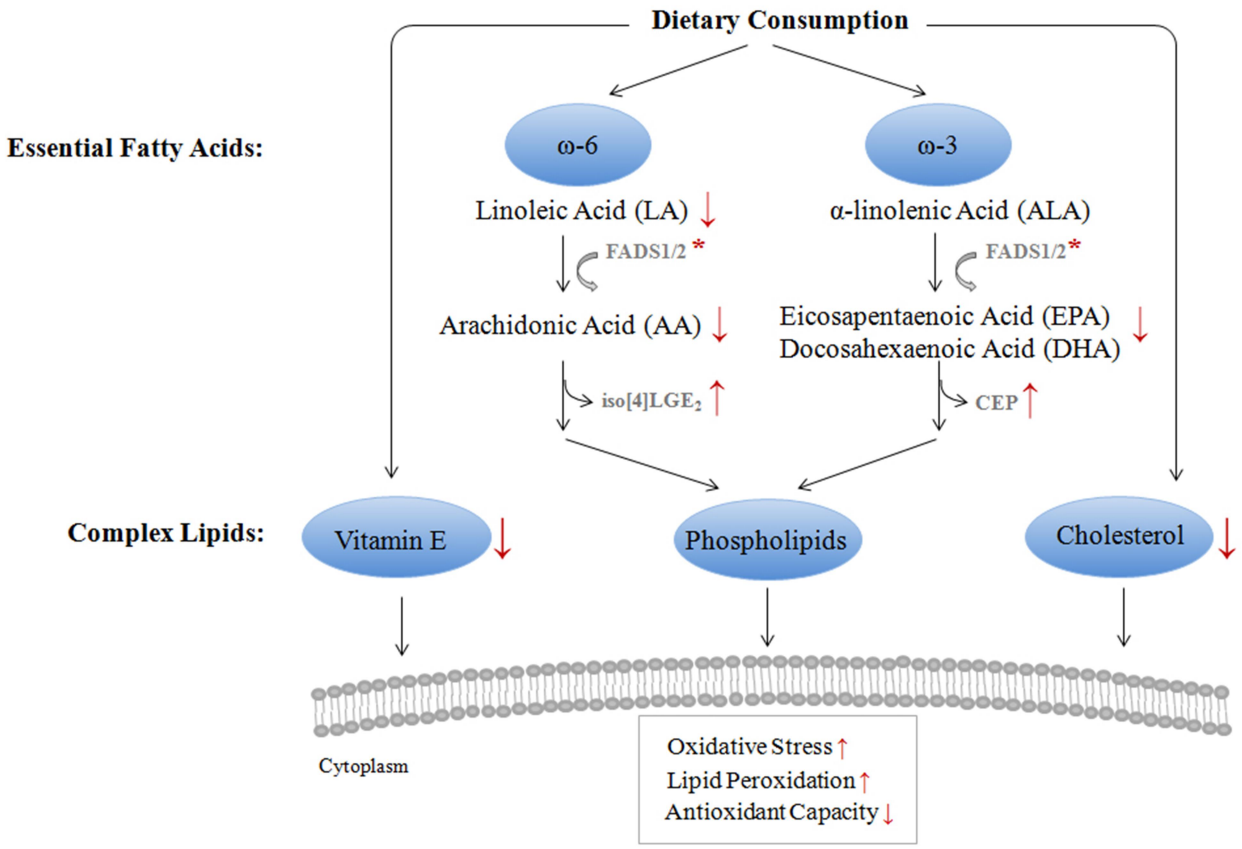

Fig. 2-1: Cell membrane lipid components implicated in ASDs.
Irregularities in PUFAs and complex lipids have been associated with ASDs (dashed arrows indicate an increase or decrease level in individuals with an ASD; asterisks indicate a link to ASDs).

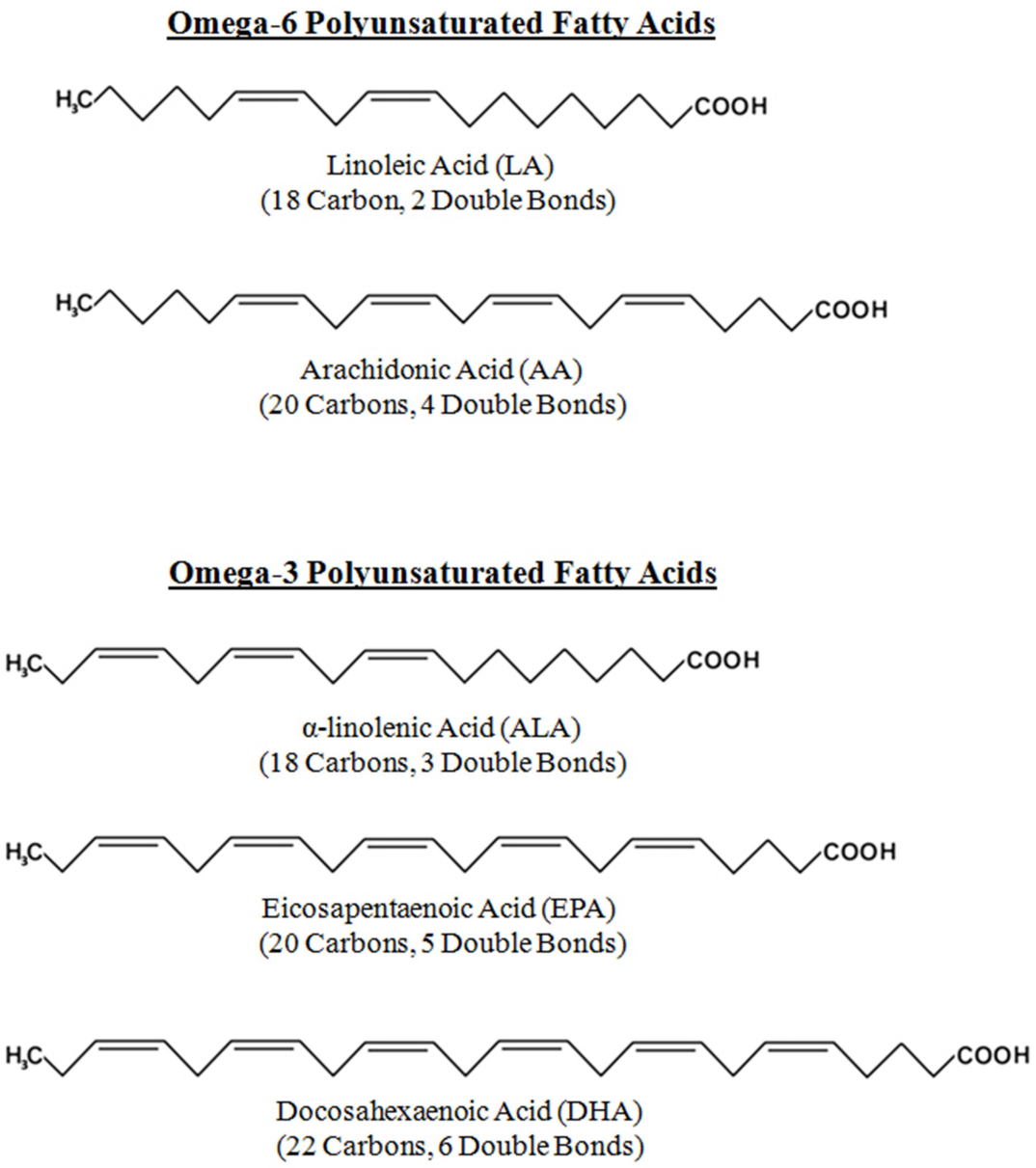

Fig. 2-2 : Omega polyunsaturated fatty acids.
The essential PUFAs are long carbon chain molecules that have an acidic (COOH) end and a methyl (CH3) end. The location of the first double bond, at the sixth or third carbon atom, determines whether the PUFA is an o-6 or o-3 fatty acid, respectively.

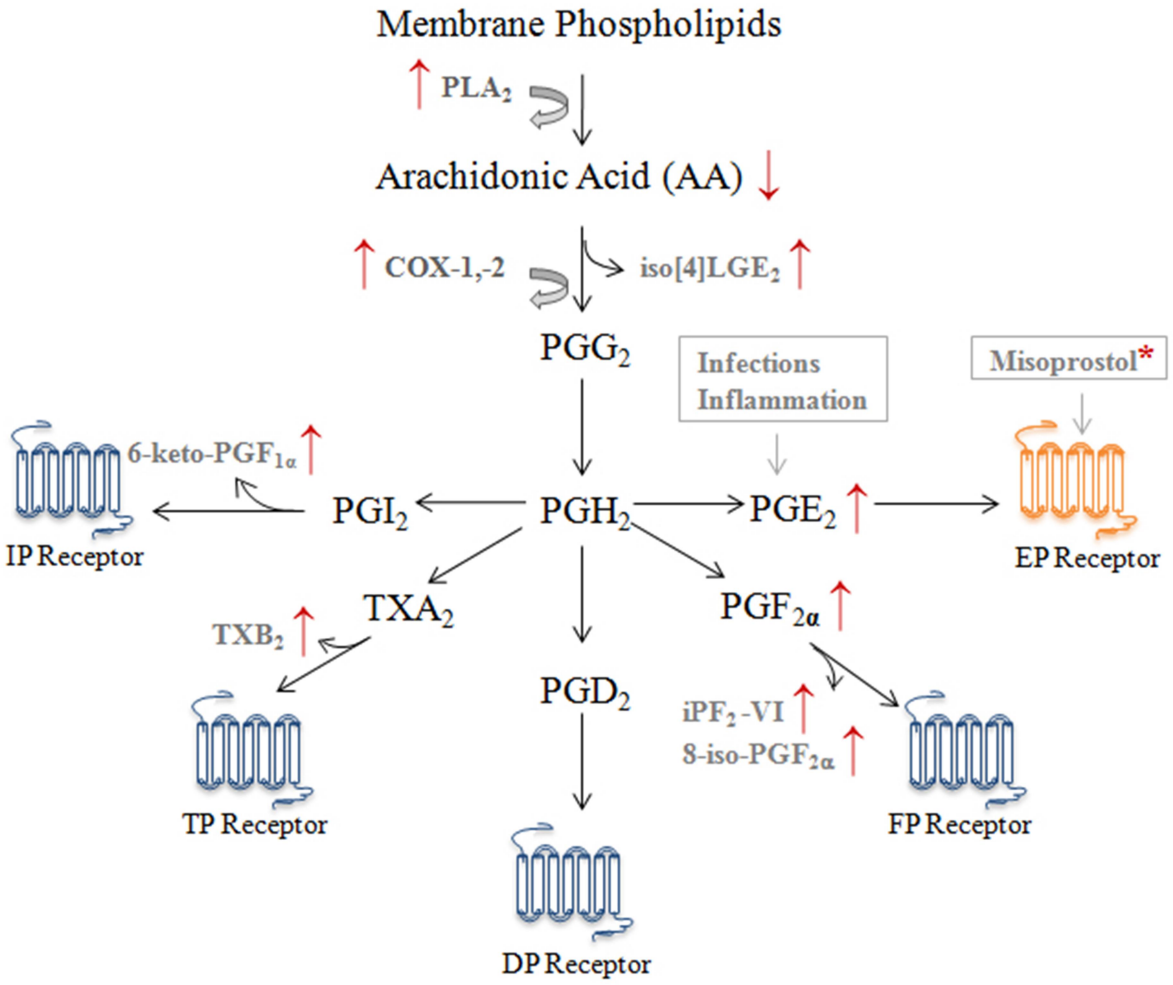

Fig. 2-3: Defects in lipid derivatives and prostanoids.
Prostanoids (PGE_2, PGF2a, PGD2, PGI2) and thromboxane A2 (TXA2) are bioactive lipid metabolites that elicit cellular events through the action of their respective receptors (EP, FP, DP, IP, and TP) (dashed arrows indicate an increase or decrease level in individuals with an ASD; asterisks indicate a link to ASDs).

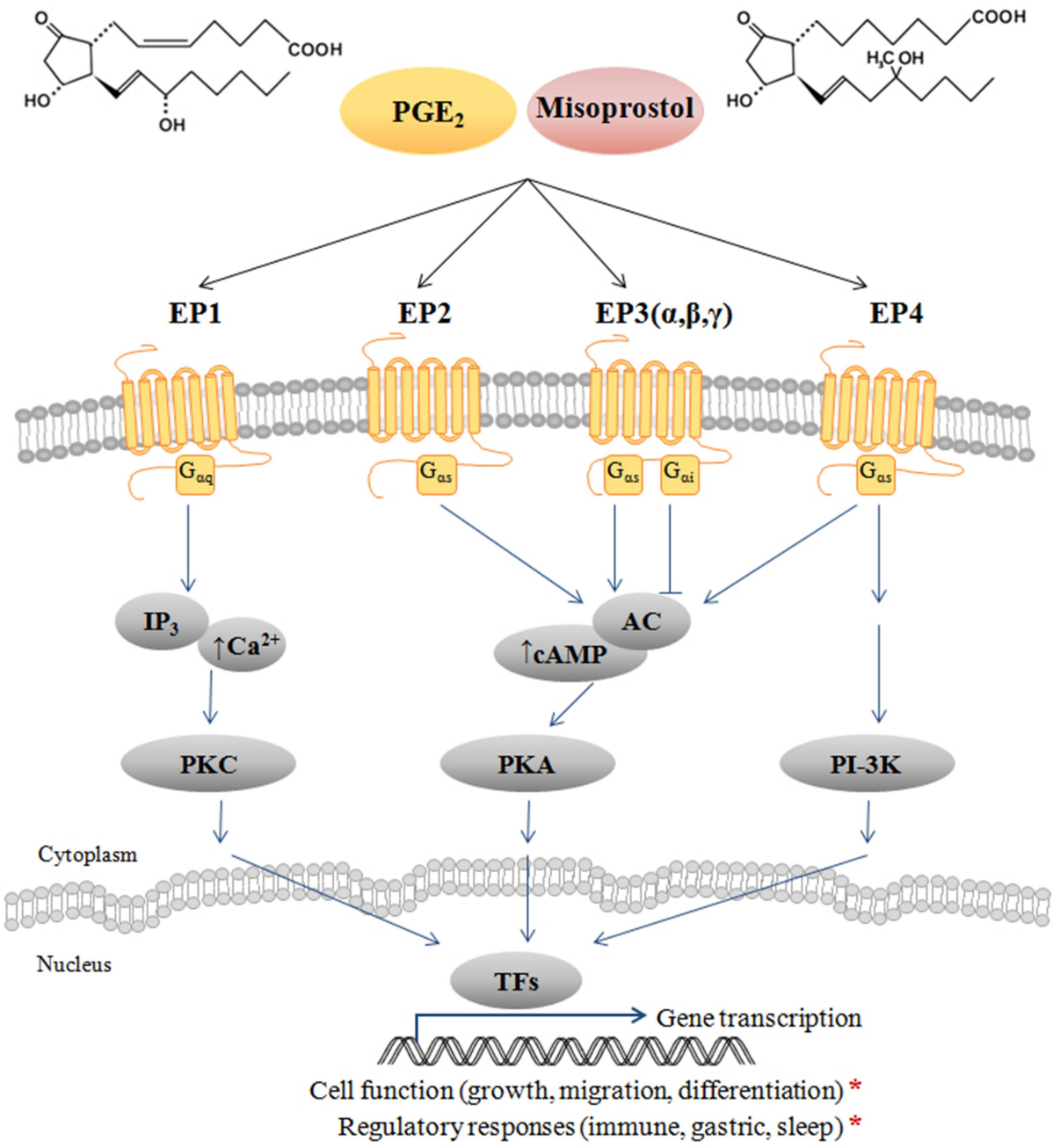

Fig 2-4: The PGE_2 signalling pathway.
Stimulation of EP1-4 via PGE_2 or misoprostol acid leads to activation of PKC, PKA, or PI-3K, resulting in gene transcription. Abnormal deviations indicated by the asterisks have been associated with ASDs.

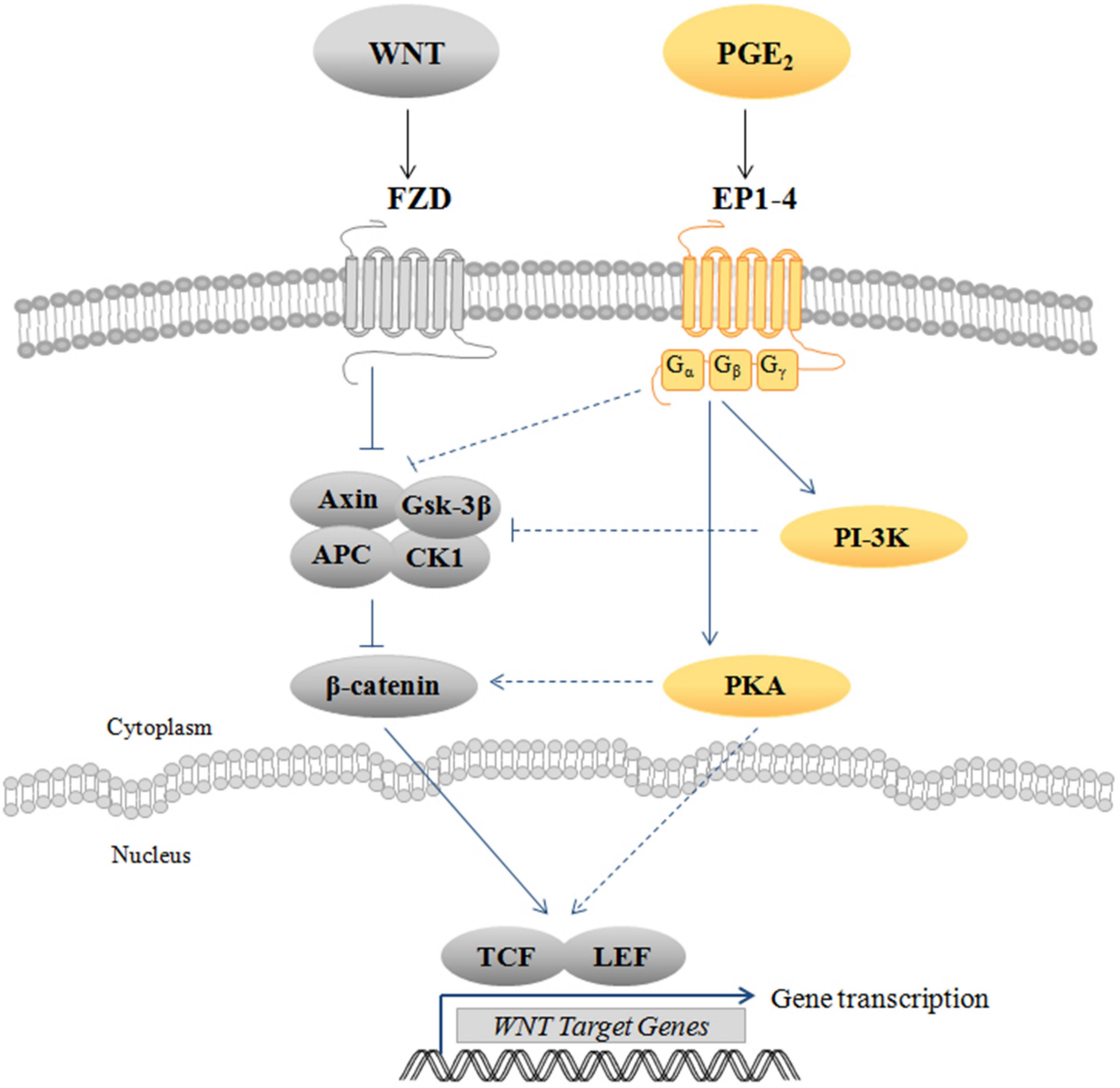

Fig. 2-5: The interaction between Wnt and PGE_2 signalling pathways.
Wnt and PGE_2 signalling have been implicated in ASDs, and an interaction between these pathways has been determined in various cell types as indicated by the dotted lines.

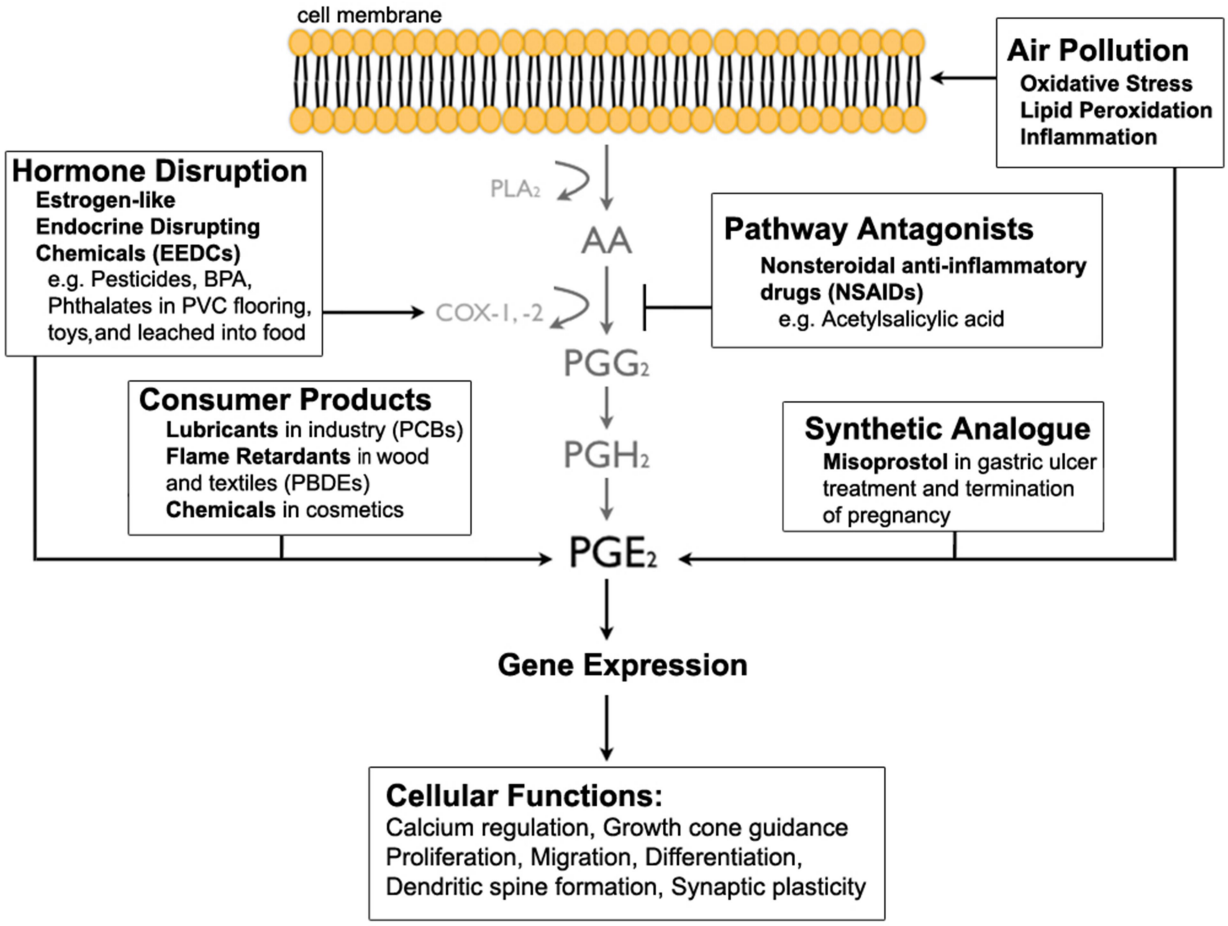

Fig. 2-6: Common environmental factors affecting the COX-2/ PGE_2 signalling pathway. Environmental factors, such as exposure to chemicals in drugs, air pollution, pesticides and consumer products, can disrupt normal lipid signalling pathways, such as the PGE_2 pathway. They can act as analogs, antagonists and EEDCs that interfere with PGE_2 signalling and result in altered gene expression, thereby influencing the function and development of brain cells.

2.10. References

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CHAPTER 3.

Study 1: Prostaglandin E2 alters Wnt-dependent migration and proliferation in neuroectodermal stem cells: implications for autism spectrum disorders.

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Contributions: Christine T. Wong designed and performed all experiments, collected samples, acquired and analyzed the data, made all figures and tables, and prepared the manuscript. Eizaaz Ahmad assisted in gene expression experiments. Dr. Hongyan Li provided administrative and technical support and proofread the manuscript. Dr. Dorota A. Crawford supervised the design of the study and was involved with editing the manuscript.

Objectives and Hypotheses: The primary aim of this first study was to investigate the potential interaction between the PGE_2 and Wnt (a crucial morphogen) signalling pathways. Our major objectives were to determine how PGE_2 affects the motility (final distance, path length, and average speed) and proliferation behaviour of Wnt-activated NE-4C stem cells. We also aimed to identify the molecules downstream from PGE_2 receptors that interfere with the Wnt signalling pathway. I hypothesized that crosstalk between the PGE_2 and Wnt signalling pathways alters the motility and proliferation of NE-4C stem cells through downstream effectors such as protein kinase A (PKA) and phosphatidylinositide 3-kinase (PI-3K).

3.1. Chapter Summary

Prostaglandin E2 (PGE_2) is a natural lipid-derived molecule that is involved in important physiological functions. Abnormal PGE_2 signalling has been associated with pathologies of the nervous system. Previous studies provide evidence for the interaction of PGE_2 and canonical Wnt signalling pathways in non-neuronal cells. Since the Wnt pathway is crucial in the development and organization of the brain, the main goal of this study is to determine whether collaboration between these pathways exists in neuronal cell types. We report that PGE_2 interacts with canonical Wnt signalling through PKA and PI-3K in neuroectodermal (NE-4C) stem cells. We used time-lapse microscopy to determine that PGE_2 increases the final distance from origin, path length travelled, and the average speed of migration in Wnt-activated cells. Furthermore, PGE_2 alters distinct cellular phenotypes that are characteristic of Wnt-induced NE-4C cells, which corresponds to the modified splitting behaviour of the cells. We also found that in Wnt-induced cells the level of β-catenin protein was increased and the expression levels of Wnt-target genes (*Ctnnb1*, *Ptgs2*, *Ccnd1*, *Mmp9*) was significantly upregulated in response to PGE_2 treatment. This confirms that PGE_2 activated the canonical Wnt signalling pathway. Furthermore, the upregulated genes have been previously associated with ASDs. Our findings show, for the first time, evidence for cross-talk between PGE_2 and Wnt signalling in neuronal cells, where PKA and PI-3K might act as mediators between the two pathways. Given the importance of PGE_2 and Wnt signalling in prenatal development of the nervous system, our study provides insight into how interaction between these two pathways may influence neurodevelopment.

3.2. Introduction

The plasma membrane phospholipids play a fundamental role in the nervous system and act as a reservoir for second messenger molecules important for the development and normal functioning of the brain. Prostaglandin E2 (PGE_2) is a bioactive fatty acid that is derived from arachidonic acid, a major structural component of plasma membrane phospholipids, through the enzymatic metabolism of cyclooxygenases-1 and -2 (COX-1,-2) and then prostaglandin synthases (Breyer et al., 2001). Extracellular stimuli such as immunological and infectious agents (Brown, 2012, Patterson, 2011, Parker-Athill and Tan, 2010), environmental toxins such as mercury and lead (Grandjean and Landrigan, 2006), and exposure to drugs including misoprostol and valproic acid (Arndt et al., 2005) can trigger the local production of PGE_2 via specific biosynthetic pathways, resulting in altered cell signal transmission that modulates biological functions such as sleep, fever, inflammation, and pain (Sugimoto and Narumiya, 2007).

The diverse action of PGE_2 is achieved through the activation of 4 different G-protein coupled E-prostanoid receptors (EP1 through 4) (Furuyashiki and Narumiya, 2011, Coleman et al., 1994). The divergent role of PGE_2 is amplified by the variety of different kinase- mediated signalling cascades that can be activated through its EP receptors, such as the protein kinase A (PKA), phosphatidylinositide 3-kinases (PI-3K), and protein kinase C (PKC) pathways (Andreasson, 2010).

During the early stages of pregnancy, there are elevated levels of COX-2 and PGE synthases, enzymes responsible for the production of PGE_2, which is indicative of the involvement of PGE_2 in prenatal development (Saint-Dizier et al., 2011). We have previously shown that the expression profiles of EP receptors during mouse embryonic development

changes depending on the embryonic stage, with EP receptor expression highest during E7 (Embryonic day 7) and E15, which corresponds to peak periods of neurogenesis (Tamiji and Crawford, 2010). It has been shown that PGE_2 plays a regulatory role in membrane excitability and synaptic transmission in neurons (Chen and Bazan, 2005). PGE_2 increases the dendritic length and complexity of Purkinje neurons, and can also alter neuronal firing activity in the developing brain (Dean et al., 2012). PGE_2 is involved in synaptic plasticity and neuroprotection (Chen and Bazan, 2005), and can also be involved in neuronal cell death and apoptosis (Miyagishi et al., 2013, Shimamura et al., 2013). Prostaglandins have also been reported to induce the differentiation of neuronal cells (Choi et al., 2001). Moreover, the inhibition of COX-2, can suppress neurogenesis and proliferation of neural progenitor cells (Goncalves et al., 2010). These studies show the important role PGE_2 can play during normal development of the nervous system. Furthermore, previous research found that PGE_2 can exert various effects on cell development, proliferation, and migration in a diversity of cell lines. It has been shown that PGE_2 stimulates cell growth and motility in osteoblasts (Raisz et al., 1993), prostate cancer cells (Tjandrawinata et al., 1997), and pancreatic stellate cells (Charo et al., 2013). The migration of vascular smooth muscle cells (Lin et al., 2012), intestinal subepithelial myofibroblasts (Iwanaga et al., 2012), dendritic cells (Joo et al., 2012), hepatocellular carcinoma cells (Mayoral et al., 2005), and mesangial cells (Jaffer et al., 1995) can all be regulated by PGE_2. However, the effects of PGE_2 on neural stem cell behaviour and movement are not well characterized. Our previous studies provide some insight into the molecular mechanisms of abnormal PGE_2 signalling on neuronal cells. We have found that exposure to PGE_2 results in the retraction of neurites and the elevation of calcium amplitude fluctuations in growth cones of differentiated Neuro-2A cells (Tamiji and Crawford, 2010, Tamiji and Crawford, 2010)

Abnormal fatty acid metabolism through the PGE_2 pathway may contribute to the pathology of neurodevelopmental disorders such as Autism Spectrum Disorders (ASDs) (Tamiji and Crawford, 2010). Abnormal levels of PGE_2 and other fatty acid metabolites have been identified as potential biomarkers for ASDs (El-Ansary and Al-Ayadhi, 2012). PGE_2 can act as an endogenous modulator for cerebellar development in the rat brain affecting social interaction and sensory behaviour, which are characteristic behaviours altered in ASDs (Dean et al., 2012). Clinical studies have shown that maternal exposure to the drug misoprostol (prostaglandin E analogue), has been associated with the development of Moebius syndrome and autistic-like symptoms (Miller, 2007, Miller et al., 2005, Bandim et al., 2003).

Current literature also provides evidence that PGE_2 signalling interacts with another crucial developmental pathway, the canonical Wnt (*wingless-related MMTV integration site*) signalling pathway in various non-neuronal cells (Wong and Crawford, 2014) such as osteocytes (Kitase et al., 2010), prostate and colon cancer cells (Liu et al., 2010), hematopoietic stem cells (Goessling et al., 2009), and mesenchymal stem cells (Kleiveland et al., 2008). Wnt signalling is tightly regulated in early development and is required for the formation of the nervous system (Ciani and Salinas, 2005). The canonical Wnt signalling pathway is composed of a network of proteins that modify cell communication and interactions with other cells. Wnt proteins bind to cell surface Frizzled (FZD) receptors, where the signal is then transduced to β-catenin, activating the transcription of Wnt-target genes. The Wnt molecules are vital to embryonic development since they can moderate cell proliferation and differentiation by participating in the determination of cell fates (Buechling and Boutros, 2011). Previous literature shows that convergence of PGE_2-dependent signalling with the canonical Wnt pathway can occur at the level of β-catenin through EP1-4 receptors, including the association of the $G_{\alpha s}$ subunit with

Axin, the stimulation of the cAMP/PKA pathway, or the phosphorylation of GSK-3β by PI-3K (Buchanan and DuBois, 2006). However, the interaction of PGE_2 and Wnt signalling is not well-characterized in the nervous system. To activate and study canonical Wnt signalling in an in vitro model system, Wnt Agonist (WntA), 2-amino-4-[3,4-(methylene- dioxy)benzylamino]-6-(3-methoxyphenyl) pyrimidine, can serve as a useful reagent. WntA is a cell-permeable pyrimidine compound that mimics the effects of Wnt by functioning through the canonical pathway via upregulating TCF-activity without inhibiting the activity of GSK-3β (Liu et al., 2005). This is important because GSK-3β plays a regulatory role in many signalling pathways other than Wnt so an agonist that blocks GSK-3β could have disparate effects in cellular models.

This study investigates the effects of PGE_2 interaction with the Wnt signalling pathway on the behaviour of murine neuroectodermal (NE-4C) stem cells. We demonstrate that PGE_2 modifies canonical Wnt signalling in NE-4C stem cells by altering components of cell motility such as final distance travelled, path length travelled, average speed of migration, as well as cell splitting behaviour. We also reveal that PGE_2 can alter the protein expression of non-phospho (active) β-catenin (Ser33/37/ Thr41), as well as the expression of specific Wnt-target genes. Interestingly, the genes implicated in our study have been previously associated with ASDs. To our knowledge, we show for the first time, that PGE_2 signalling interacts with the Wnt pathway in neural stem cells to affect cell behaviour and gene transcription. Our study furthers our understanding of the possible mechanisms by which intracellular cross-talk between PGE_2 and Wnt signalling may contribute to cell migration and proliferation during prenatal development of the nervous system.

3.3. Methods

Cell culture

Mouse NE-4C cells were obtained from American Tissue Culture Collection (ATCC) and grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 X penicillin-streptomycin mixture (Invitrogen). Cells were maintained in an incubator containing 5% CO_2 at 95% humidity 37°C. Cells were plated on 0.01% poly-L-lysine (Sigma) coated 100 mm culture plates (BD Falcon) and were subcultured at a 1:10 ratio. Supplemented MEM was changed every 2–3 days.

Cell culture treatments

NE-4C cells (ATCC) were dissociated with 0.05% trypsin-EDTA, pelleted and resuspended in complete medium as described above. The cells were plated on poly-L-lysine 0.01% (Sigma, MW 70000–150000 kDa) on 35 mm tissue culture dishes (Sarstedt). Plated cells were incubated in 5% CO_2 at 95% humidity 37°C overnight before treatment with Wnt Agonist (WntA, Calbiochem), prostaglandin E_2 (PGE_2, Sigma) and/or blockers. WntA (2 μM), PGE_2 (1 μM), H89 dihydrochloride hydrate (H89, 10 μM, Sigma), Wortmannin (WORT, 1 μM, Sigma) or an equivalent volume of vehicle were added to each well. Cells were treated for 24 hours.

Reverse transcription and real-time PCR

Total RNA was extracted from NE-4C cells using the NucleoSpin RNA/Protein Kit (Macherey-Nagel) and was reverse-transcribed into cDNA using MMuLV reverse transcriptase (New England Biolabs) according to manufacturer's instructions. Primer3

Input software (v. 0.4.0) was used to design forward and reverse primers for EP receptors and have been previously noted (Tamiji and Crawford, 2010). Selection of Wnt-target genes was determined using Custom TaqMan® Array Plates (Life Technologies) as a screening tool (data not shown). Genes that had a greater than 1.8 fold-change were selected for further validation and forward and reverse primers were designed (Table 3-1). Real-time PCR was performed using the 7500 Fast Real-time PCR system (Applied Biosystems) and the $\Delta\Delta C_T$ method was applied to calculate the expression of transcripts. Hypoxanthine phosphoribosyl transferase (*Hprt*) and Phosphoglycerate Kinase 1 (*Pgk1*) served as endogenous controls. The relative quantification (RQ) ratios were determined from the average of three technical replicates from three biological replicates.

Table 3-1: qRT-PCR Primers for undifferentiated NE-4C stem cells

Name	Primer	Primer Sequence (5'-3')	Base pair Length
Hprt	Forward	TCCATTCCTATGACTGTAGATTTTATCAG	29
	Reverse	AACTTTTATGTCCCCCGTTGACT	23
Pgk1	Forward	CAGTTGCTGCTGAACTCAAATCTC	24
	Reverse	GCCCACACAATCCTTCAAGAA	21
Ptgs2	Forward	CAGCCAGGCAGCAAATCC	18
	Reverse	TTATACTGGTCAAATCCTGTGCTCAT	26
Ctnnb1	Forward	GGACGTTCACAACCGGATTG	20
	Reverse	GAGAATAAAGCAACTGCACAAACAA	25
Ccnd1	Forward	GCACTTTCTTTCCAGAGTCATCAA	24
	Reverse	CTCCAGAAGGGCTTCAATCTGT	22
Mmp9	Forward	TCGCGTGGATAAGGAGTTCTCT	22
	Reverse	ATAGGCTTTGTCTTGGTACTGGAAGA	26

Western blot analysis

Total protein was extracted from NE-4C cells using the NucleoSpin RNA/Protein Kit (Macherey-Nagel). Samples were separated by polyacrylamide gel electrophoresis (PAGE). Primary antibodies used for EP expression levels include rabbit polyclonal anti-EP1, -EP2, -EP3, -EP4 (1:200; Santa Cruz Biotechnology). Detection of rabbit monoclonal anti-Phospho-Histone H3 (Ser10) (1:1000; Cell Signaling) was used as a measure of cell splitting behaviour. Primary antibodies used for β-catenin expression levels were rabbit monoclonal anti-non-phospho (Active) β-catenin (Ser33/37/Thr41) and rabbit polyclonal anti-phospho-β-catenin (Ser552) (1:1000; Cell Signaling). Blots were reprobed with mouse monoclonal anti-β-Actin (1:10,000; Abcam). Visualization of bound anti-rabbit and anti-mouse horseradish peroxidise-conjugated secondary antibodies was achieved by incubation with ECL Prime Western Blotting Detection Reagent (GE Healthcare) and detection by Geliance 600 Imaging System (Perkin Elmer).

Immunocytochemistry

NE-4C cells were seeded onto 35 mm culture plates containing poly-L-lysine coated coverslips and grown overnight at 37°C. The cells were fixed with 50% acetone and 50% methanol for 20 minutes at −20°C and washed with phosphate buffered saline (4.3 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH_2PO_4). Cells were then incubated with primary antibodies in PBS with 0.3% Triton-X 100 and 2% Normal Goat Serum. Cellular localization of the EP receptors was determined by incubation with anti-EP primary antibodies as described above along with mouse monoclonal anti-Lamin A + C nuclear envelope marker (1:200; Abcam), anti-58 K Golgi marker [58 K-9] (1:100;

Abcam), anti-PDI endoplasmic reticulum marker [RL90] (1:100; Abcam) or β-Actin (1:1000; Abcam) at room temperature for 1 hour. Following primary antibody incubation, cells were washed three times with PBS-T for 15 min and incubated with secondary antibodies in PBS-T and 2% NGS for 1 hour at room temperature in the dark. Secondary antibodies used were anti-rabbit fluorescein isothiocyanate (FITC) (1:100; Jackson ImmunoResearch Laboratories) and anti-mouse TexasRed (1:200; Jackson ImmunoResearch Laboratories). Cells were then washed twice with PBS-T for 10 min, followed by a 20 minute incubation of 4′,6-diamidino-2-phenylindole (DAPI) (1:2000; Molecular Probes) at room temperature. Cells were washed twice with PBS-T for 5 min and coverslips were mounted on glass microscope slides with mounting media (Vectashield). The staining was visualized and captured using an Eclipse 80i upright fluorescent microscope with DS-5MC camera (Nikon).

Time-lapse imaging and analysis

Cell behaviour was recorded using Nikon Eclipse Ti-E microscope. Three biological replicates of each treatment condition were performed (*N=3*), where an average of 150 cells were tracked. Micrographs were automatically captured every 10 minutes for a 24 hour period from a minimum of three fields. To maintain conditions physiologically suitable for the cells, an enclosed chamber was mounted to the microscope, which was equipped with CO_2 supply and temperature thermostat. Cells were kept at 5% CO_2, 95% humidity, 37°C. Measurements were completed using NIS Elements software, including a specialized tracking module. Final distance from origin, path length, and average speed were tracked and calculated from an average of 150 cells per treatment condition. The *final distance* was

defined as the distance between the initial and final positions of the cell, represented as a straight line distance. The *path length* was the total distance travelled from the initial to the final cell position. The *average speed* of a cell was calculated by dividing the total distance travelled by the time it took to travel between the two positions.

Initial and final cell counts were used to determine fold change as a measurement of proliferation. Split percentage was quantified as a measurement of proliferation behaviour. Split percentage was defined as the percentage of cells that fulfilled the complete cell cycle, which was evaluated based on whether the parent cell could successfully split into two daughter cells.

Cell viability analysis

Cells were disassociated and diluted with equal volumes of trypan blue dye (4%). Cell count averages were taken from a minimum of four hemacytometer squares to determine cell number and viability.

Statistical analysis

All numerical data were presented as mean + SEM of three individual experiments. Statistical analysis was performed using student t-test or one-way analysis of variance (ANOVA) followed by Tukey post-hoc comparisons or Dunnett t-test (2-sided). Differences were considered statistically significant at $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

3.4. Results

Expression of EP1-4 receptors in NE-4C cells

To determine whether NE-4C cells endogenously express the receptors of PGE_2, we performed real-time quantitative PCR assay, Western blot analysis, and immunocytochemistry. Our results show that in NE-4C cells, EP2 had the highest mRNA expression followed by EP3γ and EP4 receptors. Endogenous EP1 and EP3β receptor expression was considerably low in NE-4C cells, while the EP3α transcript level was nearly absent and may be considered negligible. The relative quantity (RQ = 1) values of EP1, EP2, EP3α, EP3β, EP3γ, and EP4 transcripts expression were 3, 542, 0, 1, 391, and 15, respectively (Fig. 3-1A). Western blot results confirm the expression of all four EP receptors in NE-4C cells (Fig. 3-1B). The localization of the EP receptors in NE- 4C cells was also detected with immunocytochemistry using EP1-4 specific antibodies along with antibodies against various cellular organelles including the nuclear envelope, Golgi apparatus, the endoplasmic reticulum, and β-Actin (Fig. 3-1C). Our results show that EP1 receptors were localized in the ER membrane, EP2 receptors were uniformly expressed around the nucleus and co-localized with the nuclear envelope marker, EP3 receptors were located at the plasma membrane, and EP4 receptors at the Golgi apparatus. Hence, NE-4C cells can act as an appropriate experimental model to study PGE_2 signalling.

Prostaglandin E_2 increases the cell motility of Wnt-induced NE-4C cell migration

The effect of PGE_2 on Wnt-dependent migration of NE-4C cells was determined using Nikon Eclipse Ti-E microscope with NIS Elements time-lapse tracking software over a 24 hour period. Final distance, path length, and average speed were quantified after exposure to 1 μM PGE_2, 2 μM Wnt Agonist (WntA), or 2 μM WntA with the addition of 1 μM PGE_2.

The results show that untreated NE-4C cells moved an average final distance of 65.6 μm following a 24 hour period (Fig. 3-2A). The addition of PGE_2 to the cells resulted in a final distance of 56.2 μm which was not significantly different from the untreated control (65.6 μm). WntA only treatment resulted in a significant decrease in final distance of 21.3 μm ($p = 0.00242$) when compared to the control. The addition of PGE_2 to WntA-treated cells resulted in a final distance of 45.0 μm, which is an increase by 23.6 μm ($p = 0.04371$), as compared to WntA only-treated cells. It represents a 211% increase from the WntA-regulated movement. Visualization of final distance through dispersion XY position plots clearly illustrates that PGE_2 signalling restores the Wnt-regulated suppression of cell movement (Fig. 3-2B, *WntA + PGE2*).

The quantification of path length (Fig. 3-3A) revealed the same pattern. The path length of untreated cells was 458.9 μm. As compared to untreated cells, PGE_2 only treatment did not result in a significant change (408.6 μm), but WntA treatment significantly decreased the path length to 103.3 μm ($p = 0.00189$). Addition of PGE_2 to WntA-treated cells led to a path length of 362.1 μm. This is a 350% increase ($p = 0.00928$) compared to WntA only-treated cells.

Quantification of average speed showed that PGE_2 treated cells travelled at a speed of 10.5 nm/s, which was not significantly different from untreated NE-4C cells that moved at a speed of 11.0 nm/s (Fig. 3-3B). WntA only treatment resulted in a decreased average cell speed of 1.65 nm/s ($p = 0.00065$). Addition of PGE_2 to WntA- treated cells resulted in an average speed of 7.34 nm/s. This suggests that addition of PGE_2 elevated the average speed by 439%; an increase of 5.59 nm/s ($p = 0.00946$) when compared to WntA only-treatment.

In summary, administration of PGE_2 treatment leads to significant changes in WntA-regulated cell behaviours such as final distance, path length, and average speed. PGE_2 treatment significantly restored the cell kinematic measures which were suppressed by WntA treatment.b

Prostaglandin E_2 modulates Wnt-induced cell behaviour through PKA and PI-3K kinases

Previous studies in embryonic kidney and colon cancer cells determined that the convergence of PGE_2 signalling on the Wnt pathway occurred through the activation of PKA or PI-3K (Castellone et al., 2005, Fujino et al., 2002, Sheng et al., 2001). To determine whether PGE_2 treatment alters Wnt-induced cell migration behaviour via these kinases in NE-4C cells, we used dihydrochloride hydrate (H89) to block PKA and Wortmannin (Wort) to block PI-3K. Our results show a trend across final distance, path length, and average speed (Fig. 3-2 and 3-3). With the addition of H89 to WntA + PGE_2 treated cells, all cell motility measures significantly decreased compared to the WntA + PGE_2 treated cells, resulting in movement profiles that were not statistically different from the WntA-only condition. Specifically, H89-treated cells travelled a final distance of 20.32 μm from the origin (p = 0.02477), path length of 116.01 μm (p = 0.00567), and at an average speed of 1.37 nm/s (p = 0.00073) (Fig. 3-2A and 3-2B).

With the addition of Wort to WntA + PGE_2 treated cells, there was a decreasing trend in final distance and path length but it was not significantly different from PGE_2 + WntA treated cells. Only average speed significantly decreased to 2.76 nm/s (N = 3; p = 0.00422) compared to the WntA + PGE_2 treatment. Post hoc Dunnett t-test revealed that measurements from the H89 and Wort conditions were not significantly different from the WntA-only treatment, indicating that H89 and Wort significantly diminished the effect of PGE_2 on WntA- treated cells. This indicates that PGE_2 likely acts through PKA and PI-3K to elicit effects on the WntA-dependent cell motility. However, it appears that H89 may have had a greater effect, suggesting that PGE_2 may predominately act through PKA.

Prostaglandin E_2 alters cell proliferation behaviour of NE-4C cells induced by Wnt agonist treatment

Previous literature reveals that PGE_2 may also affect cell proliferation via the Wnt signalling pathway in prostate and colon cancer cells (Liu et al., 2010) and hematopoietic (Goessling et al., 2009) and mesenchymal (Kleiveland et al., 2008) stem cells. We studied the effects of PGE_2 on NE-4C cell proliferation using NIS Elements software. The cells were exposed to 1 μM PGE_2, 2 μM WntA, or 2 μM WntA with the addition of 1 μM PGE_2. Furthermore, H89 or Wort was added to PGE_2 + WntA treated cells to determine the effective role of these kinases. The initial number of cells was compared to the final number of cells following 24 hours treatment. PGE_2 treatment led to an increase in cell number by 4.60-fold, which was not significantly different from the untreated cells that proliferated by a 4.59 fold-increase (Fig. 3-4A). Administration of WntA resulted in a fold- change of 0.86 ($p < 0.001$) which was significantly lower than untreated cells. Addition of PGE_2 to WntA-treated cells (WntA + PGE_2) resulted in a fold-change of 1.03, which was not significant from the WntA only treated condition. Although we observed lower levels of proliferation in the WntA, WntA + PGE_2 and WntA + PGE_2 + blocker conditions, we confirmed no change in cell viability between the conditions tested (Fig. 3-4B).

However, we observed distinct differences in cell phenotype between the WntA, WntA + PGE_2 and WntA + PGE_2 with H89 or Wort treatment. A majority of the cells treated with WntA adopted a shiny circular shape (indicated by black arrows, Fig. 3-4D). This was not as prevalent in the WntA + PGE_2 condition. However, the cells treated with WntA + PGE_2 and Wort blocker, adopted the shiny circular phenotype seen in the WntA condition. Cells treated with WntA +

PGE_2 and H89 blocker adopted a circular appearance as well but a smaller population of these round cells were shiny.

Our experiments showed that cell viability was not affected but a distinct shiny circular cell appearance was observed, which is characteristic of a cell just prior to splitting into two daughter cells. Therefore, we also quantified the *split percentage*, defined as the percentage of cells that successfully divided into two daughter cells during the recorded time period. As expected, the NE-4C untreated cells demonstrated a split percentage of 100% (Fig. 3-4C), indicating that all cells entering a mitotic phase resulted in cell division. A similar pattern was seen in PGE_2-treated cells (97.5%). However, treatment of WntA resulted in a significant decrease of split percentage to 0% ($p < 0.001$), where mitotic cells appeared to become arrested in a round stage denoted in Fig. 3-4D (*WntA* Image) with black arrows. The addition of 1 μM PGE_2 to WntA-treated cells produced a significant increase in split percentage to 14.7% ($p < 0.001$, Fig. 3-4C) as compared to WntA only treatment. The cells appear to resume their flat morphology. These results suggest that PGE_2 treatment can modify Wnt-induced proliferation behaviour such as split percentage. Following treatment with either H89 or Wort, cells returned to a split percentage of 0% as seen with WntA only treatment (Fig. 3-4C, -4D). This again indicates that PGE_2 likely acts on the Wnt pathway through PKA and PI-3K to modify cell proliferation.

To further confirm our results of the cell splitting behaviour, we measured the level of Phospho-Histone H3 (Ser10) (Fig. 3-5) since phosphorylation at Ser10 is tightly associated with chromosome condensation and segregation that occurs during mitosis (Liokatis et al., 2012, Nowak and Corces, 2004, Goto et al., 1999). Compared to untreated cells, PGE_2 only-treated cells did not display a significant difference. However, when compared to untreated NE-4C cells,

cells treated with WntA, WntA + PGE_2 and WntA + PGE_2 with H89 or Wort treatment led to a significance increase in Phospho-Histone H3 (Ser10) expression. RQ values were 1.35 (p = 0.033), 1.52 (p = 0.001), 1.36 (p = 0.027), and 1.58 (p = 0.005), respectively. This revealed that although cell numbers were lower under these conditions, the relative expression of Phospho-Histone H3 (Ser10) was significantly higher, indicating that a greater percentage of cells were undergoing mitosis when exposed to these treatments compared to untreated cells. This correlates with our finding that a larger proportion of cells under these conditions adopts and seems to be arrested in a round stage characteristic of cells undergoing mitosis.

Prostaglandin E_2 increases active β-catenin expression in Wnt-induced NE-4C cells

β-catenin is a key effector in the canonical Wnt signalling pathway that regulates downstream gene transcription (Cadigan and Nusse, 1997). β-catenin levels can be intricately regulated at multiple phosphorylation sites. Phosphorylation at Ser33, Ser37, and Thr41 leads to its destabilization and primes it for degradation (Wu and He, 2006), while phosphorylation at Ser552 has been correlated with β-catenin nuclear accumulation (Fang et al., 2007, He et al., 2007). We tested the levels of non-phospho-(the active form) β-catenin (Ser33/37/Thr41) and phospho-β-catenin (Ser552). The addition of PGE_2 only to NE-4C cells did not significantly change the levels of either form of β-catenin (Fig. 3-6A and -6B). However, adding PGE_2 to WntA-induced NE-4C cells lead to a significant 2.1 fold increase in non-phospho-(active) β-catenin (Ser33/37/ Thr41) levels compared to the WntA only treated condition (Fig. 3-7A). There was no significant difference in Phospho-β-catenin (Ser552) levels between the sample conditions (Fig. 3-7B), suggesting that phosphorylation of β-catenin at Ser552 is likely not involved with the behavioural differences in NE-4C cells described earlier. These results indicate

that PGE_2 may interact with the canonical Wnt signalling pathway by regulation of non-phospho- (active) β-catenin (Ser33/37/Thr41) levels.

Prostaglandin E_2 regulates expression of Wnt-target genes in Wnt-induced NE-4C cells

To investigate whether the addition of PGE_2 can influence gene transcription relevant to the canonical Wnt pathway, we screened 29 target genes using Custom TaqMan® Array Plates. We found that *Ctnnb1*, *Ptgs2*, *Ccnd1*, and *Mmp9* were differentially regulated (data not shown). Their expression was confirmed with real-time PCR using RNA derived from the same treatment conditions used for behavioural analyses, which includes 1 μM PGE_2, 2 μM Wnt Agonist (WntA), or 2 μM WntA with the addition of 1 μM PGE_2. Kinase blockers (H89 or Wort) were added to PGE_2 + WntA treated cells to determine the potential contribution of PKA and PI3K activity via PGE_2 signalling. Our real-time PCR results indicate that PGE_2 affects the expression levels of all Wnt-target genes tested (Fig. 3-8).

Ctnnb1 (beta-catenin) levels were not altered with the addition of PGE_2 when compared to untreated NE-4C cells, but cells treated with WntA showed a significant increase of RQ value 1.25 ($p = 0.0372$). Addition of PGE_2 to WntA-induced cells led to a further increase of *Ctnnb1* level to an RQ value of 1.55, which was significantly different from the WntA-only condition ($p = 0.0131$). This pattern was consistent with the expression of phospho (active) β-catenin (Ser33/37/Thr41) protein quantified earlier using Western blot analysis. Addition of H89 or Wort to PGE_2 + WntA treated cells resulted in RQ values to 0.83 and 0.60, respectively, compared to untreated cells which was a significant decrease compared to the PGE_2 + WntA condition ($p < 0.001$, $p < 0.001$). The PKA and PI3K blockers, H89 and Wort, appeared to remove the effect of PGE_2 on *Ctnnb1* expression in WntA-induced cells, while also reversing the influence on

Ctnnb1 levels from WntA-only treatment. This suggests that PKA and PI3K signalling may modify *Ctnnb1* expression through PGE_2 signalling.

NE-4C cells treated with PGE_2 alone had a significant decrease in *Ptgs2* (prostaglandin-endoperoxide synthase 2; gene encoding COX-2) mRNA levels compared to untreated cells (RQ = 0.56, $p < 0.001$), while cells treated with WntA had a significant increase of RQ value 2.99 ($p = 0.00286$). In contrast, when PGE_2 was added to WntA-induced NE-4C cells, *Ptgs2* expression was further elevated with an RQ value of 4.59 compared to untreated. This value was significantly different from the PGE_2 + WntA condition ($p = 0.015$). Addition of H89 or Wort to PGE_2 + WntA treated cells resulted in RQ values of 2.16 and 4.22, but only the H89 treatment was significantly different from the PGE_2 + WntA condition ($p < 0.001$). This suggests that the effect of PGE_2 on WntA-induced cells may be through PKA.

Expression of *Ccnd1* (cyclin D1) was also altered. Administration of PGE_2 treatment to NE-4C cells correlated with a significant increase of an RQ value to 3.68 ($p = 0.045$) compared to untreated cells, while WntA-treated cells had a significant increase of RQ value to 1.50 ($p = 0.048$). Addition of PGE_2 to WntA-activated cells was associated with a further increase of *Ccnd1* expression, with an RQ value 1.99 compared to untreated cells, which was significantly different from WntA-only treated cells ($p = 0.047$). H89 or Wort added to PGE_2 + WntA treated cells had RQ values of 0.74 and 1.42, respectively, which was significantly different from the PGE_2 + WntA condition ($p = 0.0054$, $p = 0.0078$). The blockers, H89 and Wort, seemed to attenuate the increase of *Ccnd1* levels associated with the addition of PGE_2 to WntA-induced cells.

In comparison to untreated NE-4C cells, PGE_2 treatment did not change levels of *Mmp9* (matrix metalloproteinase 9). However, when compared to WntA-induced NE-4C cells, addition

of PGE_2 treatment to WntA-treated cells caused a significant increase in expression level ($p < 0.001$). Specifically, with WntA treatment, *Mmp9* expression was significantly elevated to an RQ value of 2.19 ($p < 0.001$) compared to untreated cells, but addition of PGE_2 to WntA-induced cells resulted in a further rise of *Mmp9* expression with an RQ value of 3.00. H89 and Wort were added to PGE_2 + WntA treated cells and RQ values for *Mmp9* were 2.16 and 2.68, respectively, compared to the untreated condition. These values were significantly different from the PGE_2 + WntA condition. This indicates that the use of H89 and Wort diminished the increase in *Mmp9* expression as a result of PGE_2 treatment on WntA-induced cells.

Overall, these results demonstrate that PGE_2 can raise the expression of Wnt-target genes, specifically, *Ctnnb1*, *Ptgs2*, *Ccnd1*, and *Mmp9*, in WntA-induced NE-4C cells. Since H89 and Wort attenuated the changes caused by PGE_2, PKA and PI3K likely serve as a molecular link for the interaction between the PGE_2 and canonical Wnt signalling pathways.

3.5. Discussion

Cell migration and proliferation are crucial components of neural development. Previous studies have shown that elevated levels of PGE_2 can result in increased cell motility and proliferation in various non-neuronal cells (Aso et al., 2013, Bai et al., 2009, Yen et al., 2008, Sheng et al., 2001). Recent evidence indicates that abnormalities in cell behaviour can result from the interaction between PGE_2 with Wnt signalling pathways (Oshima and Oshima, 2013, Castellone et al., 2005). Our current study provides evidence, for the first time, for the cross-talk between these two pathways in neural stem cells. We report that PGE_2 treatment elicits changes in cell behaviour such as an increase in components of cell motility and proliferation, as well as expression of Wnt-target genes, in Wnt-activated NE-4C stem cells. Moreover, the stimulatory

effects of PGE_2 are subdued through the inhibition of downstream pathway kinases, PKA and PI-3K, suggesting that PGE_2 acts through these particular kinases to converge with the Wnt pathway.

Previous studies have shown that PGE_2 can increase or decrease the activity of canonical Wnt signalling. PGE_2 activates several components of the canonical Wnt pathway in colorectal cancer cells (Buchanan and DuBois, 2006). Specifically in these cells, PGE_2 stimulated a significant increase in the activity of Wnt transcription factors, T cell factor-4 (Tcf-4), as well as elevated protein levels of Wnt-target genes (Shao et al., 2005). PGE_2 acted through its EP2 receptor to modulate β-catenin activity of the Wnt pathway, promoting the growth of colon cancer cells (Castellone et al., 2005). Wnt activation induced by PGE_2 also contributed to abnormal proliferation resulting in enhanced gastric tumorigenesis (Oshima and Oshima, 2013). Furthermore, PGE_2–regulated Wnt signalling had a hepatoprotective effect, aiding in liver regeneration (North et al., 2010). In pre-osteoblastic cells, concentration-dependent treatment of PGE_2 modulated Wnt signalling by altering protein expression of pathway activators, β-catenin and low-density lipoprotein receptor-related protein 5/6 (LRP 5/6), as well as Wnt inhibitor, dickkopf-1 (DKK-1); low doses of PGE_2 promoted the Wnt pathway while high doses inhibited it (Liu et al., 2010). PGE_2 also modified Tcf-luciferase activity of Wnt signalling through the same dose effect (Liu et al., 2010). Additionally, in human colorectal adenoma and carcinoma cells, PGE_2 treatment upregulated the protein expression of the Wnt target gene, leucine-rich G-protein coupled receptor 5 (LGR5), which internalizes FZD co-receptor LRP6 and decreases Wnt activity (Al-Kharusi et al., 2013).

Altogether, these studies reveal that the interaction between PGE_2 and Wnt signalling can have different effects depending on the dose of PGE_2 administered and the specific cell type.

We reveal that PGE_2 increases the final distance and path length travelled, as well as the average speed of migration in Wnt-activated neuroectodermal stem NE-4C cells. We also show that PGE_2 alters the phenotype of Wnt-treated cells, which corresponds to an increase in split percentage. Aberrations in cell motility and proliferation behaviour could have detrimental effects to early development of the nervous system. This is because proper neural development requires an orchestrated system of cellular events, such as migration and proliferation, to occur over particular windows of time (Stiles and Jernigan, 2010). Careful control of these crucial neurobiological processes during prenatal development is required for the formation of complex layered structures in the brain like the cerebral cortex, hippocampus, and cerebellum (Evsyukova et al., 2013, Hatten, 1999).

Our study adds to the current body of research by showing that PGE_2 interferes with the Wnt pathway by attenuating Wnt-dependent cell behaviour in NE-4C cells. This is important because Wnt signalling is involved in a myriad of regulatory processes important for the development and organization of the nervous system (Ille and Sommer, 2005). It is thoroughly established that Wnt signalling is instrumental to normal anterior-posterior patterning of the embryo (Arkell et al., 2013). Wnt proteins are key regulators for the formation of the neural tube, as well as neuronal migration and differentiation (Ciani and Salinas, 2005, Ille and Sommer, 2005). Wnt signalling also modulates neurite outgrowth (Lu et al., 2004), axon growth and guidance (Bovolenta et al., 2006, Sanchez-Camacho et al., 2005, Zou, 2004, Lyuksyutova et al., 2003), dendritic development and arborization (Wayman et al., 2006, Rosso et al., 2005), radial migration (Zhou et al., 2004), and synapse formation and plasticity (Gogolla et al., 2009, Chen et al., 2006). Moreover, Wnt signalling is crucial in neuronal fate determination, particularly in the specification and differentiation of neuronal precursors in the midbrain (Prakash et al., 2006) and

forebrain (Zhou et al., 2006, Hirabayashi and Gotoh, 2005). Furthermore, epithelial stem cells require Wnt/β-catenin signalling for proliferation and quiescent division (Lowry et al., 2005) and the balance between re-entry and exit of the cell cycle can be altered by Wnt/β-catenin signalling (Chenn and Walsh, 2002). Additionally, aberrant cortical progenitor cell proliferation patterns and defective hippocampus development can result due to abnormal Wnt signalling (Lee et al., 2000). Interestingly, recent findings provide evidence that defective Wnt signalling could contribute to the pathogenesis of psychiatric disorders like schizophrenia and ASDs (Kalkman, 2012, Okerlund and Cheyette, 2011, Cotter et al., 1998). Specifically, *Wnt2*, located in the putative speech and language region at chromosome 7q31-33, has been identified as a susceptibility gene for autism. (Lin et al., 2012, Wassink et al., 2001). Given the importance of Wnt signalling in prenatal development and the existing interaction between Wnt and PGE_2 pathways in NE-4C stem cells, alterations in levels of PGE_2 via endogenous and exogenous means may have profound effects on nervous system development.

In addition to quantifying cell behaviour, we also demonstrate that PGE_2 can affect the expression of non-phospho (active) β-catenin (Ser33/37/Thr41). Wnt/β-catenin signalling occurs through a complex, highly regulated pathway that involves the phosphorylation of multiple sites on β-catenin, which may promote its degradation or activation and subsequent nuclear internalization. For instance, the phosphorylation of sites Ser33, 37, and Thr41 targets β-catenin for ubiquitination and proteasomal degradation (Kimelman and Xu, 2006, Liu et al., 2002). Quantification of β-catenin that is non-phosphorylated at these sites has become a common measurement for active or stabilized β-catenin expression. Phosphorylation of β-catenin at the site Ser552 has also been correlated with increased β-catenin/TCF mediated transcriptional activity (Zhao et al., 2010, Taurin et al., 2006). We found that PGE_2 treatment administered to

Wnt-activated cells increased the expression of non-phospho (active) β-catenin (Ser33/37/Thr41) protein. In contrast, the phospho-β-catenin (Ser552) levels remained unchanged. It has been established that the regulation of glycogen synthase kinase 3 beta (GSK3β) activity may control stabilization of β-catenin and increased levels of non-phospho (active) β-catenin (Ser33/37/Thr41) protein (Gao et al., 2014). It is possible that PGE_2 signalling may modify GSK3β activity but this remains to be determined. Nonetheless, the increased levels of non-phospho (active) β-catenin (Ser33/37/Thr41) quantified were in line with our gene expression results that also showed an increase in *Ctnnb1* expression as well as other Wnt-target genes. *Ctnnb1* encodes for the β-catenin protein, which can regulate cell growth and adhesion and is also a key downstream component of the canonical Wnt pathway. It has also been shown to regulate cortical size; enlarged cortices with increased cortical folds were observed in *Ctnnb1* transgenic mice (Chenn and Walsh, 2002). Interestingly, brain overgrowth and abnormal excess in number of neurons was measured in children with autism (Courchesne et al., 2011). Gene expression of *Ctnnb1* was altered in both young and adult autistic cases (Chow et al., 2012). Furthermore, de novo mutations of this gene and its relevant network have been ranked significantly as potential autism candidate genes (Krumm et al., 2014, O'Roak et al., 2012).

Within the canonical Wnt pathway, the β-catenin/TCF complex can promote the transcription of target genes including *Ptgs2* (Nunez et al., 2011), *Ccnd1* (Klein and Assoian, 2008, Shtutman et al., 1999), and *Mmp9* (Ingraham et al., 2011, Wu et al., 2007). Expression of these genes was increased as an effect of elevated PGE_2 signalling in our study, and interestingly, previous studies have reported a link between these genes and ASDs as described below. *Ptgs2*, also known as COX-2, is the key enzyme in prostaglandin biosynthesis, including the production of PGE_2. COX-2 is a crucial mediator of inflammation and prostanoid signalling

(Chen, 2010, Seibert and Masferrer, 1994). Polymorphism of *Ptgs2* has been associated with ASDs (Yoo et al., 2008). A recent clinical study proved the efficacy of a COX-2 inhibitor drug, celecoxib, as an adjunctive therapy in the treatment of autism: the treatment was superior for treating irritability, social withdrawal, and stereotypy of children with autism (Asadabadi et al., 2013).

Another gene affected was *Ccnd1*. This gene encodes for a protein in the cyclin family, which are important regulators in cell cycle progression, transcription, and neuronal function (Lim and Kaldis, 2013, Bloom and Cross, 2007). The increased levels of *Ccnd1*, as a result of added PGE_2, may be involved with the altered proliferation behaviour visualized in this study. Aberrant *Ccnd1* levels have also been associated with ASDs. In autistic rat pups (model encompassed administration of valproic acid), *Ccnd1* expression was atypical in the cerebellum compared to controls (Kim et al., 2013). Another study showed that the dysregulation of *Ccnd1* lead to abnormal cell cycle and proliferation, neuronal and network excitability and behaviour, and revealed its potential link to human neuro-cardio-facial-cutaneous and related syndromes, which are associated with developmental abnormalities, cognitive deficits, and autism (Pucilowska et al., 2012). Diminished expression of 22q11 genes, which disrupts cortical neurogenesis and cell migration, led to alterations in *Ccnd1* levels (Meechan et al., 2009). The authors explain that a developmental disruption, as such, may alter cortical circuitry and establish vulnerability for developmental disorders, including schizophrenia and autism.

Mmp9 is a membrane of the matrix metalloproteinase (MMP) family, which can target many extracellular proteins including proteases, growth factors, and adhesion molecules (McCawley and Matrisian, 2001) and are involved with the breakdown of the extracellular matrix in normal physiological processes such as embryonic development and

tissue remodelling (Vu and Werb, 2000). MMPs are also important in neuronal development, plasticity, and maintenance of neuronal health (Fujioka et al., 2012). Mmp9 has also been shown to regulate the proliferation and migration of embryonic neural stem cells (Ingraham et al., 2011) and participate in neuronal differentiation by regulating neurite elongation and neuronal cell migration (Shubayev and Myers, 2004, Chambaut-Guerin et al., 2000, Ferguson and Muir, 2000). Therefore, altered *Mmp9* expression may contribute to the deviant behaviour observed in our study. *Mmp9* has also been associated with ASDs (Abdallah and Michel, 2013). Elevated levels of MMP9 protein were found in the amniotic fluid of ASD cases compared to controls (Abdallah et al., 2012). Findings from this study provide evidence that molecular and physiological abnormalities in ASDs may begin prenatally. *Mmp9* has also been implicated in Fragile X syndrome (FXS) (Janusz et al., 2013), which is characterized by behaviours at the extreme of the autistic spectrum. Using a mouse model of fragile x (Fmr1 KO), levels of MMP9 was found to be elevated in the hippocampus of Fmr1 KO mice (Bilousova et al., 2009). Furthermore, Minocycline, a drug that inhibits MMP9 activity, has been shown to promote dendrite spine maturation and improve behavioural performance in Fmr1 KO mice (Bilousova et al., 2009). These researchers continued their work in human trials and found that Minocycline taken as a daily dose for 8 weeks led to behavioural improvements in FXS patients. This was consistent with their fmr1 KO mouse model results, indicating that MMP9 activity alters underlying neural defects that contribute to behavioural abnormalities seen in ASDs (Paribello et al., 2010).

Taken altogether, our gene expression results not only show a potential interaction of the PGE_2 and canonical Wnt pathway in the nervous system, but also provide further evidence for a link to ASDs.

We show that PGE_2 interacts with canonical Wnt signalling through PKA and PI-3K to produce the reported behavioural changes in cell motility and proliferation, as well as gene expression. Specifically, we found that inhibiting these PGE_2 downstream pathway kinases, PKA and PI-3K with H89 and Wort respectively, reduced the effect of PGE_2. This is in line with previous literature, which found that the convergence of PGE_2-dependent effects and the Wnt pathway can occur through the stimulation of PKA or PI-3K in embryonic kidney cells and colon cancer cells (Castellone et al., 2005, Fujino et al., 2002, Sheng et al., 2001). Moreover, similar stimulatory effects on cell migration induced by PGE_2 in Wnt-activated NE-4C cells from our study were also exhibited in prostate cancer cells through the activation of PI-3K (Vo et al., 2013). Our results revealed that H89 had a stronger effect than Wort, suggesting that PGE_2 may predominately act through PKA; but future studies are needed to determine which EP receptors are involved. A proposed model is provided in Fig. 3-9.

Increasing evidence for the contribution of environmental factors in the etiology of neurodevelopmental disorders like ASDs has prompted urgency to reveal their potential exogenous causes and underlying mechanisms (Landrigan et al., 2012). Environmental factors like exposure to drugs, toxins or infectious agents cause disruptions in PGE_2 signalling by increasing the levels of oxidative stress, consequent lipid peroxidation, and the immunological response; these factors and consequences that disturb normal PGE_2 signalling have all been linked to ASDs (Ecker et al., 2013). We show that increased PGE_2 signalling can modify cell migration, proliferation behaviour, and gene expression in Wnt-activated NE-4C stem cells. Aberrant cell migration and proliferation are pathophysiologic mechanisms that impact the brain broadly, and could be possible factors that contribute to

the origination of neurodevelopment disorders. Abnormalities in structure, organization, and connectivity of the brain are all indicators of irregular neural cell migration and proliferation. Local distortions in neural cytoarchitecture, dysplasia, and hypoplasia have been described in brains of autistic subjects (Wegiel et al., 2010). Moreover, structural abnormalities and atypical connectivity of the brain in ASDs have been identified by a number of research groups (Abrams et al., 2013, Aoki et al., 2013, Ecker et al., 2013, Minshew and Williams, 2007, Belmonte et al., 2004). Noteworthy, areas of the brain that would be most impacted by dysregulation in neuronal migration and proliferation— that is the cerebellum, cerebral cortex, and hippocampus— are also implicated in ASDs (Penzes et al., 2013, Cauda et al., 2011, Wegiel et al., 2010, Hashimoto et al., 1995). Despite the assumptions that can be made from our in vitro results, in vivo models must be employed to further describe the possible effects of PGE_2 and its interaction with morphogenic signalling pathways, such as Wnt, during prenatal development.

3.6. Conclusions

PGE_2 is an important lipid signalling molecule and its interaction with the Wnt signalling pathway could have significant effects on prenatal development. We showed that PGE_2 can affect Wnt-dependent cell behaviours and gene expression in neuroectodermal stem cells through PKA and PI-3K. Aberrant PGE_2 and Wnt signalling have been linked to ASDs, and altered migration and proliferation due to irregular gene expression during embryonic development in ASDs have been suggested in previous studies. Our *in vitro* study provided further evidence that these aberrations may be potential mechanisms in the genesis of neurodevelopmental disorders like ASDs.

3.7. Figures

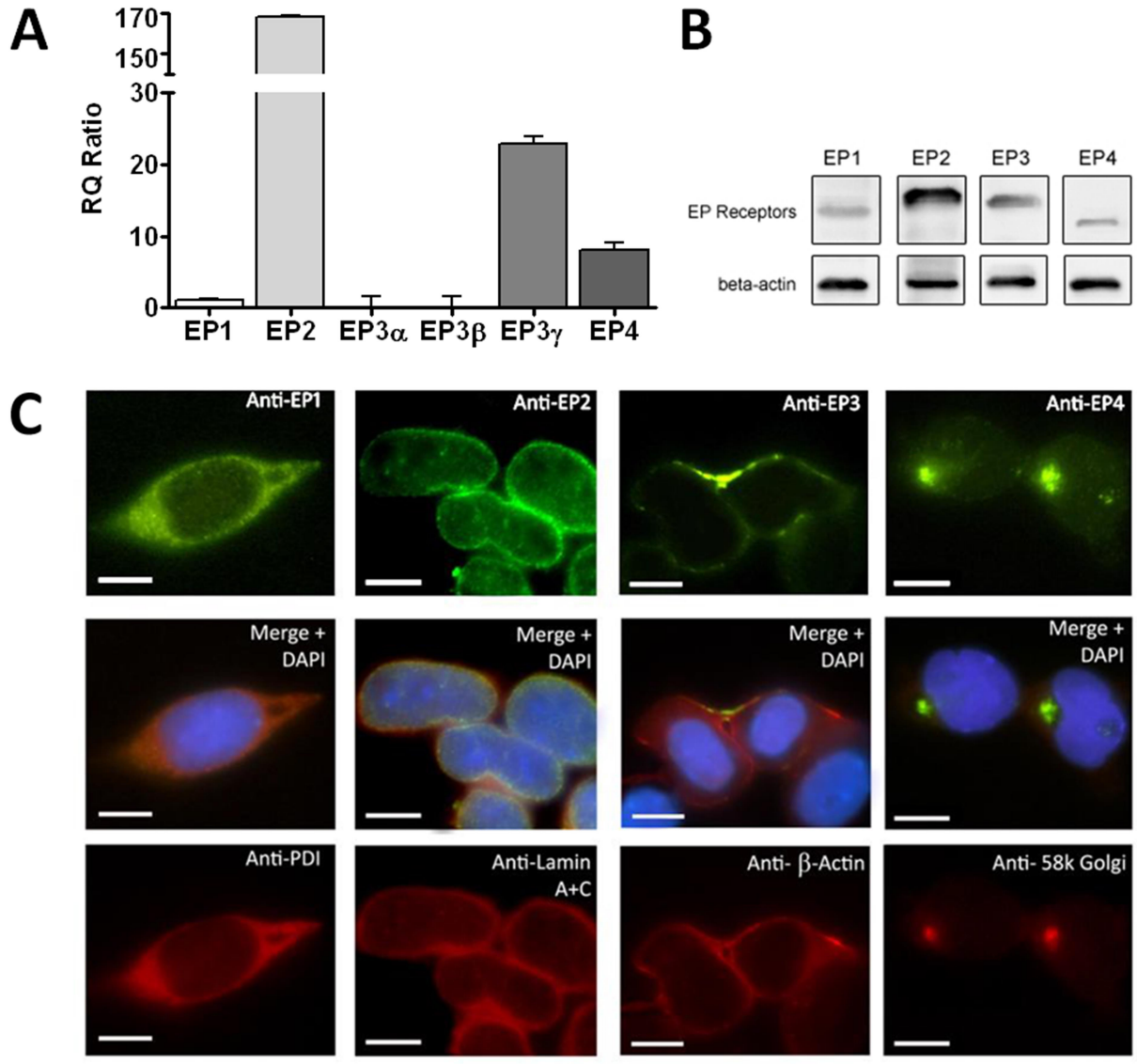

Fig. 3-1: Expression of EP receptors' mRNA and protein in NE-4C cells.
(A) Real-time PCR was used to determine the RQ value for *EP1*, *EP2*, *EP3α*, *EP3β*, *EP3γ* and *EP4* receptors, which was found to be 2, 16, 1, 2, 46 and 46 respectively. The error bars represent + SEM. (B) Western blot analysis of the EP1, EP2, EP3 and EP4 receptors expression (65, 68, 62 and 53 kDa, respectively). β-Actin was used to indicate equal loading. (C) Immunocytochemistry revealed the subcellular localization of EP1-4 receptors with specific organelles visualized through the use of anti-PDI endoplasmic reticulum marker, anti-Lamin A + C nuclear envelope marker, β-Actin cell membrane marker, and anti-58 K Golgi marker. The scale bar represents 10 μm

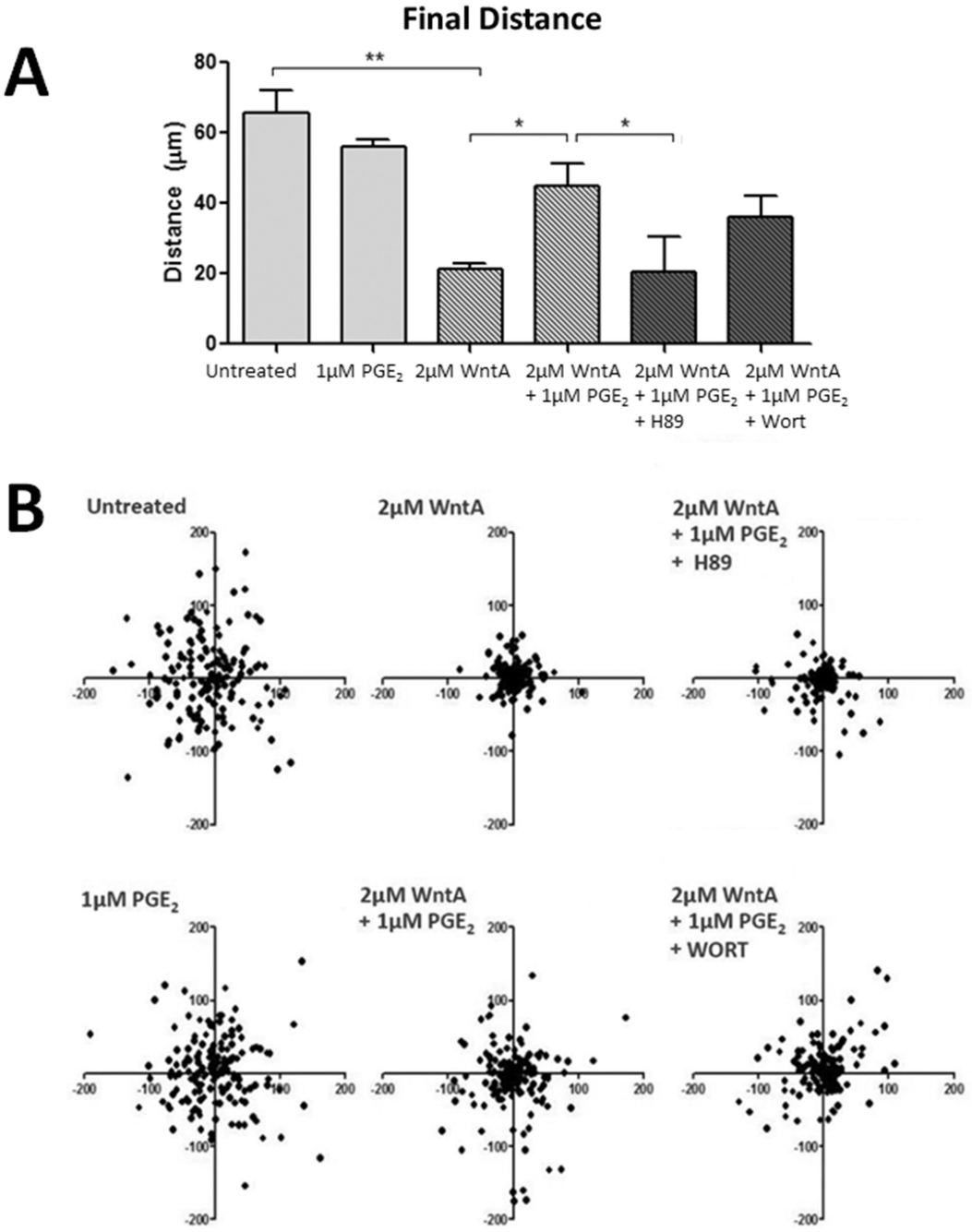

Fig. 3-2: PGE_2 -dependent effect on final distance travelled from origin.
(A) Final distance from origin was 65.6, 56.2, 21.3, 45.0 µm, respectively. The error bars represent + SEM and values were considered significant at *p<0.05, **p<0.01. (B) The Dispersion XY position plots illustrate the effect of PGE_2 on Wnt-induced behaviour, where addition of PGE_2 to Wnt-activated cells increased the final distance. Addition of H89 (PKA blocker) and Wort (PI-3K) blocker reduces the effect PGE_2. Measurements represent an average of 150 cells from three independent experiments (N = 3).

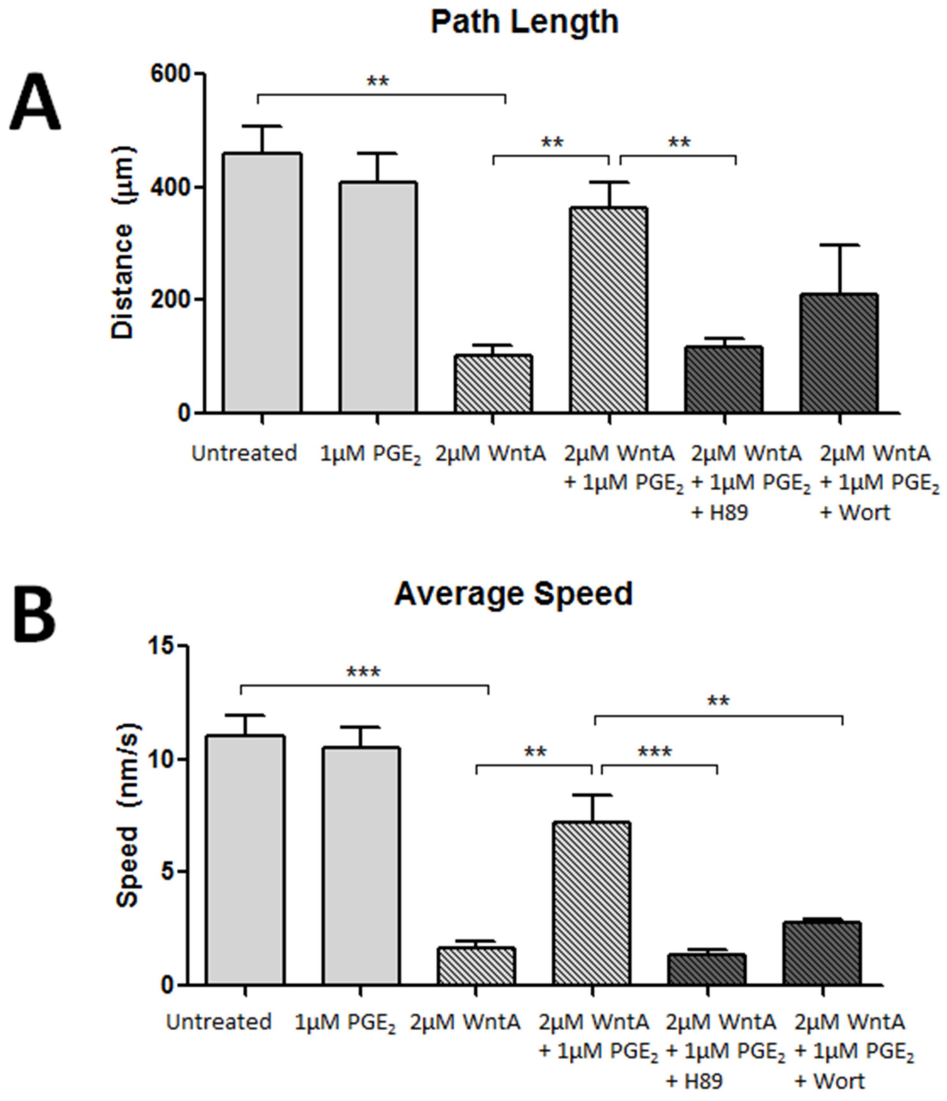

Fig. 3-3: PGE_2 -dependent effect on path length and average speed.
(A) Path length travelled was 459, 409, 103, 362 µm, respectively. (B) Average speed of migration was 11.0, 10.5, 1.7, 7.2 nm/s, respectively. The error bars represent + SEM, $^{**}p< 0.01$, $^{***}p<0.001$. Results represent an average of 150 cells from three independent experiments (N = 3).

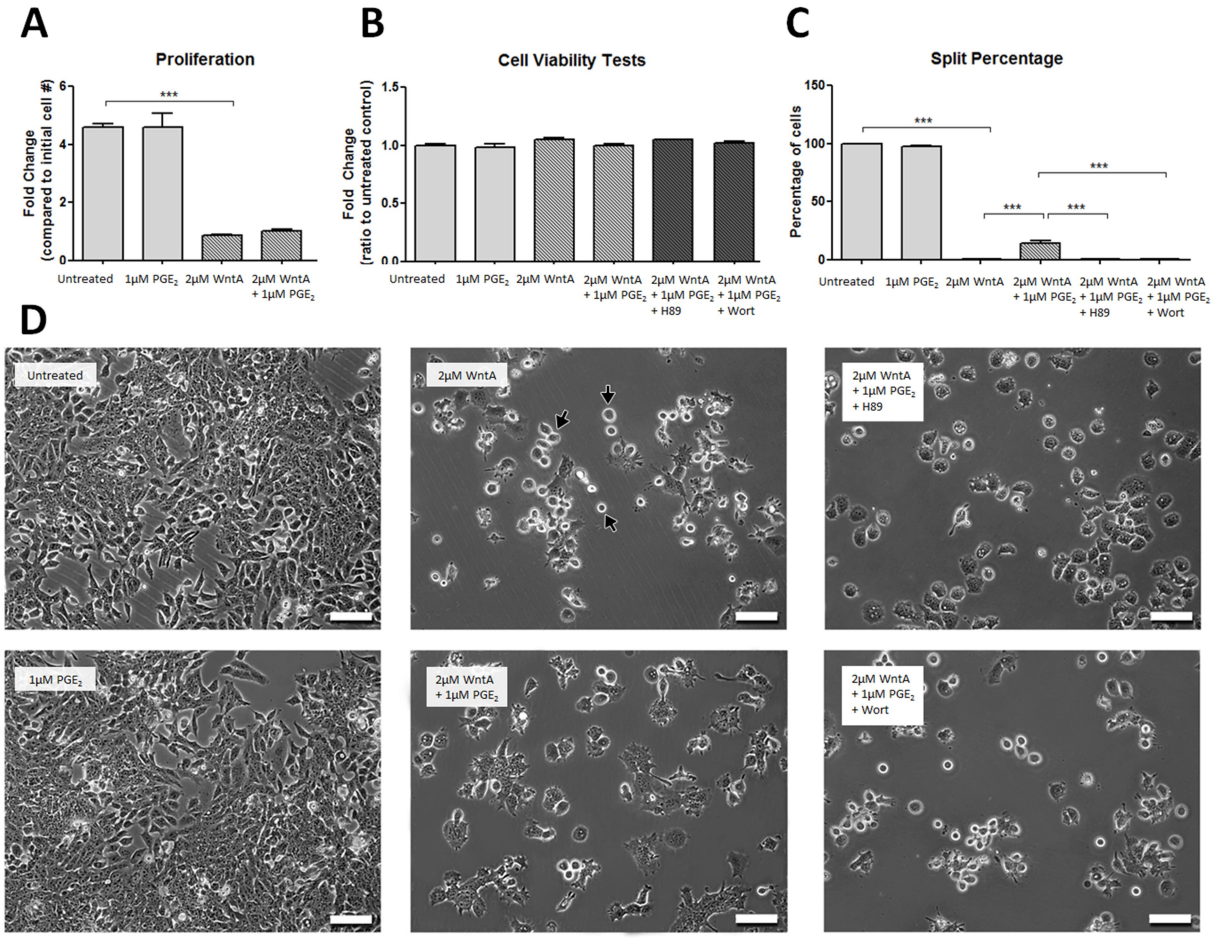

Fig. 3-4: PGE_2 -dependent effect on proliferation behaviour.
(A) Over the experimental duration of 24 hours, the number of cells changed by a fold of 4.60, 4.59, 0.86, 1.03, respectively. (B) Cell viability across treatment conditions was not significantly different. (C) Percentage of successful split ratio was 100%, 98%, 0%, 15%, 0%, and 0% respectively. The error bars represent + SEM, ***$p< 0.001$. Measurements represent an average of 150 cells from three independent experiments ($N = 3$). (D) WntA treatment resulted in an arrested state indicted by the black arrows and corresponded with a significant decrease in cell proliferation (***$p< 0.001$). Scale bar represents 100 μm.

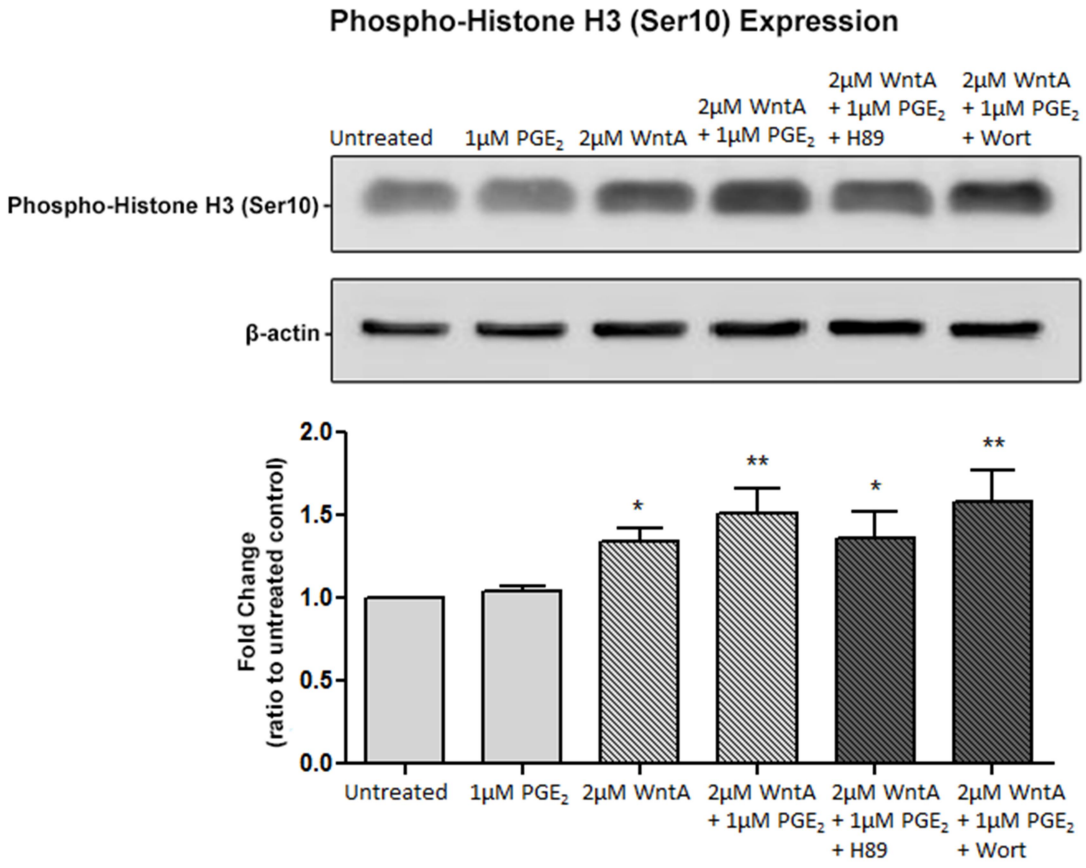

Fig. 3-5 : PGE_2 -dependent effect on phospho-histone H3 (Ser10) expression.
Western blot analysis was used to determine Phospho-Histone H3 (Ser10) protein (17 kDa). The expression of Phospho-Histone H3 (Ser10) represented in fold change was 1, 1.04, 1.35, 1.52, 1.36, and 1.58, respectively. The error bars represent + SEM and values were considered significantly different from untreated *p<0.05, **p<0.01. Average measurements represent protein from three independent experiments (N = 3). β-Actin was used to indicate equal loading.

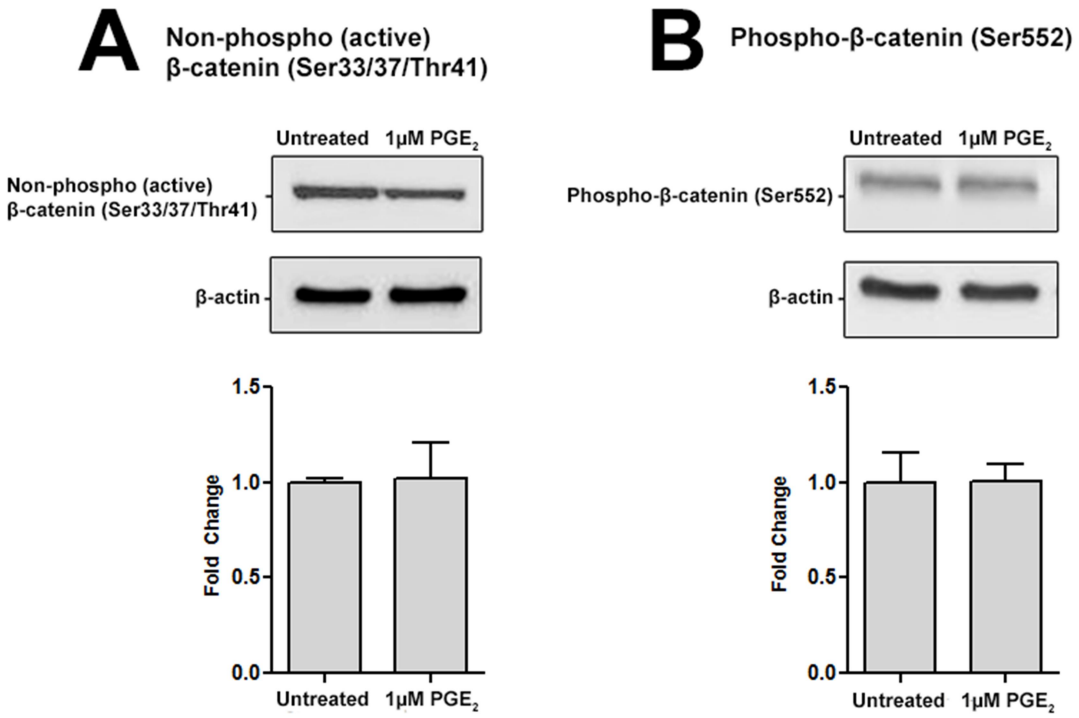

Fig. 3-6: PGE_2 -dependent effect on β-catenin expression in NE-4C cells.
Western blot analysis was used to determine two forms of active β-catenin: (A) non-phospho-(active) β-catenin (Ser33/37/Thr41) and (B) phospho-β-catenin (Ser552) (92 kDa). Addition of PGE_2 to NE-4C cells did not yield a significant difference in levels of either active form of β-catenin compared to control. The error bars represent + SEM and values were considered significantly different from control at $*p<0.05$, $**p<0.01$. Average measurements represent protein from three independent experiments ($N = 3$). β-Actin was used to indicate equal loading.

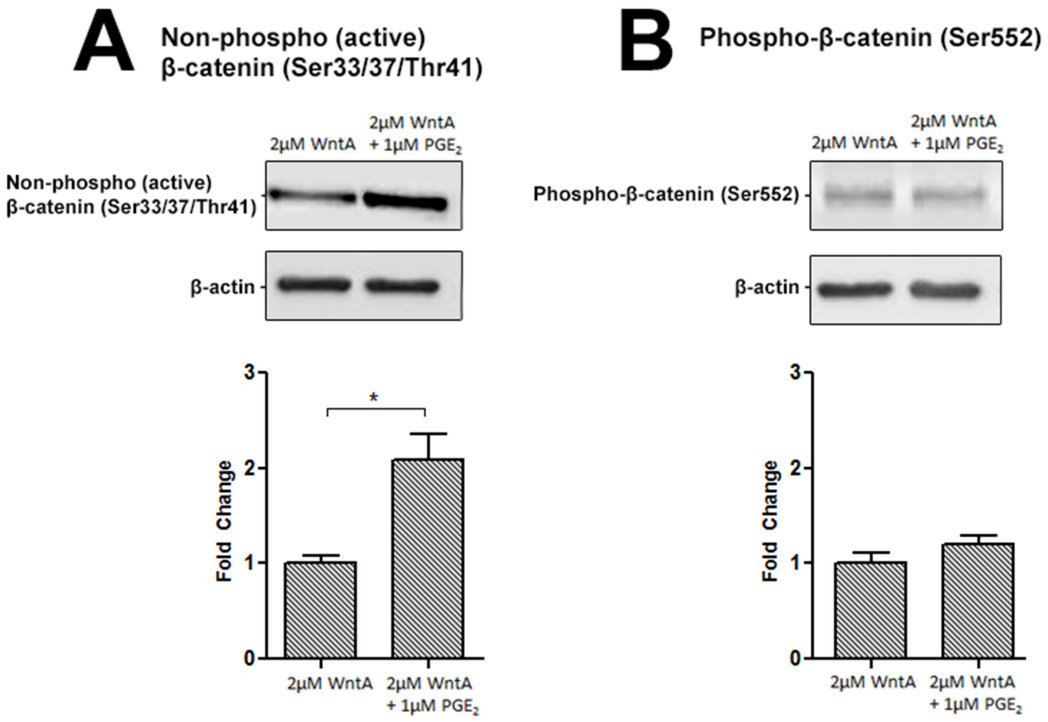

Fig. 3-7: PGE_2 -dependent effect on β-catenin expression in Wnt-activated NE-4C cells. Western blot analysis was used to determine two forms of active β-catenin: non-phospho-(active) β-catenin (Ser33/37/Thr41) and phospho-β-catenin (Ser552) (92 kDa). (A) The expression of active β-catenin represented in fold change was 1, 2.09, 1.61, and 1.98, respectively. The error bars represent + SEM and values were considered significantly different from control at $*p<0.05$. Only PGE_2 +WntA condition was significantly different from WntA only condition. (B) There was no significant difference in phospho-β-catenin (Ser552) expression between the conditions. Average measurements represent protein from three independent experiments ($N = 3$). β-Actin was used to indicate equal loading.

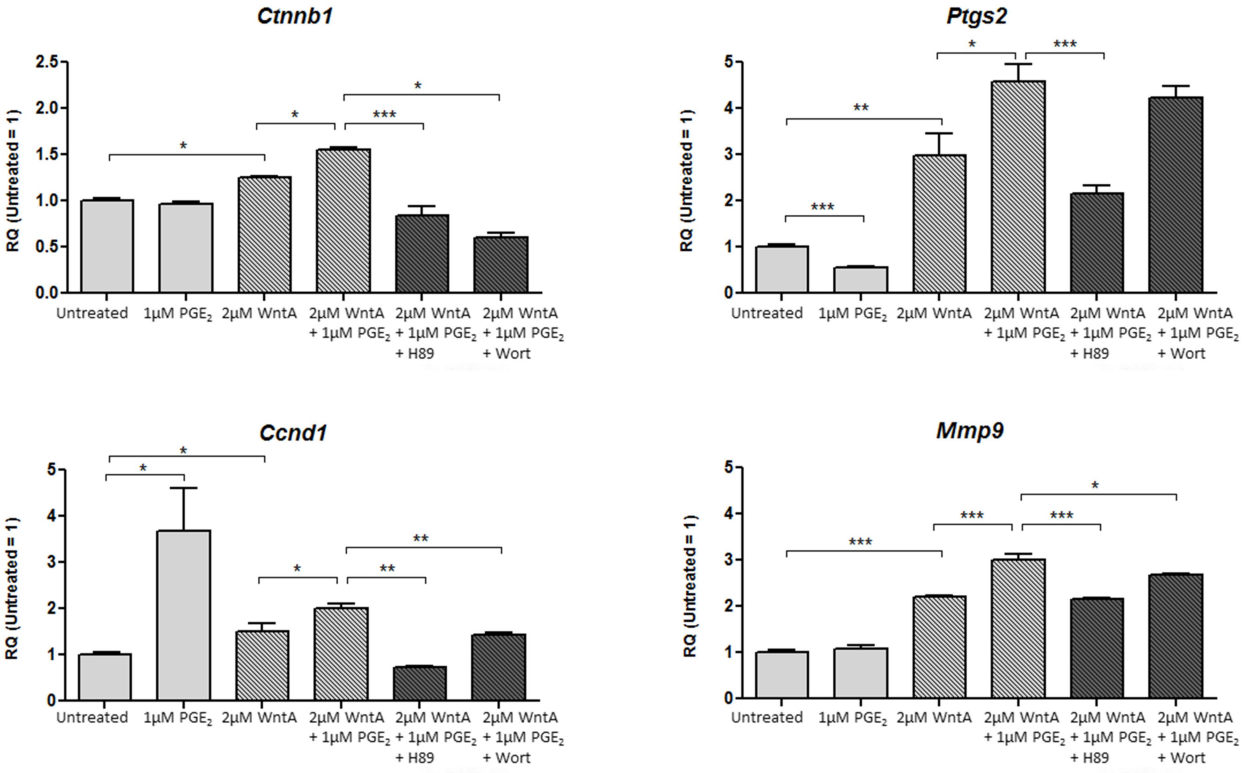

Fig. 3-8: PGE_2 -dependent effect on Wnt-target genes.
Real-time PCR was used to determine the RQ value for *Ctnnb1, Ptgs2, Ccnd1*, and *Mmp9*. The expression of *Ctnnb1* represented in fold change was 1, 0.97, 1.25, 1.55, 0.84, and 0.60, respectively. The fold change expression of *Ptgs2* was 1, 0.56, 2.99, 4.59, 2.16, and 4.22. The fold change expression of *Ccnd1* was 1, 3.68, 1.50, 1.99, 0.74, and 1.42. *Mmp9* fold change expression was 1, 1.08, 2.19, 3.00, 2.16, and 2.68, respectively. The error bars represent + SEM and values were considered significantly different from control at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Average measurements are from three independent experiments ($N = 3$).

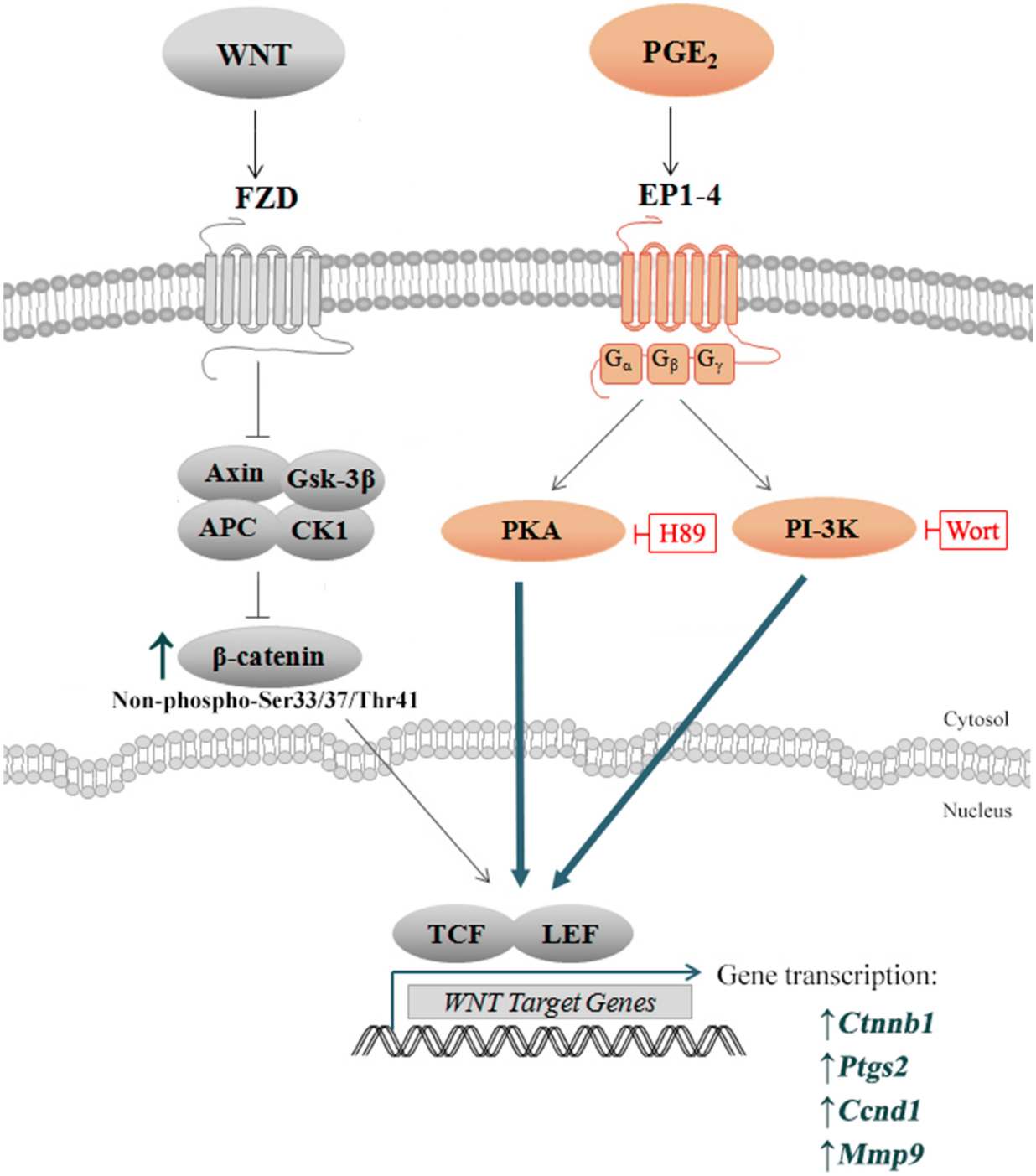

Fig. 3-9: A proposed model for PGE_2 -Wnt interactions in Wnt-induced NE-4C cells. From the compilation of our results (bolded) and other studies, a schematic model is drawn of the mechanism by which PGE_2 might interact with the canonical Wnt pathway.

3.8. References

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CHAPTER 4.

Study 2: Prostaglandin E2 promotes neural proliferation and differentiation and regulates Wnt target gene expression.

Manuscript 4 Citation (Copyright Permission in Appendix B.)**:**

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Contributions: Christine T. Wong designed and performed all experiments, collected samples, acquired and analyzed data, made all figures and tables, and prepared the manuscript. Netta Ussyshkin and Eizaaz Ahmad assisted in gene expression experiments. Ravneet Rai-Bhogal aided with collections. Dr. Hongyan Li provided technical support. Dr. Dorota A. Crawford supervised the study and edited the manuscript.

Objectives and Hypotheses: The second study was predominately completed *in vitro* with an *in vivo* component. The main purpose was to investigate whether an elevated level of PGE_2 affects the proliferation and differentiation of NE-4C stem cells into neurons. We aimed to determine if PGE_2 alters the formation of neural stem cell clusters (neurospheres), if the expression of genes involved in neuronal differentiation is affected, and if PGE_2 influences the expression of genes from the canonical Wnt signalling pathway. I hypothesized that PGE_2 would effect the differentiation of NE-4C cells by altering the progression of neurosphere formation, as well as their size and roundness. Moreover, I hypothesized that PGE_2 would change the expression of Wnt signalling regulator, β-catenin, as well as the expression of Wnt-target genes.

4.1. Chapter Summary

Prostaglandin E2 (PGE_2) is an endogenous lipid molecule that regulates important physiological functions including calcium signalling, neuronal plasticity, and immune responses. Exogenous factors such as diet, exposure to immunological agents, toxic chemicals, and drugs can influence PGE_2 levels in the developing brain and have been associated with autism disorders. The main goals of this study were to determine if changes in PGE_2 level can alter the behavior of undifferentiated and differentiating neuroectodermal (NE-4C) stem cells and whether PGE_2 signalling impinges with the Wnt/β-catenin pathway. We show that PGE_2 increases proliferation of undifferentiated NE-4C stem cells. PGE_2 also promotes the progression of NE-4C stem cell differentiation into neuronal lineage cells, which is apparent by accelerated appearance of neuronal clusters (neurospheres) and earlier expression of neuronal marker, *Mapt*. Furthermore, PGE_2 alters the expression of downstream Wnt-regulated genes previously associated with neurodevelopmental disorders. In undifferentiated stem cells, PGE_2 downregulates *Ptgs2* expression and upregulates *Mmp9* and *Ccnd1* expression. In differentiating neuronal cells, PGE_2 causes upregulation of *Wnt3*, *Tcf4*, and *Ccnd1*. The convergence of the PGE_2 and Wnt pathways is also apparent through increased expression of active β-catenin, a key signalling component of the Wnt/β-catenin pathway. The results of this study provide novel evidence that PGE_2 may influence progression of neuronal development and influence Wnt-target gene expression. We discuss how these findings could have potential implications for neurodevelopmental disorders such as autism.

4.2. Introduction

The human brain is highly composed of lipids and the availability and metabolism of these lipids are tightly integrated with healthy development, maintenance, and function of the nervous system (Lawrence, 2010). Prostaglandin E2 (PGE_2) is a bioactive lipid molecule derived from plasma membrane phospholipids through the enzymatic activity of phospholipase A2 and cyclooxygenase-1, -2 (COX-1, -2). PGE_2 binds to four E-prostanoid receptor subtypes (EP1, EP2, EP3, EP4) to regulate various functions in the developing nervous system including memory formation and synaptic plasticity (Furuyashiki and Narumiya, 2011), thermoregulation and immune response modulation (Lazarus, 2006), and neurotransmitter release (Bezzi et al. 1998). Furthermore, various studies have linked abnormalities in the PGE_2 pathway to neurodevelopmental disorders, such as Autism Spectrum Disorders (ASDs) (Wong and Crawford, 2014). Altered PGE_2 signalling due to environmental factors has been reported in many cases of ASDs (Landrigan, 2010, Tamiji and Crawford, 2010b). For example, exogenous stimuli that affect PGE_2 levels, such as immunological agents (Patterson, 2011), toxic chemicals (Schwartzer et al., 2013), and exposure to drugs (Arndt et al., 2005), have all been associated with ASDs.

Although the molecular mechanisms by which PGE_2 affects brain development are still not well understood, we have shown that an increased level of PGE_2 augments the migration of neuronal stem cells (Wong et al., 2014), retracts neuronal extensions (Tamiji and Crawford, 2010c) and elevates calcium levels in the cytosol and growth cones (Tamiji and Crawford, 2010a). Recent research shows emerging evidence for cross-talk between PGE_2 signalling and the major developmental pathway called Wingless-Type MMTV Integration Site Family (Wnt) pathway. Interestingly, the Wnt pathway has also been associated with ASDs (Kalkman 2012,

Zhang et al., 2014). Previous literature in different cell types has provided some evidence for the interaction of COX/PGE_2 signalling with the Wnt pathway via protein kinase A (PKA) and phosphoinositide 3-kinase (PI-3K) (Buchanan and DuBois, 2006, Evans, 2009, Wong et al., 2014). Wnt signalling plays a pivotal role in embryogenesis (Solis et al., 2013). Wnt ligands are present in specific concentration gradients and modulate expression of target genes that regulate cell proliferation, differentiation, and migration during development of the nervous system (Logan and Nusse, 2004).

In this study, our goal was to further determine if PGE_2 affects proliferation and neuronal differentiation of neuroectodermal (NE-4C) stem cells as well as the expression of important developmental genes. We found that PGE_2 increases the proliferation of NE-4C stem cells and accelerates their differentiation. We also determined that PGE_2 modifies the expression of downstream Wnt-regulated genes, including *Ptgs2*, *Mmp9*, *Ccnd1* in undifferentiated NE-4C stem cells and *Wnt3*, *Tcf4*, and *Ccnd1* in differentiating cells. We found that the expression of the common gene, *Ccnd1*, is also altered in the brain of C57BL/6 mouse offspring prenatally exposed to PGE_2. Finally, we examined potential implications of these findings for neuronal pathology and neurodevelopment, including the possible link to ASDs.

4.3. Materials and Methods

Cell Culture

Mouse NE-4C (ATCC CRL-2925) cells were grown in Gibco Minimal Essential Medium (MEM), supplemented with 10% Fetal Bovine Serum, 2mM L-glutamine, 1×Penicillin Streptomycin, and 1mM Sodium Pyruvate (Gibco). Cells were cultured according to ATCC culture method guidelines and maintained in an incubator at 37°C, 5% CO_2, 95% humidity. Cells were plated on 0.01% poly-L-lysine (Sigma) coated culture plates. Supplemented MEM was changed every 2 days.

Cell culture treatments of Undifferentiated NE-4C stem cells

NE-4C cells were dissociated with 0.05% trypsin-EDTA and resuspended in supplemented MEM described above. The cells were plated on poly-L-lysine (Sigma) coated 60 mm culture dishes (BD Falcon). The plated cells were then cultured in the supplemented MEM at 37°C and 5% CO_2 for 24 hours before treatment with 1μM prostaglandin E2 (PGE_2; Sigma), 10μM PGE_2, or an equivalent volume of vehicle. Cells were incubated in designated treatments for 24 hours before being lysed for collection.

Proliferation Assay

60mm cell culture plates were seeded with 4 x 10^6 cells and kept at 37°C and 5% CO_2 for 24 hours before addition of vehicle, 1μM PGE_2, or 10μM PGE_2 treatment. Following 24 hour incubation of treatment conditions, undifferentiated NE-4C stem cells were extracted and diluted with equal volumes of trypan blue dye (4%). Cell count averages were taken from a minimum of

four hemacytometer squares to determine the number of cells per treatment sample. Three independent experiments were completed for each treatment condition.

Cell Differentiation and PGE_2 treatment

NE-4C cells were dissociated with 0.05% trypsin-EDTA and plated onto poly-L-lysine (Sigma) pre-coated plates at $2.5\times10^3/cm^2$. Plated cells were cultured in supplemented MEM (as above) overnight before neuronal differentiation induction. Differentiation was induced by replacing the MEM with Serum-Free Medium (SFM); Day 0 in the differentiation schedule. SFM has been previously shown to promote and support the differentiation of stem cells into neurons (Brewer, 1995, Schulz et al., 2004). SFM contained Neurobasal Medium, 2mM L-glutamine, 1×Penicillin Streptomycin, and 1×B-27 (Gibco). SFM was subsequently replaced every other day and cells differentiated for a total of 8 days. For PGE_2-treated conditions, 1μM PGE_2 (Cayman Chemical) was added to the fresh SFM preparation with each replacement. Lysates were collected on day 0, 6, and 8 for extraction of total RNA and protein.

Microscope Imaging and Analysis

Imaging of cell behaviour was completed by Nikon Eclipse Ti-E microscope. Micrographs were taken at 10X objective from 125 fields every 48 hours for duration of 8 days (Day 0, 2, 4, 6, 8). Measurements were determined from a minimum of three independent experiments for each day during two differentiation conditions: SFM or SFM+PGE_2. Area, perimeter, and roundness of neurospheres were calculated from approximately 1,000 neurospheres per respective day. Neurospheres were manually selected using NIS-Elements software, which computed the area and perimeter of objects selected. The *area* represents the

space occupied by the neurosphere, while the *perimeter* represents the bordering length of the neurosphere. The formula used for *roundness* was: Roundness = $4\times\pi\times$Area/(Perimeter)^2. A roundness measure of 1 is indicative of a perfect circle, while a lower value is indicative of an object other than a circle. Hence the lower the value, the less round the neurosphere is; the closer the value is to 1, the closer the neurosphere is to a perfect round shape.

Polymerase Chain Reaction of Cell Differentiation Markers

Octamer-binding transcription factor (*Oct4*) and microtubule-associated protein tau (*Mapt*) were used as early and late neuronal markers. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as a positive control for measuring cDNA synthesis efficiency. 4 µg of total RNA was converted to cDNA and then amplified using gene specific primers designed using Primer3 Input software v.0.4.0. (Primer3, RRID:SCR_003139) (Table 4-1) and cycling parameters: 94°C for 1 min, 55°C for 30s, 72°C for 30s for 30 cycles followed by 72°C for 5min. The presence or absence of these markers was visualized on a 1% agarose gel. Polymerase chain reaction also confirmed an absence of the glial marker, glial fibrillary acidic protein (*Gfap)* during SFM differentiation of NE-4C stem cells (data not shown).

Table 4-1: PCR primers used for neural cell marker genes

Name	Primer	Primer Sequence (5'-3')	Base pair Length
Gapdh	Forward	TTCACCACCATGGAGAAGGC	20
	Reverse	GGCATGGACTGTGGTCATGA	20
Gfap	Forward	TCAATCAGTGCTAAGCTTCATA	22
	Reverse	TGCAGCCAGGAATAGACCTT	20
Mapt	Forward	TGAGATTGCTTGCGTTGTGG	20
	Reverse	ACAGCAACAGTCAGTGTAGA	20

Oct4	Forward	CTGGCTAAGCTTCCAAGGGC	20
	Reverse	CCAGGGTCTCCGATTTGCAT	20

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA isolation was completed using the NucleoSpin®RNA/Protein-Kit (Macherey-Nagel) and was reverse-transcribed into cDNA using MMuLV reverse transcriptase (New England Biolabs) following the manufacturer‘s instructions. Custom Taqman® Array 96 Well FAST plates (Applied Biosystems) were used to screen the expression of 29 developmental genes available by the manufacturer (*Shh*, *Gli1*, *Gli2*, *Gli3*, *Bmp2*, *Bmp4*, *Bmp7*, *Smad4*, *Wnt1*, *Wnt2*, *Wnt3*, *Wnt3a*, *Wnt5a*, *Wnt7a*, *Wnt8a*, *Ctnnb1*, *Lef1*, *Tcf7*, *Tcf3*, *Tcf4*, *Ptgs2*, *Axin1*, *Gsk3b*, *Axin2*, *Ccnd1*, *Mmp2*, *Mmp9*, *Mmp7*, *Myc*), along with 3 housekeeping genes (*18S*, *Gusb*, *Hprt*). Genes with expression ±1.5 RQ were further analyzed. All these genes were further validated using SYBER Green quantitative real-time polymerase chain reaction (qRT-PCR). Forward and reverse primers for the indicated genes (Table 4-2) were designed using Primer Express® Software v3.0. qRT-PCR was conlebducted with a 7500 Fast Real-time PCR system (Applied Biosystems) and the ΔΔCT method was used to calculate transcript expression as we previously described (Tamiji and Crawford, 2010a, Tamiji and Crawford, 2010c, Wong et al., 2014). Hypoxanthine phosphoribosyl transferase (*Hprt*) and Phosphoglycerate Kinase 1 (*Pgk1*) were used as endogenous controls. The relative quantification (RQ) ratios for each gene were determined from the average of three independent experiments. Genes that were differentially expressed due to PGE_2 treatment by an RQ of 2 or higher were further validated through protein expression analysis described below.

Table 4-2: qRT-PCR primers for gene expression quantification in NE-4C stem cells

Name	Primer	Primer Sequence (5'-3')	Base pair Length
Hprt	Forward	TCCATTCCTATGACTGTAGATTTTATCAG	29
	Reverse	AACTTTTATGTCCCCCGTTGACT	23
Pgk1	Forward	CAGTTGCTGCTGAACTCAAATCTC	24
	Reverse	GCCCACACAATCCTTCAAGAA	21
Cdh2	Forward	CCACTTATGGCCTTTCAAACACA	23
	Reverse	CCGTAGAAAGTCATGGCAGTAAACT	25
Ccnd1	Forward	GCACTTTCTTTCCAGAGTCATCAA	24
	Reverse	CTCCAGAAGGGCTTCAATCTGT	22
Mmp9	Forward	TCGCGTGGATAAGGAGTTCTCT	22
	Reverse	ATAGGCTTTGTCTTGGTACTGGAAGA	26
Ptgs2	Forward	CCGTAGAAAGTCATGGCAGTAAACT	25
	Reverse	TTATACTGGTCAAATCCTGTGCTCAT	26
Tcf4	Forward	GGGTTTGCCGTCTTCAGTCTAC	22
	Reverse	GCCTGGCGAGTCCCTGT	17
Wnt3	Forward	AATGCCATTGCGTCTTCCA	19
	Reverse	AGTGCCCTGGCTCACTACTTG	21

Protein isolation and Western blot analysis

Total protein was isolated using the NucleoSpin®RNA/Protein-Kit (Macherey-Nagel). Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Protein bands were detected using Mouse monoclonal primary anti-Cyclin D1 (DCS6) (1:2000, Cell Signaling Technology Cat#2926P Lot#10 RRID:AB_10828124) or Rabbit monoclonal primary anti-Non-phospho (Active) β-catenin (Ser33/37/Thr41) (D13A1) (1:1000, Cell Signaling Technology Cat#8814S Lot#3 RRID:AB_11127203), followed by goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10,000, Abcam Cat# ab6789 RRID:AB_955439)

or goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000, Abcam Cat# ab6721 Lot#GR150215-3, RRID:AB_955447). Blots were then reprobed with mouse monoclonal anti-β-Actin [AC-15] (1:10,000; Abcam Cat#ab6276 Lot#634267 RRID:AB_2223210) or mouse monoclonal anti-GAPDH (1:5,000, Abcam Cat#ab8245 Lot#GR232949-5 RRID:AB_2107448) for quantitative analysis. ECL Prime Western Blotting Detection Reagent (GE Healthcare) was used for visualization with Geliance 600 Imaging System (Perkin Elmer). GeneSnap and GeneTools (Perkin Elmer) software was used to capture blot images and quantify band intensities, respectively. Band intensities for proteins of interest were first normalized to GAPDH or β-Actin than compared to the control group for each respective experiment.

Animals for Ccnd1 quantification

Male and female mice (C57BL/6) were obtained from Charles River Laboratories. Upon arrival, they were maintained at the animal facility at York University, kept at a 12 h light/dark cycle, and provided with unlimited food and water. All protocols for animal procedures used in this study were approved by the Animal Care Committee (ACC) at York University. Samples were obtained from a current ongoing *in vivo* study in our laboratory (Rai-Bhogal et al., unpublished). In brief, on embryonic day 11 (E11), the pregnant females were injected subcutaneously with 0.2mg/kg concentration of 16,16-dimethyl prostaglandin E_2 ($dmPGE_2$; Cayman Chemical) in saline. Controls were injected with saline only. $dmPGE_2$ has a prolonged half-life *in vivo* since it is resistant to metabolism by prostaglandin-inactivating enzyme, 15-hydroxyprostaglandin dehydrogenase (Ohno et al., 1978). Brain tissue samples were collected from all pups of each litter, at embryonic day 16 (E16) and embryonic day 19 (E19) with

maternal treatment of saline or $dmPGE_2$. Total RNA and protein were extracted from brain tissue using the trizol (Sigma) method for further quantification of mRNA and protein quantification using qRT-PCR and western blot, respectively. At least 3 whole litters were collected from each condition. The mean value for the E16 saline condition represents 35 pups, E16 $dmPGE_2$ represents 21 pups, E19 saline represents 20 pups, and E19 $dmPGE_2$ represents 20 pups.

Statistical Analysis

All numerical data have been presented as mean with SEM bars representing a minimum of three individual experiments for each sample condition and time point of interest. For *in vitro* undifferentiated system, statistical analysis was performed with one-way ANOVA followed by Tukey post-hoc comparisons. For the *in vitro* differentiating system, two-way ANOVA followed by Tukey post-hoc tests were conducted to determine statistical significance of neurosphere characteristics and T-tests were used for Wnt-target expression. T-test was conducted to compare the $dmPGE_2$-treated condition to the saline-treated condition for each embryonic stage. A value of $*p<0.05$, $**p<0.01$, $***p<0.001$, was considered statistically significant. One-way ANOVA with Tukey post-hoc tests were used for β-catenin expression. Statistical tests were conducted using IBM SPSS Statistics 23.0 software (SPSS, RRID:SCR_002865).

4.4. Results

Prostaglandin E_2 increases proliferation of NE-4C stem cells

The NE-4C stem cell line is used in this study as an *in vitro* model for embryonic development. They derive from the brain of 9-day-old mouse embryos at the onset of neurogenesis and their behaviour is known to mimic cellular events that occur in the brain such

as proliferation, migration, aggregation and differentiation into neuronal cells (Schlett and Madarasz, 1997). To assess the effect of PGE_2 treatment on the proliferation of the stem cells, we conducted a proliferation assay (Fig. 4-1). We applied 1µM or 10µM PGE_2 concentrations, which represent a range widely tested in various neural cell types (Koch et al. 2015, Ma, 2010, Simm et al., 2016, St-Jacques and Ma, 2013, Tamiji and Crawford, 2010c). Plates were seeded with $4.0x10^5$ cells (*0hr*) and were incubated overnight to allow cells to settle. Cells were then incubated for 24 hours under each condition. The control condition (*Control 24hr*) proliferated to an average cell number of 1.80×10^6 cells (n=3). The 1µM PGE_2 treatment condition resulted in a significantly greater number of cells compared to the control condition, with an average of 2.33×10^6 cells, $F(2,6)=18.488$, n=3, $p=0.00258$. Similarly, the 10µM PGE_2 treatment condition yielded 2.19×10^6 cells, which was greater than the control condition $F(2,6)=18.488$, n=3, $p=0.0125$. However, there was no significant difference between the 1µM and 10µM PGE2 treatment conditions ($p=0.3092$).

PGE_2 treatment results in a shift in the formation of neurospheres

NE-4C stem cells are able to differentiate into neurons through formation of neural cell clusters (neurospheres) (Bez et al., 2003, Schlett et al., 1997, Tarnok et al., 2002). Moreover, the formation of neurospheres is considered a physiologically relevant *in vitro* model for studying neurogenesis or neural development (Vangipuram et al., 2008, Zhou et al., 2011). Neuronal differentiation of NE-4C stem cells was induced by SFM on Day 0 and continued for 8 days (see methods). Micrographs taken throughout the differentiation of NE-4C cells revealed that PGE_2-treated cells showed an earlier switch in morphology, causing an accelerated formation of neurospheres (Fig. 4-2). We observed that NE-4C stem cells normally undergo time and stage

specific changes prior to differentiation: proliferation (Day 2), followed by aggregation and inward migration (Day 4, 6), and neurosphere formation (Day 8) (Fig. 4-2A). On Day 2, there were no observable differences in cell proliferation between SFM and SFM+PGE_2 conditions. However, on Day 4, large aggregates migrated inwards becoming rounder in shape in the PGE_2 condition as compared to SFM-only condition. More distinct differences were noticeable on Day 6, where the SFM+PGE_2 condition showed an earlier formation of neurospheres (Fig. 4-2B) whereas clear neurosphere formation with the absence of aggregates occurred on Day 8 for the SFM-only condition (Fig. 4-2A). Treatment with PGE_2 resulted in earlier formation of aggregates and neurosphere clusters (Fig. 4-2B). Neurospheres appeared larger and rounder in the SFM+PGE_2 compared to the SFM-only condition on the same respective day. Further quantification of differences in neurosphere morphology is described below. In summary, these results show that PGE_2 also affected the differentiation process of NE-4C stem cells. We observed accelerated neurosphere formation in the PGE_2-treated condition compared to the SFM-only control, indicative of earlier initiation of differentiation.

PGE_2 alters the expression timeline of neuronal cell differentiation markers

To validate the changes in differentiation phenotype described above, we studied two commonly used cell type markers: *Oct4* as a stem cell marker (Yamada et al., 2013) and *Mapt* as a neuronal differentiation marker (Cardozo et al., 2012). *Oct4* stem cell marker was observed throughout the experiment in the SFM-only condition (Fig. 4-2C, *left*), but was only present from Day 0 to 4 in the SFM+PGE_2 condition (Fig. 4-2D, *left*) suggesting that the cells underwent an earlier molecular switch due to the treatment of PGE_2. The expression of *Mapt* neuronal marker complimented our findings for *Oct4* expression. In the SFM-only condition, *Mapt* expression

appeared on Day 8 (Fig. 4-2C, *right*) along with *Oct4*, indicating the presence of both stem cells and differentiating neuronal cells. In the PGE_2-treated cells, the *Mapt* neuronal marker was observed earlier, at Day 6 and thereafter (Fig. 4-2D, *right*). More importantly, there was also a distinct absence of *Oct4* expression on Day 6 and 8 in the PGE_2-treated condition, demonstrating that a genetic switch during differentiation occurs earlier with an increased level of PGE_2. The presence of the neuronal marker on Day 6 and 8 in the PGE_2-treated condition also suggests that PGE_2 accelerated the differentiation process of NE-4C stem cells into neuronal lineage cells.

PGE_2 increases the expression of neurosphere adhesion marker cadherin-2 (Cdh2)

The above experiments showed earlier initiation of differentiation and distinct differences in neurospheres formation in the presence of PGE_2 on Day 6 and 8. We used qRT-PCR to show the difference in expression of cadherin-2 (*Cdh2*), an important neural adhesion molecule that is commonly used as a marker for neurosphere formation (Chen et al., 2006, Sheng et al., 2012) on Day 6 and 8. Day 0 was used as a baseline for relative quantification values and statistical comparisons were made between the two conditions (SFM and SFM+PGE_2). A two-way ANOVA followed by Tukey post-hoc comparisons were conducted to examine the effect of treatment and differentiation day on the expression of *Cdh2*. There was no interaction between the effect of treatment and day, $F(1,11)=1.323$, $p=0.2745$. However, Tukey post-hoc comparisons revealed that on Day 6, the *Cdh2* expression was significantly higher in the presence of PGE_2 compared to the SFM-only condition (Fig. 4-3A, *left*). The RQ value in the SFM-only condition was 6.95±0.615 (n=3), while in the PGE_2-treated condition, it increased to an RQ of 24.33±4.83, $F(3,9)=3.387$, n=6, $p=0.0016$. On Day 8, there was no significant difference in *Cdh2* expression between the conditions, ($p=0.052$); the RQ values were

15.92±1.04 (n=3) and 23.11±2.41 (n=3) in the SFM-only and SFM-PGE_2-treated conditions, respectively.

Notably, in the SFM-only condition the formation of neurospheres continued from Day 6 to 8 as indicated by increased *Cdh2* expression $F(3,11)=1.323$, $p<0.001$ (Fig. 4-3A, *right*). This was also evident by the continuous presence of *Oct4*, the stem cell marker shown above (Fig. 4-2C, *right*). However, the *Cdh2* expression in the PGE_2-treated condition reached a plateau and with no difference from Day 6 to Day 8, suggesting saturation in the formation of neuronal clusters. These results complement our *Oct4* and *Mapt* data shown above, where no expression of *Oct4* and only the presence of *Mapt* detected on Day 6 and 8 (Fig. 4-2D) in the PGE_2-treated condition.

PGE_2 modifies the area, perimeter, and roundness of neurospheres

Since phenotype differences in the progression of differentiation were visibly apparent between the SFM-only and SFM+PGE_2 treatment conditions on Day 6 of differentiation (Fig. 4-2) we quantified the measurements of area, perimeter, and roundness of neurospheres on Day 6 and 8 (see methods for definitions). These measures are physiologically relevant since neurosphere size (area and perimeter) is correlated with cell proliferation (Moors et al., 2009) and roundness is an indicator of coalescence and cell migration (Vangipuram et al., 2008). A two-way ANOVA followed by Tukey post-hoc comparisons were conducted to examine the effect of treatment and differentiation day on the area of neurospheres. There was a significant interaction between the effect of treatment and day, $F(1,9)=92.886$, $p<0.001$. In the control SFM samples, the average area of neurosphere-like structures on Day 6 was $2.96\pm0.27 \times 10^4$ μm^2, number of neurospheres (N)=936, number of independent samples (n)=3. PGE_2-treated samples

on Day 6 had a significantly smaller neurosphere area of 2.41±0.09 $\times 10^4$ μm^2, $F(3,9)=287.460$, N=1221, n=3 $p=0.03354$ (Fig. 4-3B, *left*). On Day 8 of the SFM condition, the area of neurosphere structures was 4.78±0.25 $\times 10^4$ μm^2 (N=991, n=3) and the SFM+PGE_2 treatment condition had a significantly larger area of 6.36± 0.14 $\times 10^4$ μm^2, $F(3,9)=287.460$, N=1286, n=4, $p<0.001$. Analysis of the neurosphere area is represented in line graphs for each individual condition (Fig. 4-3B, *right*). SFM-only samples show a significant increase in area of 161% ($F(3,9)=287.460$, $p<0.001$) from Day 6 to 8 whereas in the SFM+PGE_2 samples, area increased by 264% ($F(3,9)=287.460$, $p<0.001$).

PGE_2 treatment had a similar effect on the perimeter of neurospheres as it did on the area (Fig. 4-3C, *left*). A two-way ANOVA followed by Tukey post-hoc comparisons were conducted, which examined the effect of treatment and differentiation day on the perimeter of neurospheres. There was also a significant interaction between the effect of treatment and day, $F(1,9)=56.670$, $p<0.001$. On Day 6, the perimeter in the SFM condition was 750.2±68.5µm (N=936, n=3) and became significantly smaller in the SFM+ PGE_2 condition with an average perimeter of 548.7±33.5 µm, $F(3,9)=54.318$, N=1221, n=3, $p<0.001$. On Day 8 the results were reverse. The perimeter was 795.2±10.3µm (N=991, n=3) in the SFM-only condition and increased significantly in the SFM+ PGE_2 condition to 901.7±8.4 µm, $F(3,9)=54.318$, N=1286, n=4, $p=0.018$. Analysis of trend lines (Fig. 4-3C, *right*) show that there is no significant difference in perimeter in SFM-only condition from Day 6 to Day 8 ($p=0.473$), but in the SFM+PGE_2 condition, there is a significant increase of 164%, $F(3,9)=54.318$, $p<0.001$.

A two-way ANOVA followed by Tukey post-hoc comparisons were also conducted to examine the effect of treatment and day of differentiation on the roundness of neurospheres. A significant interaction between the effect of treatment and day was found, $F(1,9)=10.104$,

p=0.01121. The roundness of the neurospheres in the SFM condition was 0.64±0.041 (N=936, n=3) on Day 6, while in the PGE_2 condition, neurospheres were significantly rounder, with a roundness ratio of 0.83±0.063, $F(3,9)=29.262$, N=1221, n=3, $p<0.001$ (Fig. 4-3D, *left*). This indicates that on Day 6, the neurospheres were closer to a perfect round shape in the PGE_2 -treated condition compared to the SFM-only condition, suggesting that cells were further along in differentiation under PGE_2 treatment. However, the roundness between treatment conditions on Day 8 was not significant (p=0.220); SFM condition neurospheres had a roundness ratio of 0.83±0.005 (N=991, n=3), while SFM+PGE_2 condition had a roundness ratio of 0.89±0.014 (N=1286, n=4). Trend lines for each individual condition (Fig. 4-3D, *right*) show that there is a significant increase in roundness of 130% in the SFM-only condition from Day 6 to Day 8 ($F(3,9)=29.262$, $p<0.001$) but there was no difference in roundness between these days in the SFM+PGE_2 condition (p=0.166).

Taken together, the PGE_2 treated samples had significantly different morphological measurements of area, perimeter, and roundness when compared to the control sample on the respective day. On Day 6, the PGE_2-treated condition had neurospheres (containing differentiating neurons only, as indicated above) that were smaller in size but rounder as compared to larger and more irregular neurospheres (containing a mixture of stem cells and differentiating neurons) seen in SFM-only control cells. Neurospheres under the PGE_2-treated condition had already undergone inward migration to form round neurospheres on Day 6, whereas in the control condition, the stage of inward migration took place on Day 8. This indicates that PGE_2 can affect the differentiation behaviour of NE-4C stem cells by modulating the shape and composition of neurospheres.

PGE_2 altered expression of Wnt signalling target genes in undifferentiated NE-4C stem cells

We previously established that NE-4C stem cells express all four of the EP receptors (Wong et al., 2014), enabling the investigation of how elevated levels of PGE_2 affect NE-4C stem cells. In this study, we tested the expression level of various developmental genes in undifferentiated NE-4C stem cells using Custom TaqMan® Arrays and determined that PGE_2 modified the expression of three downstream Wnt-target genes: *Ptgs2* (encodes for COX-2), *Mmp9* (encodes for Matrix Metalloproteinase 9), and *Ccnd1* (encodes for Cyclin D1). qRT-PCR was completed to confirm the expression level of these genes (Fig. 4-4A,-4B,-4C). One-way ANOVA followed by Tukey post-hoc comparisons were used for statistical analyses.

Treatment with 1μM and 10μM PGE_2 resulted in a reduced expression of *Ptgs2*, with RQ values of 0.56±0.012 ($F(2,6)=164.774$, n=3, $p<0.001$) and 0.89±0.029 ($F(2,6)=164.774$, n=3, $p=0.011$), respectively, compared to the base line in untreated cells (control). The effect of PGE_2 on *Ptgs2* expression appeared to be significantly more prominent with 1μM PGE_2 treatment compared to 10μM treatment ($F(2,6)=164.774$, n=3, $p<0.001$). *Mmp9* expression was significantly altered only by 10 μM PGE_2 treatment. We detected an increase of *Mmp9* expression with an RQ value of 1.37±0.041 ($F(2,6)=25.909$, n=3, $p=0.0012$) compared to control. *Ccnd1* expression increased with either 1μM or 10μM PGE_2 with RQ values of 3.68±0.93 ($F(2,6)=9.136$, n=3, $p=0.033$) and 4.08±0.25 ($F(2,6)=9.136$, n=3, $p=0.018$), respectively. *Ccnd1* expression was not significantly different between 1μM and 10μM PGE_2 treatment conditions ($p=0.868$).

The PGE_2 treatment caused more than 300% increase in *Ccnd1* expression level, which met the cutoff of RQ value 2 (see methods). The level of *Ccnd1* was further validated through protein expression analysis using western blot. When compared to the untreated control

condition, Ccnd1 protein levels increased by a fold change of 1.45±0.017 ($F(2,6)=42.260$, n=3, $p=0.00212$) and 1.66±0.089 ($F(2,6)=42.260$, n=3, $p<0.001$), when treated with 1μM or 10μM PGE_2 (Fig. 4-4D). There was no significant concentration-dependent effect ($p=0.0624$). This was consistent with the increase in the mRNA expression. The significant upregulation of Ccnd1 in NE-4C stem cells due to elevated PGE_2 is also in line with the increases in cell proliferation (as indicated in Fig. 4-1).

PGE_2 increases the expression of Wnt-target genes during neurosphere formation

Since Day 6 of NE-4C cell differentiation marked a significant phenotype difference between the SFM and SFM+1μMPGE_2 conditions, these samples were used to screen for various developmental genes using the Custom TaqMan® Array Plates (as described above). We found differentially regulated Wnt-target genes with a RQ greater than 1.5, namely, *Wnt3* (Wingless-Type MMTV Integration Site Family, member 3), *Tcf4* (transcription factor T-cell factor 4), and *Ccnd1*, which was also upregulated in undifferentiated NE-4C stem cells. qRT-PCR was used to confirm the expression of these genes in differentiating cells from Day 6 and Day 8. The SFM-only control condition for each time point of interest was considered as the basal expression for relative quantification (RQ) values, where RQ=1 (Fig. 4-5A,-5B,-5C).

Wnt3 expression on Day 6 increased with PGE_2 treatment to an RQ of 1.97±0.189 in PGE_2-treated cells ($t(4)=-7.210$, n=3, $p=0.00196$), while on Day 8 *Wnt3* expression between SFM-only and SFM+1μMPGE_2 conditions was not significant ($p=0.126546$). The RQ value for *Tcf4* expression increased to 1.93±0.104 in PGE_2-treated cells on Day 6, $t(4)=-7.210$, n=3, $p=0.00196$. *Tcf4* expression was not affected by PGE_2 on Day 8 ($p=0.0762$). RQ values for *Ccnd1* expression on Day 6 PGE_2 samples had an increased RQ of 2.32±0.208, $t(4)=-8.180$, n=3,

p=0.00382. On Day 8, *Ccnd1* expression also increased to an RQ of 2.94±0.121 in PGE_2-treated cells, $t(4)$=-8.180, n=3, p<0.001.

The expression of *Ccnd1* gene expression increased by an RQ value greater than 2 (cutoff used), thus was further confirmed by determining the level of protein expression using western blot technique. When compared to the SFM samples, the protein expression of Ccnd1 in PGE_2 treated samples significantly increased by a fold change of 1.66±0.054 ($t(4)$=-12.208, n=3, p<0.001) on Day 6 and 2.48±0.045 ($t(4)$=-32.368, n=3, p<0.001) on Day 8 (Fig. 4-5D). This trend was similar to the gene expression of *Ccnd1* and consistent with the results obtained in undifferentiated cells.

Maternal injection of PGE_2 changes Ccnd1 expression in mouse offspring

We observed that PGE_2-dependent expression of Ccnd1 mRNA and protein was increased in both undifferentiated and differentiating NE-4C stem cells, therefore we also tested its expression *in vivo*. From a separate ongoing study in our lab, we had available samples from mouse offspring affected with PGE_2 during prenatal development (Rai-Bhogal et al., unpublished). Pregnant mice were subcutaneously injected with $dmPGE_2$ (see methods) on Embryonic Day 11 (E11), which is normally the beginning of neurogenesis (Takahashi et al., 1996, Zhang and Jiao, 2014). mRNA and protein from brain tissues at E16 and E19 were used for quantification of Ccnd1 expression. We observed that the average transcript level of *Ccnd1* in $dmPGE_2$-injected mouse off-springs significantly increased at E16 by a RQ of 1.37±0.025 ($t(4)$= -14.826161, n=3 litters, p=0.00452) compared to saline-injected mice but there was no significant difference at E19 (p=0.8855) (Fig. 4-6A). Protein analysis revealed that mice prenatally exposed to $dmPGE_2$ had an increased expression of Ccnd1 at E16 with a RQ of

1.83±0.271, $t(6)$= -2.309328, n=5 litters, p=0.0369 (Fig. 4-6B). At E19, dmPGE$_2$-exposed mice had a decrease in Ccnd1 expression level with a fold change of 0.552±0.038, $t(4)$=11.88, n=3 litters, p<0.001. These results show that a single exposure to elevated levels of PGE$_2$ during an early critical time point in prenatal development can result in stage-specific changes (E16 vs E19) of Ccnd1 expression.

PGE$_2$ increases protein expression of β-catenin in differentiating NE-4C stem cells

In our previous study, we showed that PGE$_2$ can increase the level of non-phospho-(active) β-catenin (Ser33/37/Thr41) in Wnt-activated NE-4C stem cells (Wong et al., 2014). In this study we tested if PGE$_2$ also interacts with the canonical Wnt signalling pathway in differentiating NE-4C cells by regulation of active β-catenin (Ser33/37/Thr41) levels. We show that PGE$_2$ can influence the Wnt/β-catenin pathway in differentiating neuronal NE-4C cells by increasing the level of active β-catenin (Fig. 4-7). Western blot results revealed that on Day 6 of differentiation, the expression of active β-catenin increased by a fold change value (FC) of 1.42±0.0243 in SFM control cells (n=3) and 2.14±0.194 in SFM+PGE$_2$-treated cells (n=3) in comparison to Day 0 NE-4C stem cells (n=3). On Day 8 of differentiation, β-catenin protein expression had an FC of 1.75±0.166 for the SFM condition (n=3) and 1.76± 0.113 in the SFM+PGE$_2$ condition (n=3). Compared to the SFM condition, PGE$_2$ treatment resulted in a significant increase in β-catenin protein expression on Day 6 ($F(4,10)$=11.616, p=0.0156), corresponding to the stage with the observed phenotype shift shown in Figure 4-2.

4.5. Discussion

Our study investigated a general hypothesis whether PGE_2 can interfere with normal neural cell behaviour and alter expression of developmental genes in neuroectodermal NE-4C stem cells. We showed that PGE_2 can promote NE-4C stem cell proliferation and accelerate their lineage progression into neurons. We also determined that PGE_2 modified the expression level of specific downstream Wnt-target genes in undifferentiated and differentiating NE-4C stem cells. Moreover, we review the association of all the affected genes with neurodevelopmental disorders. The findings of this study show that PGE_2 may influence early neuronal development and alter expression of specific Wnt-target genes (Fig. 4-8).

We first found that PGE_2 increased cell proliferation in undifferentiated NE-4C stem cells and promoted neuronal differentiation in SFM-induced NE-4C stem cells. Disruptions in these two key biological processes could result in neurodevelopmental pathologies, such as acceleration of brain growth in early childhood (Redcay and Courchesne, 2005), minicolumn irregularities in the brain (Casanova et al., 2002, Casanova et al., 2006), and abnormalities in brain cytoarchitecture (Bailey et al., 1998), which have all been reported in autism spectrum disorders (ASDs).

We also analyzed markers for early stem cell gene *Oct4* (Okuda et al., 2004, Pesce and Scholer, 2001, Yamada et al., 2013) and late neuronal cell gene *Mapt* (Cardozo et al., 2012, Chambaut-Guerin et al., 2000) to determine the effects of PGE_2 on neuronal differentiation. Our results indicated that PGE_2 promotes earlier acquisition of neuronal characteristics. PGE_2 treatment during NE-4C differentiation also increased the expression of *Cdh2,* an essential adhesion molecular that serves as a key neurosphere marker (Chen et al., 2006, Kim et al., 2010, Sheng et al., 2012). This corresponds with the earlier appearance of neurospheres and neuronal

marker, *Mapt*, observed in the PGE_2 treatment condition. Moreover, increased levels of *Cdh2* could have potential implications *in vivo*. Alterations in *Cdh2* levels can interrupt or misguide important prenatal processes of the brain (Derycke and Bracke, 2004, Garcia-Castro et al., 2000, Radice et al., 1997, Warga and Kane, 2007). It is feasible that altered *Cdh2* expression is a mechanism through which some structural and behavioural phenotypes of ASDs emerge. For example, abnormal white matter integrity in children with ASDs (Weinstein et al., 2011) is indicative of aberrant axonal growth, which is regulated by *Cdh2* (Bekirov et al., 2008). Additionally, dysregulation of *Cdh2*, which guides left-right symmetry (Garcia-Castro et al., 2000), may contribute to asymmetrical development of the ventricles (Miles and Hillman, 2000) and altered synaptic connectivity patterns (Monk et al., 2009) recorded in ASDs. Synaptic communication and plasticity is central to learning and memory and disturbances in relevant systems have been described in ASDs (Goh and Peterson, 2012). Considering PGE_2 may play a role in the regulation of adhesion molecules such as *Cdh2*, irregular PGE_2 signalling could have serious consequences in development.

Furthermore, we found that exposure to PGE_2 affects neurosphere formation such as the area, perimeter, and roundness. In our study, PGE_2 treatment accelerated aggregation and neurosphere formation. Neurosphere size and roundness was modified by PGE_2, suggesting that cell proliferation (Moors et al., 2009) and migration (Vangipuram et al., 2008) was affected, respectively. Neurospheres are composed of differentiating neural cell clusters and are considered dynamic spherical structures that contain distinct gradients of cell proliferation and survival (Bez et al., 2003). Since the fate of neural progenitor cells is dependent on its position within the neuroepithelium and the time at which the cell initiates differentiation (Stiles and Jernigan, 2010), it can be inferred from our findings that altered PGE_2 levels may influence

neural cell fate and patterning. This could have significant implications for the developing brain. In fact, improper neural cell migration has been previously associated with autism (Wegiel et al., 2010) and schizophrenia (Deutsch et al., 2010).

Along with our cell behavioural findings, we also screened for developmental genes—belonging to the Bmp, Shh and Wnt pathway—that may be affected by PGE_2 treatment. Interestingly, in both undifferentiated and differentiating NE-4C cells, PGE_2 caused significant changes specifically in the expression of Wnt-target genes. In undifferentiated cells, PGE_2 decreased *Ptgs2* and increased *Mmp9* and *Ccnd1* expression. We also determined that a different subset of Wnt-target genes was affected by PGE_2 in differentiating NE-4C cells; PGE_2 increased the levels of *Wnt3*, *Tcf4*, and *Ccnd1*. Since *Ccnd1* was commonly affected in both undifferentiated and differentiated cells, we confirmed that *Ccnd1* expression was also altered *in vivo*. Compellingly, all of these genes have been implicated in neurodevelopmental disorders. The alteration of *Ptgs2* expression is particularly interesting. This is because the Wnt-target gene, *Ptgs2*, is also known as COX-2 and is responsible for the enzymatic biosynthesis of prostaglandins including PGE_2. Our results show that the collaboration between the PGE_2 and Wnt/β-catenin signalling pathways may occur through regulation of *Ptgs2* via a feedback loop mechanism. This adds to our previous research, which found that cross-talk occurs between the PGE_2 and Wnt/β-catenin pathways through protein kinase A (PKA) and phosphoinositide 3-kinase (PI-3K) (Wong et al., 2014) (Fig. 4-8). Furthermore, polymorphism of *Ptgs2* has been previously reported in individuals with ASDs (Yoo et al., 2008).

We established that elevated levels of PGE_2 resulted in a general upregulation of canonical Wnt/β-catenin pathway genes including, *Wnt3*, *Tcf4*, *Mmp9* and, *Ccnd1*. *Wnt3* expression is normally associated with neurogenesis (David et al., 2010), which is in line with

our results that showed PGE_2 accelerates neural stem cell differentiation and increases *Wnt3* expression. Additionally, *Wnt3* is required for normal forebrain formation (Mattes et al., 2012). *Wnt3* also regulates the development of neuromuscular (Henriquez et al., 2008) and sensory (Krylova et al., 2002) neurons. Considering atypical forebrains have been reported in autism patients (Riva et al., 2011) and motor skill deficits (Chukoskie et al., 2013, Cook et al., 2013) and sensory integration difficulties (Green et al., 2013, Hazen et al., 2014) are pervasive across age in ASDs, deviations in *Wnt3* expression due to increased PGE_2 signalling may contribute to behavioural manifestations of ASDs.

Tcf4 is a transcription factor and major downstream effector of canonical Wnt signalling (Faro et al., 2009). Abnormal increases in *Tcf4* expression due to increased PGE_2 exposure could have profound developmental effects since *Tcf4* is important in normal nervous system development (de Pontual et al., 2009). Moreover, disruptions in *Tcf4* expression have been linked to neurodevelopmental disorders, including Pitt-Hopkins syndrome (Sweatt, 2013) and schizophrenia (Navarrete et al., 2013). Additionally, the *Tcf4* gene is located on chromosome 18q21.2, which is an autism susceptibility locus.

The matrix metalloproteinase (Mmp) family is important in numerous neurobiological processes, such as learning and memory (Fujioka et al., 2012). *Mmp9* has been previously shown to increase neural stem cell proliferation and migration (Ingraham et al., 2011). Thus, the PGE_2-dependent increase in NE-4C stem cell proliferation found in this study may be influenced by the correlating elevation in *Mmp9* expression. *Mmp9* is expressed in the hippocampus, cortex and cerebellum (Yong, 2005). *Mmp9*-deficient mice show abnormalities in cell accumulation, migration, and programmed cell death in the cerebellum (Vaillant et al., 2003). Interestingly, the cerebellum, hippocampus, and cortex are all brain regions that are commonly abnormal in ASDs.

Additionally, *Mmp9* has been directly associated with ASDs (Abdallah and Michel, 2013, Abdallah et al., 2012). Therefore, increases in *Mmp9* expression due to elevated PGE_2 levels during critical prenatal periods may be disruptive to normal brain development.

Increased PGE_2 levels also resulted in upregulation of *Ccnd1* transcript and protein expression *in vitro* and *in vivo*. *Ccnd1* encodes for the Cyclin D1 (Ccnd1) protein, which has been shown to regulate cell cycle progression and neuronal function, as well as promote differentiation and neurogenesis (Lim and Kaldis, 2013, Lukaszewicz and Anderson, 2011). This supports our results, where in parallel with the increases in Ccnd1 expression, we also found that PGE_2 led to increased proliferation of undifferentiated NE-4C stem cells and accelerated neuronal differentiation in SFM-induced NE-4C stem cells.

Although previous studies in hematopoietic and colorectal cells have determined that PGE_2 can interact with Wnt signalling through β-catenin (Evans, 2009, Goessling et al., 2009, Greenhough et al., 2009), investigation into whether a similar interaction exists in neuronal cells is limited. We previously determined that PGE_2 can interact with the Wnt/β-catenin signalling pathway in undifferentiated NE-4C stem cells by modulating the expression of β-catenin and Wnt-regulated genes (Wong et al., 2014). In this study, we have now determined that PGE_2 can also increase β-catenin expression in differentiating neuronal NE-4C cells.

Normally without the Wnt ligand in an *in vitro* model, GSK-3β (part of the β-catenin destruction complex) destabilizes β-catenin by phosphorylating it at the Ser33/Ser37/Thr41 sites, which marks it for degradation (Wu and He, 2006). In this study, we saw a lower level of non-phospho-(active) β-catenin (Ser33/37/Thr41) in untreated cells compared to PGE_2-treated cells on Day 6 of differentiation. This corresponds with the stage where altered neurosphere phenotype was observed. This suggests that these PGE_2-induced changes in differentiating

neuronal cells may occur through the collaboration of both the PGE_2 and Wnt/β-catenin pathways.

Various studies have described abnormalities in either PGE_2 or Wnt signalling in ASDs (Kalkman, 2012, Landrigan, 2010, Tamiji and Crawford, 2010b, Wong and Crawford, 2014, Zhang et al., 2014). The cross-talk between PGE_2 and Wnt pathways might be important for normal early development. Exogenous stimuli that affect PGE_2 levels, including exposure to immunological agents (Patterson, 2011), toxic chemicals (Schwartzer et al., 2013), and drugs (Arndt et al., 2005), have all been associated with ASDs. Moreover, the misuse of misoprostol, a prostaglandin E analogue, during early pregnancy is linked to characteristics of ASDs and Moebius syndrome (Bandim et al., 2003). In rodent males, disrupted PGE_2 signalling via exposure to COX-inhibitors led to social and sensory abnormalities (Dean et al., 2012). This is of importance since social deficits and sensory sensitivity are frequently described in ASDs (Robertson and Simmons, 2013) and males have a higher risk of developing an ASD (Lai et al., 2014). What still remains to be determined is whether or not the described changes in PGE_2 levels due to environmental influences can result in Wnt-dependent pathologies of the developing brain.

4.6. Conclusions

Overall our current study provides molecular evidence that the PGE_2 pathway can alter signalling from the Wnt/β-catenin pathway—which are both autism candidate signalling pathways—in neural cells and can affect the expression of genes previously associated with developmental disorders. Importantly, our study provides novel evidence that changes in the level of PGE_2 may disrupt the proliferation and differentiation of neural stem cells, which

normally occurs during early development of the nervous system. *In vivo* experiments currently conducted in our lab using injection and knock-out mouse model systems may shed more light on the involvement of PGE_2 signalling in pathologies of the developing brain.

4.7. Figures

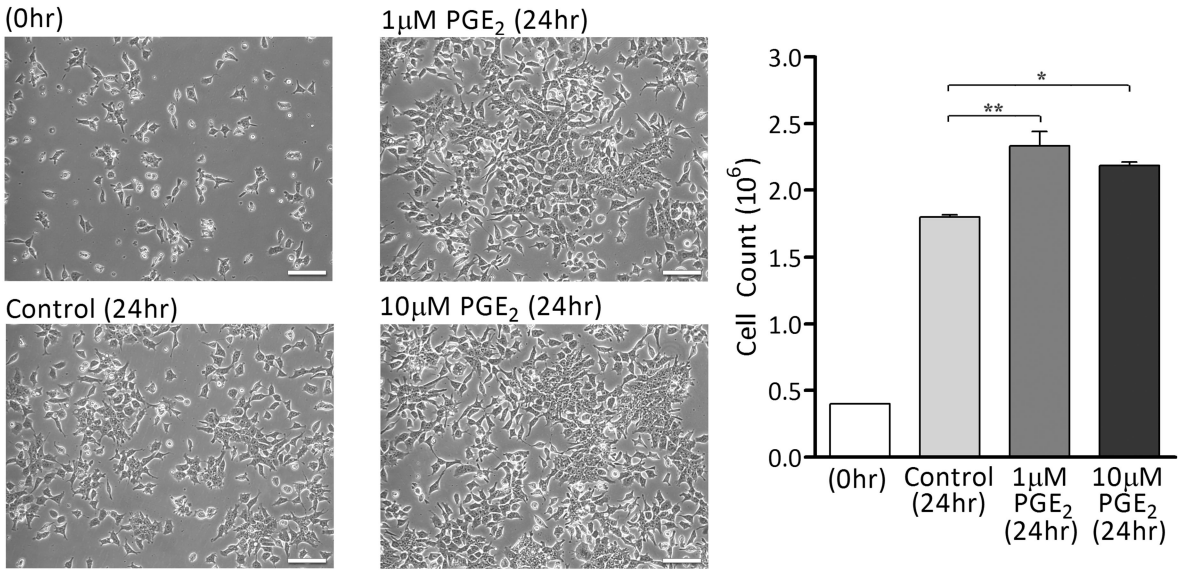

Fig. 4-1: PGE_2 increased the proliferation of NE-4C stem cells.
Representative images and quantification data of cell proliferation assay. Data are mean with SEM bars from three independent experiments. Statistical significance was assessed by one-way ANOVA, followed by Tukey's post hoc comparison. **P* <0.05, ***P* <0.01. Scale bars = 100 μm.

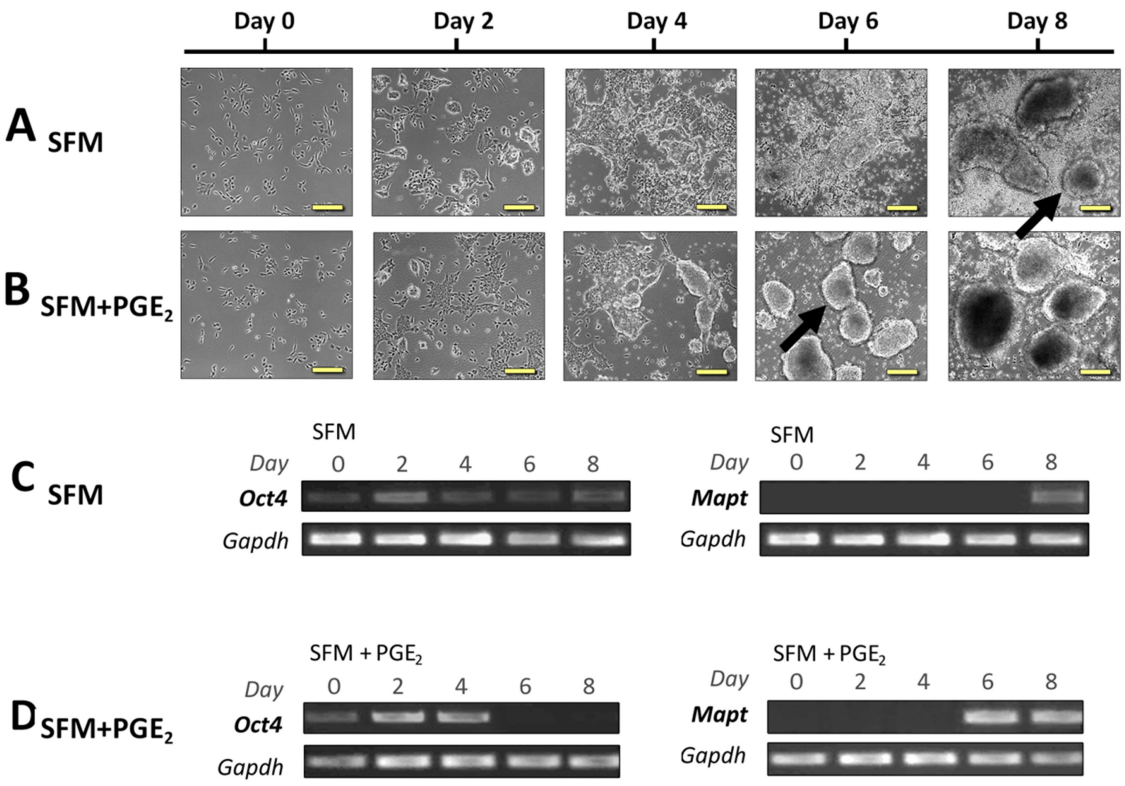

Fig. 4-2: Morphological characteristics and expression time line of neuronal markers in differentiating NE-4C cells exposed to 1 µM PGE_2.
(A,B): Morphology in SFM-only- and PGE_2-treated cells differentiated over an 8-day duration. Arrows highlight neurosphere structures. (C,D): Gene expression of early *Oct4* and late *Mapt* neuronal markers visualized via PCR for samples from days 2, 4, 6, and 8 of differentiation. *Gapdh* was used as a housekeeping gene control for PCR. Images are representative of three independent experiments. *Gfap* marker shows absence of astrocytes and confirms the presence of neuronal lineage. Whole adult mouse brain was used for specificity of the *Gfap* marker. Scale bars = 200 µm.

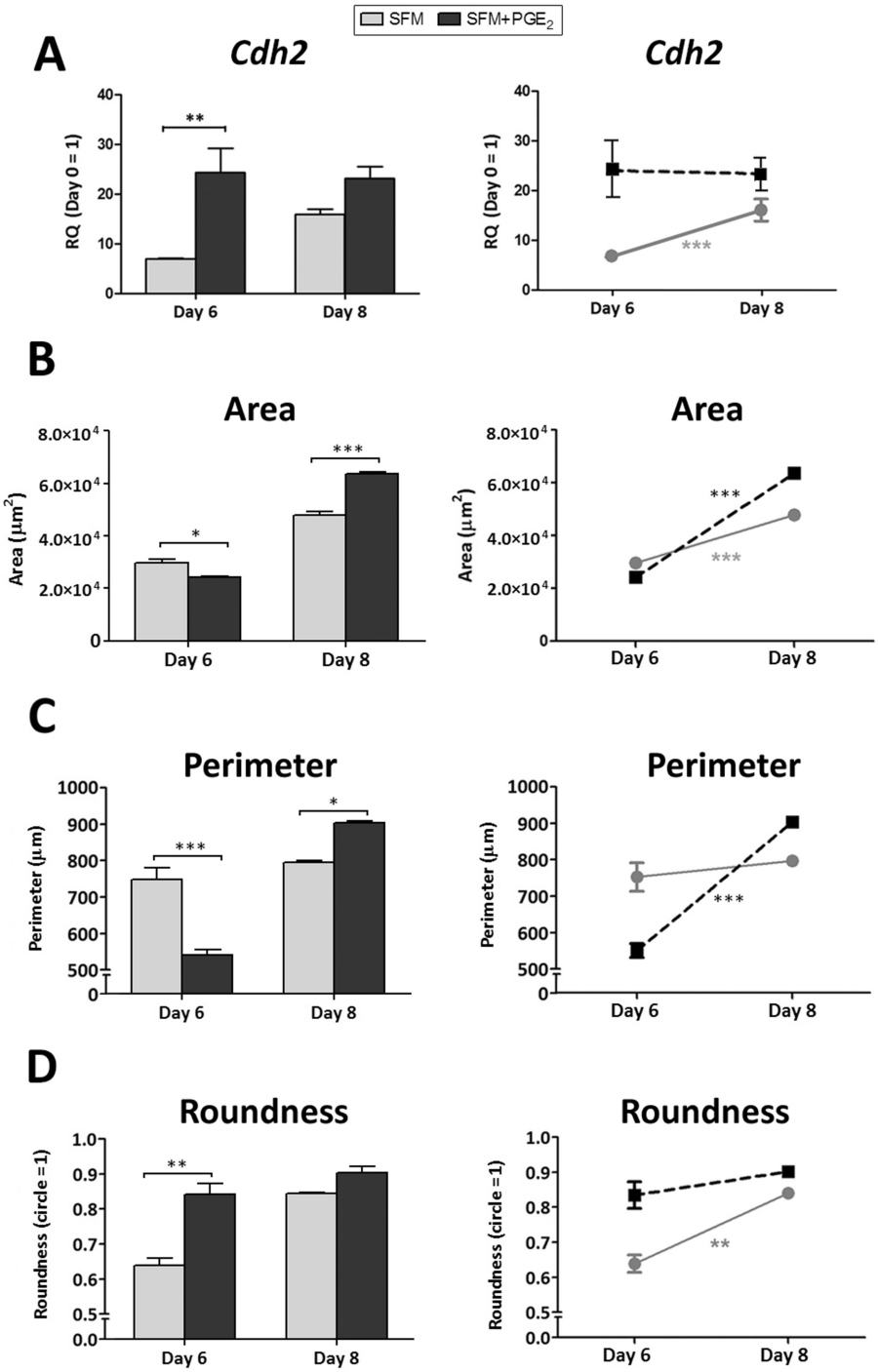

Fig. 4-3: PGE_2 affected neurosphere formation during SFM differentiation of NE-4C cells. (A): Expression of neurosphere-specific adhesion molecule, *Cdh2*, quantified by RT-PCR. The expression level is represented as RQ values compared with day 0 (RQ = 1). (B,C): PGE_2 treatment resulted in a decrease in neurosphere area and perimeter on day 6 but an increase in these parameters on day 8. (D): PGE_2-treated condition had significantly rounder neurospheres on day 6. Line graphs (A–D at right) depict changes in Cdh2 expression and the increasing trend of area, perimeter, and roundness in each individual condition from day 6 to day 8. Each average determined for neurosphere area, perimeter, and roundness represents a total of ~1,000 neurospheres, captured from a minimum of three independent experiments. Statistical significance was assessed by two-way ANOVA, followed by Tukey's post hoc comparison; error bars represent SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

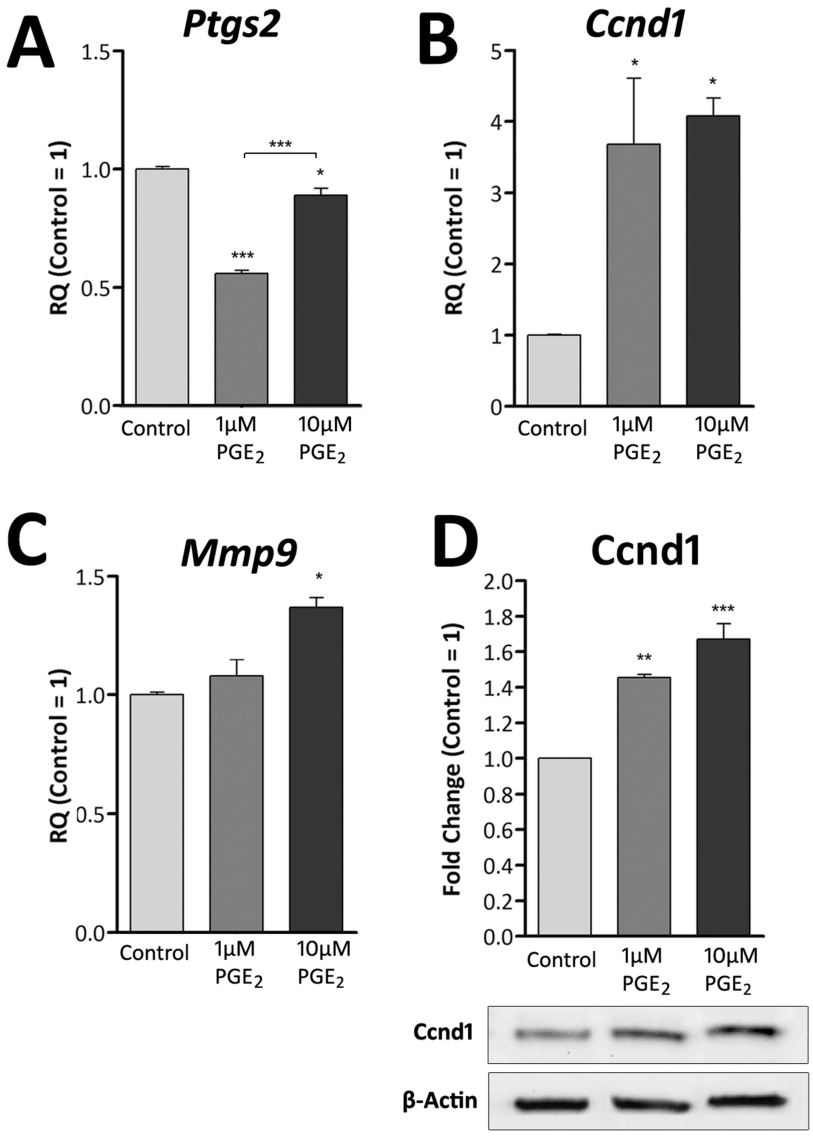

Fig. 4-4: PGE_2 affected Wnt-target genes in undifferentiated NE-4C stem cells.
Gene expression levels are displayed as RQ values compared with control untreated NE-4C stem cells. (A): Downregulation of *Ptgs2* gene expression. (B): Upregulation of *Mmp9* gene expression. (C,D): Increased expression of *Ccnd1* transcript and protein. Data are mean with SEM bars from three independent experiments. Statistical significance was assessed by one-way ANOVA, followed by Tukey's post hoc comparison. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

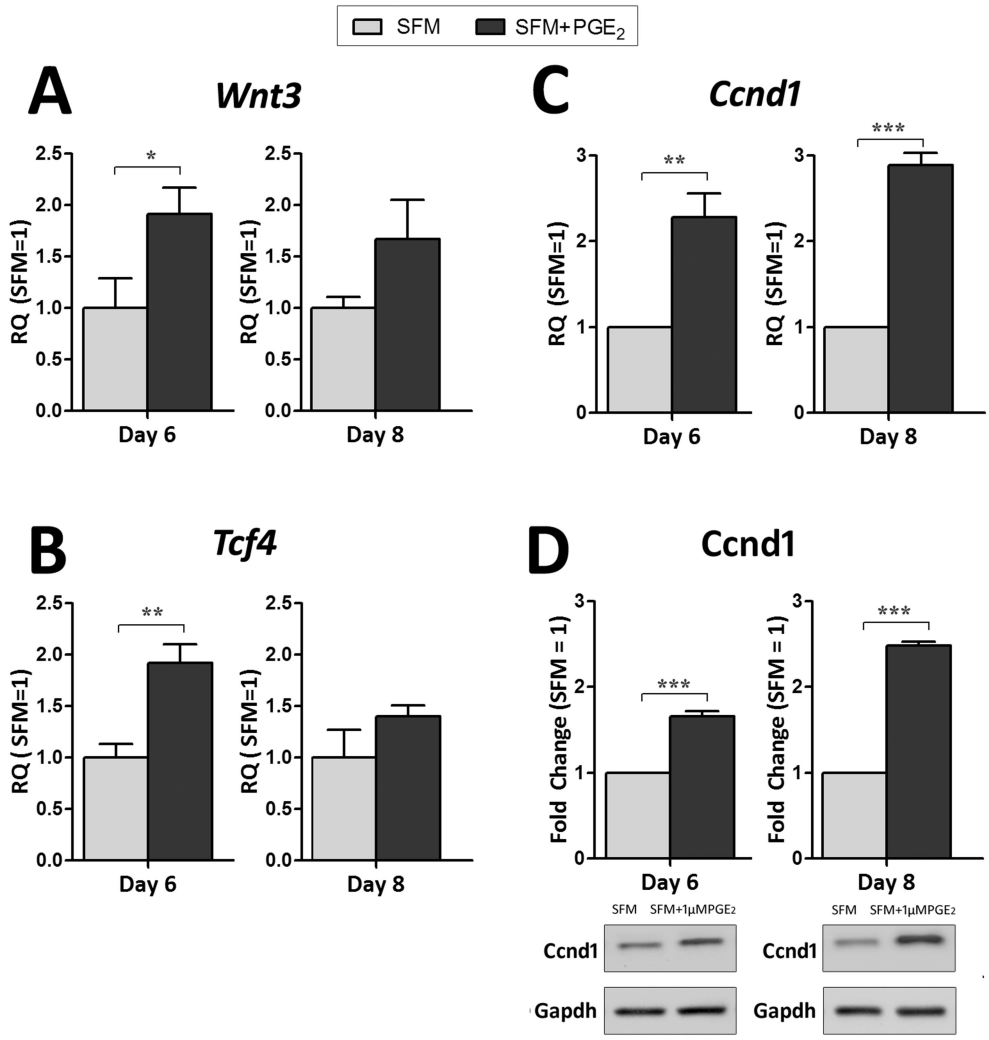

Fig. 4-5: PGE_2 increased the expression of Wnt-target genes in differentiating NE-4C stem cells. Gene expression levels are displayed as RQ values compared with the untreated SFM condition. (A): Increased *Wnt3* expression. (B): Increased *Tcf4* expression. (C,D): Elevated expression of *Ccnd1* transcript and protein. Data are mean with SEM bars from three independent experiments. Statistical significance was assessed by t-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

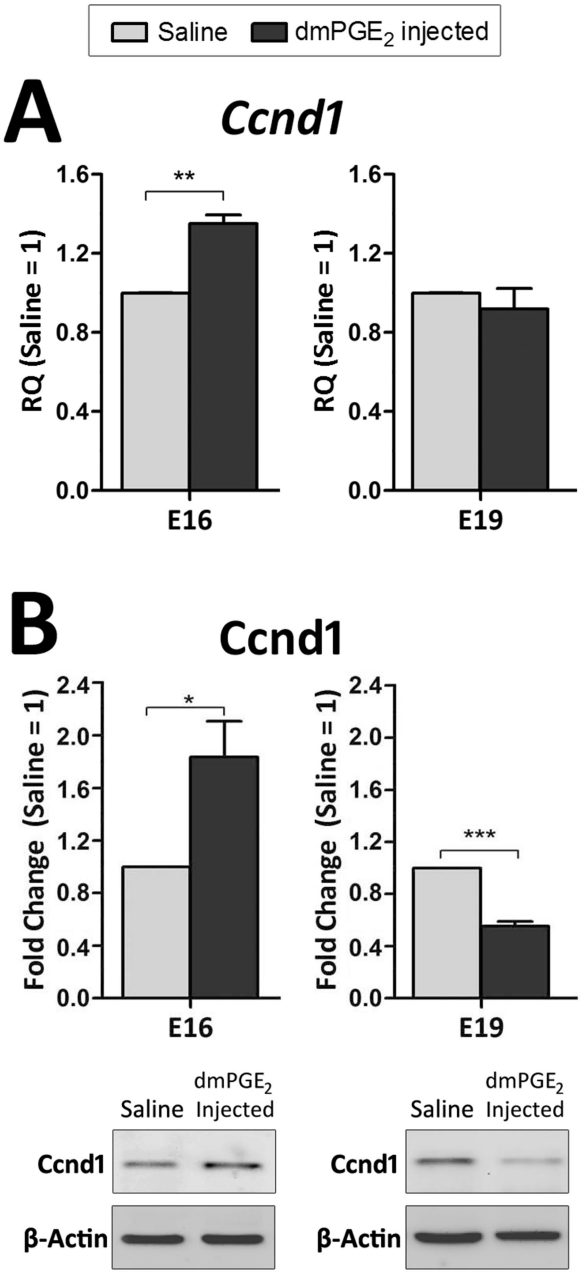

Fig. 4- 6: Ccnd1 expression in C57BL/6 mouse offspring at E16 and E19.
(A): Prenatally dmPGE$_2$-exposed mice have increased Ccnd1 expression at E16. (B): Ccnd1 protein expression increased at E16 and decreased at E19 in prenatally dmPGE$_2$-exposed mice. Data are mean with SEM bars from three independent experiments. The number of animals used in each experimental group is described in the Methods. Statistical significance was assessed by t-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

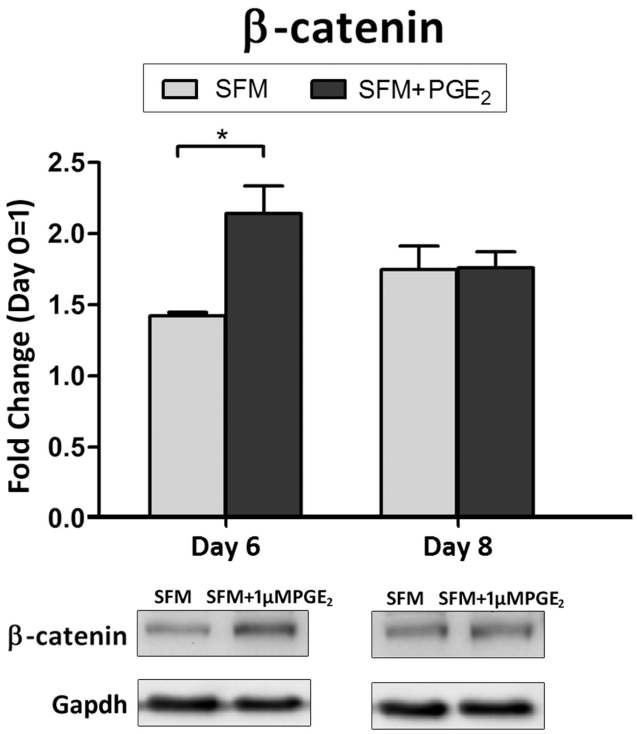

Fig. 4-7: PGE_2 increased the level of b-catenin in differentiating NE- 4C stem cells.
RQ values were compared with untreated NE-4C stem cells. Data are mean with SEM bars from three independent experiments. Statistical significance was assessed by one-way ANOVA, followed by Tukey's post hoc comparison. *$P < 0.05$.

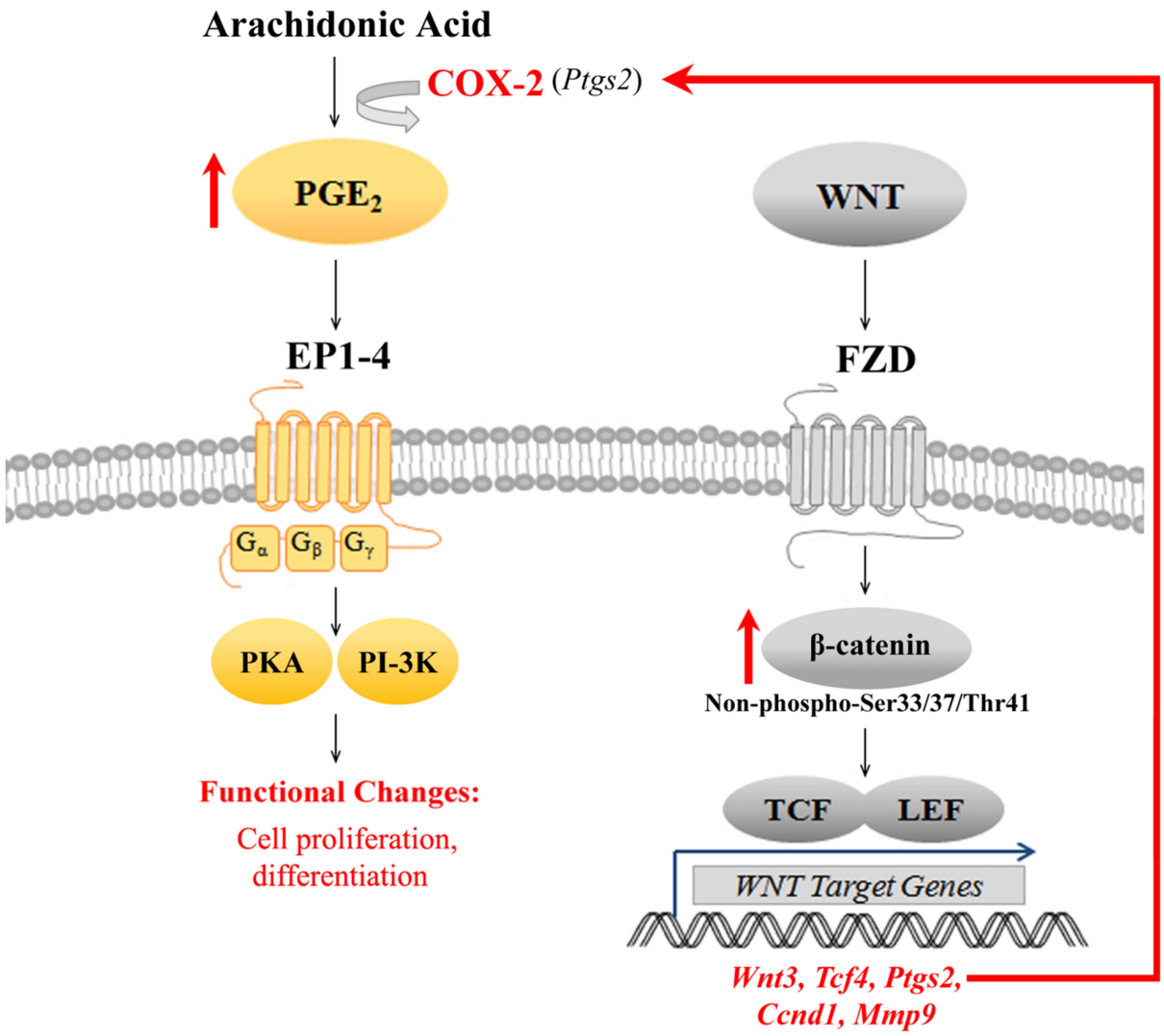

Fig. 4-8: Proposed model of the cross-talk between the PGE_2 and the Wnt/b-catenin signalling pathways. Previous literature has provided evidence for the interaction of COX-2/ PGE_2 signalling with the Wnt pathway via PKA and PI-3K (Wong et al., 2014). Our current study adds that elevated levels of PGE_2 promote the proliferation and differentiation of NE-4C stem cells and alter the expression Wnt-regulated genes through increased levels of β-catenin. A feedback loop for the regulation of PGE_2 and Wnt signalling may occur through the expression of *Ptgs2*, a Wnt-target gene that codes for COX-2 and that is responsible for the production of PGE_2.

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CHAPTER 5.

Study 3: Prenatal exposure to prostaglandin E2 leads to abnormal cell density and migration in the mouse brain

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Contributions: Christine T. Wong designed and performed all experiments, collected samples, acquired and analyzed the data, made all figures and tables, and prepared the manuscript. Isabel Bestard Lorigados assisted in the acquisition of RNA samples and data. Dr. Dorota A. Crawford supervised the design of the study and was involved with editing the manuscript.

Objectives and hypotheses: The first and second studies (Chapter 3 and 4) showed that PGE_2 affects cell movement, proliferation, and differentiation *in vitro,* which led us to our third study to determine if neuronal cell migration and density were also influenced by PGE_2 *in vivo*. Our main objectives were to study if PGE_2 alters cell density in the cerebellum, hippocampus, olfactory bulb, and neocortex, and if neocortical cell migration is also affected. I hypothesized that PGE_2 would alter neuronal cell density, neocortical migration, and the expresson of cell growth and motility genes in a sex-dependent manner.

5.1. Chapter Summary

The prostaglandin E2 (PGE_2) pathway is important for developmental processes in the brain such as neuronal proliferation and migration. Abnormal PGE_2 signalling affected by various environmental or genetic risk factors has been linked to autism spectrum disorders (ASDs). We previously found that a single maternal injection of PGE_2 during pregnancy, embryonic day 11 (E11), can lead to autism-related behaviours in male and female mouse offspring. Using the same model system in this new study, we investigated the impact of maternal PGE_2 exposure on proliferation and migration of two cell cohorts originating from E11 or E16. We quantified cell density in the cerebellum, hippocampus, olfactory bulb, and neocortex, as well as cortical cell migration at postnatal day 8. PGE_2-exposed mice compared to controls had significantly lower cell densities in the cerebellum and neocortex, while the opposite effect was observed in the olfactory bulb. Sex-dependent analyses revealed that cell density differences seen in the neocortex were specific to PGE_2-exposed females, while differences in the olfactory bulb were only detected in PGE_2-exposed males. Moreover, WT or PGE_2-exposed females had an overall greater cell density in the cerebellum, hippocampus, and neocortex compared to matched males. Normal neocortical cell migration was also disrupted, with greater cell migration in PGE_2-exposed mice. Irregular expression of cell growth and motility genes, beta-actin (*Actb*) and spinophilin (*Spn*), were also exhibited in PGE_2-exposed mice compared to controls in a sex-specific manner. Overall, our findings demonstrate that aberrant PGE_2 signalling during prenatal development may affect normal cell proliferation and migration in brain areas that have been implicated in ASDs.

5.2. Introduction

Prostaglandins are important endogenous, lipid-derived signalling molecules that have several biological roles including inflammatory regulation and various homeostatic functions. Prostaglandin E2 (PGE_2) is the most abundant prostaglandin and is also involved in neurodevelopmental processes such as cell proliferation, migration, differentiation, and synaptogenesis (Wong et al. 2016, Wong et al. 2014, Tassoni et al. 2008, Chen and Bazan 2005). PGE_2 is a derivative of arachidonic acid from cell membranes and is generated through the subsequent enzymatic activity of phospholipase A2 and cyclooxygenase-1, -2 (COX-1,-2). PGE_2 elicits its diverse functions through four receptors, EP1 through EP4 (Narumiya 2007). Abnormal levels of PGE_2 due to various genetic and environmental factors, including exposure to chemicals in pesticides, pollution, consumer products, and drugs like misoprostol and acetaminophen have been associated with an increased risk for autism spectrum disorders (ASDs) (Avella-Garcia et al. 2016, Liew et al. 2016, Wong et al. 2015, Wong and Crawford 2014, Brandlistuen et al. 2013). Maternal exposure to Misoprostol, a drug structurally analogous to PGE_2, has been linked to the neurodevelopmental conditions: ASDs and Moebius syndrome (Bos-Thompson et al. 2008, Bandim et al. 2003, Pastuszak et al. 1998).

Our previous *in vitro* studies showed the PGE_2 disrupts the proliferation, migration, and differentiation of neuronal stem cells (Wong et al. 2016, Wong et al. 2014). Furthermore, research in rodent models has determined that perinatal PGE_2 exposure can also affect brain development by influencing neuron branching, spine density, male sex behaviour, and male play behaviour (Hoffman et al. 2016, Wright and McCarthy 2009, Amateau and McCarthy 2004). We also recently determined that a single injection of PGE_2 to pregnant mice at embryonic day 11 (E11) resulted in autism-like behaviours in offspring, as well as alterations in the expression of

autism-linked genes (Rai-Bhogal et al. 2018). However, *in vivo* investigations into the potential effect of prenatal PGE_2-exposure on the cell density of ASD-associated brain regions and neuronal migration in the cortex have not yet been conducted.

In this current study, we aimed to study the sex-dependent effect of PGE_2 signalling on neural proliferation and migration by investigating postnatal day 8 (P8) mouse offspring that had maternal exposure to PGE_2 at E11. We measured the density of cells originating from two separate cohorts (E11 and E16) in various brain regions including the cerebral cortex, hippocampus, cerebellum, and olfactory bulb since they have been previously implicated in ASDs (Courchesne et al. 2019, Becker and Stoodley 2013, Courchesne et al. 2011, Brang and Ramachandran 2010, Wegiel et al. 2010, Dager et al. 2007, Raymond et al. 1996). We also examined the effect of PGE_2 on neocortical cell migration. Additionally, the expression of cell growth and migration genes—spinophilin (*Spn*), beta-actin (*Actb*), and cyclin D1 (*Ccnd1*)—was quantified. This study shows for the first time that elevated maternal PGE_2 at a single time point during pregnancy alters the density and migration of cells in various brain regions as well as transcript expression of *Spn* and *Actb* in male and female offspring.

5.3. Methods

Animals

C57bl/6 mice were obtained from Charles River Laboratories. All animals were bred and maintained at York University animal facility on a 12-hour light/dark cycle. Whole brain samples were extracted at postnatal day 8 (P8), where birth was considered postnatal day 0. P8 in mice is analogous to infanthood in humans (Auvin and Pressler 2013, Semple et al. 2013), which is when symptoms of ASDs are first observed. All experiments and protocols followed the York

University Animal Care Committee ethics guidelines and have been approved by the Research Ethics Board of York University.

Maternal Injection for PGE_2-exposed mouse model

Male and female mice were housed together for breeding overnight. Every morning, females were inspected for a vaginal plug, which was noted as embryonic day 1 (E1, Supplementary Fig. 5-S1). Upon occurrence of a vaginal plus, females were separated and housed individually for the entirety of their pregnancy. On E11, pregnant females were weighed and given a single subcutaneous injection of 0.2 µg/g concentration of 16, 16-dimethyl prostaglandin E2 ($dmPGE_2$; Cayman Chemical) diluted in saline as used in previous studies (Rai-Bhogal et al. 2018, Okamoto et al. 2011, Ma 2010, Tessner et al. 2004). The metabolic rate of $dmPGE_2$ is slower and thus considered a stable analogue of PGE_2 (Ohno et al. 1985, Steffenrud 1980). E11 was used as the injection time-point since it coincides with the beginning of neurogenesis in embryonic mice. E11 also corresponds to the time when misoprostol was taken in human cases resulting in Moebius syndrome and autism characteristics (Bandim et al. 2003, Pastuszak et al. 1998). Maternal exposure of $dmPGE_2$ at E11 was also shown in earlier studies to lead to autism-like behaviours and alteration of autism-linked gene expression (Rai-Bhogal et al. 2018, Wong et al. 2017). Herein, mouse offspring subjected to maternal exposure of $dmPGE_2$ will be referred to as "PGE_2 mice".

CldU and IdU labelling

Two cell subpopulations of different ages were labelled in each sample utilizing a method that detects sequential incorporation of different thymidine analogues (CldU and IdU) into the

dual helix of any cell actively synthesizing DNA during the specific time-point when the thymidine marker is injected (Tuttle 2010). Through this technique, the later fate of a specific cohort of cells can be studied. For both control and PGE_2-exposed animals, subcutaneous injections of 5-Chloro-2'- deoxyuridine (CldU) or 5-Iodo-2'deoxyurdine (IdU) (Sigma) were administered at 50μg/g dissolved in saline to pregnant mice. In the mouse, the neuronogenetic interval extends from embryonic day 11 (E11) through early E17 (Takahashi et al. 1995). For dual labelling of early- and late-born cohorts of neural cells, an injection of CldU at E11 and IdU at E16 were given, and animals were sacrificed at P8 (Supplementary Fig. 5-S1). For the PGE_2-exposed group, pregnant mice were given a single co-injection of CldU and PGE_2 at E11 at described concentrations. Cells born on E11 and E16 are likely neuronal cells since neurons are predominately produced from E10 to E18 in rodents, while astrocytes and oligodendrocytes are generated at later time points around E18 and postnatally, respectively (Reemst et al. 2016, Miller and Gauthier 2007, Bayer and Altman 1991).

Immunohistochemistry (IHC)

Left hemisphere brain samples were carefully extracted and fixed in 4% paraformaldehype (PFA) in PBS at 4°C for 48 hrs for histological staining. Paraffin-embedding and serial slicing from the mid-sagittal plane onwards at 4μm thickness was completed by The Centre for Phenogenomics (Toronto, Canada). Immunohistochemistry was performed as previously described (Tuttle et al. 2010). In brief, paraffin removal from samples was completed by xylene incubation, followed by rehydration through subsequent ethanol incubations. Cell permeabilization was completed by 0.2% Triton X-100 in PBS. An antigen retrieval step was performed using 0.01M pH 6.0 sodium citrate buffer followed by a 1.5N HCl incubation.

Sections were then circled using a liquid blocking super PAP pen (Cedarlane). Sections were treated with 0.25% trypsin EDTA in a pre-warmed hydration chamber at 37°C for 3 min. All subsequent steps were then completed in a hydration chamber. Samples were blocked in 5% goat serum diluted in PBS, followed by 4°C overnight incubation of the primary antibody for CldU called Rat anti-BrdU (1:100, ab6326, Abcam), diluted in 5% goat serum in PBS. Samples were then incubated in a high stringency wash of low salt TBST buffer (36 mMTris, 50mM NaCl, 0.5% tween-20; pH 8.0) at 37°C with agitation for 20 min at 225 rpm. Next, samples were incubated overnight at 4°C overnight with the primary antibody for IdU, Mouse anti-IdU (1:100, ab181664, Abcam). Secondary antibody incubation was completed in the dark with Alexa Fluor 555 goat anti-mouse (1:500, ab150118, Abcam) and Alexa Fluor 488 goat anti-rat goat anti-rat (1:500, ab150165, Abcam). Coverslips were mounted with ProLong Gold Antifade Mountant (ThermoFisher).

Cell Density and Neocortical Cell Migration Analysis

CldU and IdU staining was visualized and captured using an Eclipse 80i upright fluorescent microscope with DS-5MC camera (Nikon). The cerebellum, hippocampus, olfactory bulb, and neocortex were investigated. Estimated cell density measurements for each brain region were calculated by dividing the total number of cells counted by the area of each specific brain region. Neocortical cell migration distances from the subplate to the labelled cells were measured in the cortex. The migration percentage for each cell was calculated by taking its migration distance and dividing it by the total distance between the subplate and cortical pial surface. Image analysis was completed using NIS-Elements software (Nikon). All analyses were completed blind to the condition.

RNA Isolation and quantitative Real-Time Polymerase Chain Reaction

Right hemisphere brain samples were extracted at postnatal day 8 and homogenized in Trizol (Sigma) using a Polytron power homogenizer. The standard Trizol (Sigma) method was conducted for total RNA isolation. MMuLV reverse transcriptase (New England Biolabs, Ipswich, MA) was used following manufacturer's protocol to convert total RNA into cDNA. Quantitative real-time polymerase chain reaction (qRT-PCR) using SYBER green master mixes in a 7500 FAST RT-PCR system (Applied Biosystem, Foster City, CA) was performed on the cDNA samples. Transcript expression represented by relative quantification (RQ) values was calculated using the ΔΔCt method. Primer Express v3.0 (ThermoFisher Scientific, Waltham, MA) was used to design the primers for genes investigated: Spinophilin (*Spn*), Beta-Actin *(Actb),* Cyclin D1 *(Ccnd1)* (Table 5-1). Hypoxanthine phosphoribosyl transferase (*Hprt*) and phosphoglycerate kinase 1 (*Pgk1*) were quantified as housekeeping controls for the qRT-PCR experiments. The RQ means were calculated from the RQ values of at least three individuals from different litters determined in independent experiments.

Table 5-1: qRT-PCR primers for cell growth and migration gene expression

Name	Primer	Primer Sequence (5'-3')	Base pair Length
Hprt	Forward	TCCATTCCTATGACTGTAGATTTTATCAG	29
	Reverse	AACTTTTATGTCCCCCGTTGACT	23
Pgk1	Forward	CAGTTGCTGCTGAACTCAAATCTC	24
	Reverse	GCCCACACAATCCTTCAAGAA	21
Spn	Forward	CAAGGACTACCAGCAAAAGGAGAT	24
	Reverse	CCTGGCTAGCTCCGACTCTTC	21
Actb	Forward	GCTTCTTTGCAGCTCCTTCGT	21
	Reverse	AGCGCAGCGATATCGTCAT	19
Ccnd1	Forward	GCACTTTCTTTCCAGAGTCATCAA	24
	Reverse	CTCCAGAAGGGCTTCAATCTGT	22

Statistical Analysis

Numerical data are reported as mean±standard error of the mean (SEM), which represents the average of individuals from a minimum of three separate litters. Two-way ANOVA followed by post-hoc Bonferroni comparisons were conducted to determine if there were significance differences between PGE_2-exposed and wildtype (WT) control groups and between males and females. Independent t-test was performed to determine if there were differences in gene expression. Significance was identified for p values less than 0.05.

5.4. Results

Cerebellar Cell Density

Cells originating from E11 and E16 were counted in P8 cerebellum samples (Fig. 5-1A, 1B). Two-way ANOVA analysis on E11 cell density values (cells/mm^2) were conducted (Fig. 5-1C, $F(3,10)=27.23$, $p=0.00004$). There was a lower cerebellar cell density in PGE_2-exposed mice compared to WT controls for males (Fig. 5-1C, $p=0.005233$; WT=339.5±21.9, PGE_2=156.2±19.5) and for females (Fig. 5-1C, $p=0.000084$; WT=595.0±23.3, PGE_2=267.6±72.0). Sex differences were seen between WT mice, where males had a lower cell density than females (Fig. 5-1C, $p=0.000324$; M=339.5±21.9, F=595.0±23.3).

Two-way ANOVA analysis on cerebellar E16 cell density values were also performed (Fig. 5-1D, $F(3,10)=13.83$, $p=0.000685$). PGE_2-exposed mice also had a lower cerebellar cell density of E16-originating cells than WT controls for males (Fig. 5-1D, $p=0.01066$; WT=546.8±24.6, PGE_2=356.4±11.0). There was no significant difference between PGE_2-exposed and control females (Fig. 5-1D, $p=0.366$; WT=1652.1±332.2, PGE_2=948.3±117.5). Sex-dependent differences were seen in both the WT and PGE_2-exposed groups. WT males had a

lower cell density than WT females (Fig. 5-1D, p=0.000262). Similarly, PGE_2-exposed male also had a lower cell density than PGE_2-exposed females Sex differences were only seen between WT mice, where males had a lower cell density than females (Fig. 5-1D, p=0.02501).

Overall, PGE_2-exposed males had lower cerebellar cell densities compared to WT males for E11 and E16 cell cohorts. PGE_2-exposed females also had lower cell densities compared to WT controls for only E11-labelled cells. In general, females had greater cerebellar cell densities for E11 and E16-originating cells than males.

Hippocampal Cell Density

Hippocampal cells originating from E11 and E16 were counted in P8 samples (Fig. 5-2A, 2B). Two-way ANOVA analysis on E11 cell density values (cells/mm^2) were completed (Fig. 5-2C, F(3,11)=4.236, p=0.032). There were no significant differences in hippocampal E11 cell densities between PGE_2-exposed mice compared to WT controls for males (Fig. 5-2C, p=0.898; WT=262.4±47.4, PGE_2=288.2±65.1) and for females (Fig. 5-2C, p=0.059; WT=735.3±128.7, PGE_2=397.671±45.9). Sex differences were only seen between WT mice, where males had a lower cell density than females (Fig. 5-2C, p=0.026741).

Two-way ANOVA analysis on hippocampal E16 cell density values were also conducted (Fig. 5-2D, F(3,11)=7.135, p=0.006253). There were also no significant differences in E16 hippocampal cell densities between PGE_2-exposed mice compared to WT controls for males (Fig. 5-2D, p=0.223; WT=643.7±85.6, PGE_2=437.4±142.5) and for females (Fig. 5-2D, p=0.346; WT=1067.9±110.4, PGE_2=922.4±63.3). Sex-dependent differences were observed in the WT and PGE_2-exposed groups. WT males had a lower E16 hippocampal cell density than WT

females (Fig. 5-2D, p=0.02243). PGE_2-exposed males also had a lower cell density than PGE_2-exposed females (Fig. 5-2D, p=0.007376).

In summary, PGE_2 exposure did not have a significant impact on hippocampal cell densities for E11 and E16 cohorts. However, females had greater hippocampal cell densities for E11 and E16-originating cells compared to males.

Olfactory Bulb Cell Density

Cell densities were also determined in the olfactory bulb of P8 samples for cells originating from E11 and E16 (Fig. 5-3A, 3B). Two-way ANOVA analysis on E11 cell density values (cells/mm^2) were completed (Fig. 5-3C, F(3,14)=6.521, p=0.0055). PGE_2-exposed males had significantly higher E11 cell density compared to WT males (Fig. 5-3C, p=0.009809; WT=1475.8±253.1, PGE_2=3342.4±950.1). There was no statistical difference in E11 olfactory bulb cell densities between females (Fig. 5-3C, p=0.998; WT=725.1±67.3, PGE_2=723.4±162.2). Sex differences were not seen between WT mice (Fig. 5-3C, p=0.249571) but were present between PGE_2-exposed mice, where males had a greater cell density than females (Fig. 5-3C, p=0.001856).

Two-way ANOVA analysis on E16 cell density values were conducted (Fig. 5-3D, F(3,14)=5.389, p=0.011213). PGE_2-exposed males also had significantly higher E16 cell density compared to WT males (Fig. 5-3D, p=0.005998; WT=4=16.1±734.0, PGE_2=10963.6±3355.8). There was no difference in E16 olfactory bulb cell densities between females (Fig. 5-3D, p=0.906; WT=2887.5±238.4, PGE_2=3169.1±505.0). Sex-dependent differences were not seen in WT mice (Fig. 5-3D, p=0.60800) but were apparent between PGE_2-exposed mice, where males had a greater cell density than females (Fig. 5-3D, p=0.005135).

In short, PGE_2-exposed males had greater olfactory bulb cell densities for E11 and E16 cohorts compared to WT controls. Olfactory bulb cell densities were not affected in PGE_2-exposed females compared to female controls. Sex-dependent differences were seen in PGE_2-exposed mice for E11 and E16 olfactory bulb cell densities; males had greater cell densities compared to females.

Neocortical Cell Density

Cells originating from E11 and E16 were counted in the neocortex of P8 samples (Fig. 5-4A, 4B). Two-way ANOVA analysis on E11 cell density values (cells/mm^2) were performed (Fig. 5-4C, $F(3,14)=6.706$, $p=0.004924$). There were no significant differences in neocortical cell densities between PGE_2-exposed mice compared to WT controls for males (Fig. 5-4C, $p=0.848$; WT=227.8±24.3, PGE_2=238.7±23.8) and for females (Fig. 5-4C, $p=0.475$; WT=378.9±44.1, PGE_2=417.8±47.8). Sex differences were seen between WT mice, where males had lower neocortical cell densities than WT females (Fig. 5-4C, $p=0.007009$). Similarly, PGE_2-exposed males also had lower cell densities than PGE_2-exposed females (Fig. 5-4C, $p=0.007009$).

Two-way ANOVA analysis on neocortical E16 cell density values were also completed (Fig. 5-4D, $F(3,12)=67.459$, $p=0.000000088$). There were also no significant differences in E16 neocortical cell densities between PGE_2-exposed males compared to WT males (Fig. 5-4D, $p=0.062815$; WT=575.2±44.4, PGE_2=442.5±28.4). In contrast, PGE2-exposed females had lower cell densities than WT females (Fig. 5-4D, $p=0.001011$; WT=1254.5±53.4, PGE_2=984.3±36.2). Sex-dependent differences were observed in the WT and PGE_2-exposed groups. WT males had a lower E16 neocortical cell density than WT females (Fig. 5-4D,

p=0.000000083). Similarly, PGE_2-exposed males also had a lower cell density than PGE_2-exposed females (Fig. 5-4D, p=0.000004).

To summarize, PGE_2-exposure did not change the neocortical cell densities for E11 and E16 cell cohorts in males. However, PGE_2-exposed females had lower neocortical cell densities than WT controls for the E16 cell cohort. Sex differences were observed in neocortical cell densities, males had lower cell densities for E11 and E16-originating cells compared to females.

Neocortical Cell Migration

The neocortex develops in an 'inside-out' order, where neurons that arise earliest form the deepest cortical layers and later-produced neurons migrate past the existing neurons to upper layers (Nadarajah et al. 2003). The migration distances in the neocortex of P8 brain samples were measured from the subplate to the cells labelled at E11 (CldU-labelled) and E16 (IdU-labelled) (Fig. 5-5A). Migration percentages were calculated for each labelled cell as described in the methods.

Two-way ANOVA analysis was conducted on the migration percentages for E11-born cells of males and females (Fig. 5-5B, $F(3,7016)=76.79$, $p<0.00001$). The migration percentage of E11 cells for PGE_2-exposed males was not significantly different from WT males (Fig. 5-5B, p=0.132; WT=36.9±0.58, PGE_2=35.4±0.65). In contrast, the migration percentage of E11 cells for PGE2-exposed females was statistically greater than WT females (Fig. 5-5B p=0.00000013; WT=43.5±0.62, PGE_2=48.2±0.72). Sex differences were seen in both WT and PGE_2-exposed animals. E11-labelled cells of WT males travelled a shorter distance than E11-labelled cells of WT females (Fig. 5-5B, $p<0.00001$). Similarly, E11 cells of PGE_2-exposed males also migrated less than E11 cells of PGE_2-exposed females (Fig. 5-5B, $p<0.00001$)

Two-way ANOVA analysis was conducted on the migration percentages for E16-born cells of males and females (Fig. 5-5C, $F(3,16909)=85.32$, $p<0.00001$). The migration percentage of E16 cells for PGE_2-exposed males was significantly greater from WT males (Fig. 5-5C, $p<0.00001$; WT=81.4±0.30, PGE_2=85.8±0.25). The migration percentage of E16 cells for PGE_2-exposed females was also significantly greater from WT females (Fig. 5-5C, $p=0.01184$; WT=79.3±0.26, PGE_2=81.2±0.25). Sex differences were seen in both WT and PGE_2-exposed animals. E16-labelled cells of WT males travelled a greater distance than those of WT females (Fig. 5-5C, $p<0.00001$). Similarly, E16 cells of PGE_2-exposed males also migrated further than E16 cells of PGE_2-exposed females (Fig. 5-5C, $p<0.00001$).

In summary, PGE_2-exposure led to increased migration of E11-born cells in females and E16-born cells in males and females. Sex differences were seen in E11-labelled and E16-labelled cells. E11-labelled cells in females had greater migration percentages than E11-labelled cells in males for WT and PGE_2-exposed groups. In contrast, E16-labelled cells in males had greater migration percentages than E16-labelled cells in females for WT and PGE_2-exposed groups.

Abnormal expression of Spn and Actb in PGE_2-exposed mouse offspring

The expression of cell proliferation and migration genes, including spinophilin (*Spn)*, beta-actin (*Actb)*, and cyclin D1 (*Ccnd1)*, were quantified in PGE_2-exposed male and female mice and compared to WT controls. Brain samples from postnatal day 8 were collected for analysis. Gene expression profiles were determined using quantitative RT-PCR analysis (Table 5-1).

In comparison to WT males (RQ=1), only the expression of *Spn* out of the three genes was significantly different in PGE_2-exposed males (Table 5-2). Specifically, PGE_2-exposed males

had decreased expression of *Spn* by half (*t(4)*=4.540, *p*=0.045; RQ=0.487±0.11) compared to controls. The expression of *Actb* (*t(4)*=1.721, *p*=0.227; RQ=0.753±0.14) and *Ccnd1* (*t(4)*=2.875, *p*=0.103; RQ=0.725±0.10) were not statistically different in males.

In contrast, the expression of *Spn* (*t(4)*=-4.371, *p*=0.049, RQ=1.525±0.12) and *Actb* (*t(4)*=-5.389, *p*=0.033, RQ=1.309±0.06) were both increased in PGE_2-exposed females compared to WT females (RQ=1) (Table 5-2). No statistical differences were found in the expression of *Ccnd1* (*t(4)*=-2.179, *p*=0.161; RQ=1.198±0.091). In summary, PGE_2 exposure led sex-specific changes in the expression of *Spn* and *Actb*.

5.5. Discussion

The manifestation of ASD phenotypes is believed to result from a cascade of prenatal pathogenic processes, including irregular proliferation and migration of neural cells (Courchesne et al. 2019). Thus, in this study we aimed to determine how cell density and migration could be affected by prenatal exposure to PGE_2. We found that a single maternal injection of PGE_2 during pregnancy led to abnormalities in mouse offspring at P8. Specifically, PGE_2-exposed offspring displayed aberrant cell density in ASD-associated brain regions including the cerebellum, hippocampus, olfactory bulb, and neocortex. Sex-dependent alterations in neocortical migration of cells originating at E11 or E16 and irregular expression of cell growth and motility genes (*Spn*, *Actb*) were also affected in PGE_2-exposed offspring. Quantified cells are most probably neurons since neurogenesis occurs from E10 to E18, whereas astrocytes and oligodendrocytes are generated later in development (Reemst et al. 2016, Miller and Gauthier 2007, Bayer and Altman 1991).

We found that PGE_2-exposed males and females had a lower density of E11-labelled cells in the cerebellum compared to sex-matched controls. PGE_2-exposed males also exhibited a lower density of E16-labelled cerebellar cells than controls. The cerebellum is important in motor movement and perception (Manto et al. 2012) and is also involved with executive functioning and social cognition, indicating that the cerebellum has a predominant role in non-motor processes relevant to ASDs (Van Overwalle et al. 2014, Buckner 2013, Tedesco et al. 2011, Strick et al. 2009). One of the most consistently reported abnormalities of the brain in ASD cases is a decreased number of Purkinje cells in the cerebellum (Skefos et al. 2014, Whitney et al. 2009, Yip et al. 2007, Bauman and Kemper 2005, Bailey et al. 1998, Ritvo et al. 1986). Moreover, cerebellar dysplasia and a reduction in total cerebellar volume in children and adults with an ASD have been reported (Wegiel et al. 2010, Hallahan et al. 2009, Carper and Courchesne 2000). Our findings showing decreased cerebellar cell density at E11 and E16 complement previous studies that reported reduced Purkinje cell numbers and volume of the cerebellum in ASD patients. Moreover, anatomical deviations of the cerebellum may contribute to motor difficulties in ASDs (Esposito and Venuti 2008, Ming et al. 2007, Vernazza-Martin et al. 2005) and abnormal social cognition (Fishman et al. 2014). Interestingly, we have also previously found that an increase or decrease of PGE_2 signalling during prenatal development can lead to atypical motor ability and sociability in mice (Wong et al. 2019, Wong et al. 2017).

The hippocampus is important in learning and memory as well as cognitive flexibility and social behaviour (Rubin et al. 2014). We did not find a significant difference in hippocampal cell densities for E11 or E16 cell cohorts between PGE_2-exposed and matched control mice. Cell densities have not been well investigated in ASDs but previous studies in infants and children have reported hippocampal shape deformation, increased or decreased hippocampal volume,

altered neuron morphology, decreased dendritic branching, and reduced hippocampal functional connectivity in individuals with an ASD (Cooper et al. 2017, Dager et al. 2007, Sparks et al. 2002, Aylward et al. 1999, Raymond et al. 1996). We did find that females had greater hippocampal cell densities compared to males within PGE_2-exposed and control groups. Sex differences have been recorded for decades in the general population in regards to spatial and object memory—males outperform in spatial memory while females outperform in object memory—however, the biological etiology of these sex differences is currently inconsistent or not well understood (Koss and Frick 2017). Future memory-based behavioural testing in PGE_2-exposed mice may determine potential correlations between altered hippocampal densities and behavioural outcomes.

We also observed that PGE_2-exposed males had greater E11 and E16 cell densities in the olfactory bulb compared to matched controls. In contrast, PGE_2 exposure did not affect olfactory bulb cell densities in females. Although often overlooked, olfaction can affect one's social life, behaviours, emotions, memory, and thought (Boesveldt et al. 2017, Kadohisa 2013, Sarafoleanu et al. 2009). The olfactory bulb is a large structure in the mouse and is important for processing many social signals (Lin et al. 2005). Perhaps abnormal cell densities in the olfactory bulb could be correlated to atypical sensory processing, particularly olfactory processing, present in ASDs (Tonacci et al. 2017, Wicker et al. 2016). Sex differences were specific to PGE_2-exposed mice, with greater E11 and E16 olfactory bulb cell densities in males compared to females. No sex differences were observed in control mice. This pattern is dissimilar to the sexual dimorphism seen in general human samples, where women had a greater number of neurons and glia in the olfactory bulb than men (Oliveira-Pinto et al. 2014).

We also reported that PGE_2 exposure in males and females were correlated with lower neocortical cell densities and increased cellular migration compared to matched controls. Sex differences were also observed: neocortical cell densities were greater in females compared to males in both control and PGE_2-exposed groups. Interestingly, in both PGE_2-exposed and controls groups, cells originating from E11 travelled a greater distance in females compared to males, while cells originating from E16 travelled a greater distance in males compared to females. Abnormal cell proliferation and migration measured in our study could lead to incorrect formation and circuitry of the neocortex, which may contribute to impairments in cognition and increased susceptibility to neurodevelopmental disorders (Rubenstein 2011, Valiente and Marin 2010). Several anatomical irregularities in the cortex have been reported in ASDs: increased neuron numbers, decreased interneuron numbers, disorganization of neurons and layering, disrupted minicolumn cell organization, and abnormal neuronal migration (Hashemi et al. 2017, Stoner et al. 2014, Courchesne et al. 2011, Wegiel et al. 2010, Hutsler et al. 2007, Buxhoeveden et al. 2006, Casanova et al. 2002). Sex differences in the general human population are varied: cell density in cortical layer II and IV is higher in females than males (Witelson et al. 1995), while other studies have found greater cortical neuronal numbers in males than females (Rabinowicz et al. 2002, de Courten-Myers 1999). Similar to research in humans, our results also demonstrate abnormal migration and cell numbers in the cortex of PGE_2-exposed mice as well as sex-dependent differences.

In addition to atypical cellular densities and cortical migration, PGE_2-exposed mice also exhibited altered gene expression of spinophilin (*Spn*) and beta-actin (*Actb*) in whole brain samples at postnatal day 8. *Spn* expression was decreased in PGE_2-exposed males compared to sex-matched controls. In contrast, PGE_2-exposed females had greater *Spn* expression than

controls. *Actb* expression was increased in PGE_2-exposed females compared to sex-matched controls but not statistical difference was observed in PGE_2-exposed males compared to controls. Spinophilin and beta-actin are both important components in the cytoskeleton and are involved in embryonic development of the nervous system. Spinophilin is required for various functions including dendritic spine morphology, cell adhesion, cell growth, and neuronal migration (Sarrouilhe et al. 2006, Feng et al. 2000). Similarly, beta-actin influences cell growth and motility, wiring of neuronal circuitry through growth cone development, and synaptogenesis (Tondeleir et al. 2014, Cheever et al. 2012, Bassell et al. 1998). Beta-actin may also play a role in cognitive and hyperactive behaviours (Cheever et al. 2012). Atypical sex-dependent expression of *Spn* and *Actb* observed in PGE_2-exposed mice may contribute to sex-specific abnormal cell densities and migration also quantified in this study, and could lead to abnormal brain connectivity and synaptic plasticity, which have been observed in ASD cases (Alaerts et al. 2016, Mottron et al. 2015). Gene expression analyses were conducted on whole brain samples but future investigations on region-specific expression of these genes will offer further details on how *Spn* and *Actb* may impact cell density and migration.

5.6. Conclusions

In conclusion, our results demonstrate that a single dose of maternal PGE_2 exposure might affect the fate of current and later dividing cells in offspring by contributing to sex-specific dysregulation of neocortical migration and cell densities in the cerebellum, hippocampus, olfactory bulb, and neocortex. Specific cell types—neurons, interneurons, or glia—that may be affected in PGE_2-exposed mice remain to be determined. Moreover, the potential effect on synaptic density, spine morphology, and dendritic spine density, which have

all been implicated in ASDs (Tang et al. 2014, Hutsler and Zhang 2010), could also be investigated in PGE_2-exposed mice. Overall, the findings of our study provide new evidence that prenatal PGE_2 exposure during early development may disrupt important neuronal processes that could influence pathogenesis of neurodevelopmental disorders such as ASDs.

5.7. Figures

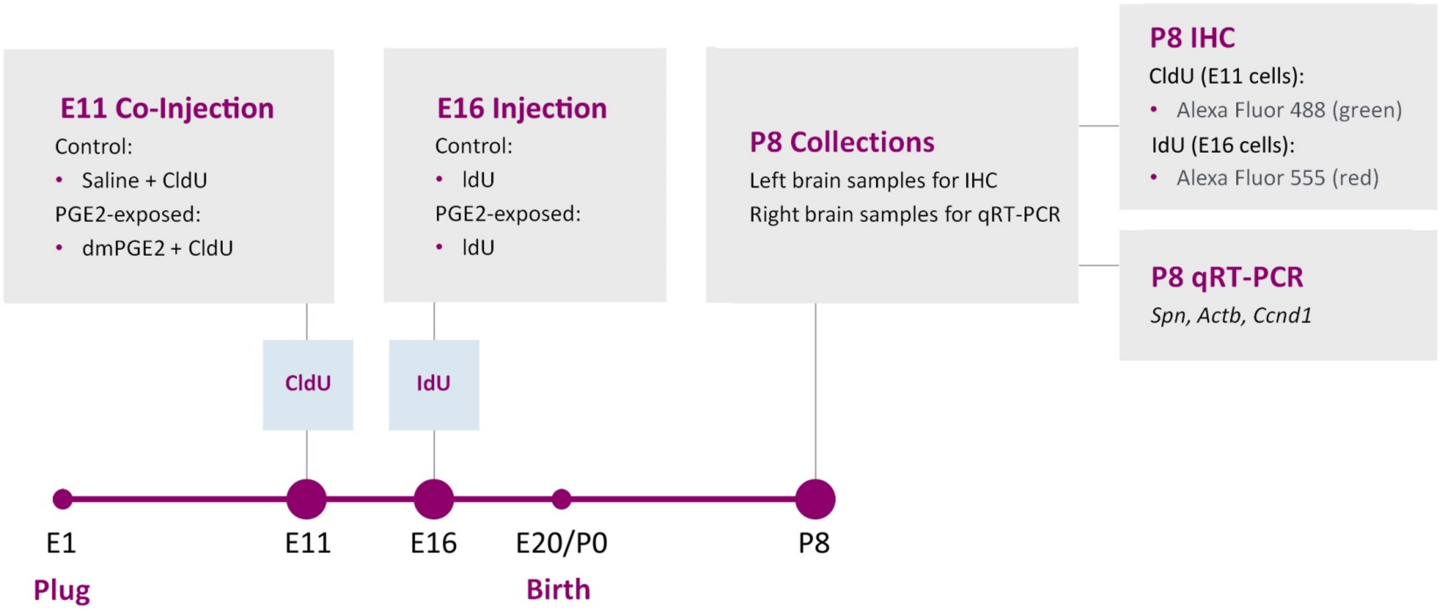

Fig. 5-S1: Methods timeline for cell density and migration quantification

Females were inspected every morning for a vaginal plug. Appearance of vaginal plug was noted as embryonic day 1; females were separated and housed individually for the entirety of their pregnancy. CldU and IdU labelling technique was used to identify and quantify two separate cell cohorts. On E11, control and PGE_2-exposed pregnant females were given a subcutaneous injection of 5-Chloro-2'- deoxyuridine (CldU) in saline. PGE_2-exposed pregnant females were given a single co-injection of CldU and 16, 16-dimethyl prostaglandin E2 ($dmPGE_2$). On E16, control and PGE_2-exposed pregnant females were given a subcutaneous injection of 5-Iodo-2'deoxyurdine (IdU). Birth occurs on the 20^{th} day of gestation (E20) also recorded as postnatal day 0 (P0) Left and right brain samples were extracted on P8 for fluorescent immunohistochemistry and qRT-PCR, respectively.

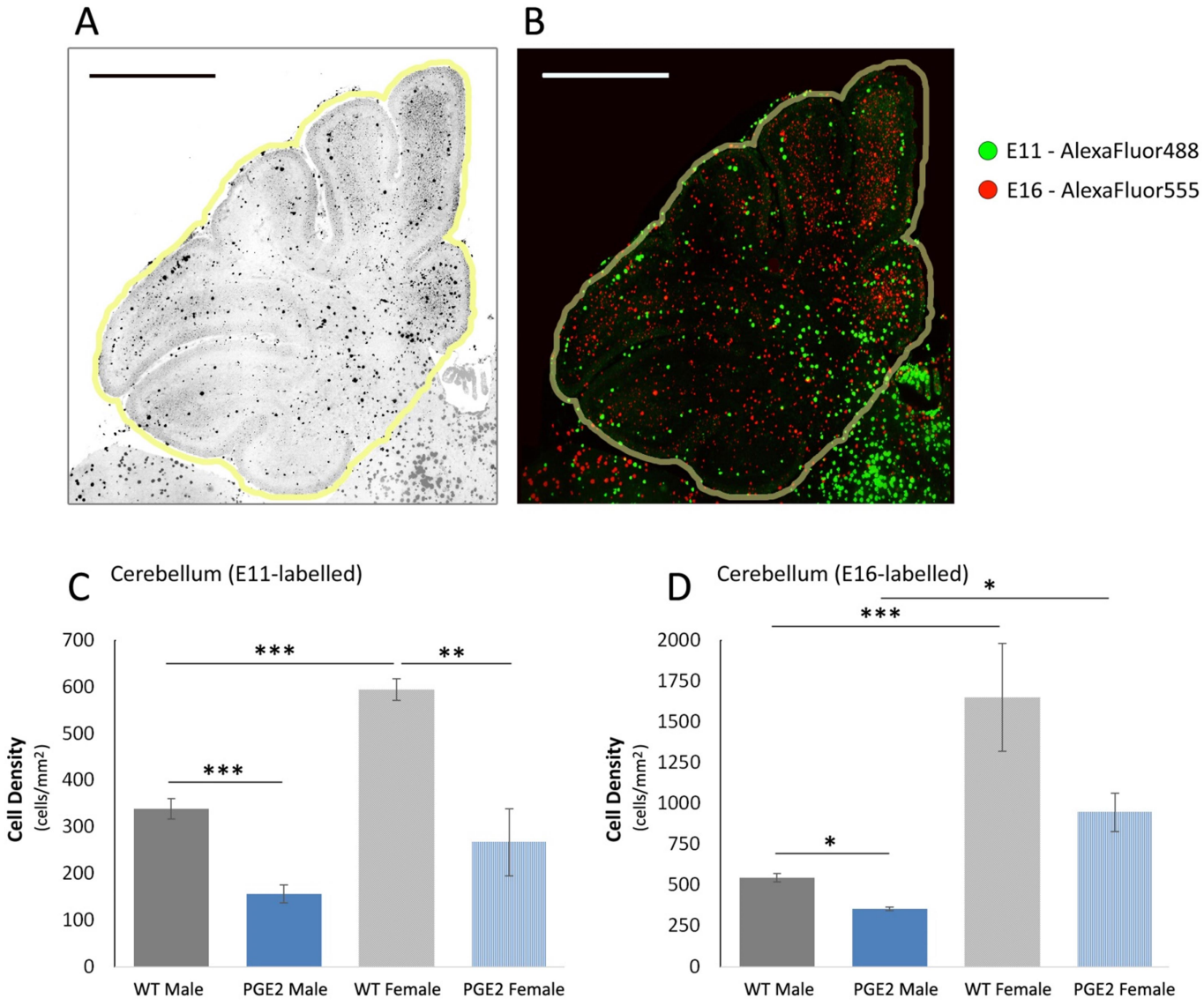

Fig. 5-1: E11 and E16 Cohort-labelled cell densities in the Cerebellum.

(A) Greyscale image and (B) Immunoflourescent image of cerebellum indicated by yellow outline. (C) Decreased cell density of E11-labelled cells in the cerebellum of PGE_2-exposed mice. Sex differences were only observed between control mice. (D) Decreased cell density of E16-labelled cells in the cerebellum of PGE_2-exposed males. Cerebellar E16 cell densities were greater in females than males. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001. Scale bar represents 500µM.

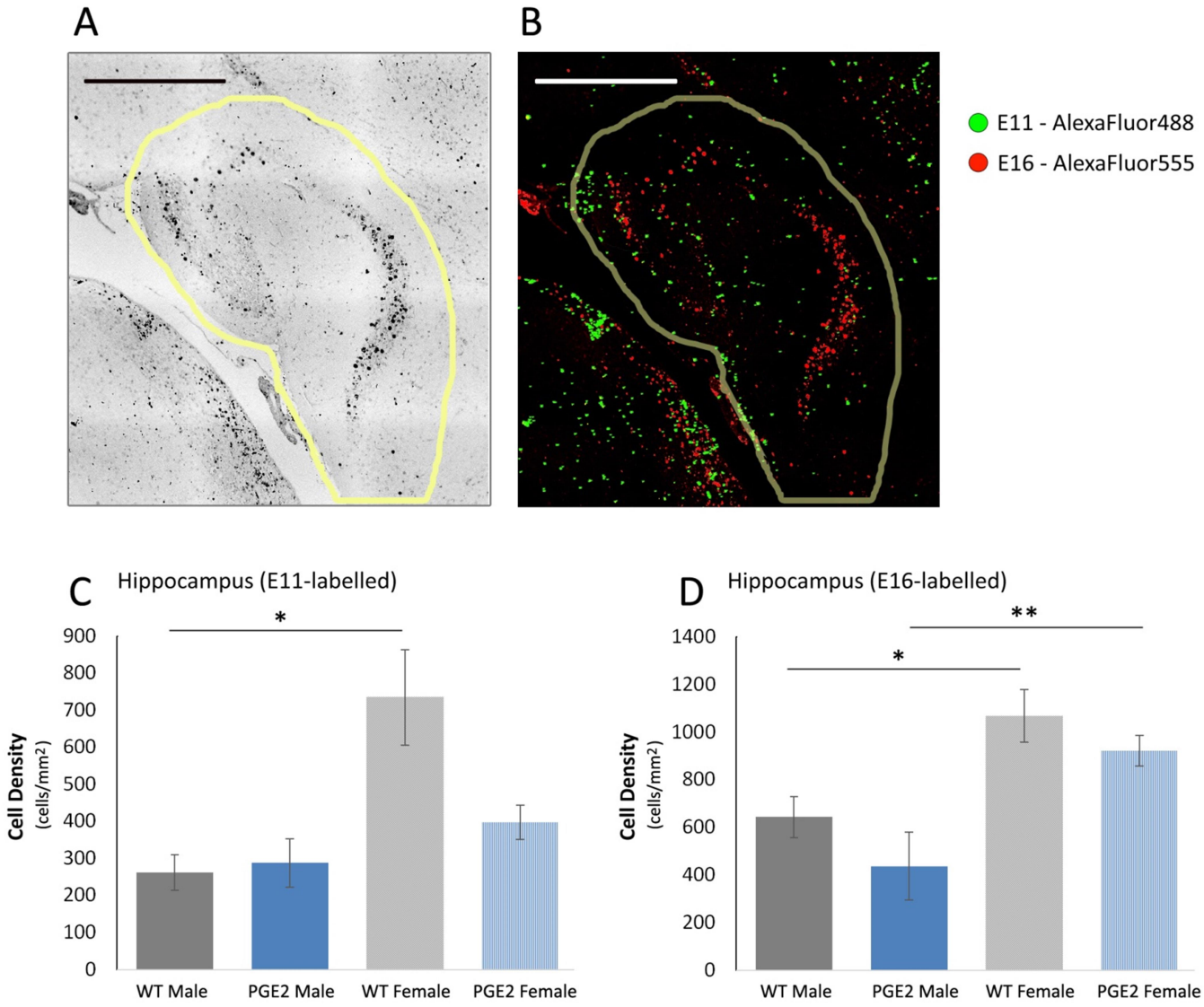

Fig. 5-2: E11 and E16 Cohort-labelled cell densities in the Hippocampus.

(A) Greyscale image and (B) Immunoflourescent image of hippocampus indicated by yellow outline. (C) PGE_2 exposure did not have a statistical effect on hippocampal cell density for E11-labelled cells compared to controls. In the control group, there was a higher E11-labelled cell density in females compared to males. (D) PGE_2 exposure did not have a statistical effect on hippocampal E16 cell density. Within the control and PGE_2-exposed group, there was a greater E16-labelled cell density in females compared to males. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001. Scale bar represents 500µM.

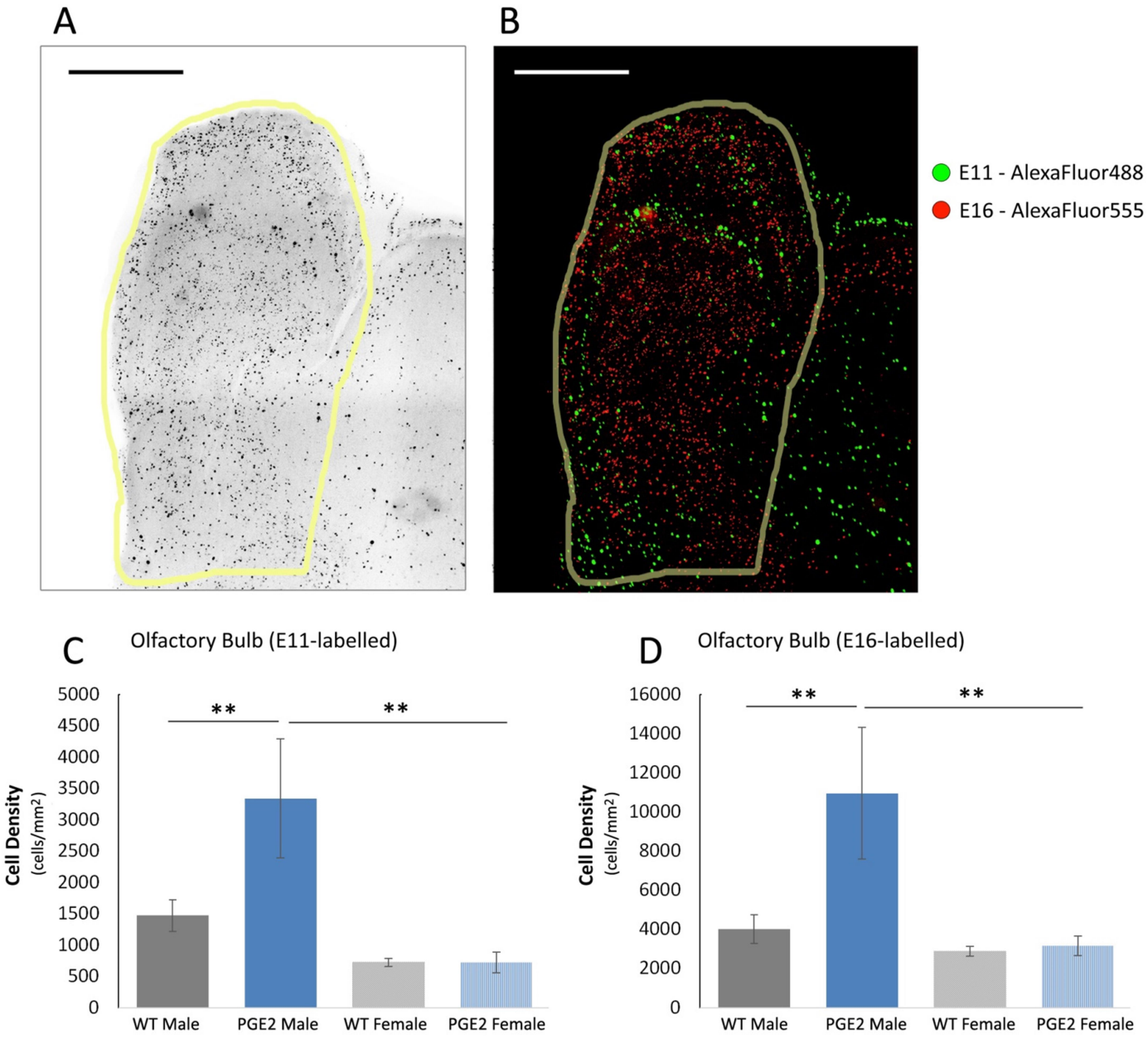

Fig. 5-3: E11 and E16 Cohort-labelled cell densities in the Olfactory Bulb.

(A) Greyscale image and (B) Immunoflourescent image of olfactory bulb indicated by yellow outline. (C) Increased cell density of E11-labelled cells in the olfactory bulb of PGE_2-exposed males. In the PGE_2-exposed group, olfactory bulb E11 cell density was greater in males than females. (D) Similarly, there was increased cell density of E16-labelled cells in the olfactory bulb of PGE_2-exposed males. Olfactory bulb E16 cell density was greater in PGE_2-exposed males than females. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001. Scale bar represents 500µM.

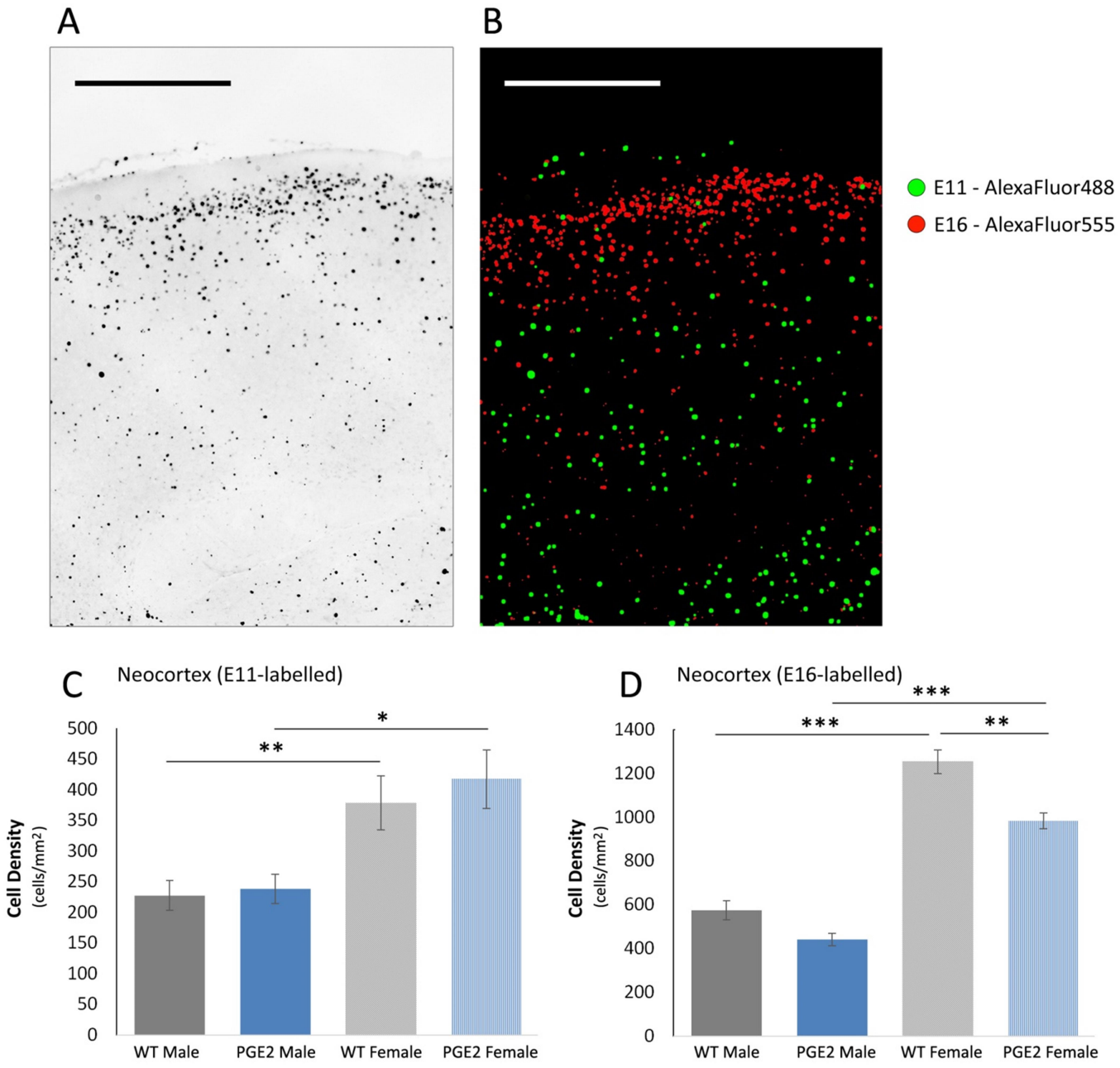

Fig. 5-4: E11 and E16 Cohort-labelled cell densities in the Neocortex.

(A) Greyscale image and (B) Immunoflourescent image of a representative portion of the neocortex. (C) PGE_2 exposed did not significantly affect cell density of E11-labelled cells in the neocortex. Within control and PGE_2-exposed groups, females had a greater E11 cell density than males. (D) Decreased cell density of E16-labelled cells in the neocortex of PGE_2-exposed females. Neocortical E16 cell densities were greater in females than males. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001. Scale bar represents 500µM.

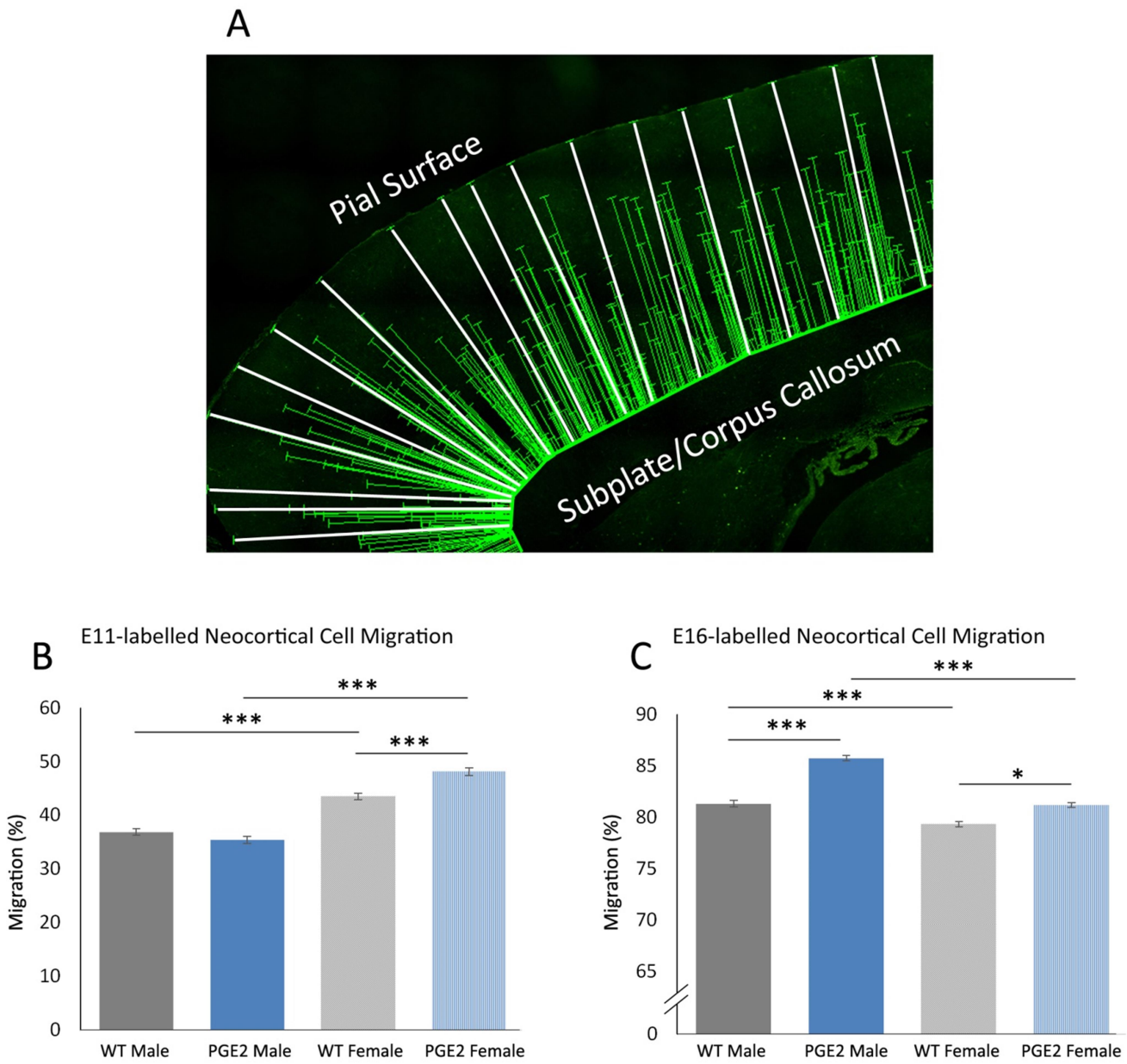

Fig. 5-5: E11 and E16 Cohort-labelled cell migration in the Neocortex.

(A) Neocortical cell migration distances from the subplate to the labelled cells were measured in the cortex (E11-labelled cells shown here). The migration percentage for each cell was calculated by taking its *migration distance* (indicated by green lines) and dividing it by the *total distance* between the subplate/corpus callosum and pial surface (indicated by white lines). (B) Increased neocortical cell migration of E11-labelled cells in PGE_2-exposed females. In the control and PGE_2-exposed group, E11-labelled cell migration was greater in females than males. (C) Increased neocortical cell migration of E16-labelled cells in PGE_2-exposed males and females compared to sex-matched controls. E16-labelled cells migrated further in males compared to females within control and PGE_2-exposed groups. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

Table 5-2 : Growth and migration gene expression in PGE_2-exposed mice.

Gene expression analysis were completed on postnatal day 8 samples as shown as RQ units for male and female PGE_2 groups relative to respective controls (RQ=1). Values represent the mean of at least 3 independent individuals. *p<0.05, **p<0.01, ***p<0.001

PGE_2-exposed compared to WT controls (RQ=1)		
Males	**RQ Mean Values**	***p*-values**
Spn	**0.487**	**0.045***
Actb	0.753	0.227
Ccnd1	0.725	0.103
Females	**RQ Mean Values**	***p*-values**
Spn	**1.526**	**0.049***
Actb	**1.309**	**0.033***
Ccnd1	1.198	0.161

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CHAPTER 6.

Study 4: Augmented microglial density and abnormal morphology in mice with defective cyclooxygenase-2/prostaglandin E2 signalling

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Contributions: Christine T. Wong designed and performed all experiments, collected samples, acquired and analyzed the data, made all figures and tables, and prepared the manuscript. Josee Smith assisted in the collection of mouse samples and helped with acquiring data. Dr. Dorota A. Crawford supervised the design of the study and was involved with editing the manuscript.

Objectives and Hypotheses: The fourth study was comprised of *in vivo* experiments with the main purpose of studying the effect of elevated PGE_2 and COX-2-deficiency on microglia. The objectives were to determine whether elevated PGE_2 or COX-2-deficiency can influence microglial density and morphology. The cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus were investigated. I hypothesized that both exposure to PGE_2 or lack of COX-2 activity would disrupt microglial density, activation state, branch density, and process length in the mouse brain in a sex-dependent and region-specific manner. This was the first study to determine that aberrations to the COX-2/PGE_2 signalling pathway during prenatal development could alter microglia in the brain.

6.1. Chapter Summary

Cyclooxygenase-2 (COX-2) and its enzymatic product, prostaglandin E2 (PGE_2), have key roles in inflammation and normal developmental processes in the brain, including synaptic plasticity and masculinization. The COX-2/PGE_2 lipid signalling pathway is susceptible to numerous environmental agents associated with autism spectrum disorders (ASDs). Our recent studies in mice determined that an elevated level of maternal PGE_2 during pregnancy or COX-2-deficiency leads to autism-related behaviours in offspring that is sex-dependent. Our previous research has also provided some insight into possible molecular mechanisms by which defects in PGE_2 signalling may affect brain development. In this study, we aim to further investigate the impact of abnormal signalling of the COX-2/PGE_2 pathway on microglial density and morphology in the developing brain. In the healthy brain, microglia play a well-established role in inflammation but can also influence many neurodevelopmental processes including synaptic modification. We demonstrate that both maternal exposure to PGE_2 or lack of COX-2 activity affect microglial density, activation state, branch density, and process length in the mouse brain at postnatal day 8 in a sex-dependent and region-specific manner. Overall, PGE_2-exposed mice exhibited higher microglial density with a greater percentage of amoeboid microglia and fewer ramified microglia, which was further demonstrated by decreased branching and process lengths. COX-2-deficient mice also had an elevated microglial density but with a lower percentage of amoeboid microglial cells and more ramified microglia. Greater branching and increased process lengths were also found in COX-2-deficient mice. Our findings in two separate mice models of ASDs provide further evidence that abnormal COX-2/PGE_2 signalling can contribute to brain pathology by disrupting microglial density and morphology.

6.2. Introduction

Prostaglandin E2 (PGE_2) is a bioactive signalling molecule derived from cell membrane lipids from the enzymatic activity of cyclooxygenase-1, and -2 (COX-1, 2). In the brain, COX-2 expression is particularly important since it can be both constitutive and inducible. Its constitutive expression contributes to normal brain functions while its inducible expression results as a response to intrinsic and extrinsic stressors (Minghetti, 2004). COX-2 regulates the production of PGE_2, which is best known as a potent inflammatory mediator but is also the main prostanoid in the developing nervous system, involved in synaptic plasticity, cerebellar development, and masculinization (Dean et al., 2012, Dean et al., 2012, Wright and McCarthy, 2009, Chen and Bazan, 2005).

Growing clinical and molecular research provides evidence that abnormal COX-2/PGE_2 signalling is linked to autism spectrum disorders (ASDs) (Wong et al., 2015, Wong and Crawford, 2014, Tamiji and Crawford, 2010). For instance, various genetic factors and environmental insults such as maternal infections, pollution, pesticides, and drugs capable of affecting the level of PGE_2 have been linked to ASDs. Furthermore, prenatal use of common over-the-counter drugs that inhibit COX enzymatic activity, such as acetaminophen, has been linked to behavioural disorders including, ADHD and autism (Bauer et al., 2018, Ystrom et al., 2017, Avella-Garcia et al., 2016). Current findings from our lab in murine models have determined for the first time that an elevated level of maternal PGE_2 during pregnancy or COX-2-deficiency leads to autism-related behaviours manifested in a sex-dependent manner (Wong et al., 2019, Wong et al., 2017). Our previous research has also revealed possible molecular mechanisms by which abnormal COX-2/PGE_2 signalling may affect brain development including dysregulation of intracellular calcium level, proliferation and migration of neuronal

stem cells, neuronal differentiation, and expression of ASD-linked genes (Rai-Bhogal et al., 2018, Davidson et al., 2016, Wong et al., 2016, Wong et al., 2014, Tamiji and Crawford, 2010). Moreover, we showed that COX-2-deficient mice exhibit differential expression of various ASD-associated genes and deficits in pathways involved in synaptic transmission, dendritic spine formation, and immune response (Wong et al., 2019, Rai-Bhogal et al., 2018)

Interestingly, current research shows that in the healthy brain, PGE_2 can also regulate neonatal microglia activation (Chen et al., 2018, Cantaut-Belarif et al., 2017, Quan et al., 2013). Microglia are major resident immune cells in the central nervous system and are key mediators of neuroinflammatory processes. Microglia are also essential in the development and wiring of synaptic circuitry (Ziebell et al., 2015, Paolicelli and Gross, 2011, Tremblay et al., 2010, Wake et al., 2009). In addition, dysfunction of microglia has been implicated in ASDs (Kim et al., 2018, Edmonson et al., 2016, Koyama and Ikegaya, 2015, Takano, 2015, Rodriguez and Kern, 2011). However, the molecular mechanisms that might affect microglial dysfunction in ASDs are not known.

In this study, we aim to examine whether abnormal COX-2/PGE_2 signalling can disrupt microglial density and morphology during early development. Two mouse model systems of ASDs recently established in our lab were used here: offspring exposed to PGE_2 during pregnancy at embryonic day 11 (E11) and homozygous COX-2^- knockin (KI) mice deficient in the PGE_2-producing enzyme (Wong et al., 2019, Wong et al., 2017). Our data collected from pups on postnatal day 8 (P8) brains revealed that irregular COX-2/PGE_2 signalling in both models alters microglial density and morphology in various neuroanatomic locations that have been previously implicated in ASDs, including the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus in a sex-dependent manner. These findings demonstrate that

abnormal PGE_2 levels during prenatal development affects microglia in mouse offspring that display autism-relevant behaviours. These results add to our previous studies by providing evidence that abnormal COX-2/PGE_2 signalling might lead to some cases of ASDs by affecting microglia function in the developing brain.

6.3. Methods

Animals in PGE_2 -exposed model: C57bl/6 wildtype mice and maternal Injection of PGE_2

Wildtype C57bl/6 (WT-C57) mice were obtained from Charles River Laboratories. Animals were maintained on a 12-hour light/dark cycle at York University animal. Ethics guidelines of the York University Animal Care Committee were followed for all experiments and protocols, which were approved by the Research Ethics Board of York University.

Overnight breeding was completed by housing male and female mice together. Females were inspected for a vaginal plug every morning, which was recorded as embryonic day 1 (E1). If a vaginal plug was found, females were separated and housed individually for their entire pregnancy. On E11, a single subcutaneous injection of 0.2 μg/g concentration of 16, 16-dimethyl prostaglandin E2 (dmPGE_2; Cayman Chemical) diluted in saline or saline vehicle (for WT-C57 mice) was administered to pregnant females, as done in previous studies (Rai-Bhogal et al., 2018, Wong et al., 2017, Okamoto et al., 2011, Tessner et al., 2004). dmPGE_2 has a slower metabolic rate and is a stable analogue of PGE_2 (Ohno et al., 1985, Steffenrud, 1980). E11 marks the start of neurogenesis in mice and also corresponds with the time that misoprostol, a drug analogous to PGE_2, was taken in human cases resulting in Moebius syndrome and autism characteristics (Bandim et al., 2003, Pastuszak et al., 1998). Maternal exposure of dmPGE_2 at E11 has also been recently shown to result in autism-like behaviours and altered expression of

autism-linked genes in offspring (Rai-Bhogal et al., 2018, Wong et al., 2017). Mouse offspring exposed to maternal dmPGE$_2$ will be referred to as "PGE$_2$ mice".

COX-2$^-$ knock-in model: 129S6 Wildtype mice and COX-2$^-$ knock-in mice

Ptgs2^{Y385F} founder mice, also known as B6.129S6(FVB)-*Ptgs2$^{tm1.1Fun}$*/J mice or *Ptgs2* knockin (COX-2$^-$ KI) mice, were obtained from Jackson Laboratory (stock #008101; Queen's University, laboratory of C. Funk) backcrossed for at least 5 generations to wild-type 129S6/SvEvTac (WT-S6) mice acquired from Taconic Laboratory. *Ptgs2^{Y385F}* mice are a genetic mouse model created by a targeted point mutation of the *Ptgs2* gene, specifically a Y385F amino acid substitution. This results in complete inhibition of COX-2 activity but downstream peroxidase activity remains intact (Yu et al., 2006, Yu and Funk, 2006). Since homozygous COX-2$^-$ KI females are infertile, heterozygous females were bred with homozygous or heterozygous males to produce COX-2$^-$ KI male and female offspring for brain sampling. All WT-S6 and COX-2$^-$ KI mice were bred and maintained under the same conditions at York University on a 12-hours light/dark cycle.

Genotyping

Polymerase chain reaction (PCR) analysis was conducted for genotyping analysis of the COX-2 gene using primer sequences detailed by the Jackson Laboratory (*Ptgs2*; Table 6-1). DNA extraction was completed on individual tail samples, which were denatured in alkaline lysis buffer (25mM NaOH) at 95°C for 30 min, followed by addition of neutralization buffer (Tris-HCl). Standard PCR was performed in an Eppendorf Mastercycler according to the Jackson Laboratory *Ptgs2$^{tm1.1Fun}$* protocol (The Jackson Laboratory, 2016).

Table 6-1: PCR Primer set for *Ptgs2* Genotype Analysis

Primer	Name	Primer Sequence (5'-3')	Base pairs	COX-2$^-$	Wild-type
Forward	Mus Ptgs2IMR7834	ACCAGTCTCTCAATGAGTAC	20 bp	585 bp	493 bp
Reverse	Mus Ptgs2IMR7835	AGAATGGTGCTCCAAGCTCTAC	22 bp		

Brain Exaction

Whole brain samples were extracted on postnatal day 8 (P8), where birth was considered postnatal day 0. P8 in mice is analogous to infanthood in humans (Pressler and Auvin, 2013, Semple et al., 2013), which is the time when ASD symptoms are first observed. Brain samples were collected and fixed in 4% (w/v) paraformaldehyde (PFA) in PBS at 4°C for 48 hours in preparation for histological staining.

Immunohistochemistry

P8 brain samples were carefully extracted, prepared, and then delivered to The Centre for Phenogenomics (Toronto, Canada) for paraffin-embedding and 4μm serial slicing from the mid-sagittal plane. Paraffin removal from samples was completed by xylene incubation, followed by subsequent ethanol incubations for rehydration. Antigen retrieval was performed by boiling samples in 0.01M pH 6.0 sodium citrate buffer for 20 minutes in a microwave. To prevent endogenous peroxidase activity, slides were then incubated with 0.3% H_2O_2 in methanol for 30 minutes. Next, sections were circled using a liquid blocking super PAP pen (Cedarlane). All subsequent steps were then performed in a hydration chamber. Blocking was completed in 5% goat serum and 0.1% Triton X-100 diluted in PBS for 30 minutes. Slides were then incubated overnight at 4°C in primary antibody for Iba1 called Rabbit anti-Iba1 (1:1000, Wako, #019-19741) diluted in 5% goat serum in PBS. Secondary antibody staining was completed with

biotinylated anti-rabbit IgG antibody (1:200, Vector Laboratories) for 1 hour, followed by 1 hour incubation with Elite ABC Reagent (Vectastain Avidin Biotin Complex kit, Vector laboratories). Immunostaining was developed by incubating samples with 3,3'-Diaminobenzidine (DAB) substrate solution (SIGMAFAST DAB tablets, D4168, Sigma) in 0.3% ammonium nickel sulfate hexahydrate and Milli-Q water. Slides were then dehydrated in ascending EtOH washes followed by xylene incubation. Permount mounting medium was used to coverslip slides.

Microglial Cell Density and Morphological Analysis

Microglia-specific staining was captured using an Eclipse Ti microscope (Nikon). The cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus were investigated using light microscopy and visualized at 20x magnification with automated image stitching (Fig. 1). Estimated cell density measurements for each brain region were calculated by dividing the total number of cells by the area of interest. Microglial cells were characterized morphologically. Quiescent microglia are thought to have a ramified morphology with branches processes that are long and thin; while activated microglia have enlarged cell bodies that are characteristically round and amoeboid in shape (Kloss et al., 2001; Stence et al., 2001; Schwarz et al., 2012; Schafer et al., 2012). Activation state (Amoeboid or Ramified), number of primary branches and longest length of processes of each microglial cell were quantified using NIS-Elements software (Nikon). Primary branches were defined as branches that protruded directly from the soma. Length of processes were measured from the surface of the soma. All analyses were completed blind to the condition.

Statistical Analysis

Numerical data are reported as mean±standard error of the mean (SEM), which represents quantification from a minimum of three individuals from at least three separate litters. Two-way ANOVA followed by post-hoc Bonferroni pairwise comparisons were conducted to determine if there were significant differences in microglial characteristics between experimental groups of interest and respective wildtype (WT) control groups, and between males and females. Significance was determined for p values less than 0.05.

6.4. Results

Altered Microglial Density in PGE_2-exposed offspring mice

Changes in microglial density have been previously shown to influence key developmental processes including neurogenesis, neuron migration, formation of axons, and synaptic pruning (Askew 2018, Cunningham 2013, Squarzoni 2014, Rodriguez 2010, Antony 2011, Paolicelli 2011). In this study, microglial density was quantified in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus at postnatal day 8 (P8) (Fig. 6-1) to determine whether differences occurred between PGE_2-exposed mice and wild-type C57bl/6 (WT-C57) mice. Males and females were also examined independently. Two-way ANOVA analysis on microglial density values (cells/mm^2) was conducted for each brain region.

Results from the cerebellum (Fig. 6-2, $F(3,8)=2.913$, $p=0.100727$) show that the cerebellar microglial density of PGE_2 mice (males and females combined) was statistically greater than WT-C57 mice ($p=0.035251$, WT=73.1±11.5, PGE_2=116.1±12.9). Males and females of each group were also independently investigated. The cerebellum of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had microglial densities of 62.3±17.0, 101.2±4.0,

83.8±16.1, 131.0±24.3, respectively. Post hoc comparisons revealed no significant differences between groups within the same sex or between sexes (p>0.05).

In the hippocampus (Fig. 6-2, F(3,8)=1.757, p=0.232827), there were no significant differences in microglial density between PGE_2 mice as compared to WT-C57 mice (p=0.23429, WT=143.0±12.3, PGE_2=161.3±9.1). The hippocampus of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had microglial densities of 129.0±23.4, 148.3±4.3, 157.0±4.2, 174.4±15.0, respectively, with no significant sex-dependent differences between these groups (p>0.05).

In the olfactory bulb (Fig. 6-2, F(3,8)=3.279, p=0.079612), microglial density of PGE_2 mice was statistically greater than WT-C57 mice (p=0.018863, WT=83.1±10.4, PGE_2=174.3±28.0). No significant differences were observed in the olfactory bulb of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females, which had microglial densities of 69.5±12.7, 154.1±19.4, 96.7±13.8, 194.5±56.0, respectively (p>0.05).

Results from the prefrontal cortex (Fig. 6-2, F(3,8)=2.148, p=0.172289) showed no statistical differences in microglial density between PGE_2 mice and WT-C57 mice (p=0.324361, WT=88.8±11.3, PGE_2=100.6±6.3). We also did not see significant sex differences. The prefrontal cortex of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had microglial densities of 74.8±4.3, 88.9±3.8, 102.9±20.5, 112.3±6.8, respectively. Post hoc comparisons showed no significant differences between these groups (p>0.05).

In the thalamus (Fig. 6-2, F(3,8)=4.686, p=0.035839), microglial density of PGE_2 mice was statistically greater than WT-C57 mice (p=0.043477, WT=65.4±5.2, PGE_2=86.0±6.6). The thalamus of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had microglial densities of 50.1±5.2, 74.4±6.6, 80.7±2.2, 97.7±16.0, respectively. There were significantly

greater microglial density in the thalamus of WT-C57 females compared to WT-C57 males (p=0.044505) but this difference was not observed between PGE_2 males and PGE_2 females (p>0.05).

Overall, PGE_2-exposed mice (males and females combined) at P8 had higher microglial densities in the cerebellum, olfactory bulb, and thalamus compared to WT-C57 mice. PGE_2-exposure did not affect microglial densities in the prefrontal cortex and hippocampus. When each sex was analyzed independently, statistical differences were generally not found between males and females, with the exception of WT-C57 males and females, where females had a greater microglial density than males in the thalamus.

Altered Microglial Density in COX-2$^-$ KI mice

Microglial densities were also investigated in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus in COX-2$^-$ KI mice and wild-type 129S6 (WT-S6) mice at P8. Males and females were also examined separately. Two-way ANOVA analysis on microglial density values (cells/mm^2) was conducted on each area.

Similar to the PGE_2-exposed animals (males and females combined), results for cerebellar microglial density (Fig. 6-3, $F(3,8)$= 5.200, p=0.027716) showed that COX-2$^-$ KI mice was statistically greater than WT-S6 mice (p=0.006634, WT=56.7±8.2, COX-2=87.5±8.0). Males and females of each group were also independently investigated. The cerebellum of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial densities of 50.7±3.0, 94.7±10.1, 62.6±3.4, 80.4±12.9, respectively. Interestingly, post hoc comparisons revealed that COX-2$^-$ males had a significantly higher density of microglia in the cerebellum than WT-S6 males (p=0.006417). No statistical difference was found between WT-S6 females and

COX-2$^-$ females (p=0.17641). Sex-dependent differences in microglial density were not found in the cerebellum (p>0.05).

For hippocampal microglial density (Fig. 6-3, F(3,8)=0.301, p=0.823721), there were no significant differences between COX-2$^-$ KI mice and WT-S6 mice (p=0.708032, WT=129.5±10.8, COX-2=134.8±7.1). The hippocampus of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial densities of 132.4±18.0, 142.8±13.4, 126.5±15.8, 126.9±3.0, respectively, with no significant differences within groups of the same sex or between sexes (p>0.05).

In the olfactory bulb (Fig. 6-3, F(3,8)=5.536, p=0.023632), microglial density of COX-2$^-$ KI mice was statistically greater than WT-S6 mice (p=0.024542, WT=98.1±4.6, COX-2=115.2±6.6). The olfactory bulb of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial densities of 105.8±6.3, 104.6±6.7, 90.4±3.0, 125.8±7.8, respectively. COX-2$^-$ females had a significantly higher density of microglia in the olfactory bulb than WT-S6 females (p=0.003708). No statistical difference was found between WT-S6 males and COX-2$^-$ males (p=0.894199). Sex-dependent differences were seen in COX-2$^-$ mice, where females had a greater microglial density than males (p=0.041503) but a sex-dependent effect was not present in WT-S6 mice (p=0.116908).

In the prefrontal cortex (Fig. 6-3, F(3,8)=24.377, p=0.000223), microglial density of COX-2$^-$ KI mice was statistically greater than WT-S6 mice (p=0.000077, WT=66.6±4.4, COX-2=100.2±5.9). The prefrontal cortex of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial densities of 73.5±5.2, 88.2±1.9, 59.7±4.9, 112.2±5.3, respectively. COX-2$^-$ females had a significantly higher density of microglia in the prefrontal cortex than WT-S6 females (p=0.000038). No statistical difference was found between WT-S6 males and COX-

2^- males (p=0.052043). Sex-dependent differences were seen in COX-2^- mice, where females had a greater microglial density than males (p=0.00574) but a sex-dependent effect was not present in WT-S6 mice (p=0.063866).

In the thalamus (Fig. 6-3, $F(3,8)$=1.266, p=0.349611), microglial density of COX-2^- KI mice was not statistically different than WT-S6 mice (p=0.415009, WT=63.1±6.4, COX-2=70.1±5.8). The thalamus of WT-S6 males, COX-2^- males, WT-S6 females, and COX-2^- females had microglial densities of 71.6±10.0, 75.4±9.9, 54.5±5.3, 64.7±6.1, respectively, with no significant differences between these groups (p>0.05).

Overall, COX-2^- KI mice at P8 had higher microglial densities in the cerebellum, olfactory bulb, and prefrontal cortex compared to WT-S6 mice. Disruption of COX-2 activity did not affect microglial densities in the hippocampus and thalamus. When each sex was analyzed independently, statistical differences were found in various regions between WT-S6 and COX-2^- mice, including the cerebellum (males), olfactory bulb (females), and prefrontal cortex (females). Moreover, sex-dependent differences were not present in WT-S6 but were found in the olfactory bulb and prefrontal cortex of COX-2^- KI mice, with greater microglial densities in females than males.

Altered Amoeboid and Ramified Microglial Morphology in PGE_2-exposed offspring mice

Microglia were characterized into their two main morphological phenotypes—active amoeboid cells or quiescent ramified cells (Fig. 6-4)—in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus at P8 brain samples of PGE_2-exposed mice and wild-type C57bl/6 (WT-C57) mice.

Amoeboid Morphology Percentages

The percentage of amoeboid microglia to total microglia identified in each specified areas was calculated. Two-way ANOVA analysis on active amoeboid microglia percentages was conducted on each brain region of interest.

In the cerebellum (Fig. 6-4, $F(3,8)=19.568$, $p=0.000484$), active amoeboid microglia morphology percentages of PGE_2 mice in general was not statistically different from WT-C57 mice when males and females were averaged together ($p=0.20356$, WT=22.1±4.6, PGE_2=25.0±2.7). Investigating males and females of each group separately showed that the cerebellum of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had active microglia morphology percentage values of 32.0±0.9, 30.2±2.2, 12.1±2.6, 19.8±2.3, respectively. There were no difference between WT males and PGE_2 males ($p>0.05$) but PGE_2 females had significantly greater microglial activated morphology compared to WT females ($p=0.033994$). Moreover, females had statistically lower cerebellar microglial activated morphology than males within the WT groups ($p=0.000158$) and the PGE_2 groups ($p=0.007888$).

For hippocampal active amoeboid microglia percentages (Fig. 6-4, $F(3,8)=0.351$, $p=0.789928$), there were no significant differences between PGE_2 mice and WT-C57 mice ($p=0.73294$, WT=9.6±2.7, PGE_2=10.8±2.2). The hippocampus of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had amoeboid morphology percentages of 11.8±3.6, 9.7±4.5, 7.1±4.3, 11.8±1.7, respectively, with no significant differences between these groups ($p>0.05$).

Olfactory bulb active amoeboid microglia percentages (Fig. 6-4, $F(3,8)=2.961$, $p=0.097613$) showed no significant differences between PGE_2 mice and WT-C57 mice ($p=0.286967$, WT=17.5±4.0, PGE_2=21.6±2.2). The olfactory bulb of WT-C57 males, PGE_2

males, WT-C57 females, and PGE_2 females had amoeboid morphology percentages of 23.6±5.2, 25.3±1.5, 11.4±3.9, 18.0±3.0, respectively. WT-C57 males had greater microglial activation in the olfactory bulb compared to WT-C57 females (p=0.046131) but there were no other significant differences between these groups (p>0.05).

There were no statistical differences in prefrontal cortex active amoeboid microglia percentages (Fig. 6-4, $F(3,8)$=4.546, p=0.038563) between PGE_2 mice and WT-C57 when both sexes were combined (p=0.361248, WT=9.5±2.8, PGE_2=12.3±3.1). The prefrontal cortex of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had active microglia percentages of 14.5±3.7, 17.6±1.9, 4.5±0.2, 7.0±4.0, respectively. Post hoc comparisons confirmed no significant differences between WT and PGE_2-exposed mice within the male or female groups (p>0.05). However, post hoc analysis showed that males had greater microglial activation compared to females within the WT groups (p=0.039795) and PGE_2 groups p=0.032334).

Interestingly, percentage of active amoeboid microglia morphology in the thalamus (Fig. 6-4, $F(3,8)$=4.364, p=0.04247) were overall statistically greater in PGE_2 mice than WT-C57 mice (p=0.016325, WT=5.7±1.3, PGE_2=11.5±1.7). The thalamus of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had active microglial percentages of 8.3±0.8, 11.6±2.4, 3.0±0.6, 11.3±2.8, respectively. There was greater microglial activation in the thalamus of PGE_2-exposed females compared to WT-C57 females (p=0.015239). There were no statistical differences between the remaining comparisons (p>0.05).

Overall, PGE_2-exposed mice at P8 had higher percentages of active amoeboid microglia morphology in the cerebellum and thalamus compared to WT-C57 mice in a sex-dependent manner, with differences between females groups reaching significance. PGE_2-exposure did not

affect overall microglial activation percentages in the olfactory bulb, prefrontal cortex and hippocampus. When each sex was analyzed independently, a general trend appeared where males had a greater percentage of active microglia compared to females in the cerebellum, olfactory bulb, and prefrontal cortex.

Ramified Morphology Percentages

The percentage of ramified microglia to total microglia identified in each specified areas was also calculated. Two-way ANOVA analysis on ramified microglia percentages was conducted on each brain region of interest.

In the cerebellum (Fig. 6-4, $F(3,8)=19.568$, $p=0.000484$), ramified microglia morphology percentages of PGE_2 mice was not statistically different from WT-C57 mice when males and females were combined ($p=0.20356$, WT=77.9±4.6, PGE_2=75.0±2.7). Investigating males and females of each group separately showed that the cerebellum of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had ramified microglia morphology percentage values of 68.0±0.9, 69.8±2.2, 87.9±2.6, 80.2±2.3, respectively. There were no difference between WT males and PGE_2 males ($p>0.05$) but PGE_2 females had significantly less ramified morphology compared to WT females ($p=0.033994$). Moreover, females had statistically greater cerebellar microglial ramified morphology than males within the WT groups ($p=0.000158$) and the PGE_2 groups ($p=0.007888$).

For hippocampal ramified microglia percentages (Fig. 6-4, $F(3,8)=0.351$, $p=0.789928$), there were no significant differences between PGE_2 mice and WT-C57 mice ($p=0.73294$, WT=90.5±2.7, PGE_2=89.2±2.2). The hippocampus of WT-C57 males, PGE_2 males, WT-C57

females, and PGE_2 females had ramified morphology percentages of 88.2±3.6, 90.3±4.5, 92.9±4.3, 88.2±1.7, respectively, with no significant differences between these groups (p>0.05).

For olfactory bulb ramified microglia percentages (Fig. 6-4, $F(3,8)=2.961$, p=0.097613), there were no significant differences between PGE_2 mice and WT-C57 mice (p=0.286967, WT=82.5±4.0, PGE_2=78.4±2.2). The olfactory bulb of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had ramified morphology percentages of 76.4±5.2, 74.7±1.5, 88.6±3.9, 82.0±3.0, respectively. WT-C57 males had fewer ramified microglia in the olfactory bulb compared to WT-C57 females (p=0.046131) but there were no other significant differences between these groups (p>0.05).

In the prefrontal cortex (Fig. 6-4, $F(3,8)=4.546$, p=0.038563), no differences were found for ramified microglia percentages between PGE_2 mice and WT-C57 (p=0.361248, WT=90.5±2.8, PGE_2=87.7±3.1). The prefrontal cortex of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had ramified microglia percentages of 85.5±3.7, 82.4±1.9, 95.5±0.2, 93.0±4.0, respectively. Post hoc comparisons confirmed no significant differences between WT and PGE_2-exposed mice within the male or female groups (p>0.05). However, post hoc analysis showed that males had fewer ramified microglia compared to females within the WT groups (p=0.039795) and PGE_2 groups p=0.032334).

For percentages of ramified microglia morphology in the thalamus (Fig. 6-4, $F(3,8)=4.364$, p=0.04247), it was statistically less in PGE_2 mice than WT-C57 mice (p=0.016325, WT=94.3±1.3, PGE_2=88.5±1.7). The thalamus of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had ramified microglia percentages of 91.6±0.8, 88.4±2.4, 97.0±0.6, 88.7±2.8, respectively. There was less ramified microglia in the thalamus of PGE_2-

exposed females compared to WT-C57 females (p=0.015239). There were no statistical differences between the remaining comparisons (p>0.05).

Overall, the results of ramified microglia percentages complimented the amoeboid percentages. PGE_2-exposed mice at P8 had fewer ramified microglia compared to WT-C57 in the cerebellum (females) and thalamus (sexes combined and females only). PGE_2-exposure did not affect overall ramified percentages in the olfactory bulb, prefrontal cortex and hippocampus but when each sex was analyzed separately, a general trend appeared where males had a lower percentage of ramified microglia compared to females in the cerebellum, olfactory bulb, and prefrontal cortex.

Altered Amoeboid and Ramified Microglial Morphology in COX-2$^-$ KI mice

Amoeboid Microglial Morphology

The percentage of active amoeboid microglia morphology was also analysed in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus at P8 in the COX-2$^-$ KI mice and wild-type 129S6 (WT-S6) mice. Two-way ANOVA analysis on active amoeboid microglia percentages was conducted on each brain area.

In the cerebellum, there were no significant differences in active amoeboid microglia morphology percentages (Fig. 6-5, F(3,8)=0.9335, p=0.46807). The cerebellar active microglia percentages of WT-S6 mice, COX-2$^-$ mice, WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 31.8±6.8, 22.1±4.9, 26.4±4.1, 27.3±8.9, 37.2±13.6, 17.0±3.5, respectively.

In the hippocampus, there were also no significant differences in active amoeboid microglia morphology percentages (Fig. 6-5, F(3,8)=0.6900, p=0.583399). WT-S6 mice, COX-2$^-$ mice, WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had hippocampal

active microglia percentages values of 17.9±3.7, 10.8±4.0, 16.4±5.7, 13.8±8.2, 19.3±5.8, 7.9±1.5, respectively.

In the olfactory bulb, there were no significant differences in active amoeboid microglia morphology percentages (Fig. 6-5, $F(3,8)=0.962$, $p=0.456247$). WT-S6 mice, COX-2$^-$ mice, WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial activation values in the olfactory bulb of 22.8±3.8, 16.3±4.3, 20.0±6.7, 20.8±7.8, 25.7±4.3, 11.9±3.4, respectively.

In the prefrontal cortex (Fig. 6-5, $F(3,8)=4.832$, $p=0.033271$), the microglial activation of COX-2$^-$ KI mice was statistically less than WT-S6 mice when both sexes were considered ($p=0.028123$, WT=15.2±1.8, KI=9.4±1.9). The prefrontal cortex of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had active microglia percentages of 12.9±1.6, 12.9±0.8, 17.4±3.1, 6.0±2.4, respectively. Post hoc comparisons showed that COX-2$^-$ KI females had a statistically lower percentage of active microglia than WT-S6 females ($p=0.005492$), but there were no other significant differences between the groups ($p>0.05$).

In the thalamus, there were no significant differences in active amoeboid microglia morphology percentages (Fig. 6-5, $F(3,8=0.814$, $p=0.521383$). The thalamic active microglia percentages of WT-S6 mice, COX-2$^-$ mice, WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 9.8±0.6, 9.6±2.5, 9.9±1.1, 12.3±4.4, 9.8±0.8, 6.8±1.9, respectively.

Overall, COX-2$^-$ KI mice at P8 had a lower percentage of active microglia only in the prefrontal cortex compared to WT-S6 mice, which was specific to female mice. Disruption of COX-2 did not statistically affect the percentage of amoeboid microglia in the cerebellum, hippocampus, olfactory bulb, and thalamus. COX-2$^-$ KI did not lead to sex-dependent effects on microglial activation.

Ramified Microglial Morphology

The percentage of ramified microglia morphology was also determined in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus at P8 in the COX-2$^-$ KI mice and wild-type 129S6 (WT-S6) mice. Two-way ANOVA analysis on ramified microglia percentages was conducted on each brain area.

In the cerebellum, there were no significant differences in ramified microglia morphology percentages (Fig. 6-5, $F(3,8)=0.9335$, $p=0.46807$). The cerebellar active microglia percentages of WT-S6 mice, COX-2$^-$ mice, WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 68.2±6.8, 77.9±4.9, 73.6±4.1, 72.7±8.9, 62.8±13.6, 83.0±3.5, respectively.

In the hippocampus, there were also no significant differences in ramified microglia morphology percentages (Fig. 6-5, $F(3,8)=0.6900$, $p=0.583399$). WT-S6 mice, COX-2$^-$ mice, WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had hippocampal ramified microglia percentages values of 82.1±3.7, 89.2±4.0, 83.6±5.7, 86.2±8.2, 80.7±5.8, 92.1±1.5, respectively.

In the olfactory bulb, there were no significant differences in ramified microglia morphology percentages (Fig. 6-5, $F(3,8)=0.962$, $p=0.456247$). WT-S6 mice, COX-2$^-$ mice, WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial activation values in the olfactory bulb of 77.2±3.8, 83.7±4.3, 80.0±6.7, 79.2±7.8, 74.3±4.3, 88.1±3.4, respectively.

In the prefrontal cortex (Fig. 6-5, $F(3,8)=4.832$, $p=0.033271$) the percentage of ramified microglia in COX-2$^-$ KI mice was statistically greater than WT-S6 mice ($p=0.028123$, WT=84.8±1.8, COX-2=90.6±1.9). The prefrontal cortex of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had active microglia percentages of 87.1±1.6, 87.1±0.8, 82.6±3.1, 94.0±2.4, respectively. Post hoc comparisons showed that COX-2$^-$ females had a

statistically more ramified microglia than WT-S6 females (p=0.005492), but there were no other significant differences between the groups (p>0.05).

In the thalamus, there were no significant differences in ramified microglia morphology percentages (Fig. 6-5, F(3,8=0.814, p=0.521383). The thalamic active microglia percentages of WT-S6 mice, COX-2$^-$ mice, WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 90.2±0.6, 90.4±2.5, 90.1±1.1, 87.7±4.4, 90.2±0.8, 93.2±1.9, respectively.

Overall, COX-2$^-$ KI mice at P8 had a greater percentage of ramified microglia only in the prefrontal cortex compared to WT-S6 mice, specifically in female mice. Disruption of COX-2 did not statistically affect the percentage of ramified microglia in the cerebellum, hippocampus, olfactory bulb, and thalamus. No statistical differences in ramified microglial percentages were quantified between males and females within the WT-S6 or COX-2$^-$ KI groups.

PGE_2-exposed offspring mice display altered number of primary microglial branches

It is well established that the morphology of microglia and their function are highly connected (Cho and Choi, 2017, Karperien et al., 2013). Although basic microglia function can be informed through the phenotypic categorization of cells as ramified or amoeboid, quantification of more subtle structural features including branching and process length could help explain the functional complexities of microglia, particularly during development (Karperien et al., 2013, Tremblay, 2011). To determine if PGE_2 exposure could affect microglial morphology, we quantified *branch density* in microglia (defined as the number of *primary branches* directly protruding from the soma) in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus at P8 (Fig. 6-6). Two-way ANOVA analysis on the number of

branches per microglia was conducted in each brain region comparing PGE_2-exposed mice and wild-type C57bl/6 (WT-C57).

In the cerebellum (Fig. 6-6, $F(3,989)=8.538$, $p=0.000013$), the number of branches per microglia in PGE_2 mice was not significantly different than WT-C57 mice ($p=0.291890$, WT=1.8±0.1, PGE_2=2.0±0.1). When males and females within each group were independently investigated, the average branch numbers per microglia in the cerebellum of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females were 1.6±0.1, 1.7±0.1, 1.9±0.1, 2.2±0.1, respectively. Post hoc comparisons revealed that PGE_2 females had greater microglial branching compared to WT-C57 females ($p=0.010423$). Females also had a greater number of microglial branches than males within both the WT-C57 ($p=0.031729$) and PGE_2 groups ($p=0.000037$).

Hippocampal microglial branching (Fig. 6-6, $F(3, 1026)=3.936$, $p=0.00831$) was significantly less in PGE_2 mice compared to WT-C57 mice ($p=0.015154$, WT=2.4±0.1, PGE_2=2.2±0.1). The average number of microglia branches in the hippocampus of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females were 2.3±0.1, 2.1±0.1, 2.5±0.1, 2.3±0.1, respectively, but there were no significant differences between these groups ($p>0.05$).

For olfactory bulb microglial branching (Fig. 6-6, $F(3, 1043)=4.214$, $p=0.005659$), there were no significant difference between PGE_2 mice and WT-C57 mice ($p=0.284694$, WT=2.1±0.1, PGE_2=2.0±0.1). The average number of branches per microglia in the olfactory bulb of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females were 1.8±0.1, 1.8±0.1, 2.1±0.1, 2.0±0.1, respectively. WT-C57 females had greater microglial branching than WT-C57 males ($p=0.015432$). However, there were no other significant differences between these groups ($p>0.05$).

Prefrontal cortex microglial branch numbers (Fig. 6-6, $F(3, 1267)=2.148$, $p<0.0001$) showed no statistical differences between PGE_2 mice and WT-C57 mice ($p=0.203660$, WT=2.1±0.1, PGE_2=2.2±0.1). The prefrontal cortex of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had microglial branch numbers of 2.0±0.1, 2.0±0.1, 2.2±0.1, 2.5±0.1 respectively. PGE_2 females had greater microglial branching compared to WT-C57 females ($p=0.000803$), and also greater microglial branching when compared to PGE_2 males ($p<0.0001$).

For microglial branching in the thalamus (Fig. 6-6, $F(3,1718)=2.786$, $p=0.039456$), it was statistically less in PGE_2 mice than WT-C57 mice ($p=0.010983$, WT=2.2±0.04, PGE_2=2.1±0.04). The thalamus of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had microglial branch numbers of 2.3±0.1, 2.1±0.1, 2.2±0.04, 2.1±0.1, respectively. There was decreased microglial branching in the thalamus of PGE_2 males compared to WT-C57 males ($p=0.027548$). There were no statistical differences between the remaining comparisons ($p>0.05$).

Overall, PGE_2-exposed mice at P8 had decreased microglial branching in the hippocampus and thalamus compared to WT-C57 mice. Within the thalamus, decreased microglial branching was prominent in PGE_2 males compared to WT-C57 males. PGE_2-exposure did not seem to statistically affect microglial branching in the cerebellum, olfactory bulb, and prefrontal cortex. However, when each sex was analyzed independently, PGE_2 females had greater branching compared to WT-C57 females in the cerebellum and prefrontal cortex. Moreover, sex differences revealed that statistically greater microglial branching was seen in females compared to males in the cerebellum (WT-C57 and PGE_2 groups), olfactory bulb (WT-C57), and prefrontal cortex (PGE_2).

COX-2$^-$ KI mice display increased microglial branch density

To determine if deficient COX-2 activity could affect microglial branch density, the number of primary branches per microglia was quantified in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus of P8 COX-2$^-$ KI mice. Whether differences occurred between COX-2$^-$ KI mice and wild-type 129S6 (WT-S6) mice, and between males and females, was evaluated. Two-way ANOVA analysis on the number of primary branches per microglia was conducted on each brain area.

In the cerebellum (Fig. 6-7, $F(3,1214)=15.631$, $p<0.0001$), the number of branches per microglia in COX-2$^-$ KI mice was significantly greater than WT-S6 mice ($p<0.0001$, WT=1.3±0.04, COX-2=1.6±0.04). When males and females within each group were independently investigated, the average branch numbers per microglia in the cerebellum of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 1.5±0.1, 1.6±0.1, 1.1±0.1, 1.5±0.1, respectively. Post hoc comparisons revealed that COX-2$^-$ females had greater microglial branching compared to WT-S6 females ($p<0.0001$). Males had a greater number of microglial branches than females, which was statistically different in WT-S6 mice ($p<0.0001$).

Hippocampal microglial branching (Fig. 6-7, $F(3, 816)=5.368$, $p=0.001157$) was significantly greater in COX-2$^-$ KI mice compared to WT-S6 mice ($p=0.000079$, WT=2.0±0.1, COX-2=2.3±0.04). The average number of microglia branches in the hippocampus of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 2.0±0.1, 2.4±0.1, 2.0±0.1, 2.3±0.1, respectively. Both COX-2$^-$ KI males ($p=0.004880$) and COX-2$^-$ females ($p=0.005218$) had statistically higher microglial branches compared to sex-specific WT-S6 controls.

For olfactory bulb microglial branching (Fig. 6-7, $F(3, 1487)=0.501$, $p=0.68191$), there was no significant difference in between COX-2$^-$ KI mice and WT-S6 mice ($p=0.29900$,

WT=1.8±0.04, COX-2=1.9±0.03). The average number of branches per microglia in the olfactory bulb of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 1.8±0.1, 1.9±0.1, 1.8±0.1, 1.9±0.1, respectively, with no significant differences between these groups (p>0.05).

In the prefrontal cortex (Fig. 6-7, F(3, 1092)=6.817, p=0.000149), there was statistically higher microglial branching in COX-2$^-$ KI mice compared to WT-S6 mice (p=0.000060, WT=1.8±0.04, COX-2=2.1±0.04). The prefrontal cortex of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial branch numbers of 1.9±0.1, 2.1±0.1, 1.7±0.1, 2.0±0.1, respectively. COX-2$^-$ males (p=0.041613) and COX-2$^-$ females (p=0.000286) had greater microglial branching compared to sex-specific WT-S6 controls. Males had a greater number of microglial branches than females, which was statistically different in WT-S6 mice (p=0.02295).

Microglial branching in the thalamus (Fig. 6-7, F(3, 1493)=8.165, p<0.0001) of COX-2$^-$ KI mice was not statistically different than WT-S6 mice (p=0.204093, WT=2.0±0.04, COX-2=2.1±0.04). WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial branch numbers of 2.2±0.1, 2.0±0.1, 1.8±0.1, 2.1±0.1, respectively. However, analyizing males and females independently revealed an increase in microglial branching in the thalamus of COX-2$^-$ females compared to WT-S6 females (p=0.000186). WT-S6 males had greater microglial branches than WT-S6 females (p<0.0001).

Overall, COX-2$^-$ KI mice at P8 had increased microglial branching in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus compared to WT-S6 mice. COX-2-deficiency did not seem to affect microglial branching in the olfactory bulb. Analysis on sex

differences revealed statistically greater microglial branching in WT-S6 males compared to WT-S6 females in the cerebellum, prefrontal cortex, and thalamus.

PGE_2-exposed offspring mice display decreased microglial branch lengths

In addition to the number of microglial branches, the length of microglial branches has also been shown to be indicative of their function and state (Cho and Choi, 2017, Karperien et al., 2013). The length of the longest process (μm) was measured for each identified microglia (Fig. 6-8). To determine if maternal exposure to PGE_2 affects microglial branch lengths, measurements were conducted in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus at P8. We examined differences between PGE_2-exposed mice and wild-type C57bl/6 (WT-C57) mice, and between males and female using two-way ANOVA analysis on the microglial branch lengths in each brain region.

In the cerebellum (Fig. 6-8, $F(3,989)=14.754$, $p<0.0001$), the branch lengths of microglia in PGE_2 mice were not significantly different than WT-C57 mice ($p=0.87625$, WT=10.0±0.3, PGE_2=9.6±0.3). Males and females within each group were also independently investigated. The average branch length per microglia in the cerebellum of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females were 8.0±0.5, 8.4±0.4, 10.9±0.3, 10.6±0.3, respectively. Post hoc comparisons revealed that females had greater microglial branch lengths compared to males within the WT-C57 ($p<0.0001$) and PGE_2 groups ($p<0.0001$).

Hippocampal microglial branch lengths (Fig. 6-8, $F(3,1026)=15.431$ $p<0.0001$) were significantly less in PGE_2 mice compared to WT-C57 mice ($p<0.0001$, WT=15.4±0.3, PGE_2=13.2±0.3). The average microglial branch length in the hippocampus of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females were 14.0±0.4, 14.0±0.5, 16.4±0.4, 12.6±0.4,

respectively. PGE_2 females had shorter hippocampal microglial branch lengths compared to WT-C57 females (p<0.0001). WT-C57 females had greater branch lengths than WT-C57 males (p<0.0001). In contrast, PGE_2 females had shorter branch lengths than PGE_2 males (p=0.020297).

In the olfactory bulb (Fig. 6-8, F(3, 1043)=7.627, p<0.0001), there was no significant difference in microglial branch lengths between PGE_2 mice and WT-C57 mice (p=0.719, WT=10.8±0.3, PGE_2=10.1±0.3). The average branch length per microglia in the olfactory bulb of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females were 8.9±0.5, 10.3±0.4, 11.6±0.3, 9.9±0.4, respectively. PGE_2 females had greater microglial branch lengths than WT-C57 females (p<0.0001). WT-C57 females also had longer microglial branches compared to WT-C57 males (p<0.0001).

For the average branch lengths of microglia in the prefrontal cortex (Fig. 6-8, F(3, 1267)=13.563, p<0.0001), PGE_2 mice had shorter branch lengths than WT-C57 mice (p<0.0001, WT=15.6±0.3, PGE_2=13.6±0.3). The prefrontal cortex of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had microglial branch lengths of 14.0±0.5, 13.9±0.4, 16.9±0.5, 13.3±0.4, respectively. PGE_2 females had shorter microglial branch lengths compared to WT-C57 females (p<0.0001) in the prefrontal cortex. WT-C57 females also had greater microglial branch lengths compared to WT-C57 males (p<0.0001).

In the thalamus (Fig. 6-8, F(3,1718)=25.87, p<0.0001), microglial branch lengths of PGE_2 mice was also statistically shorter than WT-C57 mice (p<0.0001, WT=16.8±0.3, PGE_2 =14.1±0.2). The thalamus of WT-C57 males, PGE_2 males, WT-C57 females, and PGE_2 females had microglial branch lengths of 15.3±0.5, 14.8±0.4, 17.6±0.3, 13.4±0.3, respectively. There were decreased microglial branch lengths in the thalamus of PGE_2 females compared to WT-C57

females (p<0.0001). WT-C57 females also had longer microglial branch lengths compared to WT-C57 males (p<0.0001). In contrast, PGE_2 females had shorter microglial branch lengths compared to PGE_2 males in the thalamus (p=0.005841).

Overall, PGE_2-exposed mice at P8 had decreased microglial branch lengths in the hippocampus, prefrontal cortex, and thalamus compared to WT-C57 mice. Closer examination of each group by independently investigating both sexes revealed that PGE_2 females had shorter microglial branch lengths than WT-C57 females in the mentioned areas as well as in the olfactory bulb. PGE_2-exposure did not seem to statistically affect average microglial branch length in the cerebellum. Sex difference analyses within the WT-C57 group showed that statistically longer microglial branch lengths were seen in females compared to males in all areas quantified: cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Sex difference analyses within the PGE_2 group also showed females had increased microglial branch lengths in the cerebellum compared to males. However, shorter microglial branch lengths were found in PGE_2 females compared to PGE_2 males in the hippocampus and thalamus.

COX-2$^-$ KI mice show increased microglial branch lengths

To determine if COX-2-deficiency could affect microglial morphological characteristics, the length of the longest branch (μm) of each microglia was quantified in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus of COX-2$^-$ KI mice at P8. Potential differences between COX-2$^-$ KI mice and wild-type 129S6 (WT-S6) mice, and between males and female, were examined. Two-way ANOVA analysis on the microglial branch lengths was conducted on each brain area.

In the cerebellum (Fig. 6-9, $F(3,1214)=17.405$, $p<0.0001$) the longest branch length per microglia in COX-2$^-$ KI mice was significantly longer than WT-S6 mice ($p<0.0001$, WT=6.7±0.2, COX-2=9.6±0.3). Males and females within each group were also independently investigated. The average branch length per microglia in the cerebellum of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 6.6±0.3, 9.1±0.3, 6.9±0.3, 9.3±0.4, respectively. Post hoc comparisons revealed that COX-2$^-$ males ($p<0.0001$) and COX-2$^-$ females ($p<0.0001$) had greater microglial branch lengths compared to sex-specific controls. Males and females did not have significant different cerebellar microglial branch lengths within the WT-S6 or COX-2$^-$ KI groups ($p>0.05$).

Hippocampal microglial branch lengths (Fig. 6-9, $F(3,816)=18.592$, $p<0.0001$) were significantly greater in COX-2$^-$ KI mice compared to WT-S6 mice ($p<0.0001$, WT=10.7±0.4, COX-2=13.6±0.3). The average microglial branch length in the hippocampus of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 9.5±0.5, 14.6±0.5, 13.0±0.8, 12.8±0.4, respectively. COX-2$^-$ males had longer hippocampal microglial branch lengths compared to WT-S6 males ($p<0.0001$). WT-S6 females had greater branch lengths than WT-S6 males ($p=0.000046$). In contrast, COX-2$^-$ females had shorter branch lengths than COX-2$^-$ males ($p=0.005239$).

In the olfactory bulb (Fig. 6-9, $F(3, 1487)=3.478$, $p=0.015456$), microglial branch lengths were longer in COX-2$^-$ KI mice compared to WT-S6 mice ($p=0.002238$, WT=10.0±0.2, COX-2=11.1±0.3). The average branch length per microglia in the olfactory bulb of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females were 9.6±0.6, 11.2±0.4, 10.6±0.3, 11.0±0.3, respectively. COX-2$^-$ males had greater microglial branch lengths than WT-S6 males ($p=0.003560$). There were no other significant differences between the groups ($p>0.05$).

For average branch lengths of microglia in the prefrontal cortex (Fig. 6-9, $F(3, 1092)=19.742$, $p<0.0001$), it was greater in COX-2$^-$ KI mice than in WT-S6 mice ($p<0.0001$, WT=11.5±0.3, COX-2=14.8±0.3). The prefrontal cortex of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial branch lengths of 11.0±0.4, 15.0±0.5, 12.1±0.5, 14.7±0.4, respectively. COX-2$^-$ males ($p=0.000065$) and COX-2$^-$ females ($p<0.0001$) had longer microglial branch lengths in the prefrontal cortex compared to sex-specific WT-S6 controls. There were no differences in prefrontal cortex microglial branch lengths between males and females in the COX-2$^-$ KI or WT-S6 control groups ($p=0.05$).

In the thalamus (Fig. 6-9, $F(3,1493)=10.983$, $p<0.0001$), COX-2$^-$ KI mice had statistically longer microglial branches than WT-S6 mice ($p=0.005908$, WT=13.4±0.3, COX-2=14.7±0.3). The thalamus of WT-S6 males, COX-2$^-$ males, WT-S6 females, and COX-2$^-$ females had microglial branch lengths of 12.2±0.4, 15.2±0.4, 14.9±0.5, 14.2±0.4, respectively. There were significantly increased microglial branch lengths in the thalamus of COX-2$^-$ males compared to WT-S6 males ($p<0.0001$). WT-S6 females also had longer microglial branch lengths compared to WT-S6 males ($p<0.0001$).

Overall, an opposite trend was seen in the COX-2$^-$ KI mouse model compared to PGE_2-exposed model. COX-2$^-$ KI mice had increased microglial branch lengths compared to WT-S6 mice in all the areas examined: cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Elevated microglial branch lengths were pronounced in COX-2$^-$ males. Sex difference analyses within the WT-S6 group showed that statistically longer microglial branch lengths were seen in females compared to males in the hippocampus and thalamus. However, shorter microglial branch lengths were found in COX-2$^-$ females compared to COX-2$^-$ males in the hippocampus.

6.5. Discussion

In this study, using two distinct mice model systems we show for the first time that abnormal COX-2/PGE_2 signalling during development affects microglial density and morphology in mice at P8. Offspring from the PGE_2-exposed model, where a single maternal injection of PGE_2 was administered during pregnancy at E11, elevated microglial density as well as an increase in amoeboid and decrease in ramified morphology, with region-specific alterations in the number of microglial branches and decreased process lengths. In the COX-2-deficient model, COX-2^- KI mice also had a higher density of microglia but a lower percentage of cells were in the active amoeboid state and a higher percentage of cells were ramified. Microglia in COX-2^- KI mice also showed increased branching and elevated process lengths compared to control mice in a region-specific and sex-dependent manner.

These findings could have implications for brain development and pathologies of neurodevelopmental disorders such as autism spectrum disorders (ASDs). As previously mentioned, defects in the COX-2/PGE_2 signalling pathway due to genetic or environmental risk factors have been documented in many clinical cases of ASDs, resulting in an increase or decrease in the level of PGE_2 or its metabolites (Wong et al., 2015, Wong and Crawford, 2014, Tamiji and Crawford, 2010). Investigations into the molecular impact of abnormal PGE_2 levels on neurodevelopment have only recently begun. For example, current *in vitro* and *in vivo* research from our lab (Rai-Bhogal et al., 2018, Rai-Bhogal et al., 2018, Davidson et al., 2016, Wong et al., 2016, Wong et al., 2014, Tamiji and Crawford, 2010) and others (Hoffman et al., 2016, Dean et al., 2012, Dean et al., 2012, Wright and McCarthy, 2009) provide insight on the impact disrupted PGE_2 levels (high or low) have in the developing brain. More recently, we also confirmed that PGE_2-exposed and COX-2-deficient mice both exhibit autism-like behaviours

that are sex-dependent (Wong et al., 2019, Wong et al., 2017). Below, we discuss the effect of abnormal PGE_2 levels on microglial morphology in two model systems and potential physiological and pathological implications for the developing brain.

PGE_2 exposure and COX-2-deficiency led to increased microglial density

First, we showed that an abnormal level of PGE_2 in either the PGE_2-exposed or COX-2-deficient model resulted in increased microglial density. Microglial density was increased in the cerebellum, olfactory bulb, and thalamus of PGE_2-exposed mice and in the cerebellum, olfactory bulb, and prefrontal cortex of COX-2-deficient mice. In the healthy brain, microglial cells are present in all regions (Lawson et al., 1990) and their density may increase in response to immune mediators (Chan et al., 2007, Rezaie et al., 2005). In addition to its role in innate immunity, microglia also have developmental functions in the CNS including involvement in neuronal activity and communication with astrocytes (Eyo and Wu, 2013, Tremblay et al., 2010, Wake et al., 2009, Pocock and Kettenmann, 2007, Nimmerjahn et al., 2005). Research shows that an increase in microglial density was correlated with disrupted proliferation of neural progenitors and inhibition of neuronal differentiation in the early postnatal developing brain and adult brain (Appel et al., 2018, Smith et al., 2014). Elevated microglial density has also been previously linked to abnormal neuron dendrites with reduced dendritic spine density (Appel et al., 2018). Interestingly, greater microglial density was demonstrated in the cerebral cortex, prefrontal cortex and cerebellum in individuals with autism, from childhood to into early adulthood (Suzuki et al., 2013, Tetreault et al., 2012, Morgan et al., 2010, Vargas et al., 2005, Blaylock, 2004), which were also seen in our PGE_2-exposed and COX-2-deficient models in this study.

PGE_2-exposure changes morphological features of microglia: increased amoeboid phenotype, decreased branching, and decreased process lengths

In addition to increased microglia density, the microglia in PGE_2-exposed mice had increased amoeboid phenotype, decreased branching, and decreased process lengths in various brain regions. Retraction and thickening of processes represents a shift towards activated amoeboid microglial morphology (Ransohoff and Perry, 2009). These morphological changes have been associated with irregular genesis, development, and function of neurons (Appel et al., 2018, Smith et al., 2014). Furthermore, amplified microglial activation along with decreased branching and process lengths have been morphologically characterized in autism (Suzuki et al., 2013, Tetreault et al., 2012, Morgan et al., 2010, Vargas et al., 2005, Blaylock, 2004). These differences in microglial morphology may contribute to disrupted neuronal proliferation, migration, and differentiation we previously described in this abnormal PGE_2 model (Wong et al., 2019), Rai-bhogal et al., unpublished).

COX-2-deficient changes morphological features of microglia: increased ramified phenotype, increased branching, and increased process lengths

In contrast, elevated microglial density in the COX-2-deficient model was accompanied by a lower percentage of microglia with amoeboid morphology. An increase in ramified morphology (with statistical significance in the prefrontal cortex), together with increased branching and process lengths was characterized in $COX\text{-}2^-$ KI mice compared to control mice in all regions investigated.

Interestingly, ramified microglia previously categorized as “resting” microglia have emerged as one of the most functionally and structurally dynamic cells in the nervous system

(Tremblay, 2011). They have roles in neurogenesis, differentiation of astrocytes, oligodendrocytes, and neurons, synaptic development, as well as axonal pruning and myelination (Menassa and Gomez-Nicola, 2018, Ji et al., 2013, Miron et al., 2013, Schafer et al., 2012, Paolicelli et al., 2011, Sierra et al., 2010). Ramified microglial cells are highly motile; they continually survey the environment and screen the complete brain parenchyma once every few hours (Nimmerjahn et al., 2005). They are important effectors of neuronal circuit reorganization by interacting and eliminating neuronal synaptic structures (Tremblay, 2011). An abnormal increase in ramified microglia could contribute to deficiencies in normal neuron-microglia communication, leading to impaired functional brain connectivity and abnormal social behaviour (Zhan et al., 2014), which are hallmarks of ASDs (Ha et al., 2015, Kana et al., 2014). Moreover, under non-pathological conditions, there is a gradual decrease in amoeboid microglia with a concomitant increase in ramified microglia as postnatal brain development progresses (Kaur et al., 2017, Zusso et al., 2012, Prinz and Mildner, 2011, Schlegelmilch et al., 2011). An untypical elevated percentage of ramified microglia could signify accelerated microglial development, which has been associated with autism (Hanamsagar et al., 2017). Thus, the increase in ramified microglia and related morphological changes quantified in COX-2^{-} KI mice could disturb the diverse, developmental functions of these cells.

Sex differences in PGE_2 exposure and WT-C57 mouse model

Clear sex differences were observed in the PGE_2-exposed and WT-C57 model system. In general, control males had a significantly higher percentage of morphologically active amoeboid microglia with less branch numbers and shorter process lengths compared to control females. Our findings are in line with past research that described greater amoeboid morphology in males,

indicative of increased microglial activation or possibly a more immature phenotype (Villa et al., 2018, Hanamsagar et al., 2017, Werling et al., 2016, Lenz et al., 2013, Schwarz et al., 2012). Female microglia have been found to reach a more mature, ramified microglial phenotype earlier compared to males (Hanamsagar et al., 2017).

Interestingly, microglial morphological differences were identified most frequently in PGE_2-exposed females when compared to controls. In the cerebellum and thalamus, PGE_2 exposure to females resulted in a greater proportion of morphologically active amoeboid microglia, previously described as a more "male" microglial morphologic profile (Lenz et al., 2013). This finding complements existing work completed in the preoptic area, which showed PGE_2 treatment masculinized and increased the active microglial morphologic profile in females (Lenz et al., 2013). Our results offer new evidence that PGE_2 levels could influence male specificity.

Sex differences in COX-2-deficient and WT-S6 mouse model

We found that COX-2-deficiency led to various effects on microglial density and morphology, which were both sex-dependent and region-specific. For example, COX-2^- males displayed increased microglial density in the cerebellum and increased branch lengths in the hippocampus. On the other hand, COX-2^- females had increased microglial density in the olfactory bulb and prefrontal cortex, as well as decreased amoeboid and increased ramified microglia in the prefrontal cortex. Our diverse findings allude to a complex interaction between sex and microglia. Indeed, the well-documented sexually dimorphic impact on microglia is multifaceted and encompasses differences in homeostatic and inflammatory-induced transcriptional and translational expression profiles, maturation, function, and immune reactivity

to environmental challenges (Dubbelaar et al., 2018, Guneykaya et al., 2018, Thion et al., 2018, Villa et al., 2018, Wood, 2018, Hanamsagar et al., 2017, Mangold et al., 2017, Crain et al., 2013). How these sex-dependent differences are programmed remains to be determined. One explanation may be though the action of estradiol, an important steroid sex hormone that influences sex differentiation in the brain (McCarthy, 2008). COX-2/PGE_2 signalling and estradiol can interact to effect neuron morphology and function, microglial activity, neuroinflammatory responses, and sex behaviour (Pedersen and Saldanha, 2017, Shridharan et al., 2016, Stacey et al., 2016, Lenz et al., 2013, Dean et al., 2012, Schwarz and McCarthy, 2008, Amateau and McCarthy, 2004, Ospina et al., 2004). It is possible that crosstalk between abnormal COX-2/PGE_2 signalling and sex-dependent levels of estradiol might contribute to the sex-specific changes in microglial density and morphology observed in our study.

6.6. Conclusions

Although the role of microglia in neuroinflammation in the adult brain has been studied extensively, the involvement of microglia during neurodevelopment is being progressively recognized. Our study shows that modification of PGE_2 levels results in augmented sex-dependent differences in microglial density and altered microglial morphology in ASD-associated brain regions including the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Our findings provide further evidence that microglia could respond to environmental and genetic insults in a sex-dependent manner. As described in our previous review articles, various genetic and environmental risk factors that influence the level of PGE_2 during prenatal development have been associated with ASDs (Wong et al., 2015, Wong et al., 2014, Tamiji and Crawford, 2010). The results in this current study provide new evidence that

prenatal deviations in COX-2/PGE_2 signalling can also disrupt microglial function, which in turn may affect normal brain development.

6.7. Figures

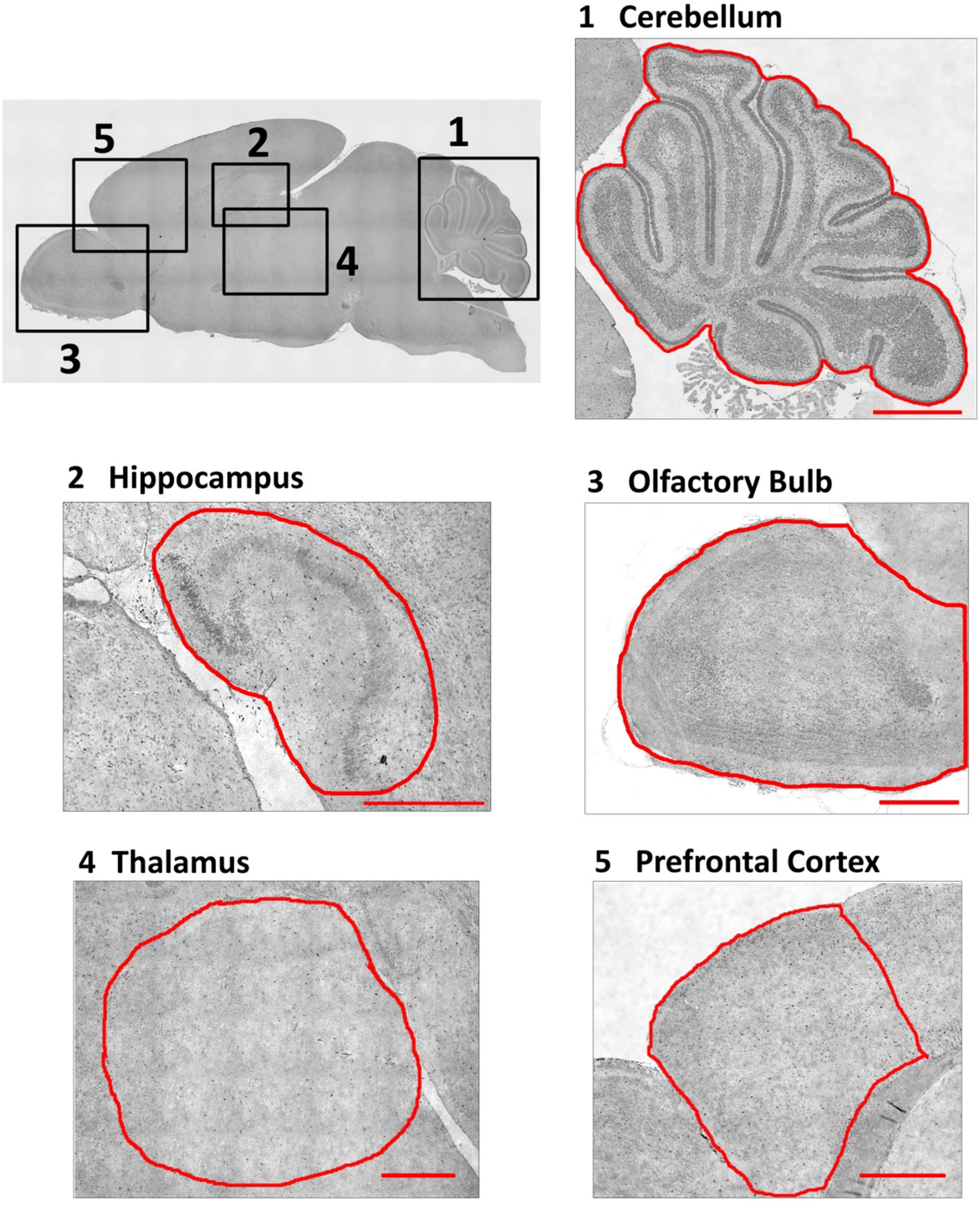

Fig. 6-1: Brain areas visualized and quantified. Sagittal slices of brain samples were used to quantify the (1) cerebellum, (2) hippocampus, (3) olfactory bulb, (4) thalamus, and (5) prefrontal cortex (outlined in red). Scale bar represents 500µm.

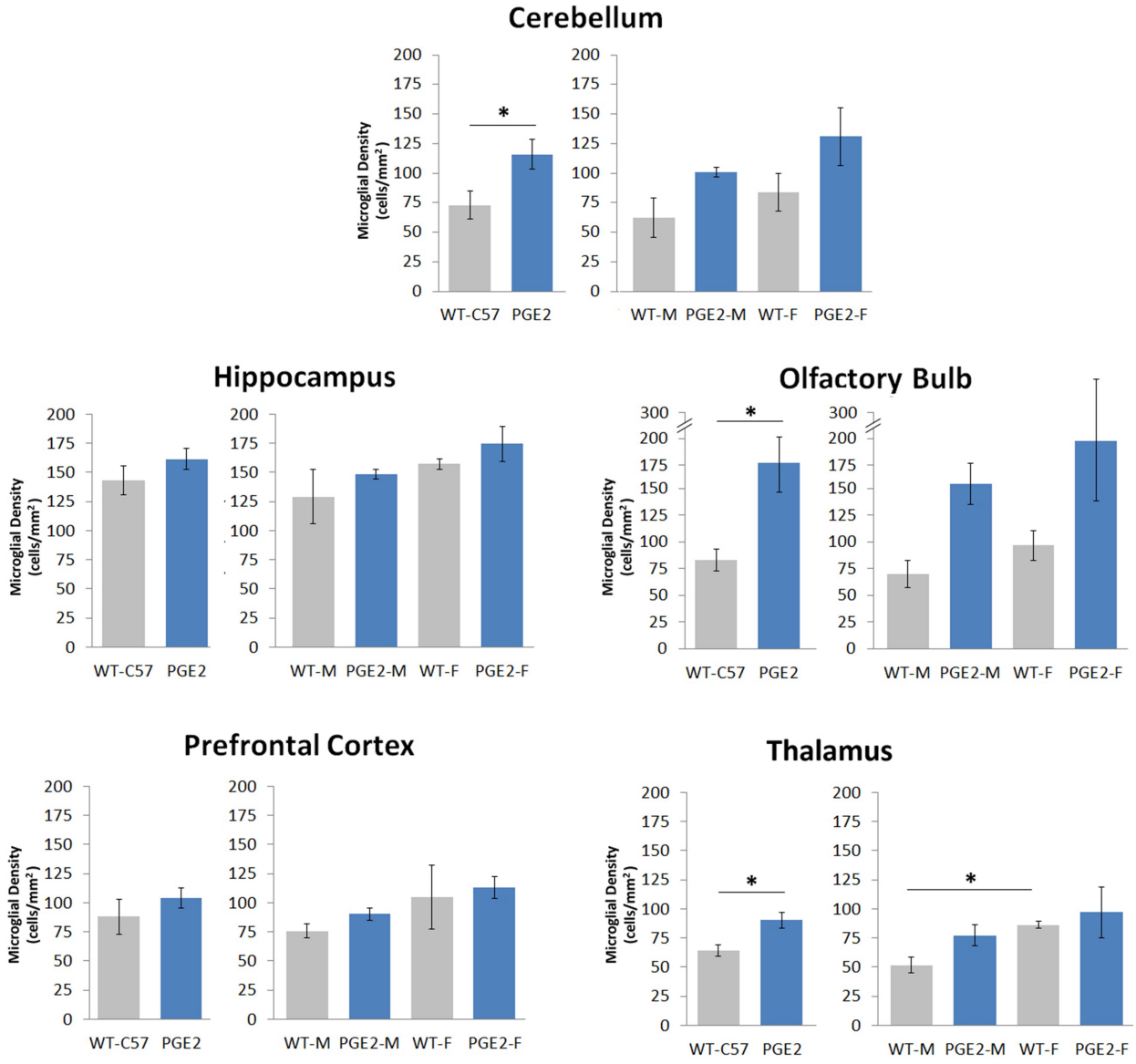

Fig. 6-2: Microglial cell densities in PGE_2-exposed mice.

Microglial densities were quantified in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

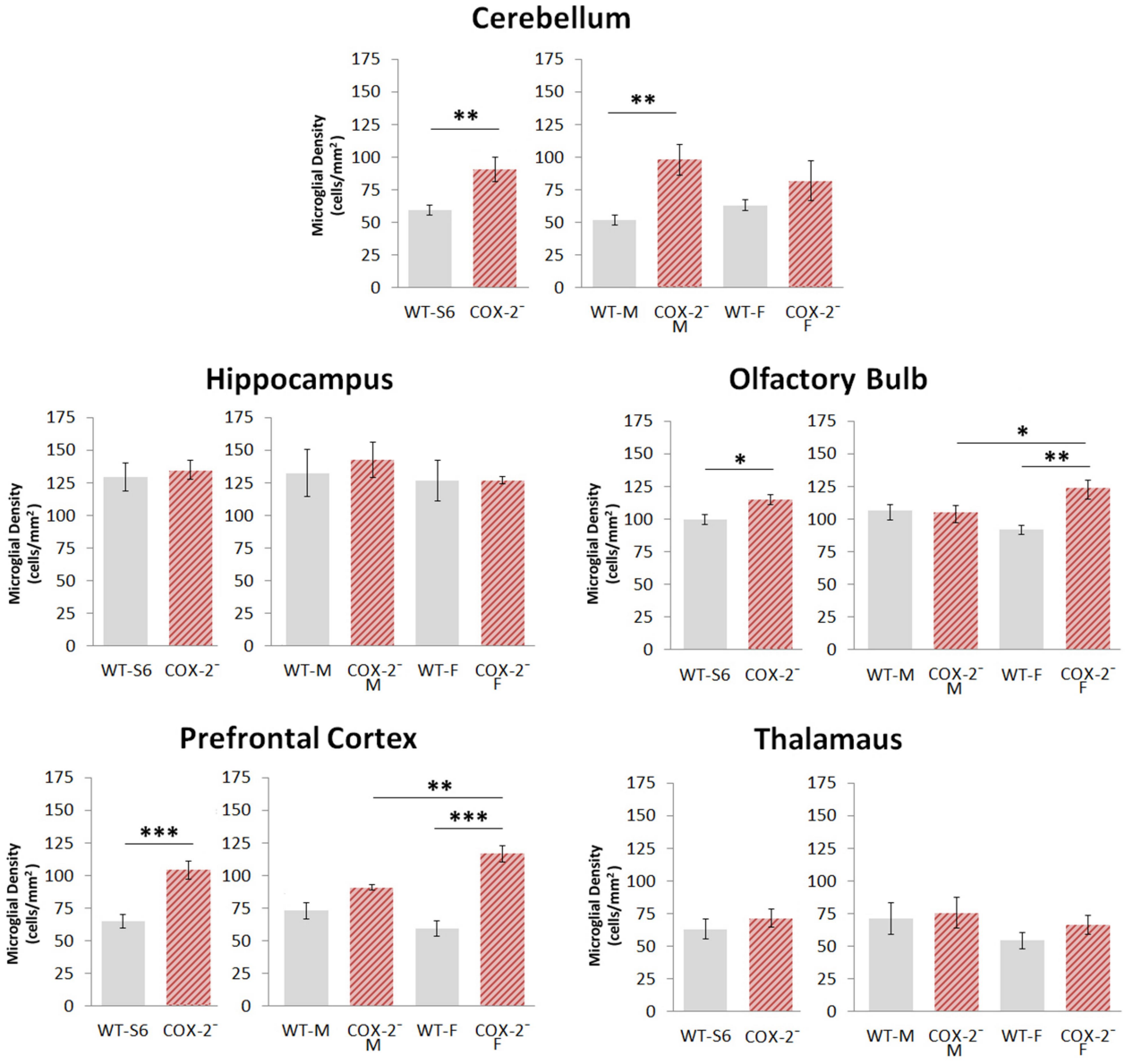

Fig. 6-3: Microglial densities in COX-2⁻ mice.

Microglial densities were quantified in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

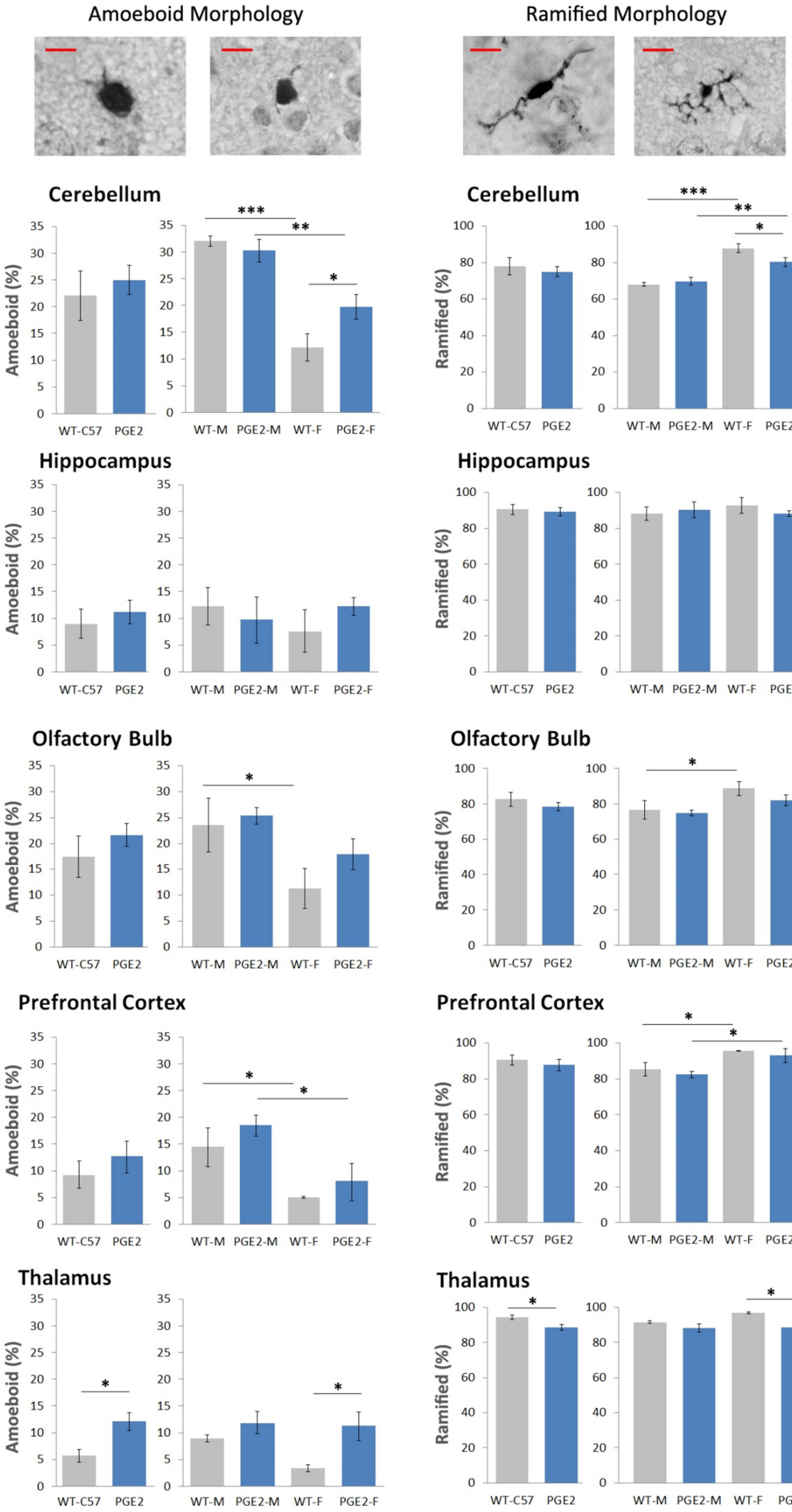
Amoeboid Morphology
Ramified Morphology
Cerebellum
Amoeboid (%)
Ramified (%)
WT-C57
PGE2
WT-M
PGE2-M
WT-F
PGE2-F
***
**
*
Hippocampus
Olfactory Bulb
Prefrontal Cortex
Thalamus

Fig. 6-4: Microglial amoeboid/ramified morphology percentages in PGE_2-exposed mice.

Stained microglia were characterized as ramified or active amoeboid cells. The percentage of amoeboid microglia was determined in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Amoeboid percentages for each area were calculated by taking the number of amoeboid microglia and dividing it by the total number of microglia counted. Ramified percentages were also determined. Scale bars represent 10μm in examples of amoeboid and ramified microglia. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

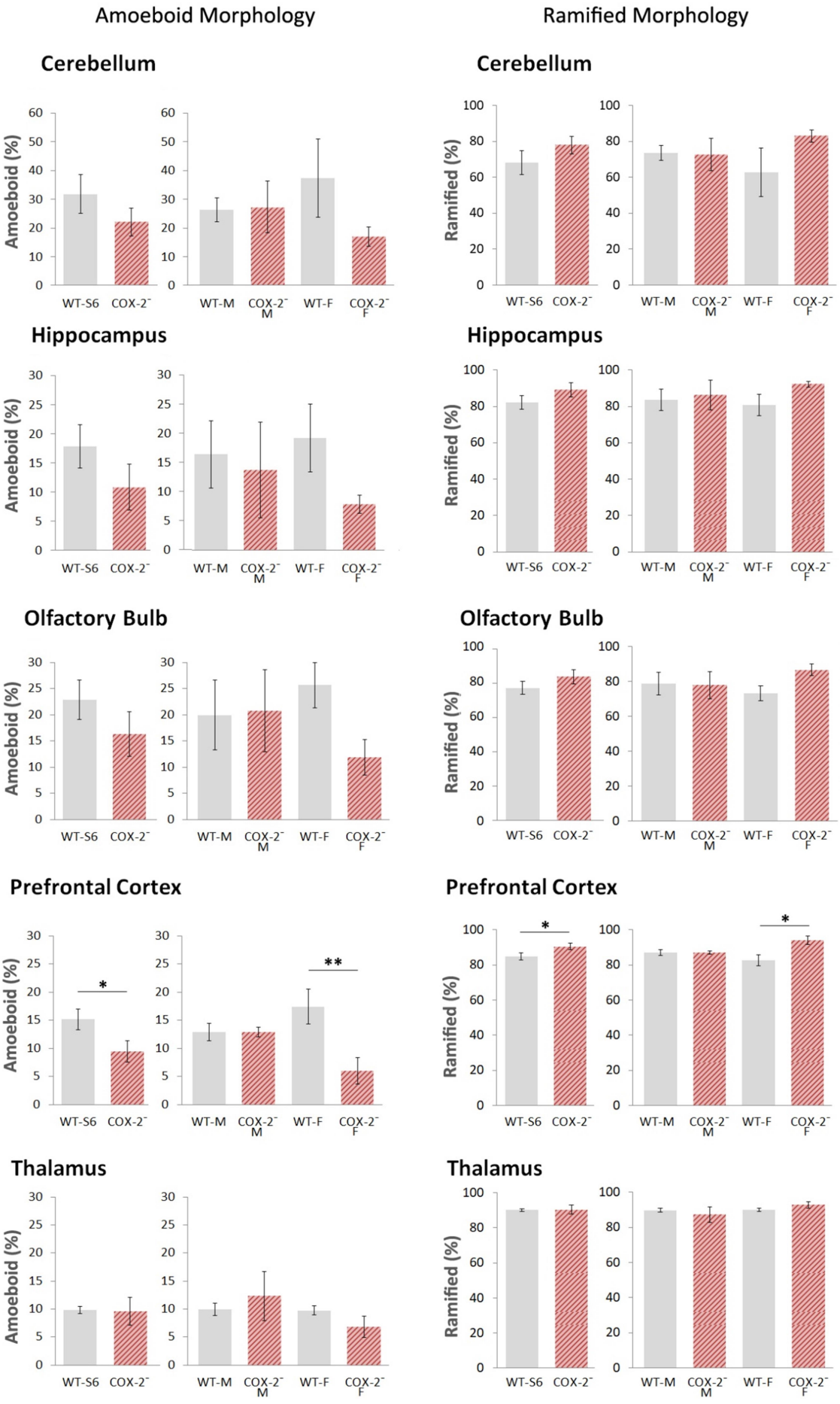
Amoeboid Morphology
Ramified Morphology
Cerebellum
Cerebellum
Hippocampus
Hippocampus
Olfactory Bulb
Olfactory Bulb
Prefrontal Cortex
Prefrontal Cortex
Thalamus
Thalamus
Amoeboid (%)
Ramified (%)
WT-S6
COX-2-
WT-M
COX-2- M
WT-F
COX-2- F
*
**

Fig. 6-5: Microglial amoeboid/ramified morphology percentages in COX-2$^-$ mice.

The percentage of amoeboid microglia was determined in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Amoeboid percentages for each area were calculated by taking the number of amoeboid microglia and dividing it by the total number of microglia counted. Ramified percentages were also determined. Scale bars represent 10μm in examples of amoeboid and ramified microglia. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

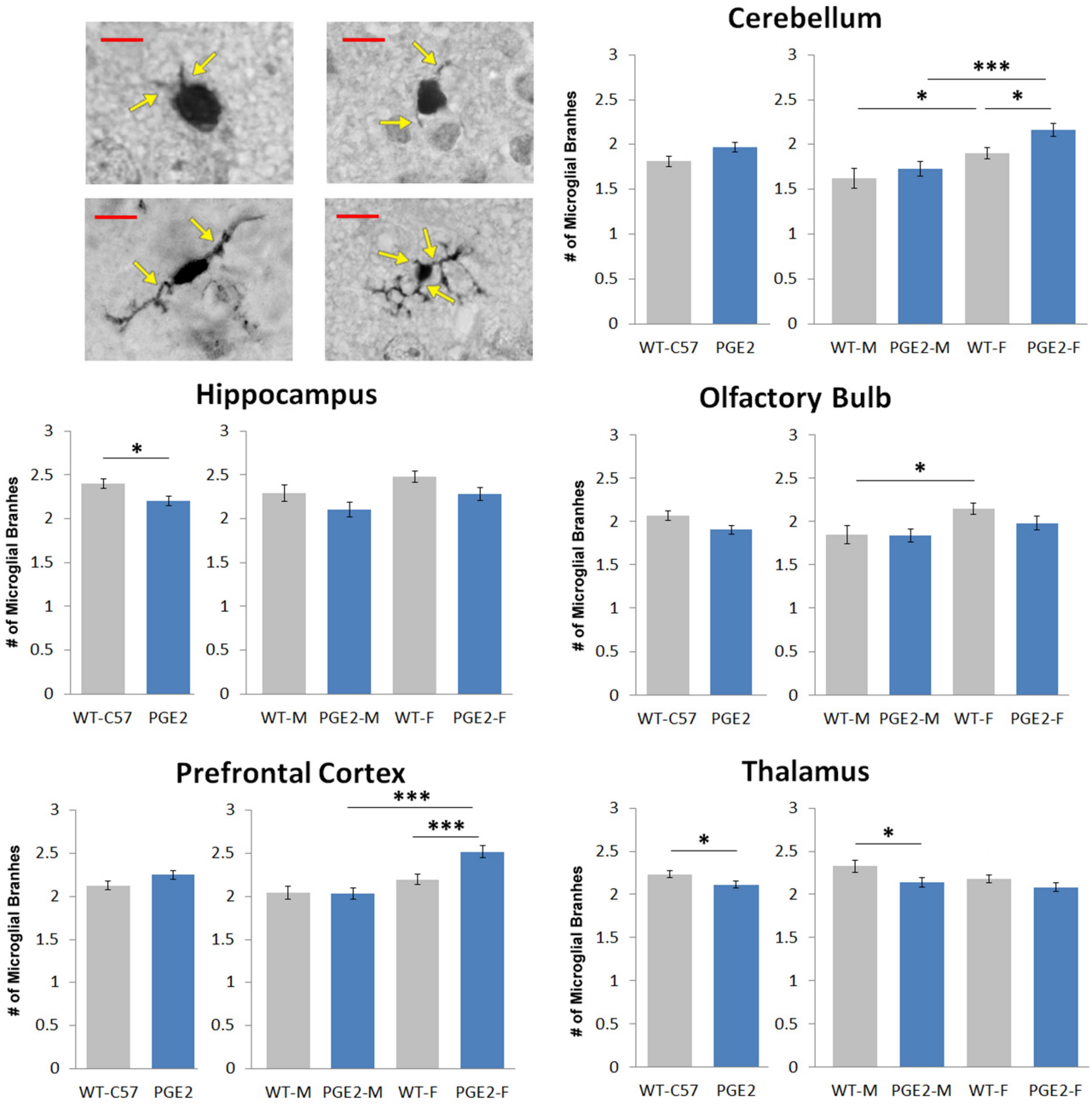

Fig. 6-6: Microglial branching in PGE_2 -exposed mice.

The average number of primary branches per microglia was determined in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. In the example of branch number quantification, arrows signify the number of branches counted; scale bar represents 10µm. Means represent quantifications from at least 3 independent animals for each experimental group. A total of 6063 cells were quantified. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

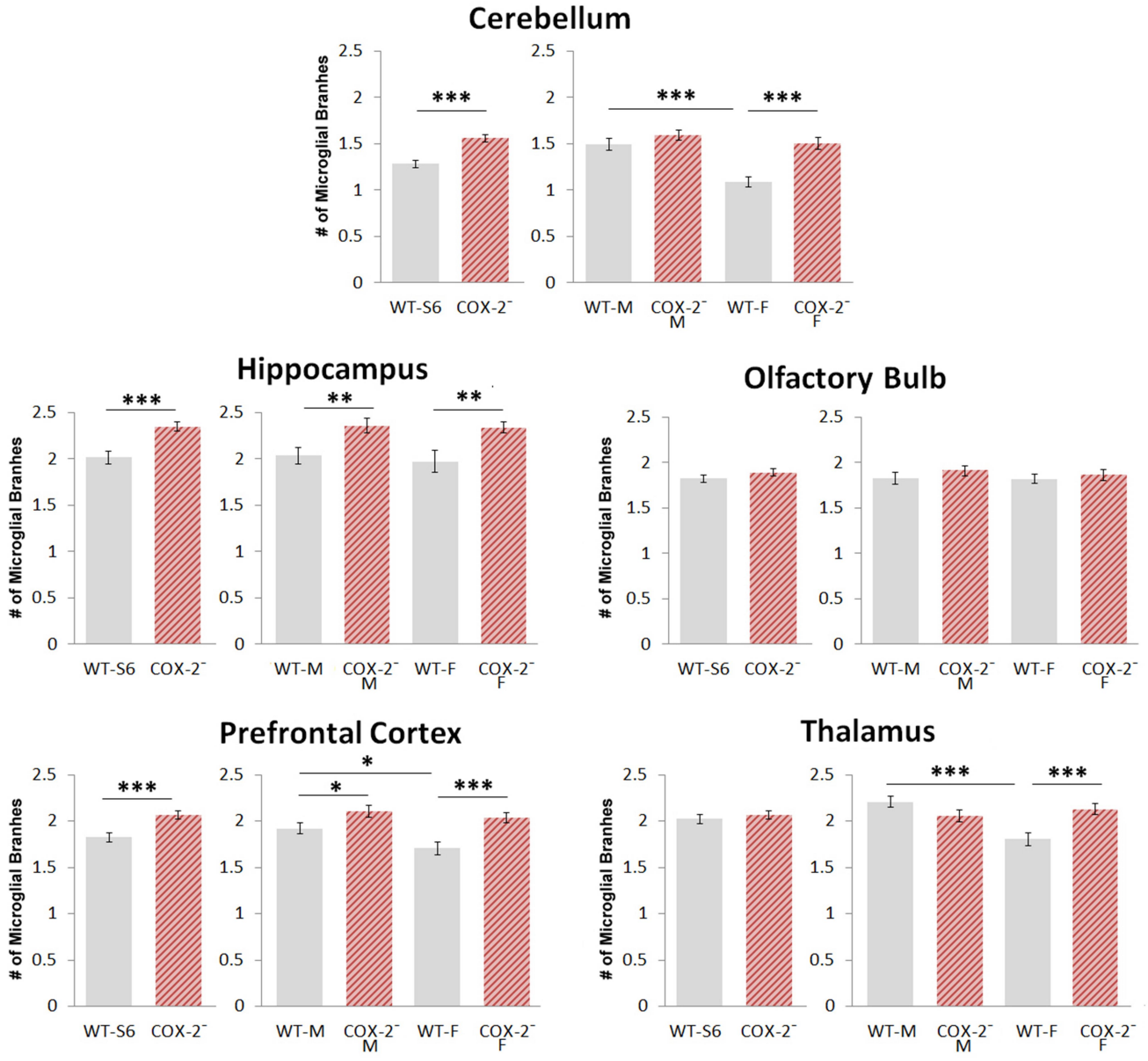

Fig. 6-7: Microglial branching in COX-2⁻ mice.

The average number of primary branches per microglia was determined in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Means represent quantifications from at least 3 independent animals for each experimental group. A total of 6122 cells were quantified. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

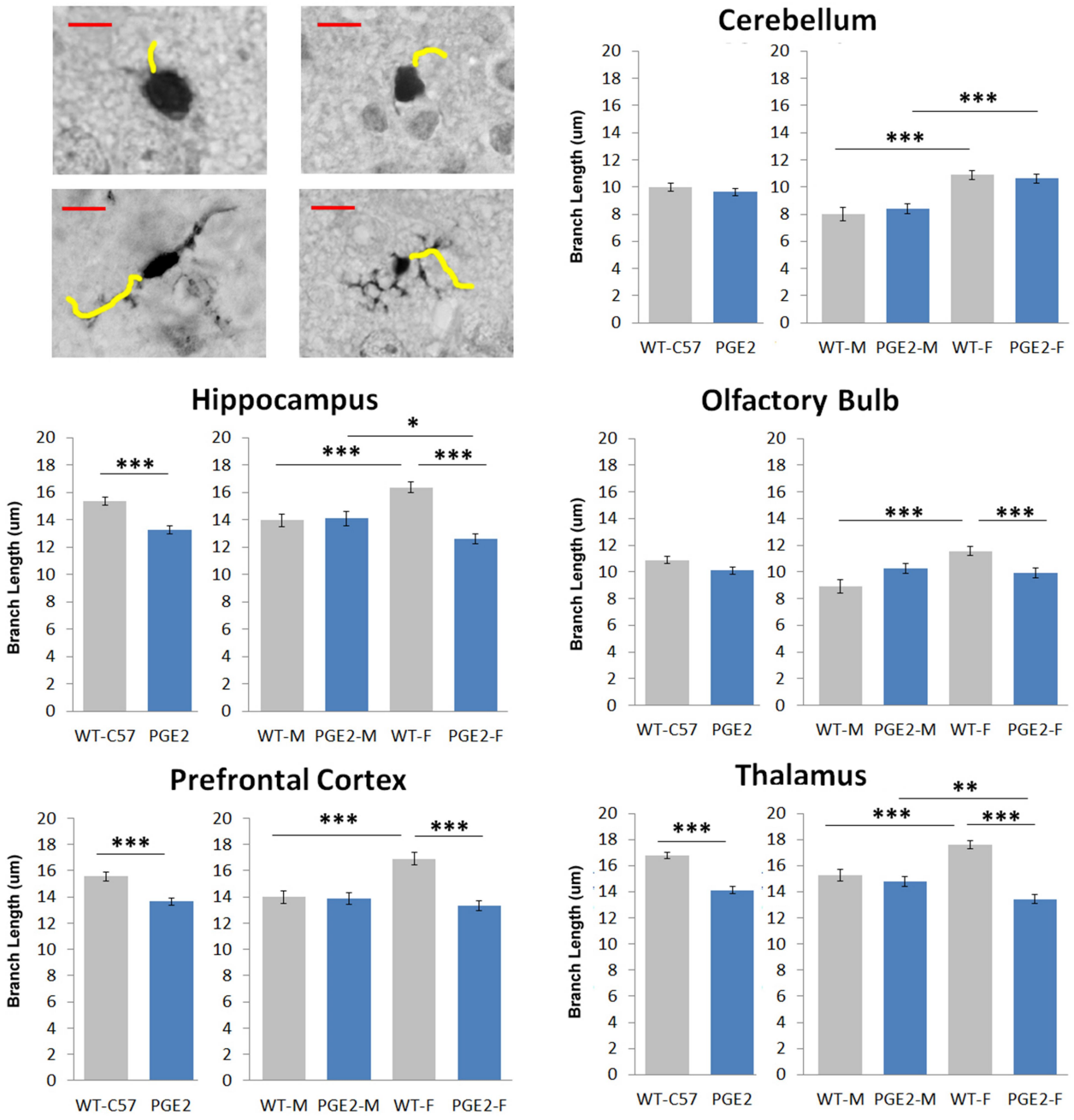

Fig. 6-8: Microglial branch lengths in PGE_2 -exposed mice.

The average of the longest branch length per microglia was determined in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. In the example of branch length quantification, yellow line signifies the longest branch quantified; scale bar represents 10µm. Means represent quantifications from at least 3 independent animals for each experimental group. A total of 6063 cells were quantified. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

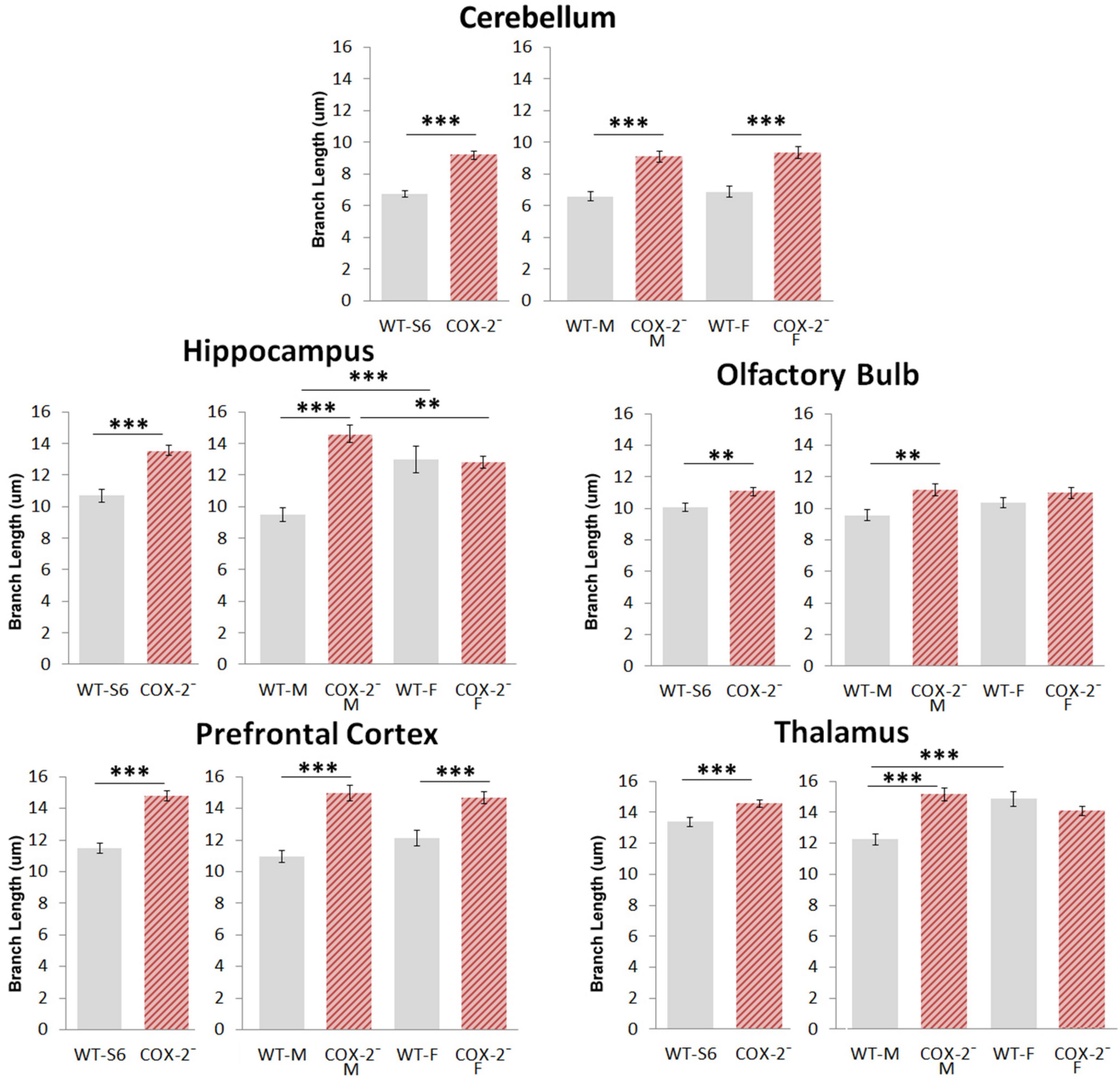

Fig. 6-9: Microglial branch lengths in COX-2⁻ mice.

The average of the longest branch length per microglia was determined in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Means represent quantifications from at least 3 independent animals for each experimental group. A total of 6122 cells were quantified. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

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CHAPTER 7.

Study 5: Autism-related Behaviours in the Cyclooxygenase-2-deficient Mouse Model

Manuscript 7 Citation (Copyright Permission in Appendix B.)**:**

Wong, C. T., Bestard Lorigados I., Crawford DA. (2019) Autism-related behaviors in the cyclooxygenase-2-deficient mouse model. *Genes Brain Behaviour*. 18:e12506.

Contributions: Christine T. Wong designed and performed experiments, collected samples, acquired and analyzed the data, and prepared the manuscript. Isabel Bestard Lorigados assisted in acquiring RNA samples and data. Dr. Dorota A. Crawford supervised the design of the study and was involved with editing the manuscript.

Objectives and Hypotheses: The fifth study was completed *in vivo* and its main objective was to investigate whether COX-2-deficiency could contribute to autism-related behaviours. The aims of this study were to characterize the behaviours of young and adult COX-2-deficient mice in the open field test, marble test, inverted screen test, and three chamber sociability test. I hypotheizsed that COX-2-deficient mice would display autism-related behaviours in a sex- and age-specific manner. Specially, I hypothesized that COX-2-deficient mice would show elevated hyperactive, repetitive, and anxiety-linked behaviours, motor deficits, and social abnormalities. In addition, I hypothesized that COX-2-deficient mice would have altered expression of autism-linked genes: *Wnt2, Glo1, Grm5,* and *Mmp9.* This was the first study to show that irregularities in the COX-2/PGE_2 signalling pathway could lead to the manifestation of autism-associated behaviours.

7.1. Chapter Summary

Prostaglandin E2 (PGE_2) is an endogenous lipid molecule involved in normal brain development. Cyclooxygenase-2 (COX-2) is the main regulator of PGE_2 synthesis. Emerging clinical and molecular research provides compelling evidence that abnormal COX-2/PGE_2 signalling is associated with autism spectrum disorder (ASD). We previously found that COX-2 knockout mice had dysregulated expression of many ASD-implicated genes belonging to important biological pathways for neurodevelopment. The current study is the first to demonstrate the connection between irregular COX-2/PGE_2 signalling and autism-related behaviours in male and female COX-2-deficient knockin, COX-2^-, mice at young (4-6 weeks) or adult (8-11 weeks) ages. Autism-related behaviours were prominent in male COX-2^- mice for most behavioural tests. In the open field test, COX-2^- mice travelled more than controls and adult male COX-2^- mice spent less time in the center indicating elevated hyperactive and anxiety-linked behaviours. COX-2^- mice also buried more marbles, with males burying more than females, suggesting increased anxiety and repetitive behaviours. Young male COX-2^- mice fell more frequently in the inverted screen test revealing motor deficits. The three-chamber sociability test found that adult female COX-2^- mice spent less time in the novel mouse chamber indicative of social abnormalities. In addition, male COX-2^- mice showed altered expression of several autism-linked genes: *Wnt2, Glo1, Grm5,* and *Mmp9.* Overall, our findings offer new insight into the involvement of disrupted COX-2/PGE_2 signalling in ASD pathology with age-related differences and greater impact on males. We propose that COX-2^- mice might serve as a novel model system to study specific types of autism.

7.2. Introduction

Autism spectrum disorder (ASD) is a neurodevelopment disorder phenotypically characterized by difficulties in social interaction, abnormal communicatory behaviours, and the presence of restricted and repetitive behaviours. Many individuals with ASD also experience motor difficulties (Travers et al., 2017), sensory sensitivity (Posar and Visconti, 2017), and anxiety (Zaboski and Storch, 2018). Epidemiological data reveals that ASD is one of the most common neurodevelopment conditions in children, affecting 1 in 68 (Christensen et al., 2016, Christensen et al., 2018, Lyall et al., 2017, Xu et al., 2018). The prevalence of ASDs has a strong sex bias, with significantly higher diagnosis in males compared to females (Loomes et al., 2017). Sex also plays a role in the clinical presentation of ASDs through varied symptomology (Halladay et al., 2015, Mandy et al., 2012, Van Wijngaarden-Cremers et al., 2014). For example, in males with ASD, attention deficit-hyperactivity disorder (ADHD) is more prevalent (Willcutt, 2012) and repetitive behaviours are more severe than females (Supekar and Menon, 2015, Szatmari et al., 2012, Tillmann et al., 2018). The manifestation of these deficient behaviours in ASDs likely arises from complex interactions between genes and environment that result in improper brain development (Chaste and Leboyer, 2012, Lai et al., 2014, Yu et al., 2015).

Current research on the developing brain has provided substantial evidence for the link between ASDs and abnormal signalling of the lipid mediator, prostaglandin E2 (PGE_2) (Tamiji and Crawford, 2010, Wong and Crawford, 2014, Wong et al., 2015). PGE_2 is the major lipid molecule in the nervous system. In the healthy brain, it is synthesized from arachidonic acid (AA) that is released from cell membranes through the activity of phospholipase A2 (PLA2) (Sang and Chen, 2006). AA is further converted to PGE_2 and other prostanoid metabolites by two enzymes, cyclooxygenase-1 and -2 (COX-1 and COX-2) (Figure 7-1). However, PGE_2 is the

predominant metabolite of COX enzymatic activity. COX-1 is constitutively expressed in most tissues and in the brain primarily by microglia (Choi et al., 2006, Hoozemans, 2001, Schwab et al., 2000). In contrast, COX-2 expression is typically inducible in the periphery by inflammation, but has constitutive expression in the kidney, intestinal tract, female reproductive system, and brain, where it is mainly localized in neurons (Brock et al., 1999, Hoozemans, 2001, Kirkby et al., 2016, Maslinska et al., 1999, Rouzer and Marnett, 2009, Yang and Chen, 2008). COX-2 expression levels are significantly higher in the whole brain, particularly in the hippocampus and cerebral cortex, compared to its expression in peripheral tissues (Kirkby et al., 2016). In COX-2-deficient mice the endogenous level of PGE_2 in the brain is reduced by half compared to wild-type mice, indicating that COX-2 contributes significantly to PGE_2 production in the nervous system (Ayoub et al., 2004, Bosetti et al., 2004, Engstrom et al., 2012, Li et al., 2010). Normal COX-2/PGE_2-mediated signalling is involved in fundamental brain functions such as dendritic spine formation, synaptic plasticity, and memory and learning (Burks et al., 2007, Chen and Bazan, 2005, Chen et al., 2002, Sang and Chen, 2006, Yang and Chen, 2008).

Various abnormalities in key components of the COX-2/PGE_2 pathway due to both genetic and environmental influences have been implicated in clinical studies on ASDs (Wong and Crawford, 2014, Wong et al., 2015). For instance, increased and decreased ratios of AA to omega-3 and omega-6 fatty acids (Bell et al., 2010, El-Ansary et al., 2011, Jory, 2016), increased PLA2 activity (Tostes et al., 2013), decreased total AA, and increased PGE_2 levels (Brigandi et al., 2015) have been reported in blood samples of human patients with ASD. Moreover, polymorphism of *Ptgs2*, the gene that encodes the COX-2 enzyme, has been associated with ASDs and its relevant behaviours (Yoo et al., 2008). Furthermore, misuse of the drug misoprostol—a PGE_2 analogue —for the termination of pregnancy was linked to elevated risk of

ASD (Bandim et al., 2003). We also previously reported that PGE_2 signalling in the developing brain is especially vulnerable to various environmental insults such as inflammation, oxidative stress, pollution, heavy metals, pesticides, and commonly used over-the-counter drugs like acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs) including acetylsalicylic acid (ASA), which all have been linked to ASD (Masarwa et al., 2018, Tamiji and Crawford, 2010, Wong and Crawford, 2014, Wong et al., 2015).

Emerging molecular evidence offers insight into possible mechanisms by which abnormal COX-2/PGE_2 signalling may affect brain development and lead to autism. For example, *in vitro* studies in our lab have shown that elevated levels of PGE_2 can modify intracellular calcium dynamics and increase neural proliferation, migration, and differentiation in neural stem cells and differentiating neurons (Davidson et al., 2016, Wong et al., 2014, Wong et al., 2016). The expression of autism-linked genes including *Ptgs2*, *Mmp9*, *Ctnnb1*, and *Wnt3* was also altered as a result of increased PGE_2 levels in these model systems (Wong et al., 2014, Wong et al., 2016). Time-sensitive postnatal elevation or reduction of PGE_2 levels have been shown to disrupt normal dendritic arborization in the cerebellum (Dean et al., 2012) and lead to reduced reciprocal social behaviour in male rats under both circumstances (Dean et al., 2012, Hoffman et al., 2016, McCarthy and Wright, 2017). Our recent whole genome microarray study of gene expression in male COX-2-deficient knockout mice found differential expression of many ASD-associated genes and deficits in various biological pathways involved in neuronal function during prenatal development such as synaptic transmission, long term potentiation, axon guidance, and dendritic spine remodelling (Rai-Bhogal et al., 2017). Interestingly, earlier studies have shown that mice lacking COX-2 enzymatic activity, including knockin COX-2^{-} and knockout COX-$2^{-/-}$ mice, exhibit irregularities in kidney morphology, gastrointestinal function,

and fertility (Lim et al., 1997, Loftin et al., 2001, Seta, 2009). However, the phenotypical consequences of COX-2 deficits in the brain have not yet been tested.

In this study, we use homozygous COX-2$^-$ knockin mice to investigate for the first time the behavioural manifestations of abnormal COX-2/PGE_2 signalling. We provide further evidence to existing clinical and molecular research indicating the COX-2/PGE_2 signalling pathway as a candidate autism pathway by demonstrating the relationship between abnormal COX-2/PGE_2 signalling and autism-related behaviour in COX-2$^-$ mice. We used the open field, marble burying, inverted screen, and three-chamber sociability tests to characterize sex- and age-dependent ASD behaviours. We observed significant differences in hyperactivity, anxiety, repetitive behaviour, motor ability, and social behaviour in male and female mice at young (4-6 weeks) or adult (8-11 weeks) ages. Additionally, we found significant alterations in the expression of autism-linked genes including *Wnt2, Glo1, Grm5,* and *Mmp9*. Our results suggest that the experimental COX-2$^-$ mouse model may be useful for studying the connection between abnormal lipid signalling and developmental disorders, such as autism.

7.3. Methods

Animals

Ptgs2^{Y385F} founder mice, also known as B6.129S6(FVB)-*Ptgs2*$^{tm1.1Fun}$/J mice, were purchased from Jackson Laboratory (stock #008101; Queen's University, laboratory of C. Funk) and backcrossed for at least 5 generations to wild-type 129S6/SvEvTac (WT) mice purchased from Taconic Laboratory. *Ptgs2*^{Y385F} mice are a genetic mouse model for selective COX-2 inactivation, which was created by a targeted point mutation of the *Ptgs2* gene, resulting in a Y385F amino acid substitution. Although this mutation results in the complete inhibition of

COX-2 activity, downstream peroxidase activity remains intact (Yu et al., 2006). Mice were bred to generate homozygous *Ptgs2* knockin (COX-2$^-$ KI) mice for behavioural testing. Homozygous COX-2$^-$ KI females are infertile, thus heterozygous females were bred with homozygous or heterozygous males to generate COX-2$^-$ KI male and female offspring. All WT and COX-2$^-$ KI mice were bred and maintained in group housing under the same conditions at York University throughout the study on a 12-hours light/dark cycle. Behavioural testing was conducted during the light phase. Mouse subjects were divided into two separate age cohorts: young (4-6 weeks old) and adult (8-11 weeks old). All equipment was thoroughly cleaned with antiseptic clinicide for disinfection and deodorization, followed by water wipes between testing of individual mice. Whole brain samples from separate animals were collected at postnatal day 8 (where birth was denoted as postnatal day 0) for gene quantification. All experiments and protocols were approved by the Research Ethics Board of York University and completed according to the York University Animal Care Committee ethics guidelines.

Genotyping

Genotyping analysis was performed via polymerase chain reaction (PCR) analysis of the COX-2 gene using primer sequences provided by the Jackson Laboratory (*Ptgs2*; Table 7-1). DNA extraction was completed on individual ear punch samples, which were denatured in alkaline lysis buffer (25mM NaOH) at 95°C for 30 min, followed by addition of neutralization buffer (Tris-HCl). Standard PCR was performed in an Eppendorf Mastercycler according to the Jackson Laboratory *Ptgs2*$^{tm1.1Fun}$ protocol (The Jackson Laboratory, 2016).

Table 7-1: PCR Primer set for COX-2$^-$ KI Genotype Analysis

Primer	Name	Primer Sequence (5'-3')	Base pairs	COX-2$^-$	Wild-type
Forward	Mus Ptgs2IMR7834	ACCAGTCTCTCAATGAGTAC	20 bp	585 bp	493 bp
Reverse	Mus Ptgs2IMR7835	AGAATGGTGCTCCAAGCTCTAC	22 bp		

Open Field Test

Changes in activity and anxiety levels were evaluated using standard methods as described previously (Bailey and Crawley, 2009, Seibenhener and Wooten, 2015). Mice were individually placed in a novel open chamber (40 cm L x 40 cm W x 40 cm H) with a center region designated as a 10 x 10 cm^2. The test mouse was placed in the center of the apparatus and allowed to roam freely for a testing duration of 10 min. Mouse movements were recorded by a Sony Cyber-shot DSC-W800 20.1 MP camera. Ambulatory activity was defined as the total pathlength travelled and was analyzed by an automated tracking program (NIS Elements Advanced Research Software). Anxiety-linked behaviour, investigated by determining the time spent in the center, was manually measured with a stopwatch by replaying the recorded video at a later date. Manual measurements were completed blind to the condition. The open field test was conducted on a total of 82 animals.

Marble Burying Test

Changes in repetitive behaviour and anxiety levels were evaluated through the marble burying test in line with previously described methods (Angoa-Perez et al., 2013, Chang et al., 2017, Deacon, 2006, Kazdoba et al., 2016, Wohr et al., 2013). The trials were recorded using a Sony Cyber-shot DSC-W800 20.1 MP camera. The apparatus was a clean standard polycarbonate mouse cage (28.5 cm L x 17.5 cm W x 12 cm H) filled with bedding measuring a height of 3.5 cm. Twenty black glass marbles (15 mm) were arranged in the cage in a 4 by 5

array. The test mouse was individually placed in the center of the apparatus and allowed to bury marbles freely for a duration of 30 minutes. Once the trial was completed, the quantity of marbles that were fully buried was determined. Videos were later analyzed manually for time spent digging and grooming, which were completed blind to the test condition. The marble burying test was conducted on a total of 104 animals. 56 animals were recorded for analysis of digging and grooming behaviour.

Inverted Screen Test

To measure changes in strength, mice were individually placed in the center of a 34 x 38 cm rectangular made of a wire mesh grid (1 mm thickness) with 13 mm squares surrounded by a wooden perimeter frame with 1.5 cm in thickness and 4 cm in height (Bonetto et al., 2015, Deacon, 2013). The screen was then slowly inverted over a 55 cm tall container filled with 30 cm of foam padding for a maximum duration of 10 min. Whether or not the mouse fell during the test was noted and recorded as a binary result (1 = mouse fell, 0 = mouse did not fall). The fall percentage in each experimental group was then calculated by averaging the values for each test subject and multiplying by 100: Fall percentage of experimental group = (sum of values for each experimental group) ÷ (number of test subjects in the group) x 100. The inverted screen test was conducted on a total of 87 animals.

Three-Chamber Sociability Test

The three-chamber test was used to measure sociability as previously described (Chang et al., 2017, Kaidanovich-Beilin et al., 2011, Kazdoba et al., 2016, Silverman et al., 2010, Wohr et al., 2013). The sociability apparatus (60 cm L x 45 cm W x 26 cm H) was made from clear acrylic walls and divided into three chambers equal in area. The two dividing walls had openings

(10 cm x 10 cm) with removable doors that restricted or permitted access into the adjacent chambers. Testing consisted of two phases. In the first phase, the test mouse was individually placed in the center chamber and allowed to explore only the center chamber for 5 minutes. An inverted black wire-mesh cylinder container (10.5 cm D x 16 cm H) was placed in the middle against the lateral edge of each outer chamber. A weighted hockey puck was placed on top of each cylinder to prevent tipping. A novel mouse (4 weeks old, matched for sex) was placed inside one of the two cylinders. The second phase was recorded using a Sony Cyber-shot DSC-W800 20.1 MP camera mounted overhead and initiated by the removal of the barrier doors, allowing the test mouse to freely roam and explore all three chambers for 10 minutes. Videos were later analyzed for time spent in each chamber and time spent sniffing or touching the cylinders, which were completed blind to the test condition. The three-chamber sociability test was conducted on a total of 59 animals.

RNA Isolation and quantitative Real-Time

RNA for gene expression experiments was isolated from whole brain tissues collected at the postnatal day 8, a stage analogous to the infant stage in humans (Pressler and Auvin, 2013, Semple et al., 2013) during which the earliest signs of ASD-related behaviours have been observed (Koterba et al., 2014, Wagner et al., 2018). Total RNA isolation was completed via standard Trizol (Sigma) method and reverse-transcribed into cDNA with MMuLV reverse transcriptase (New England Biolabs, Ipswich, MA) following manufacturer's protocol. SYBER green quantitative real-time polymerase chain reaction (qRT-PCR) on a 7500 FAST RT-PCR system (Applied Biosystem, Foster City, CA) was conducted and the ΔΔCt method was used to calculate transcript expression (Wong et al., 2014). Autism-associated genes were selected based on our recent findings in prenatal COX-2 knockout mice (Rai-Bhogal et al., 2017) and previous

research completed in human studies (refer to discussion). Primer pairs for tested genes—*Wnt2, Glo1, Grm5, Mmp9* (Table 7-2)—were designed using Primer Express v3.0 (ThermoFisher Scientific, Waltham, MA). Hypoxanthine phosphoribosyl transferase (*Hprt*) and phosphoglycerate kinase 1 (*Pgk1*) were used as housekeeping controls. Relative quantification (RQ) mean for each gene was calculated from the RQ values of three litters determined in three independent experiments.

Table 7-2: qRT-PCR Primers for autism-associated genes in COX-2$^-$ KI mice

Name	Primer	Primer Sequence (5'-3')	Base pair Length
Hprt	Forward	TCCATTCCTATGACTGTAGATTTTATCAG	29
	Reverse	AACTTTTATGTCCCCCGTTGACT	23
Pgk1	Forward	CAGTTGCTGCTGAACTCAAATCTC	24
	Reverse	GCCCACACAATCCTTCAAGAA	21
Wnt2	Forward	GCCCTGATGAACCTTCACAAC	21
	Reverse	TGACACTTGCATTCTTGTTTCAA	23
Glo1	Forward	GGATTTGGTCACATTGGGATTG	22
	Reverse	CGTCATCAGGCTTCTTCACA	20
Grm5	Forward	CATGGAGCCTCCGGATATAATG	22
	Reverse	GTATCCAAGAGGAGTGACAACC	22
Mmp9	Forward	TCGCGTGGATAAGGAGTTCTCT	22
	Reverse	ATAGGCTTTGTCTTGGTACTGGAAGA	26

Statistical Analysis

All behavioural data are graphically presented as mean±standard error of the mean (SEM) representing individuals from a minimum of three separate litters for each condition and time point. Three-way ANOVA followed by Bonferroni-adjusted pair-wise comparisons was performed on the open field test, marble burying test, and three-chamber sociability test to determine if behavioural changes were dependent on condition (wild-type vs. knockin), sex

(male vs. females), or age (young vs. adult). The Kruskal-Wallis H test followed by post hoc pair-wise comparisons was conducted on the inverted screen test. Independent t-test was used to analyze potential gene expression differences and have been reported as mean±SEM. *P* values less than 0.05 were considered significant.

7.4. Results

Hyperactive locomotor and Anxiety-linked Behaviours: Open Field Activity

The open field test was used to evaluate ambulatory activity and anxiety-linked behaviour in a novel environment for 10 min duration (Fig. 7-1A). Ambulatory activity was determined by measuring total pathlength travelled (Fig. 7-1B), while anxiety-related behaviour was measured by the time spent in the center of the apparatus (Fig. 7-1C). Three-way ANOVA analysis was completed on pathlength values (Fig. 7-1B, $F(7,73)=13.645$, $p<0.001$). Pair-wise comparisons revealed that COX-2$^-$ mice had significantly increased ambulatory activity compared to WT controls. Specifically, this increase in pathlength travelled (cm) by COX-2$^-$ mice compared to WT was seen in young males (Fig. 7-1B, $p=0.0456$), adult males (Fig. 7-1B, $p=0.000003$), young females (Fig. 7-1B, $p=0.000056$), and adult females (Fig. 7-1B, $p=0.012$).

There were no male vs. female differences in pathlength travelled within the young WT (Fig. 7-1B, $p=0.131$) or adult WT groups (Fig. 7-1B, $p=0.178$). A sex difference was also not observed between young COX-2$^-$ males and females (Fig. 7-1B, $p=0.964$). However, adult COX-2$^-$ males travelled a greater pathlength than adult COX-2$^-$ females (Fig. 7-1B, $p=0.000039$).

Similarly, WT animals did not show age differences in pathlength travelled within the male groups (Fig. 7-1B, $p=0.092$). No age differences were seen in COX-2$^-$ males (Fig. 7-1B, $p=0.204$). Although no age differences were observed in WT females (Fig. 7-1B, $p=0.121$), age-

dependent differences were seen in COX-2$^-$ females: young female COX-2$^-$ mice travelled a longer pathlength than adult female COX-2$^-$ mice (Fig. 7-1B, p=0.000428).

Anxiety-linked behaviour was measured by the amount of time spent (s) in the center of the apparatus (Fig. 7-1C). Three-way ANOVA analysis was completed on the time spent in the center (Fig. 7-1C, $F(7,73)$=3.225, p=0.005). There was no difference for time in center between young WT and COX-2$^-$ males (Fig. 7-1C, p=0.559). However, adult COX-2$^-$ males spent significantly less time in the center than adult WT males (Fig. 7-1C, p=0.001). There was no apparent difference in young COX-2$^-$ females (Fig. 7-1C, p=0.249) or adult COX-2$^-$ females (Fig. 7-1C, p=0.889) compared to respective WT controls.

Sex differences in anxiety-like behaviour were seen in WT animals, specifically in the adult group, where WT males spent more time in the center than WT females (Fig. 7-1C, p=0.000191). No sex differences were observed between young WT males and females (Fig. 7-1C, p=0.658). There were also no sex differences in COX-2$^-$ mice for the young (Fig. 2c, p=0.995) or adult groups (Fig. 7-1C, p=0.952).

Age differences were observed in the WT male group; young mice spent less time in the center than adult mice (Fig. 7-1C, p=0.015117). In contrast, no age differences were found within COX-2$^-$ males (Fig. 7-1C, p=0.134). No age differences were seen within the WT female groups (Fig. 7-1C, p=0.804) or COX-2$^-$ female groups (Fig. 7-1C, p=0.064).

In summary, ambulatory behaviour was significantly elevated in COX-2$^-$ mice compared to WT across all groups, with sex- and age-dependent differences only seen in COX-2$^-$ mice. Anxiety-linked behaviour was decreased only in adult COX-2$^-$ males compared to matched WT mice, with sex- and age-dependent differences only seen in WT groups.

Anxiety-linked and Repetitive Behaviours: Marble Burying Test Results

The marble burying test was used to assess anxiety-linked and repetitive behaviour over 30 min (Fig. 7-2A). Only the number of marbles completely buried was counted and the total time spent digging or grooming was also measured. Three-way ANOVA analysis was completed on marble burying counts (Fig. 7-2B, $F(7,96)=11.125$, $p<0.001$). Computed pair-wise comparisons determined that COX-2$^-$ mice fully buried significantly more marbles than WT controls. An increased number of marbles buried was seen in young COX-2$^-$ males (Fig. 7-2B, $p=0.00157$), adult COX-2$^-$ males (Fig. 7-2B, $p=0.000345$), young COX-2$^-$ females (Fig. 7-2B, $p=0.0483$), and adult COX-2$^-$ females (Fig. 7-2B, $p=0.012$) compared to respective controls.

Sex differences were seen in the number of marbles buried in both the WT and COX-2$^-$ groups. In WT animals, no sex differences were seen between males and females in the young group (Fig. 7-2B, $p=0.283$), but adult males were found to bury more marbles than adult females (Fig. 7-2B, $p=0.00458$). Similarly, COX-2$^-$ males buried more marbles than COX-2$^-$ females and this was seen in young animals (Fig. 7-2B, $p=0.0338$) and adults (Fig. 7-2B, $p=0.00468$).

Age-dependent differences were only seen in males, specifically, adult mice significantly buried more marbles than young mice and this was seen within WT males (Fig. 7-2B, $p=0.00208$) and within COX-2$^-$ males (Fig. 7-2B, $p=0.002670$). No significant difference was seen in marbles buried between young and adult females within WT (Fig. 7-2B, $p=0.269$) or COX-2$^-$ groups (Fig. 7-2B, $p=0.079$).

Three-way ANOVA analysis was completed on digging behaviour (Fig. 7-2C, $F(7,48)=4.874$, $p<0.001$). The pattern seen in the time spent digging (s) corresponded with the number of marbles buried, whereby COX-2$^-$ mice spent significantly more time digging than WT controls. This increased digging behaviour was seen in young COX-2$^-$ males (Fig. 7-2C,

p=0.0254), adult COX-2$^-$ males (Fig. 7-2C, p=0.000329), and young COX-2$^-$ females (Fig. 7-2C, p=0.0446). No significant differences in digging behaviour were seen between WT and COX-2$^-$ mice in adult females (Fig. 7-2C, p=0.0682). There were no significant sex or age differences within test groups seen in time spent digging (Fig. 7-2C, p>0.05). Three-way ANOVA analysis was also completed on grooming behaviour (F(7,48)=1.325, p=0.260). There were no statistical differences in the time spent grooming (s) for all group comparisons (no figure shown, p>0.05; total mean=103.2±8.2).

In overview, COX-2$^-$ mice buried significantly more marbles and spent more time digging than WT controls. Sex- and age-dependent differences were also seen in the number of marbles buried, with males and adults burying the most marbles. The greatest number of marbles were buried by adult COX-2$^-$ males. No differences were seen in time spent grooming.

Motor Strength: Inverted Screen Test

To assess changes in motor strength, mice were subjected to the inverted screen test, which ran for a duration of 10 min (Fig. 7-3A). Fall percentage (%) was determined for each experimental group as described in the methods (Fig. 7-3B). Kruskal-Wallis H test analysis was conducted on binary fall values (1 = mouse fell, 0 = mouse did not fall) (Fig. 7-3B, H(7)=19.867, p=0.00356). Young COX-2$^-$ males (KI) fell significantly more (86%) than young WT males (25%) (Fig. 7-3B, p=0.0231) but there was no statistical difference between adult WT and COX-2$^-$ males (Fig. 7-3B, p=0.143). In the females groups, although there was a trend that COX-2$^-$ mice had a greater fall percentage than WT mice, differences did not reach statistical significance between young WT and COX-2$^-$ females (Fig. 7-3B, p=0.530) or between adult WT and COX-2$^-$ females (Fig. 7-3B, p=0.0674).

When investigating potential sex differences in fall percentages, males fell more often than females. No sex differences were seen in young WT male and female mice (Fig. 7-3B, p=0.819) but adult WT males (55%) fell more frequently compared to adult WT females (9%) (Fig. 7-3B, p=0.0253). Similarly no statistical sex differences in fall values were observed in young COX-2$^-$ mice (Fig. 7-3B, p=0.0679) but males (83%) fell more often than females (43%) in the adult COX-2$^-$ (Fig. 7-3B, p=0.0381). Fall percentage values were not significantly different between the age groups tested (p>0.05).

To summarize, only young COX-2$^-$ mice had a higher fall percentage than age-matched WT mice. Sex differences were observed within adult WT and adult COX-2$^-$ groups, where males fell more frequently than females. No age differences in fall percentage were observed within WT or COX-2$^-$ mice.

Social Behaviour: Three-Chamber Sociability Test

Social behaviour was evaluated with the three-chamber sociability test for a duration of 10 minutes (Fig. 7-4A). Regardless of sex or age, we found that both genotype groups spent the most time exploring the novel mouse chamber, indicative of their preference for social proximity (Fig. 7-4B, -4C).

Three-way ANOVA analysis was conducted on the time spent (s) in the novel object chamber (Fig. 7-4B, -4C, F(7,51)=0.840, p=0.559). Pair-wise comparisons on the time spent in the novel object chamber revealed no significant differences between groups (p>0.05). Three-way ANOVA analysis was also conducted on the time spent (s) in the novel mouse chamber (Fig. 7-4B,-4C, F(7,51)=2.085, p=0.062). There were no statistical differences in the time spent in the novel mouse chamber between young WT and COX-2$^-$ males (Fig. 7-4B, p=0.0.532) or

between young WT and COX-2$^-$ females (Fig. 7-4B, p=0.271). In the adult groups, there was also no significant difference between adult WT and COX-2$^-$ males (Fig. 7-4C, p=0.327) but adult COX-2$^-$ females spent statistically less time in the novel mouse compared to WT females (Fig. 7-4C, p=0.011). The time spent sniffing the novel mouse (F(7,51)=0.877, p=0.531) or the novel object (F(7,51)=0.659, p=0.705) was not statistically different between groups (data not shown).

Overall, we observed that young and adult WT or COX-2$^-$ mice spent more time exploring the novel mouse chamber compared to the novel object chamber. A statistical difference was only seen in adult female mice spending less time in the novel mouse chamber compared to age-matched controls. There were no sex- or age-dependent differences observed.

Abnormal expression of autism-linked genes in COX-2$^-$ mice

Our previous microarray analysis study in COX-2-deficient mice at two prenatal stages and our past *in vitro* work collectively found differential expression of the following genes—*Wnt2 Glo1, Grm5,* and *Mmp9* (Rai-Bhogal et al., 2017, Rai-Bhogal et al., 2018, Wong et al., 2014)—which were all previously linked to ASD (Abdallah et al., 2012, Junaid et al., 2004, Marui et al., 2010, Skafidas et al., 2014). The gene expression levels were determined in whole brain samples collected at postnatal day 8, which is analogous to the infant stage in humans (Pressler and Auvin, 2013, Semple et al., 2013), using quantitative RT-PCR analysis (Table 7-2).

In comparison to WT males (RQ = 1), expression of all genes tested was significantly different in COX-2$^-$ males (Table 7-3). Specifically, COX-2$^-$ males had statistically elevated expression of *Wnt2* (*t(6)=-15.65, p*=0.00057; RQ=1.423±0.027) and decreased expression of *Glo1* (*t(6)=4.187p*=0.025; RQ=0.781±0.052), *Grm5* (*t(6)=4.835, p*=0.017; RQ=0.727±0.056),

and *Mmp9* (*t(6)=12.508, p*=0.009; RQ=0.540±0.044). In contrast, only one of the genes showed altered expression in COX-2$^-$ females compared to WT females (RQ = 1); *Glo1* was also significantly decreased compared to controls (*t(6)=3.445, p*=0.041, RQ=0.627±0.108) . Overall, our analyses revealed that the observed dysregulation of ASD gene expression was predominantly found in males.

7.5. Discussion

In this study, we describe for the first time autism-related behaviours in COX-2$^-$ KI male and female mice and propose that these mice may serve as a novel experimental model system for studying ASDs. We observed sex-specific and age-specific differences that have never been described before in this animal model. The results presented in this study along with published clinical data (Tamiji and Crawford, 2010, Wong and Crawford, 2014, Wong et al., 2015) as well as *in vitro* and *in vivo* molecular research (Dean et al., 2012, Hoffman et al., 2016, Rai-Bhogal et al., 2017, Rai-Bhogal et al., 2018, Wong et al., 2014, Wong et al., 2016) strengthen the evidence that abnormal COX-2/PGE_2 signalling may influence brain development and contribute to ASD pathology. We determined that male and female COX-2$^-$ KI mice lacking activity from the PGE_2-producing enzyme display behaviours linked to autism, specifically in hyperactivity, anxiety, repetitive behaviours, motor strength, and social interaction (Table 7-4). For the majority of behavioural assessments conducted, autism-related behaviours were particularly pronounced in male COX-2$^-$ mice.

Results from the open field test revealed that COX-2$^-$ mice have elevated ambulatory activity and adult COX-2$^-$ males spend less time in the center, which are analogous to greater hyperactivity and anxiety behaviours respectively (Seibenhener and Wooten, 2015). These

behaviours seem to be comparable to symptoms commonly reported in ASD cases (Matson et al., 2013, van Steensel and Heeman, 2017). For example, a number of cross-sectional studies have reported that 40-55% of children with ASD also had attention deficit-hyperactivity disorder (ADHD) (Gadow et al., 2005, Gadow et al., 2004, Gordon-Lipkin et al., 2018) or anxiety disorders (de Bruin et al., 2007, Simonoff et al., 2008, van Steensel et al., 2011). Our analysis on sex differences in the open field test revealed that COX-2$^-$ males showed increased movement or hyperactivity compared to COX-2$^-$ females, which is reminiscent of a bias in the prevalence for ADHD in males with ASD (Willcutt, 2012). We also found that anxiety-linked behaviour, indicated by decreased time spent in the center of the open field test, was significantly elevated specifically in adult COX-2$^-$ males compared to sex- and age-matched controls. This relationship between increasing age and anxiety is also seen in clinical studies on ASDs and ADHD, where older patients had a higher prevalence of anxiety disorder than younger patients (Gordon-Lipkin et al., 2018). In contrast to our results, previous work utilizing postnatal subcutaneous injections of a COX-2 inhibitor in a rat model found no alterations in activity output in the open field test (Dean et al., 2012). This signifies that behavioural outcomes of COX-2-deficiency may manifest through aberrations of COX-2/PGE_2 signalling that occur prenatally as opposed to postnatal time points.

The marble burying test showed that COX-2$^-$ animals buried a higher number of marbles compared to wild-type controls representing increased repetitive and anxiety-related behaviours, which are characteristic symptoms of autism (Gotham et al., 2013, Matson et al., 2009, van Steensel and Heeman, 2017). Sex differences were also seen in control and COX-2$^-$ animals: males buried more marbles than females suggesting that males show higher levels of anxiety-related and repetitive behaviours. This male-specific sex difference in anxiety and repetitive

behaviours has also been reported in general and ASD-affected human populations (Evans et al., 2018, Supekar and Menon, 2015, Szatmari et al., 2012). Moreover, age-specific differences were only seen in males, where adult mice buried more than young mice. It appears differences in the marble burying test were seen predominately in COX-2$^-$ animals and particularly in males, which parallels the strong male prevalence in ASD (Loomes et al., 2017).

We also found that young male COX-2$^-$ mice fell at a higher rate during the inverted screen test indicating that these animals have less motor strength compared to controls. Analyses reported by Kern et al. (2011) found that hand grip strength in children with ASD was correlated to the severity of the disorder. Motor deficits are also comorbid symptoms of autism (Deacon, 2013, McPhillips et al., 2014). For example, hypotonia, defined as a decrease in muscle tone, has been commonly characterized in young children with ASD (Ming et al., 2007). Hypotonia also seems to improve across development since it is less prevalent in older children with ASD (Ming et al., 2007). Interestingly, this age-related motor impairment is also observed in our age-dependent results, where only young COX-2$^-$ animals were affected but not adults. Impairments in motor function involving more complex motor skills such as reaching, grasping, precision gripping, and interpersonal motor coordination have also been reported in the autism population (Curioni et al., 2017, David et al., 2009, Kaur et al., 2018, Libertus et al., 2014, Sacrey et al., 2014, Wang et al., 2015). Our results signify that COX-2$^-$ mice display motor abnormalities that might be akin to deficits observed in some cases of ASDs.

Control and COX-2$^-$ mice both had a preference for social novelty in the three-chamber sociability test. However, adult female COX-2$^-$ mice spent less time in the novel mouse chamber compared to adult female controls. This suggests that COX-2$^-$ mice can have sex- and age-specific social abnormalities, which have also been reported in human cases of ASD. For

example, a recent study by Lawson et al. (2018) found that females with ASD exhibited more social impairments compared to matched males. Females with Smith-Magenis syndrome, a complex genetic neurodevelopmental syndrome with autism features, also display a greater number of abnormal social symptoms (Nag et al., 2018).

It is also important to note that in our study, upon weaning, animals were housed with litter mates of the same test group (genotype), which facilitates normal enriched social interaction. In contrast, previously published studies in other autism rodent models have shown that individually caged animals exhibit greater behavioural abnormalities compared to socially enriched animals caged with other animals (Depino et al., 2017, Toyoshima et al., 2018). This suggests that the differences we observed in the COX-2$^-$ mice are more conservative and that our results may represent a robust observation of autism-related behaviours in this model system.

To follow-up on the observed autism-associated behaviours in COX-2$^-$ mice, we investigated the expression of autism-linked genes—*Wnt2*, *Glo1, Grm5,* and *Mmp9* (Abdallah et al., 2012, Junaid et al., 2004, Marui et al., 2010, Skafidas et al., 2014). We previously reported that ASD-linked genes were affected in COX-2 knockout mice during prenatal development (Rai-Bhogal et al., 2017). All genes tested in our current study were differentially expressed in postnatal day 8 COX-2$^-$ KI mice compared to controls. Interestingly, we found an increased expression of *Wnt2* and decreased expression of *Glo1, Grm5,* and *Mmp9* predominantly in male offspring.

Wnt2 was elevated in COX-2$^-$ male mice in comparison to wild-type mice. Wnt2 is an active signalling molecule of the Wnt pathway, which has been connected to ASDs (Kalkman, 2012, Zhang et al., 2014). *Wnt2* is found in an autism susceptibility locus (7q31-33) and mutations in *Wnt2* have been found in patients with ASD, correlating to speech delay (Lin et al.,

2012, Wassink et al., 2001). The elevated *Wnt2* levels we measured in COX-2$^-$ mice may contribute to altered sociability also observed in this study. Furthermore, this finding provides additional support for an interaction between the COX-2/PGE_2 and Wnt signalling pathways, which has already been demonstrated in our lab in neuronal cell cultures and prenatal COX-2-deficient male mice (Rai-Bhogal et al., 2017, Wong et al., 2014, Wong et al., 2016).

In contrast, transcript expression of glyoxalase 1 (*Glo1*), a detoxification enzyme (Distler and Palmer, 2012), was found to be decreased in COX-2$^-$ male and female mice. Single nucleotide polymorphisms in the *Glo1* gene have been identified in ASD patients (Junaid et al., 2004). Dysregulated *Glo1* expression in mice has been associated with increased anxiety (Hovatta et al., 2005). Taking into account this correlation between *Glo1*, autism, and anxiety, the decreased *Glo1* expression that we quantified in COX-2$^-$ mice may contribute to the increased anxiety-related behaviours found in this study. Glutamate metabotropic receptor 5 (*Grm5*) expression was also decreased in COX-2$^-$ male mice. Abnormal *Grm5* expression has been previously found in ASD (Skafidas et al., 2014). Moreover, a deletion within the *Grm5* gene has been linked to ADHD (Elia et al., 2010, Elia et al., 2011), which commonly occurs in children with autism (Antshel et al., 2013). It is feasible that the hyperactive behaviour displayed by COX-2$^-$ mice in our study may be resulting from reduced *Grm5* expression in the brain. Lastly, expression of matrix metalloproteinase 9 (*Mmp9*) was found to be lower in COX-2$^-$ male mice. *Mmp9* is important in synaptogenesis, axonal pathfinding, and cortical plasticity (Reinhard et al., 2015, Vafadari et al., 2016). Atypical Mmp9 levels have been quantified in amniotic fluid samples from ASD cases (Abdallah and Michel, 2013, Abdallah et al., 2012). Mmp9 also regulates the levels of a synaptic neuroligin-1 (Peixoto et al., 2012), a gene that has been associated with ASD cases (Glessner et al., 2009, O'Roak et al., 2012). Altered levels of *Mmp9*

found in COX-2$^-$ mice during infanthood would affect early developmental processes, which may contribute to autism-like behaviours observed in this study.

This study reveals that the abnormal expression of autism-linked genes can persist in COX-2-deficient mice during postnatal development. This complements our existing results from a microarray study conducted on prenatal stages of COX-2 knockout animals (Rai-Bhogal et al., 2017). Our past study found that prenatal COX-2 knockout male mice (at both embryonic day 16 and 19) exhibit gene expression abnormalities and deficits in protein-interaction networks involved in a number of key biological processes including regulation of immune responses, synaptic transmission, cell morphogenesis, and the Wnt signalling pathway (Rai-Bhogal et al., 2017). Building on this earlier finding, irregularities in these crucial processes may contribute to the autism-linked behaviours in the COX-2-deficient mice described here.

Previous literature has also revealed additional atypical phenotypes in COX-2-deficient mice associated with the global inactivation of COX-2. For example, the differential expression of constitutive COX-2 in various peripheral tissues contributes to tissue-specific irregularities, including renal abnormalities, gastric dysfunction, and infertility (Kirkby et al., 2016, Rouzer and Marnett, 2009, Seta et al., 2009, Wallace and Devchand, 2005). COX-2-deficient mice also exhibit defects in inflammatory responses and skeletal muscle development (Bondesen et al., 2004, Otis et al., 2005, Ricciotti and Fitzgerald, 2011, Rouzer and Marneet, 2009). The hindered formation of muscle in COX-2-deficient mice may explain the reduced motor strength in COX-2$^-$ KI mice observed in this study. All these reported phenotypes could be relevant to developmental disorders such as ASDs, and thus should be evaluated together in the future.

Furthermore, our analysis of social behaviour was limited to the three-chamber sociability test. Behavioural assays in subsequent studies could include additional tests investigating

ultrasonic vocalizations, reciprocal social interactions, social novelty, and social approach. Moreover, gene expression analyses were completed on whole brain samples from postnatal day 8 but examining region-specific expression of these affected autism-associated genes across other developmental stages may provide new details regarding their impact on behavioural outcomes.

7.6. Conclusions

In conclusion, the results from this study provide the first behavioural evidence that aberrant COX-2/PGE_2 signalling in the developing brain may result in autism-related behaviours manifested in postnatal life. COX-2^- mice exhibited increased hyperactivity, anxiety, repetitive behaviour, motor deficit, and social abnormality as seen in many clinical cases of ASDs. Prior research from clinical studies in ASD individuals and from *in vitro* and *in vivo* studies, have found that alterations in the COX-2/PGE_2 pathway change neuronal cell behaviour as well as differential expression of ASD-related genes and proteins. Our current study provides further proof that a connection between abnormal COX-2/PGE_2 signalling and autism may exist. Our results suggest that mouse models with disrupted COX-2/PGE_2 signalling, such as the COX-2-deficient mouse model, may be of specific interest for the study of ASDs. This study also reveals the importance of including both sexes in molecular and behavioural experiments in order to obtain a complete picture of autism specific phenotypic changes. Moreover, behavioural differences are also specific to age and therefore should be a considered factor as well.

7.7. Figures

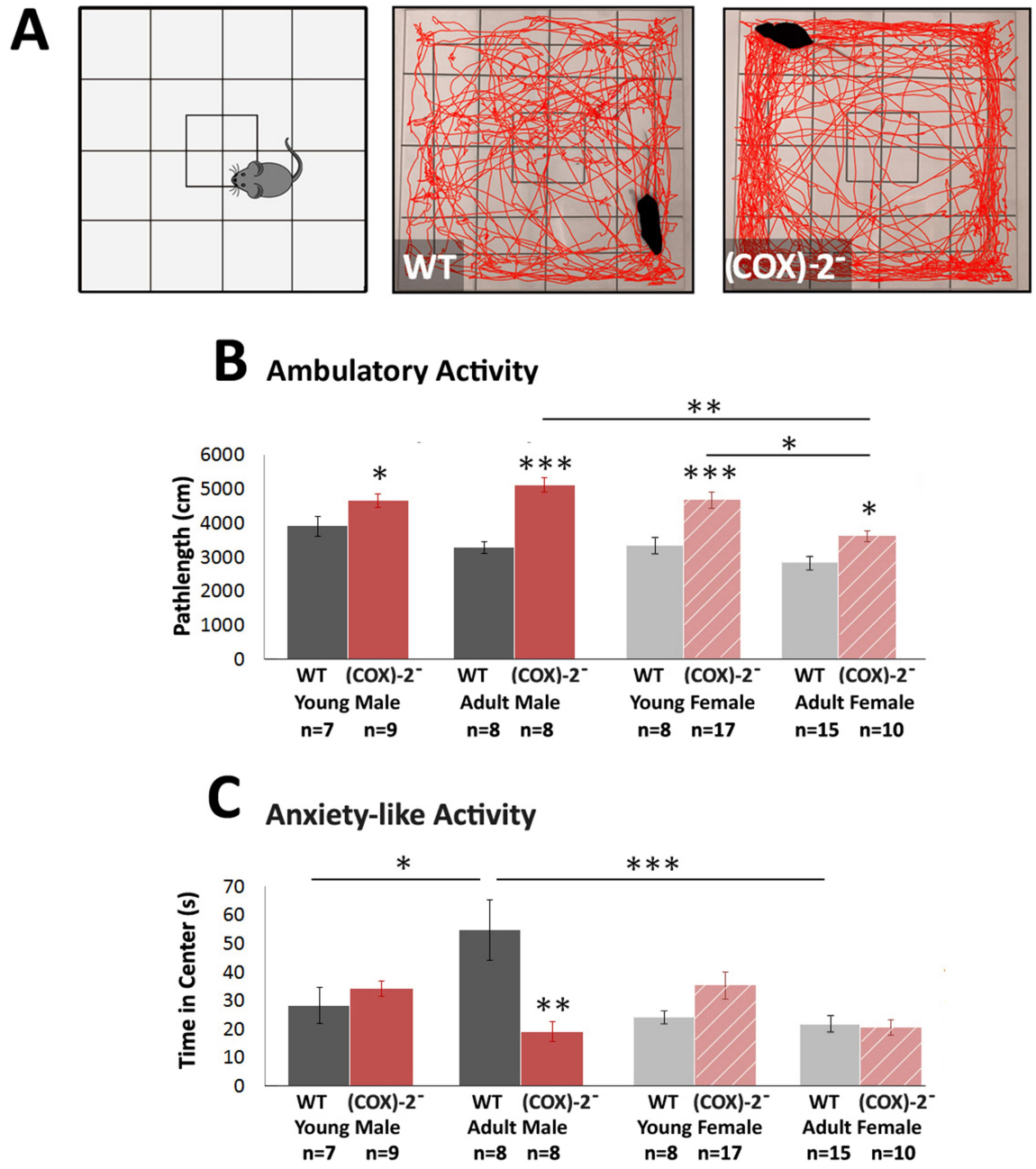

Fig. 7-1: Ambulatory activity and anxiety-linked behaviour in COX-2$^-$ mice in the open field test. (A) An automated tracking program determined the pathlength travelled indicated in red. (B) Pathlength results showed that COX-2$^-$ mice travelled more than WT controls in male and female groups. Sex- and age-dependent differences were only seen in COX-2$^-$ mice. Adult male COX-2$^-$ mice spent less time in the center than matched controls. (C) Measurements for time spent in center revealed no differences in anxiety-linked behaviour in female mice. Sex- and age-dependent differences in anxiety-related behaviour were found only in WT groups. Animals from at least 3 different litters were tested for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

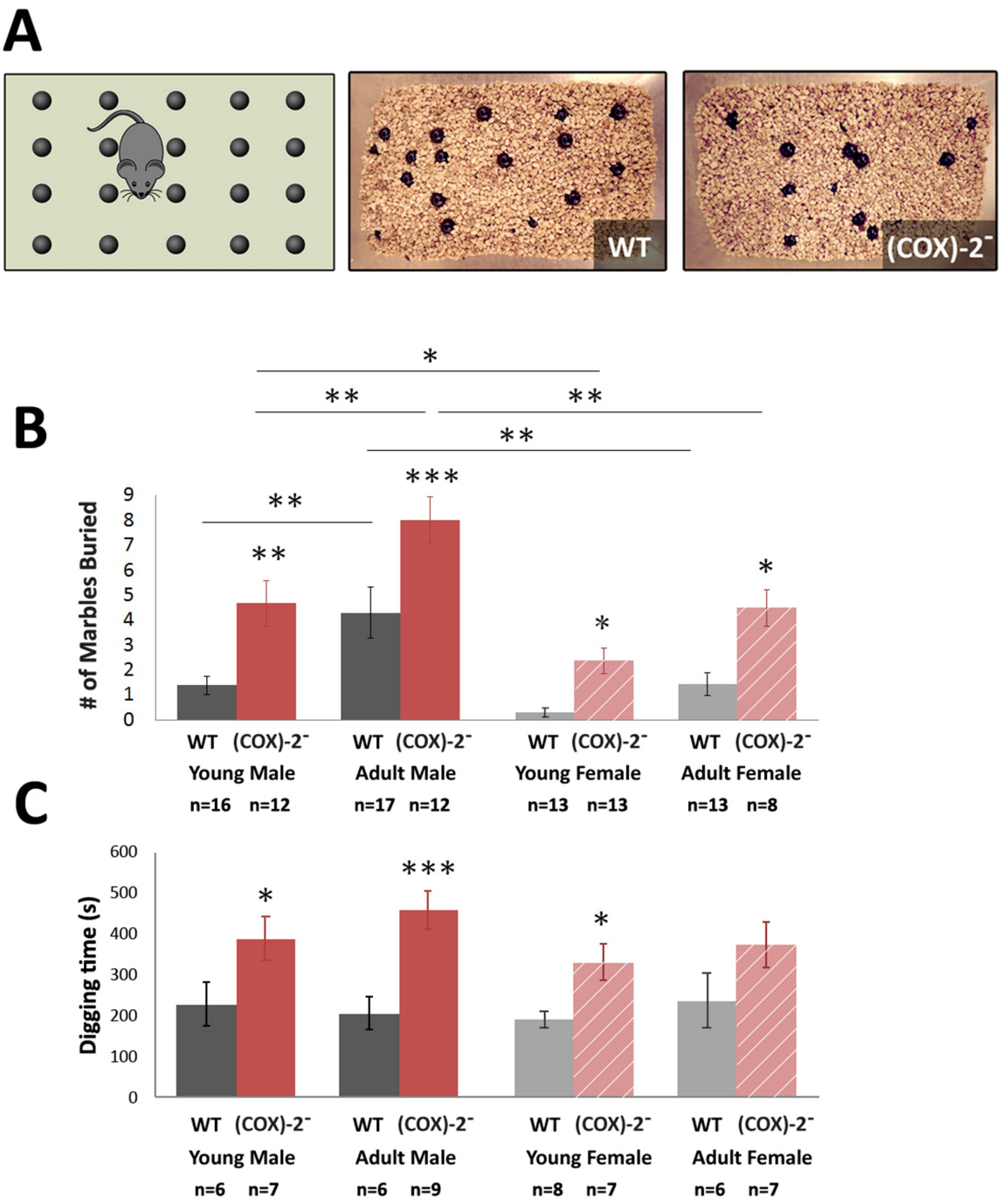

Fig. 7-2: Repetitive and anxiety-linked behaviour in COX-2$^-$ mice in the marble burying test. (A) Video recordings were taken and total marbles buried were counted. (B) Male COX-2$^-$ mice buried more marbles than controls. Female COX-2$^-$ mice buried more marble than WT mice. Sex differences were seen in adult WT and COX-2$^-$ mice. Age differences were seen in WT and COX-2$^-$ males. (C) Male COX-2$^-$ mice spent more time digging than controls. Young female COX-2$^-$ mice spent more time digging than controls. No sex- or age-dependent differences were observed in digging times. Animals from at least 3 different litters were tested for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

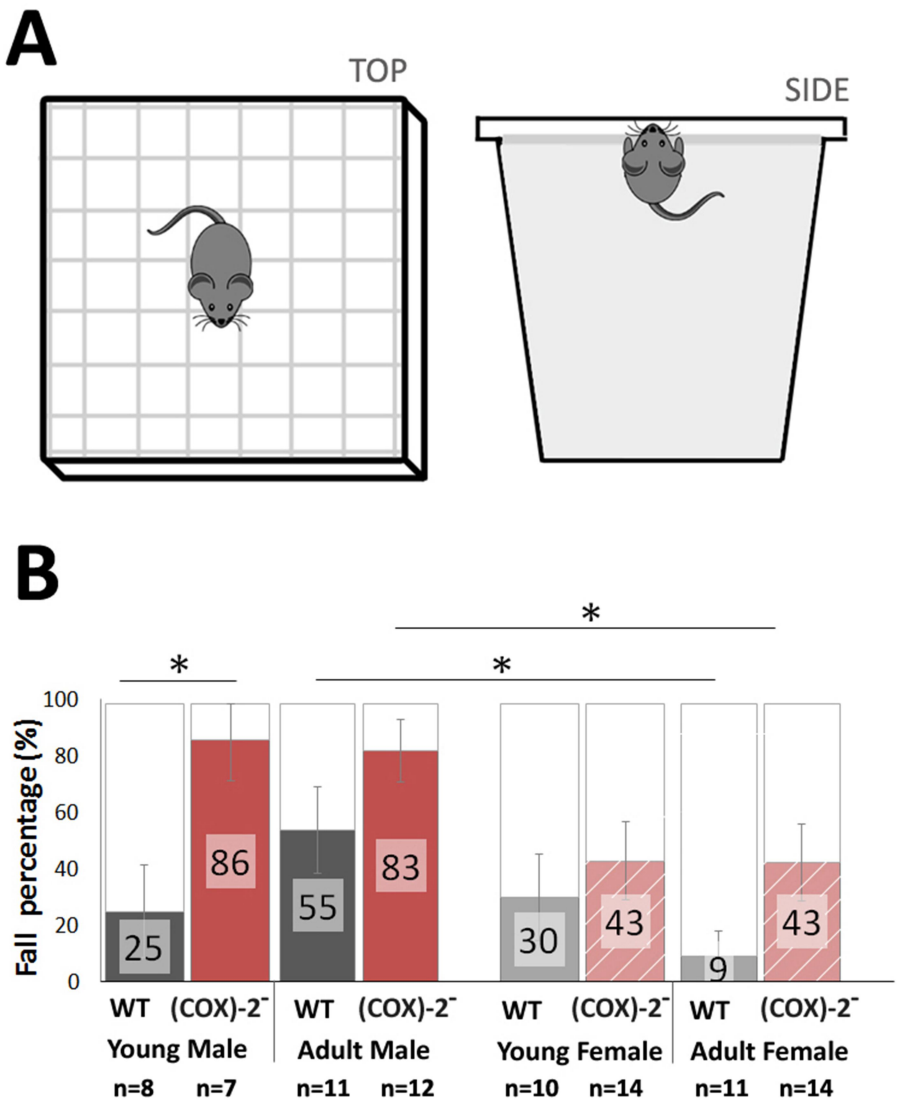

Fig. 7-3: Motor ability was measured in COX-2$^-$ mice using the inverted screen test.

(A) For the inverted screen test, whether or not the mouse subjects fell was recorded. (B) Young male COX-2$^-$ mice fell at a higher percentage than WT controls. No difference in motor strength in female mice. Males fell more frequently than females within adult WT, young or adult COX-2$^-$ groups. Animals from at least 3 different litters were tested for each experimental group. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

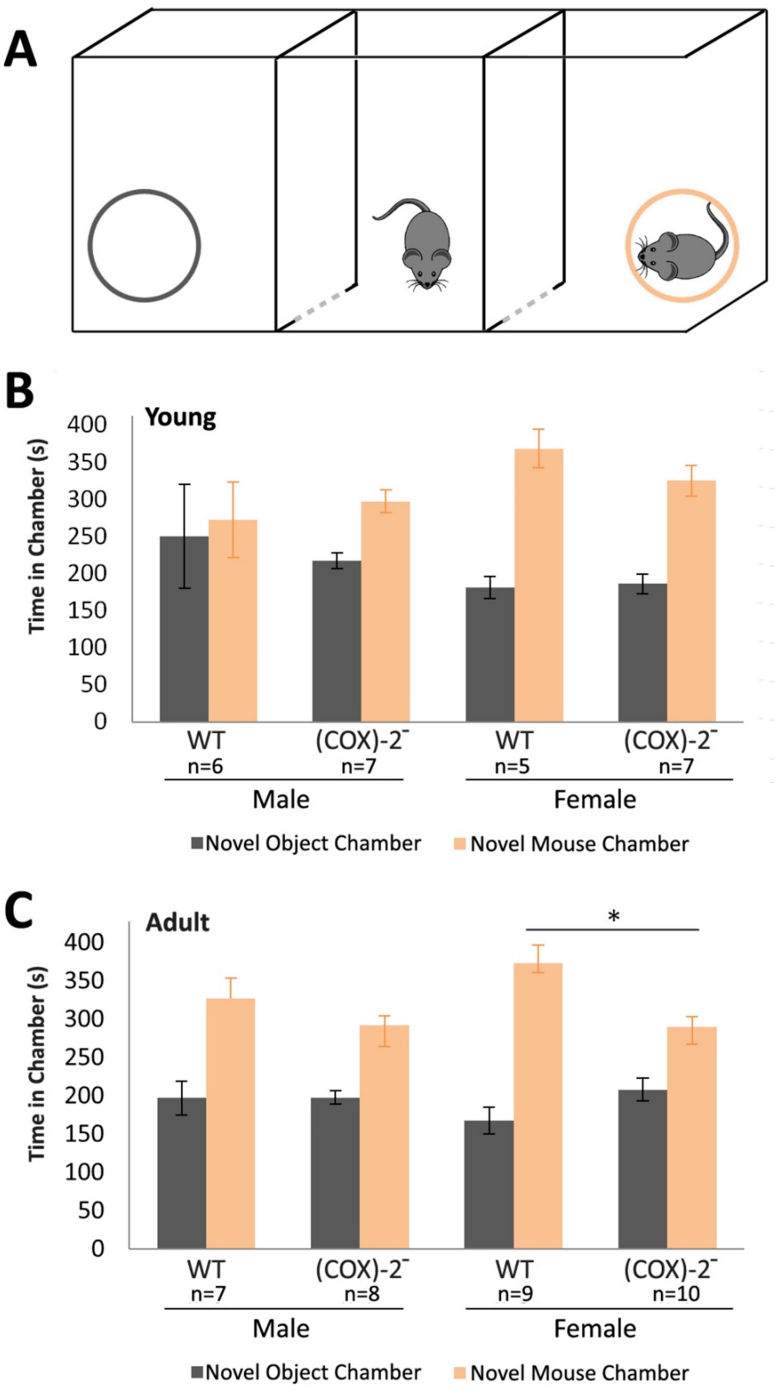

Fig. 7-4: Sociability behaviour was determined in COX-2⁻ mice using the three-chamber test. (A) Video recordings were taken and the time spent in the novel object chamber and novel mouse chamber was recorded. (B) Young mice showed preference for social proximity indicated by spending the greatest amount of time in the novel mouse chamber. No significant differences were observed between groups for time spent within the novel object chamber or time spent within the novel mouse chamber. (C) Adult mice also preferred social proximity but adult female COX-2⁻ mice spent significantly less time in the novel mouse chamber (orange bars) than matched WT controls. Animals from at least 3 different litters were tested for each experimental group. Data are presented as mean ±SEM, *p<0.05.

Table 7-3: Expression of autism-linked genes for COX-2$^-$ mice

Gene expression analysis on autism-linked genes were completed on postnatal day 8 samples as shown as RQ units for male and female COX-2$^-$ groups relative to respective controls (RQ=1). Values represent the mean of individuals from at least 3 independent litters. *p<0.05, **p<0.01, ***p<0.0001

COX-2$^-$ compared to WT controls : Autism-Linked Genes		
Males	**RQ Mean Values**	***p*-values**
Wnt2	**1.4231**	**0.00057*** **
Glo1	**0.7814**	**0.025***
Grm5	**0.7268**	**0.017***
Mmp9	**0.5404**	**0.009** **
Females	**RQ Mean Values**	***p*-values**
Wnt2	1.1540	0.281
Glo1	**0.6274**	**0.041***
Grm5	0.9096	0.588
Mmp9	1.0623	0.815

Table 7-4 : Summary of behavioural findings for COX-2$^-$ mice

Results represent the overall significant behavioural comparisons of COX-2$^-$ mice to respective WT controls, where ↑ = Increased; ↓ = Decreased; - = not significant. Each finding is a representation of individuals from at least 3 independent litters.

COX-2$^-$ mice compared to WT controls: Behavioural Results				
Behaviour	**COX-2$^-$ Male**		**COX-2$^-$ Female**	
	Young	**Adult**	**Young**	**Adult**
Open Field Test *Hyperactivity* *(pathlength)*	↑	↑	↑	↑
Open Field Test *Anxiety-like Behaviour* *(time in center)*	-	↓	-	-
Marble Burying Test *Anxiety-like & Repetitive Behaviour* *(marbles buried)*	↑	↑	↑	↑
Marble Burying Test *Anxiety-like & Repetitive Behaviour* *(digging time)*	↑	↑	↑	↑
Inverted Screen Test *Motor Deficits* *(fall percentage)*	↑	-	-	-
3 Chamber Test *Social behaviour* *(time spent in novel mouse chamber)*	-	-	-	↓

7.8. References

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CHAPTER 8.

Study 6: Maternal exposure to prostaglandin E2 leads to autism-like behaviours in mouse offspring

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Contributions: Christine T. Wong performed experiments, collected samples, acquired and analyzed the data, and prepared the manuscript. Isabel Bestard Lorigados assisted in acquiring RNA samples and data. Dr. Dorota A. Crawford supervised the study and edited the manuscript.

Objectives and Hypotheses: The sixth study was completed *in vivo* and its main purpose was to investigate whether prenatal PGE_2 exposure can influence autism-related behaviours. The aims of this study were to quantify the behaviours of young and adult PGE_2-exposed mice in the three chamber test, open field test, marble test, and inverted screen test. I hypothesized that PGE_2-exposed mice would display social abnormalities, elevated hyperactive, repetitive, and anxiety-related behaviours, and motor deficits in a sex- and age- specific manner. I also hypothesized altered expression of autism-linked genes (*Wnt2, Tcf4, Glo1*) in PGE_2-exposed mice.

8.1. Chapter Summary

Healthy brain development relies on important lipid molecules, such as Prostaglandin E2 (PGE_2). PGE_2 signalling can influence key neurobiological processes including neuronal proliferation, differentiation, and synaptogenesis. Disruption of PGE_2 levels by various environmental risk factors or genetic causes have been linked to Autism Spectrum Disorders (ASDs). Our recent study in mice lacking COX-2, the PGE_2 producing enzyme, already revealed distinct sex- and age-specific autism-related behaviours in offspring. The current study is the first to demonstrate that a single maternal injection of PGE_2 during pregnancy, can also lead to manifestation of different autism-related behaviours in male and female C57bl/6 mouse offspring at young (4-6 weeks) or adult (8-11 weeks) ages. In the three chamber test, only adult PGE_2-exposed males spent more time in the novel object chamber but both PGE_2-exposed males and females interacted longer with the novel object as opposed to the novel mouse, indicative of social abnormalities. PGE_2-exposed male and female mice buried more marbles and only young PGE_2-exposed females spent the most time grooming, suggesting increased repetitive and anxiety-like behaviours. In the open field test, only young PGE_2-exposed male and female mice travelled more and spent less time in the center than controls suggesting elevated hyperactive and anxiety-like behaviours. PGE_2-exposed mice didn't display differences in motor strength; however, young PGE_2-exposed male and female mice had greater defecation during the inverted screen test revealing increased anxiety. In addition, we detected sex-dependent downregulation of key developmental genes associated with autism such as *Wnt2, Tcf4* (in males), and *Glo1* (in females). Overall, our findings add new evidence for the connection between abnormal COX-2/PGE_2 signalling and ASD pathology. This study demonstrates that changes in the level of PGE_2 during a critical time in prenatal development can lead to appearance of distinct sex- and

age-dependent autism behaviours. We suggest that PGE_2-exposed mice could be a useful experimental model system for studying certain types of autism disorders.

8.2. Introduction

The brain is composed of a high concentration of lipids, which are important for both structural integrity and biological processes. The major bioactive lipid mediator is prostaglandin E2 (PGE_2). The PGE_2 signalling pathway is typically known for its role in regulation of inflammation and immune responses (Ricciotti and FitzGerald, 2011). PGE_2 also plays an essential role in the development of the nervous system, including processes such as cell proliferation, synaptogenesis, and learning and memory (Tassoni et al., 2008, Chen and Bazan, 2005). PGE_2 is synthesized from arachidonic acid (AA) that is released from cell membrane phospholipids through the enzymatic activity of phospholipase A2 (PLA2). AA is then converted to PGE_2 by cyclooxygenase-1 or -2 (COX-1 or COX-2), enzymes constitutively expressed in the brain mainly in microglia or neuronal cells, respectively.

Epidemiological, clinical, and molecular studies on humans and rodent models have provided convincing evidence that the PGE_2 signalling pathway might be an autism candidate pathway (Wong et al., 2015, Wong and Crawford, 2014, Tamiji and Crawford, 2010). Individuals with autism have increased or decreased AA:Omega fatty acid ratios in blood samples, decreased total AA, increased PLA2 activity, and elevated COX-2 and PGE_2 levels (Qasem et al., 2018, Yui et al., 2016, Brigandi et al., 2015, Tostes et al., 2013, El-Ansary et al., 2011, Bell et al., 2010). Abnormalities in various components of the PGE_2 signalling pathway due to genetic or environmental causes have also been associated with cases of Autism Spectrum Disorders (ASDs). For example, genetic abnormalities of key enzymes, including polymorphism

of the genes encoding PLA2 and COX-2, have been found in individuals with autism (Liu et al., 2017, Yoo et al., 2008). Many environmental factors that affect PGE_2 levels, including exposure to heavy metals, pollution, maternal infection, pesticides, or over the counter medications such as acetylsalicylic acid and acetaminophen, have all been associated with ASDs (Masarwa et al., 2018, Wong et al., 2015). Moreover, prenatal exposure to misoprostol, a synthetic analogue of PGE_2, during early gestation to terminate pregnancy resulted in neurodevelopmental defects such as Möbius Syndrome and autism-related behaviour (Miller et al., 2004, Bandim et al., 2003, Stromland et al., 2002). Studies in human and animal models of autism show that these environmental risk factors, including misoprostol, are capable of modifying the expression of many genes involved in key developmental processes and lead to manifestation of autism-related behaviour (Dufour-Rainfray et al., 2011, Miller et al., 2009, Stromland et al., 2002). Interestingly, gene expression analysis on ASD patients have uncovered dysregulation in the immune response, cell communication, and neuronal differentiation (Gupta et al., 2014, Garbett et al., 2008), which are processes influenced by PGE_2 signalling.

Subsequent molecular studies conducted in cell lines and animal models complement the human studies by providing further evidence for a connection between the PGE_2 signalling pathway and neuronal pathology that may lead to autism. For example, we found that in neuronal cell cultures, an elevated level of PGE_2 increases calcium levels in the cytosol and growth cones, and affects neural stem cell proliferation, neuronal migration and differentiation (Davidson et al., 2016, Wong et al., 2016, Wong et al., 2014, Tamiji and Crawford, 2010). Increased levels of PGE_2 also led to altered expression of autism-linked genes such as *Ptgs2*, *Mmp9*, *Ctnnb1*, and *Wnt3* in neural stem cells and differentiating neurons (Wong et al., 2016, Wong et al., 2014). Moreover, evidence from our recent study in mice has found that maternal exposure to

exogenous PGE_2 during early pregnancy led to abnormal expression of autism-associated genes, including *Wnt2* and *Wnt3a*, in offspring across crucial prenatal developmental time points (Rai-Bhogal et al., 2018). Similarly, whole genome microarray analysis in male mice lacking the PGE_2 producing enzymes (either COX-1 or COX-2) also found differential expression of numerous ASD-linked genes and defects in several biological processes that influence neurodevelopment including axonal pathfinding, synaptic transmission, synaptic plasticity, and learning and memory (Rai-Bhogal et al., 2017). Other researchers have reported that both an increase or decrease in PGE_2 levels at specific postnatal time-points also result in irregular cerebellar development (Dean et al., 2012) and decreased reciprocal social behaviour in male rats (McCarthy and Wright, 2017, Hoffman et al., 2016, Dean et al., 2012). Interestingly, our recent behavioural study in COX-2-deficient mice revealed sex-and age-specific autism-related behaviours, including increased hyperactivity, anxiety, and repetitive behaviour, as well as defects in motor ability and social interaction (Wong et al., 2019).

In the present study, we aimed to investigate the correlation between maternal exposure to exogenous PGE_2 during a critical time in pregnancy and manifestation of sex-specific ASD-like behaviour in young (4-6 weeks old) and adult (8-11 weeks old) mouse offspring. For this purpose, we characterized autism-associated behaviours using the three-chamber sociability, marble burying, open field, and inverted screen tests. We observed that PGE_2-exposed offspring showed abnormal social behaviour, repetitive and anxiety-related behaviour, and hyperactivity. Additionally, we found significant alterations in the expression of autism-linked genes including *Wnt2, Tcf4,* and *Glo1*. Most importantly, in line with reports observed in clinical studies, our data shows that a single exposure to exogenous PGE_2 at a critical time point in development can result in postnatal manifestation of ASD-like symptoms. Our findings also suggest that mice

prenatally exposed to an environmental risk factor that can affect the fetal level of PGE_2 in the brain may serve as an important experimental model to investigate the pathogenesis of ASDs. This novel study provides evidence for the existence of distinct male and female behavioural traits and genetic markers at different developmental ages. The sex-and age-dependent differences are particularly important in research on ASDs and thus should always be examined independently.

8.3. Methods

Animals

Male and female C57bl/6 mice were purchased from Charles River Laboratories. All animals were bred and maintained via group housing at York University animal facility on a 12-hour light/dark cycle. Mice were separated into groups by treatment, sex and age. Mouse subjects were divided into either treatment (see maternal injections under methods) or vehicle control groups, into male or female groups, and into young (4-6 weeks old) or adult (8-11 weeks old) groups. Between testing of individual mice, behavioural equipment was disinfected and deodorized with antiseptic clinicide and thoroughly cleaned with water. All behavioural tests were administered by the same female researcher to avoid increased stress levels in mice reported when handling is done by male experimenters (Sorge et al., 2014). For gene quantification experiments, brain samples were collected at postnatal day 8, where birth was considered postnatal day 0. Postnatal day 8 in mice is of particular interest since it is analogous to infanthood in humans (Pressler and Auvin, 2013, Semple et al., 2013), where symptoms of ASDs can be first detected. Subjects from each experimental group included mice from at least 3 different litters. All experiments and protocols followed the York University Animal Care

Committee ethics guidelines and have been approved by the Research Ethics Board of York University.

Maternal Injections

Male and female mice were housed together overnight for breeding. Females were checked every morning for the presence of a vaginal plug. When a plug was observed, this was noted as embryonic day 1 and females were then housed individually for the duration of their pregnancies. On embryonic day 11 (E11), pregnant females were weighed in the morning and given a single subcutaneous injection of 0.2 μg/g concentration of 16, 16-dimethyl prostaglandin E2 ($dmPGE_2$; Cayman Chemical) diluted in saline as used in previous studies (Rai-Bhogal et al., 2018, Okamoto et al., 2011, Tessner et al., 2004). $dmPGE_2$ has a metabolic rate that is slower than PGE_2, and is considered a stable analogue of PGE_2 that stays active for a longer time period (Steffenrud, 1980, Ohno et al., 1978). Control animals were given saline-only injections. Administration of $dmPGE_2$ exposure was conducted on E11 since this time-point marks the onset of neurogenesis in embryonic mice (Zhang and Jiao, 2015). E11 also corresponds to the time that the drug analogous to PGE_2, called misoprostol, was taken in human cases resulting in Moebius syndrome and autism characteristics (Bandim et al., 2003, Pastuszak et al., 1998). Maternal exposure of $dmPGE_2$ at E11 was also previously shown to result in altered expression of autism-linked genes during prenatal development (Rai-Bhogal et al., 2018). Herein, mouse offspring subjected to maternal exposure of $dmPGE_2$ as described above are referred to as "PGE_2-exposed mice".

Three-Chamber Sociability Test

Sociability was evaluated using the three-chamber test as previously described (Kaidanovich-Beilin et al., 2011, Silverman et al., 2010). The sociability apparatus (60 cm L x 45 cm W x 26 cm H) was constructed with clear acrylic walls creating three chambers that were equal in area. Access into adjacent chambers was restricted or permitted by removable doors that covered openings (10 cm x 10 cm) on the two dividing walls. There were two phases in the three-chamber test. The test mouse was placed in the centre chamber and allowed to explore only the centre chamber for 5 minutes in the first phase. An inverted black wire-mesh cylindrical container (10.5 cm D x 16 cm H) was placed in the middle against the lateral wall of each outer chamber. A weighted hockey puck was placed on top of each cylinder to prevent tipping. A novel mouse (4 weeks old, sex matched) was placed inside one of the two cylinders. The second phase began with the removal of the barrier doors and the test mouse was then allowed to freely explore all three chambers for 10 minutes. Trials for the second phase were recorded by a Sony Cyber-shot DSC-W800 20.1 MP camera mounted overhead. Recorded videos were replayed and manually analyzed for time spent in each chamber and time spent interacting with (sniffing or touching) the cylinders. These measurements were completed blind to the experimental test groups. The three-chamber sociability test was conducted on a total of 53 animals.

Marble Burying Test

Anxiety and repetitive behaviour was investigated using the marble burying test as previously described (Angoa-Pérez et al., 2013, Deacon, 2006). A clean standard mouse cage (28.5 cm L x 17.5 cm W x 12 cm H) was used as the testing apparatus. Bedding measuring a height of 3.5 cm was added and twenty black glass marbles (15mm diameter) were placed in the

cage in a 4 by 5 arrangement. The mouse subject was placed in the centre of the apparatus and allowed to move freely for the duration of 30 minutes. The behaviour were recorded using a Sony Cyber-shot DSC-W800 20.1 MP camera. Upon completion of the test, the number of completely buried marbles was counted. Recorded videos were later manually analyzed to measure the time spent digging or grooming. Manual measurements were completed blind to the test condition of the mouse subject. The marble burying test was conducted on a total of 62 animals. 56 animals were recorded for analysis of digging time behaviour.

Open Field Test

Ambulatory activity and anxiety-like behaviour was determined using standard open field test methods as previously described (Seibenhener and Wooten, 2015). The mouse subject was placed in the middle of an empty open chamber (40 cm L x 40 cm W x 40 cm H) with a centre region outlined by a 10 x 10 cm^2 square. Overhead video recording of free roaming behaviour by the test mouse for a duration of 10 minutes was completed with a Sony Cyber-shot DSC-W800 20.1 MP camera. Ambulatory activity was investigated by determining the total pathlength travelled, which was analyzed by an automated tracking program from the NIS Elements Advanced Research Software. Anxiety-like behaviour was observed by measuring the time spent in the centre. Times were quantified manually with a stopwatch by replaying the recorded video at a future date. These measurements were completed without given information on the experimental group of the mouse subject. The open field test was conducted on a total of 58 animals.

Inverted Screen Test

Motor strength was evaluated using the inverted screen test. The mouse subject was placed in the centre of a 34 x 38 cm rectangular wire mesh grid (1 mm thickness) composed of 13 mm squares and enclosed by a wooden frame of 1.5 cm thickness and 4 cm height (Bonetto et al., 2015, Deacon, 2013). The framed wire grid was then inverted slowly over a 55 cm tall container with foam padding (30 cm height) for a maximum duration of 10 minutes. The number of fecal boli produced during the test was noted. Whether or not the mouse fell was recorded as a binary result (1 = mouse fell, 0 = mouse did not fall). The fall percentage in each experimental group was then calculated as follows: Fall percentage of experimental group = (sum of values for each experimental group) ÷ (number of test subjects in group) x 100. The inverted screen test was conducted on a total of 54 animals.

RNA Isolation and quantitative Real-Time

Whole brain samples were collected at postnatal day 8 and homogenized in Trizol (Sigma) using a Polytron power homogenizer. Standard Trizol (Sigma) method was followed to isolate total RNA, which was then reverse-transcribed into cDNA with MMuLV reverse transcriptase (New England Biolabs, Ipswich, MA) using manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted on the cDNA samples using SYBER green master mixes in a 7500 FAST RT-PCR system (Applied Biosystem, Foster City, CA). The ΔΔCt method was used to calculate transcript expression and determine relative quantification (RQ) values (described in Wong et al. 2014). Autism-implicated genes were chosen based on current findings from human studies (refer to discussion) and our recent study in embryonic COX-$2^{-/-}$ mice (Rai-Bhogal et al., 2018). Primer Express v3.0 (ThermoFisher

Scientific, Waltham, MA) was used to design the primers for genes investigated: *Wnt2, Tcf4, Glo1, Grm5* (Table 8-1). Hypoxanthine phosphoribosyl transferase (*Hprt*) and phosphoglycerate kinase 1 (*Pgk1*) served as housekeeping controls for the qRT-PCR experiments. The RQ means were calculated from the RQ values of three different litters determined in three independent experiments.

Table 8-1: qRT-PCR Primers for autism-linked genes in PGE_2-exposed mice

Name	Primer	Primer Sequence (5'-3')	Base pair Length
Hprt	Forward	TCCATTCCTATGACTGTAGATTTTATCAG	29
	Reverse	AACTTTTATGTCCCCCGTTGACT	23
Pgk1	Forward	CAGTTGCTGCTGAACTCAAATCTC	24
	Reverse	GCCCACACAATCCTTCAAGAA	21
Wnt2	Forward	GCCCTGATGAACCTTCACAAC	21
	Reverse	TGACACTTGCATTCTTGTTTCAA	23
Tcf4	Forward	GGGTTTGCCGTCTTCAGTCTAC	22
	Reverse	GCCTGGCGAGTCCCTGTT	18
Glo1	Forward	GGATTTGGTCACATTGGGATTG	22
	Reverse	CGTCATCAGGCTTCTTCACA	20
Grm5	Forward	CATGGAGCCTCCGGATATAATG	22
	Reverse	GTATCCAAGAGGAGTGACAACC	22

Statistical Analysis

All numerical data are reported as mean±standard error of the mean (SEM), which encompasses the average of individuals from a minimum of three separate litters for each condition and time point. Three-way ANOVA followed by Bonferroni pair-wise comparisons was conducted for the open field test, marble burying test, and three-chamber sociability test to analyze potential behavioural differences dependent on condition (control vs. PGE_2-exposed), sex (male vs. females), or age (young vs. adult). The Kruskal-Wallis H test followed by post hoc pair-wise comparisons was conducted on fall percentages in the inverted screen test. Independent

t-test was performed to determine if there were differences in gene expression. Significance was determined for p values less than 0.05.

8.4. Results

Three-Chamber Sociability Test of Social Behaviour

The three-chamber sociability test was used to characterize changes in social behaviour (Fig. 8-1A). The time spent in the novel object, centre, or novel mouse chamber was recorded and reported in seconds.

Three-way ANOVA analysis was completed on the total times spent inside the chamber with the novel object (Fig. 8-1B, $F(7,45)=4.986$, $p=0.000309$). Pair-wise comparisons on the time spent in the novel object chamber revealed no significant differences between young wild-type (WT) and PGE_2-exposed males ($p=0.088$). However, adult PGE_2-exposed males spent significantly more time in the novel object chamber compared to adult WT males ($p=0.000007$; WT=191.3±6.10, PGE_2=283.2±14.2). Female WT or PGE_2 mice did not show differences in the time spent inside the novel object chamber ($p>0.05$).

Three-way ANOVA analysis was also completed on the total times spent inside the chamber with the novel mouse (Fig. 8-1C, $F(7,45)=3.806$, $p=0.002515$). Similarly, significant differences were not found between young WT and PGE_2-exposed males ($p=0.261$) but adult PGE_2-exposed males occupied the novel mouse chamber for a significantly shorter duration than adult WT males ($p=0.000562$; WT=307.7±12.7, PGE_2=203.0±13.0). Statistical analyses on female mice found that only young PGE_2-exposed females spent significantly less time in the novel mouse chamber compared to young WT controls ($p=0.0291$; WT=306.9±15.8, PGE_2=243.5±38.2).

Sex differences were only observed between adult PGE_2-exposed mice: males spent more time in the novel object chamber (Fig. 8-1B, p=0.00348; Male (M)=283.2±14.2, Female (F)=222.5±7.11) and less time in the novel mouse chamber (Fig. 8-1C, p=0.01390; M=203.0±13.0, F=281.3±16.9) compared to females.

Age-dependent comparisons were also completed and statistical differences were only seen between PGE_2-exposed males, where adult males spent more time inside the novel object chamber (Fig. 8-1B p=0.00001; Young (Y)=185.5±23.8, Adult (A)=283.2±14.2) and less time in the novel mouse chamber than young males (Fig. 8-1C, p=0.000135; Y=330.8±27.3, A=203.0±13.0).

The time spent interacting with the novel object or the novel mouse was also quantified by measuring the amount of time spent sniffing or touching. Three-way ANOVA analysis on the time interacting with the novel object was completed (Fig. 8-1D, F(7,45)=8.74, p=0.00000096). Pair-wise comparisons showed that all PGE_2-exposed animals spent more time sniffing or touching the novel object compared to respective controls. This was seen in young PGE_2-exposed males (p=0.000825; WT=60.1±4.0, PGE_2=95.0±8.5), adult PGE_2-exposed males (p=0.000002; WT=48.7±4.5, PGE_2=101.2±11.5), young PGE_2-exposed females (p=0.006152; WT=53.7±7.1, PGE_2=81.7±8.2), and adult PGE_2-exposed females (p=0.010211; WT=51.4±6.2, PGE_2=77.5±6.0). No sex- or age-dependent differences were found between groups for times spent sniffing or touching the novel object (p>0.05).

Three-way ANOVA analysis on the time spent sniffing and touching the novel mouse was also conducted (Fig. 8-1E, F(7,45)=9.031, p= 0.00000065). PGE_2-exposed animals spent less time interacting with the novel mouse than respective WT controls. This was seen in young PGE_2-exposed females (p=0.000073; WT=140.1±9.3, PGE_2=65.5±7.9), adult PGE_2-exposed

males (p=0.000014; WT=119.4±13.4, PGE_2=35.8±4.3), and adult PGE_2-exposed females (p=0.00087; WT=111.0±11.6, PGE_2=50.0±5.9). There were no statistical differences for time spent interacting with the novel mouse between young WT and PGE_2-exposed males (p=0.1019; WT=115.7±18.6, PGE_2=87.2±9.4). There were no sex- or age-dependent differences between experimental groups for the times spent sniffing and touching the novel mouse (p>0.05).

In summary, the three-chamber sociability test revealed that both young and adult PGE_2-exposed animals displayed abnormal social behaviour, however, adult PGE_2-exposed mice spent the most time in the object chamber. When compared to WT controls, adult PGE_2-exposed mice spent overall significantly more time occupying the novel object chamber and interacting with (sniffing or touching) the novel object, while also spending significantly less time inside the novel mouse chamber and sniffing or touching the novel mouse. Some sex- and age-dependent differences were also found but only in the PGE_2-exposed groups.

Marble Burying Test of Anxiety-linked and Repetitive Behaviours

To assess repetitive and anxiety-like behaviour, the marble burying test was administered (Fig. 8-2A). The number of marbles that were completed buried after the 30 minute trial was recorded. The total time spent digging and grooming were also measured. Three-way ANOVA analysis was conducted on marble burying counts (Fig. 8-2B, F(7,54)=9.623, p=0.000000105). Pair-wise comparisons determined that most PGE_2-exposed groups buried significantly more marbles compared to matched WT controls including young PGE_2-exposed males (p=0.000002; WT=1.6±0.625, PGE_2=7.9±1.79), young PGE_2-exposed females (p=0.000103; WT=0.6±0.400, PGE_2=5.6±1.21), and adult PGE_2-exposed females (p=0.0062; WT=2.1±0.588,

PGE_2=5.1±0.581). No statistical differences were seen between adult PGE_2-exposed and WT males (p=0.309; WT=4.4±0.0.766, PGE_2=5.6±0.429).

There were no sex-dependent differences between males and females within the young WT (p=0.325) or young PGE_2-exposed (p=0.0960) groups. Sex differences were seen between adult WT males and females, where males buried significantly more marbles than females (p=0.0270; M=4.4±0.0.766, F=5.6±0.429). However, there were no differences between adult PGE_2-exposed males and females (p=0.694) indicating that in contrast to the sex difference seen in WT controls, adult PGE_2-exposed females buried a similar number of marbles as PGE_2-exposed males. Age differences were found exclusively between young and adult WT males, with adults burying more marbles than young animals (p=0.0101; Y=1.6±0.625, A=4.4±0.0.766). This age-dependent difference was not observed across age groups between young and adult PGE_2-exposed males (p=0.0673), as young PGE_2-exposed males buried a large number of marbles and this was persistent in adult PGE_2-exposed males. No statistical differences were seen across age groups between young and adult WT females (p=0.137) or PGE_2-exposed females (p=0.703).

Three-way ANOVA analysis was also completed on digging behaviour (Fig. 8-2C, $F(7,48)=4.708$, p=0.000441). The time spent digging (s) complemented our results regarding the number of marbles buried whereby PGE_2-exposed mice spent more time digging than WT controls. Statistically increased time of digging behaviour was seen in young PGE_2-exposed males (p=0.00104; WT=206.1±19.7, PGE_2=390.3±49.6) and adult PGE_2-exposed females (p=0.00133; WT=218.8±25.3, PGE_2=387.9±24.3) in comparison to respective matched controls. No significant differences in digging time were seen between adult WT and PGE_2-exposed males

(p=0.470; WT=343.3±49.2, PGE_2=389.4±62.3) or between young WT and PGE_2-exposed females (p=0.363; WT=220.8±46.2, PGE_2=279.0±60.9).

Sex-dependent differences in digging time were only seen between adult WT mice. Specifically, adult WT males spent more time digging than adult WT females (Fig. 8-2C, p=0.0348; M=343.3±49.2, F=218.8±25.3). Age-dependent differences were only found between young and adult WT males, with adult mice spending significantly more time digging than young mice (Fig. 2C, p=0.0229; Y=206.1±19.7, A=343.3±49.2).

Three-way ANOVA analysis was also completed on grooming behaviour (Fig. 8-2D, $F(7,48)=5.452$, p=0.000120). Young PGE_2-exposed females were found to spend statistically more time grooming than matched females (p=0.000485; WT=67.3±9.0, PGE_2=129.4±8.3). No significant differences in grooming behaviour were found between respective controls and young PGE_2-exposed males (p=0.546; WT=68.7±6.0, PGE_2=77.3±8.5), adult PGE_2-exposed males (p=0.159; WT=34.0±4.6, PGE_2=57.8±8.3), or adult PGE_2-exposed females (p=0.639; WT=52.8±12.4, PGE2=58.9±13.5).

Sex-dependent differences in grooming behaviour were only seen in young PGE_2-exposed animals, where males spent statistically less time grooming than females (Fig. 8-2D, p=0.00205; M=77.3±8.5, F=129.4±8.3). Age-dependent differences in grooming behaviour were seen between young and adult WT males (p=0.0279; Y=68.7±6.0, A=34.0±4.6), with adult males spending less time grooming than young males. Grooming differences between age groups were also found in PGE_2-exposed females. Young PGE-exposed females spent a greater time grooming than adult PGE_2-exposed females (p=0.000042; Y=129.4±8.3, A=58.9±13.5).

To summarize, the marble burying test revealed that PGE_2-exposed mice buried more marbles and spent more time digging than WT controls. Sex and age differences were found in

WT groups for marble burying but were not present in PGE_2-exposed mice. On average, young PGE_2-exposed males buried the greatest number of marbles and spent the most time digging. The time spent grooming was greatest in young PGE_2-exposed females and reached statistical significance when compared to young PGE_2-exposed males and across age groups when compared to adult PGE_2-exposed females. Young WT males spent significantly more time grooming than adult WT males. In contrast, the increased grooming behaviour seen in young PGE_2-exposed males was persistent in the adult group.

Open Field Test of Hyperactive locomotor and Anxiety-like Behaviours

To evaluate ambulatory activity and anxiety-like behaviour in a novel environment, the open field test was used (Fig. 8-3A). Movement behaviour was assessed by measuring the total pathlength (cm) that was travelled (Fig. 8-3B), while anxiety-like behaviour was determined by quantifying the time spent in the centre of the apparatus (Fig. 8-3C).

Pathlength values underwent three-way ANOVA analysis followed by pair-wise comparisons (Fig. 8-3B, $F(7,50)=6.638$, $p=0.000015$). Young PGE_2-exposed males were found to travel a greater pathlength than WT male controls ($p=0.000083$; WT=4179±188, PGE_2=5414±318). There was no significant difference in ambulatory activity between adult PGE_2-exposed males and WT controls ($p=0.835$; WT=4308±148, PGE_2=4247±142). Young PGE_2-exposed females also travelled a longer pathlength than WT female controls ($p=0.000083$; WT=3720±68.4, PGE_2=5079±70.0) and there were no statistical differences between adult WT and PGE_2-exposed females ($p=0.705$; WT=4052±280, PGE_2=3937±293). There were no statistical sex-dependent differences in pathlength travelled between males and females in all groups tested ($p>0.05$). Age-dependent differences in ambulatory activity were observed

between young and adult PGE_2-exposed males (p=0.000411, Y=5414±318, A=4247±142) and PGE_2-exposed females (p=0.000393, Y=5079±70.0, A=3937±293). In both comparisons, young mice travelled a greater pathlength than adult mice. In contrast, no age-dependent differences were seen within WT males (p=0.654) or WT females (p=0.301).

Three-way ANOVA analysis was conducted on the amount of time spent in the centre of the apparatus (s) to evaluate anxiety-like behaviour (Fig. 8-3C, $F(7,51)$=2.586, p=0.02318). Young PGE_2-exposed males spent less time in the centre than WT males (p=0.01496; WT=50.38±12.4, PGE_2=20.89±2.86) and this pattern was also observed between young PGE_2-exposed females and WT females (p=0.00412; WT=52.67±11.9, PGE2=17.85±3.18). No significant differences were detected between adult PGE_2-exposed males and WT controls (p=0.366; WT=24.89±5.34, PGE_2=34.78±6.74) or between adult PGE_2-exposed females and WT controls (p=0.840; WT=26.87±5.59, PGE_2=29.19±7.12). There were also no sex-dependent differences for the time spent in the centre between all groups tested (p>0.05). Age-dependent differences in anxiety-like behaviour were only found between young and adult WT males (p=0.022573; Y=50.38±12.4, A=24.89±5.34) and between young and adult WT females (p=0.03727; Y=52.67±11.9, A=26.87±5.59). In both cases, young WT mice spent more time in the centre than adult WT mice. In contrast, this age-dependent difference was not seen in PGE_2-exposed males (p=0.241) or PGE_2-exposed females (p=0.304), which is the result of decreased times in the centre for both young PGE_2-exposed males and females.

The main findings from the open field test were specific to the young cohorts: young PGE_2-exposed males and PGE_2-exposed females travelled a greater pathlength and spent less time in the centre compared to respective WT controls. This indicates that young PGE_2-mice show increased hyperactivity and greater anxiety-linked behaviour in the open field test.

Ambulatory activity remained consistent across age groups in WT animals. However, ambulatory activity was decreased in adult PGE_2-exposed animals compared to young PGE_2-exposed animals. Adult WT animals spent significantly less time in the centre compared to young WT animals indicating increased anxiety-linked behaviour in adults. In contrast, PGE_2-exposed animals exhibited constant elevated anxiety-like behaviour in both young and adult groups. No sex-dependent differences were characterized in the open field test.

Inverted Screen Test of Motor Strength and Stress-linked Behaviour

The inverted screen test was administered to assess changes in motor strength and stress-related behaviour (Fig. 8-4A). To evaluate motor strength, fall percentages (%) were calculated for each experimental group as described in the methods section. The Kruskal-Wallis H test was conducted on fall percentage values to determine potential statistical differences (Fig. 8-4B, $H(7)=19.271$, $p=0.00738$). Fall percentage was not statistically different between young WT and PGE_2-exposed males ($p=0.999$; WT=50±18.9, PGE_2=50±22.4) or adult WT and PGE_2-exposed males ($p=0.916$; WT=75.0±25.0, PGE_2=77.8±14.7). Similarly, differences in fall percentage were no statistically significant between young WT and PGE_2-exposed females ($p=0.429$; WT=12.5±12.4, PGE_2=0.0±0.0) or adult WT and PGE_2-exposed females ($p=0.089$; WT=33.3±21.1, PGE_2=0.0±0.0). Sex differences in fall percentages were only observed in adult-PGE_2-exposed animals, where males fell more often (78%) than females (0%) ($p= 0.001604$; M=77.8±14.7, F=0.0±0.0). No significant differences in fall percentages were found between the age groups tested ($p>0.05$).

The number of fecal boli produced by the mouse subject during the inverted screen test was recorded to assess stress-related behaviour (see methods). Three-way ANOVA analysis was

completed on fecal boli values (Fig. 8-4C, $F(7,46)=3.269$, $p=0.00769$). Young PGE_2-exposed males defecated more frequently than WT controls ($p=0.00379$; WT=1.3±0.42, PGE_2=3.6±0.60). The number of fecal boli were not statistically different between adult WT or PGE_2-exposed males ($p=0.752$; WT=2.5±0.65, PGE_2=2.3±0.49). There were also no significant differences between young WT and PGE_2-exposed females ($p=0.140$; WT=1.3±0.42, PGE_2=2.5±0.50) or adult WT and PGE_2-exposed females ($p=0.575$; WT=3.2±0.60, PGE_2=3.6±0.53). No significant sex differences were observed ($p>0.05$). Comparisons between age groups revealed that young WT females had fewer fecal boli than adult WT females ($p=0.0121$; Y=1.3±0.42, A=3.2±0.60). There were no other differences in defecation between age groups ($p>0.05$).

Overall, PGE_2-exposure did not statistically affect fall outcomes in the inverted screen test. However, adult PGE_2-exposed males were found to fall more often than adult PGE_2-exposed females suggesting potential abnormality in motor strength in adult PGE_2-exposed males. The increased number of fecal boli in young PGE_2-exposed males compared to WT controls implies a potential for elevated stress levels in young PGE_2-exposed males during the inverted screen test. Adult WT females produced higher number of fecal boli than young WT females suggesting that WT females may experience more stress with increased age. In contrast, it was found that young and adult PGE_2-exposed females had an elevated number of fecal boli across both age groups.

Abnormal expression of Wnt2 and Glo1 in PGE_2-exposed mouse offspring

Our previous *in vitro* study and *in vivo* studies on abnormal PGE_2/COX signalling mouse models collectively found differential expression of the genes: *Wnt2*, *Tcf4*, *Glo1,* and *Grm5* (Rai-Bhogal et al., 2018, Rai-Bhogal et al., 2017, Wong et al., 2014), which have all been previously

associated with ASDs (Skafidas et al., 2014, Abdallah et al., 2012, Marui et al., 2010, Junaid et al., 2004). The expression of these autism-linked genes was quantified in PGE_2-exposed mice and compared to WT controls. Total brain samples from postnatal day 8, the time analogous to infanthood in humans (Pressler and Auvin, 2013, Semple et al., 2013), were collected for analysis. Gene expression profiles were determined using quantitative real-time RT-PCR analysis (Table 8-1).

In comparison to WT males (RQ = 1), the expression of *Wnt2* and *Tcf4* was significantly different in PGE_2-exposed males (Table 8-2). Specifically, PGE_2-exposed males had statistically decreased expression of *Wnt2* ($t(4)$=6.282, p=0.02441; RQ=0.8859±0.018) and *Tcf4* ($t(4)$=4.803, p=0.00862; RQ=0.5781±0.087) compared to controls. The expression of *Glo1* ($t(4)$=1.739, p=0.15702; RQ=0.8618±0.079), and *Grm5* ($t(4)$=1.831, p=0.20863; RQ=0.8575±0.078) were not statistically different. In contrast, only one of the genes showed altered expression in PGE_2-exposed females compared to WT females (RQ = 1); *Glo1* was also significantly decreased compared to controls ($t(4)$=35.563, p=0.00079, RQ=0.6259±0.011) (Table 3). No statistical differences were found in the expression of *Wnt2* ($t(4)$=-1.266, p=0.33298; RQ=1.1072±0.085), *Tcf4* ($t(4)$=-2.067, p=0.17462; RQ=1.3067±0.148), and *Grm5* ($t(4)=I$2.072, p=0.17402; RQ=1.2689±0.130) between PGE_2-exposed females and WT controls. Overall, we found sex-dependent dysregulation of ASD gene expression in PGE_2-exposed offspring.

8.5. Discussion

Our present study demonstrates for the first time that autism-like behaviours can result from a maternal injection of PGE_2 at a single time-point during pregnancy in mice. We propose that prenatally PGE_2-exposed mice may serve as a novel experimental model system for studying

specific types of ASDs. The findings described in this study add new and convincing evidence that abnormal PGE_2 signalling may impact brain development and contribute to autism symptomatology. We report that offspring of PGE_2-injected mice exhibit complex behavioural alterations linked to autism at different postnatal ages, including abnormal sociability, increased repetitive behaviours, hyperactivity, and anxiety (Table 8-3). We also provide novel evidence for distinct sex- and age-specific behavioural differences in this animal model and emphasize the importance of such analyses in research related to disorders such as autism.

The three-chamber sociability test showed that PGE_2-exposed male mice spent more time in the novel object chamber than the novel mouse chamber, suggesting object preference over social preference. This behaviour manifested at a later age during adulthood. PGE_2-exposed males and females also spent more time interacting with the object and less time interacting with the novel mouse compared to respective controls. These results are comparable to the tendency of children with autism to show preference for solitary object play over social play (Memari et al., 2015, Volkmar et al., 2014). Similar to the male bias for decreased social interaction observed in the three-chamber test, clinical studies have reported that male individuals with ASD are less likely to engage in social interactions (Hiller et al., 2016, Lai et al., 2015, McLennan et al., 1993) while females with ASD are thought to be more socially motivated (Sedgewick et al., 2016, Lai et al., 2015, Head et al., 2014). Although PGE_2-exposed and control females displayed social preference by spending the most time inside the novel mouse chamber, PGE_2-exposed females spent significantly less time interacting with the novel mouse and more time interacting with the novel object. This social discrepancy between PGE_2-exposed and control females was only made apparent through the analysis of object and social interaction. This might be akin to the finding that girls with autism stay in close proximity to peers, masking their social

challenges, while boys with autism tended to play alone (Dean et al., 2017, Lai et al., 2015). Similarly, women with ASD have also been described to have better superficial socio-communicatory abilities, which may conceal their social difficulties (Lai et al., 2011).

Results from the marble burying test demonstrated that PGE_2-exposed mice buried a greater number of marbles and spent more time digging than WT controls. In particular, young PGE_2-exposed males buried the most marbles and spent the most time digging, analogous to increased ASD-associated repetitive and anxiety-like behaviours. Studies on children and adolescents with ASD have also found that restrictive and repetitive behaviours are more prominent in males with ASD than their female counterparts (Mandy et al., 2012, Sipes et al., 2011). We also observed that young PGE_2-exposed females exhibited the most grooming behaviour among all groups. Increased grooming behaviour is an indication of elevated stress and anxiety (Smolinsky et al., 2009). Anxiety disorders are a common comorbidity of ASDs (van Steensel et al., 2011). Moreover, young children with ASD, especially girls, have more anxiety symptoms than older children and boys (Wijnhoven et al., 2018). The increased anxiety-like behaviour observed in young PGE_2-exposed mice appears to resemble these clinical findings of co-occurring anxiety symptoms with higher risk in young females with ASD.

The open field test revealed that both males and females PGE_2-exposed mice at young age displayed increased ambulatory activity, indicative of hyperactivity (Seibenhener and Wooten, 2015). Our age-dependent finding seems to parallel studies on attention deficit hyperactivity disorder (ADHD) and ASDs, which have reported that symptoms of ADHD are often exhibited by individuals with ASD, particularly young children (Leitner, 2014, Sikora et al., 2012). Additionally, young PGE_2-exposed mice also spent a decreased amount of time in the centre during the open field test suggestive of greater anxiety (Seibenhener and Wooten, 2015).

As mentioned above, young children with ASD show more symptoms of anxiety compared to older children with ASD (Wijnhoven et al., 2018). This age-dependent difference was also seen in the open field test, where young PGE_2-exposed mice exhibited elevated anxiety-like behaviour.

There were no significant differences in fall percentages between PGE_2-exposed and control mice for the inverted screen test, demonstrating that motor strength of offspring was not affected by maternal PGE_2 exposure. Motor deficits are comorbid symptoms to ASDs (Travers et al., 2017, Ming et al., 2007) but irregularities in motor strength were not found in PGE_2-exposed mice. However, young PGE_2-exposed males and females mice produced a significantly greater number of fecal boli during the inverted screen test. Increased defecation in mice has been shown to be induced by elevated levels of psychological stress or anxiety (Monnikes et al., 1993). We found that the age-dependent difference of anxiety-like behaviour, specific to young PGE_2-exposed mice, was also demonstrated in the inverted screen test as well as the marble burying test and open field test discussed earlier. Our behavioural results signify that the offspring of PGE_2-exposed mother mice display social abnormalities, repetitive behaviours, hyperactivity, and elevated anxiety that might be analogous to atypical behaviours observed in ASDs.

In addition to investigating behaviours of PGE_2-exposed mice, we also examined the expression of known autism-associated genes including *Wnt2*, *Tcf4*, *Glo1*, and *Grm5* (Gabriele et al., 2014, Skafidas et al., 2014, Sweatt, 2013, Kalkman, 2012). Interestingly, the expression of three of these key developmental genes, *Wnt2*, *Tcf4*, and *Glo1*, was affected in postnatal day 8 in PGE_2-exposed mice, with different effects in males and females. Specifically, we found decreased expression of *Wnt2* and *Tcf4* in PGE_2-exposed males and decreased *Glo1* expression in PGE_2-exposed females compared to respective controls.

The Wnt2 (Wingless/integrated 2) ligand is an important activator of the Wnt signalling pathway, which is crucial in brain development (Noelanders and Vleminckx, 2017, Sousa et al., 2010) and has been associated with ASDs (Kalkman, 2012). Tcf4, short for transcription factor 4, is a key protein initiated through canonical Wnt signalling (Cadigan and Waterman, 2012) and has been linked to ASDs and schizophrenia (Kwan et al., 2016, Sweatt, 2013). Various genetic knockout mouse models targeting Wnt pathway molecules have ASD-related behavioural deficits, such as impaired sociability and repetitive behaviours (Belinson et al., 2016, Dong et al., 2016, Kwan et al., 2016, Lugo et al., 2014). Decreased *Wnt2* and *Tcf4* levels measured in PGE_2-exposed male mice may contribute to abnormal sociability and repetitive behaviours that were also prominent in PGE_2-exposed males. Moreover, these results provide further evidence for an interaction between the PGE_2 and WNT signalling pathways in the brain, which has been previously shown in our lab in cell and animal model systems (Wong et al., 2019, Rai-Bhogal et al., 2018, Wong et al., 2016, Wong et al., 2014).

Glo1 (Glyoxalase 1) expression and its translated protein is important for the enzymatic detoxification of reactive oxygen species (Distler and Palmer, 2012). A specific GLO1 allele, C332 (Ala111), which results in the reduction of glyoxalase activity, has been associated with increased autism vulnerability (Gabriele et al., 2014). Decreased *Glo1* expression in PGE_2-exposed females could result in elevated oxidative stress, which has been linked to ASDs (Rossignol and Frye, 2014, Meguid et al., 2011, Chauhan and Chauhan, 2006). Oxidative stress has also been implicated in the genesis of anxiety (Hassan et al., 2014). In line with the connection between oxidative stress and anxiety, irregular *Glo1* transcript and protein expression has been associated with anxiety-like behaviour in mice (Distler et al., 2012, Hovatta et al., 2005, Kromer et al., 2005). It may be possible that the diminished expression of *Glo1* in PGE_2-exposed

females may have influenced the increase of anxiety-like behaviours characterized in these mice (demonstrated in the marble burying test, open field test, and inverted screen test).

Lastly, it is interesting that the abnormal behaviours manifested in prenatally PGE_2-exposed mice resemble those from our previous study in a decreased PGE_2 model, namely, in COX-2-deficient mice (Wong et al., 2019). The PGE_2-exposure and COX-2-deficient experimental models resulted in social defects, repetitive behaviours, hyperactivity, and anxiety-like behaviour. Social abnormalities and anxiety-like behaviours were more distinct in PGE_2-exposed mice of this current study, while motor deficits were exclusive to COX-2-deficient mice. Collectively, these studies suggest that elevated or decreased PGE_2 signalling might lead to consequent ASD-like pathologies. Other studies have reported that postnatal increase or decrease in PGE_2 levels also contribute to social irregularities in male rodents (Hoffman et al., 2016, Dean et al., 2012). The findings from our study and others' are comparable to evidence from clinical studies, where increased PGE_2 signalling—for example, from exposure to maternal inflammation (Madore et al., 2016) or pesticides (De Felice et al., 2016, Holzman, 2014, Shelton et al., 2014)—or diminished PGE_2 signalling—from acetaminophen use (Masarwa et al., 2018, Parker et al., 2017, Schultz and Gould, 2016)—have been associated with increased risk for ASDs. Altogether, results from these various studies strengthen the evidence that the COX-2/PGE_2 signalling pathway is an autism candidate pathway.

8.6. Conclusions

In conclusion, our study found that prenatally PGE_2-exposed mice exhibit abnormal sociability, repetitive behaviour, anxiety, and hyperactivity, as well as aberrant expression of ASD-linked genes in a sex- and age-dependent manner. We provide novel evidence that

disrupted COX-2/PGE_2 signalling, which could occur through different environmental or genetic causes, may influence the development of ASD-like behaviours. Moreover, our study highlights that the maternal environment or prenatal exposure to risk factors that can affect the level of PGE_2 during critical stages of development may result in a spectrum of ASD symptomatology. Our study also shows that it is imperative that molecular and behavioural research be conducted in both males and females at various developmental ages, which is currently underrepresented in the literature.

8.7. Figures

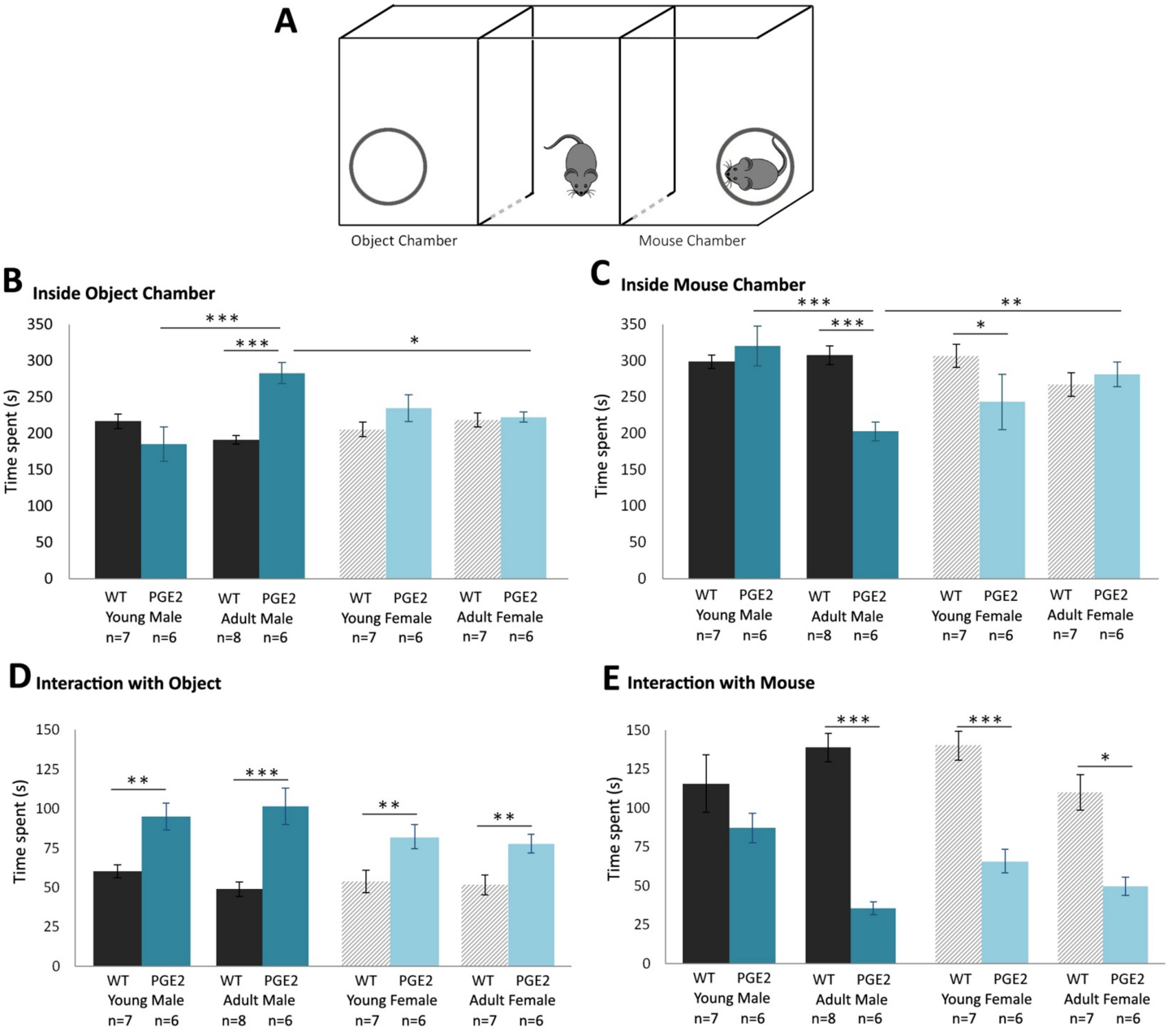

Fig. 8-1: Sociability behaviour was determined in PGE_2-exposed mice using the three-chamber test. (Figure description on following page).

Fig. 8-1: Sociability behaviour was determined in PGE_2-exposed mice using the three-chamber test. (A) Video recordings were taken and the time spent in the chambers or interacting with the novel object or novel mouse was measured. (B) Adult PGE_2 -exposed males spent significantly more time in the object chamber. (C) Young PGE_2-exposed males and females spent significantly less time in the novel mouse chamber than controls. Sex differences were only observed between adult PGE_2-exposed mice. Age-dependent differences were only seen in PGE_2-exposed males. (D) PGE_2-exposed animals spent more time interacting with the object than WT controls. (E) PGE_2-exposed mice spent less time interacting with the mouse compared to WT controls. n represents the number of animals tested in each experimental group, originating from at least 3 different litters. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

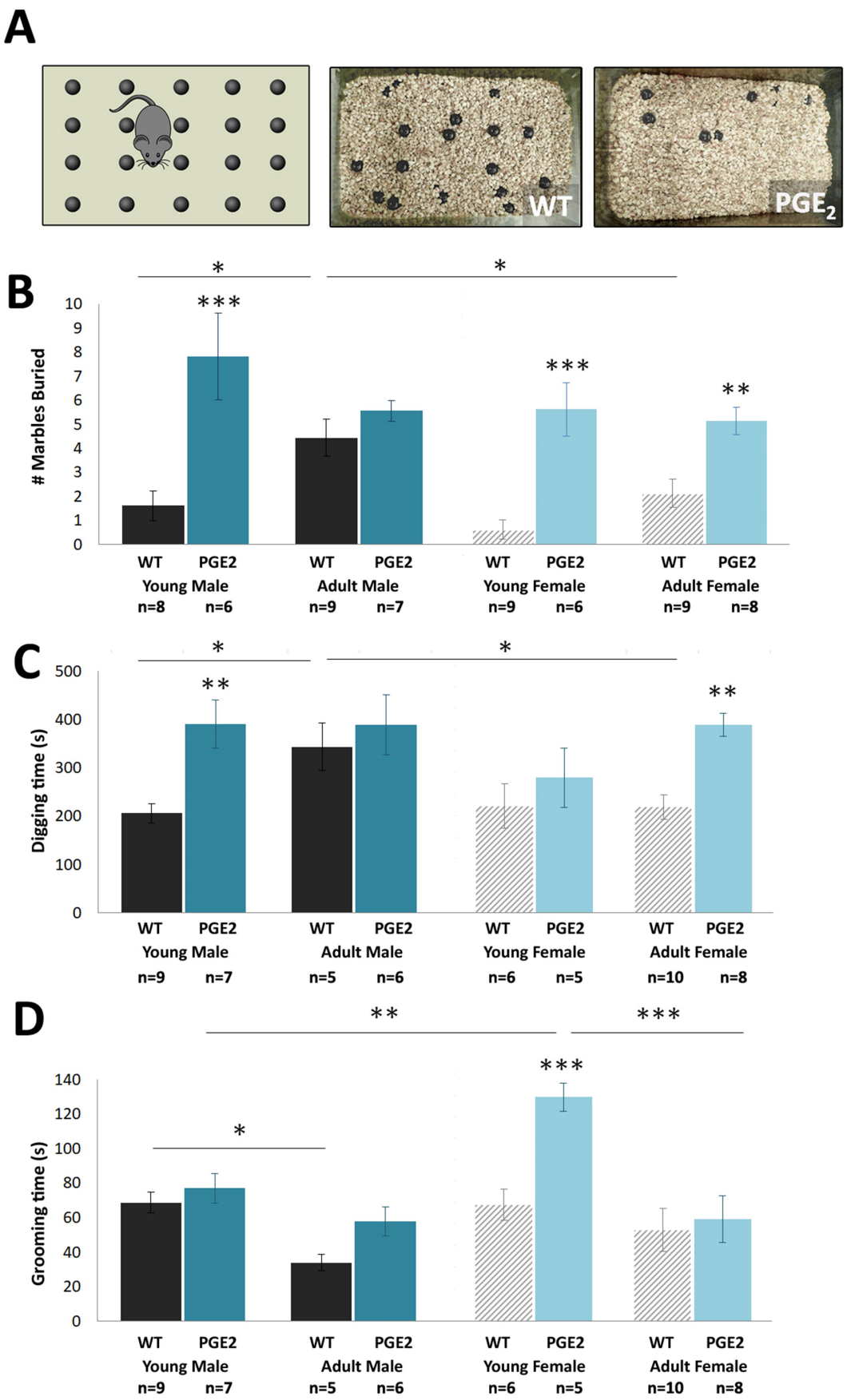

Fig. 8-2: Repetitive and anxiety-linked behaviour in PGE_2-exposed mice in the marble burying test. (Figure description on following page).

Fig. 8-2: Repetitive and anxiety-linked behaviour in PGE_2-exposed mice in the marble burying test. (A) Video recordings were taken and total marbles buried were counted. (B) PGE_2-exposed mice buried more marbles than WT controls. (C) Young PGE_2-exposed males and adult PGE_2-exposed females spent more time digging than controls. Sex differences were seen in adult WT mice. Age differences were seen in WT males. (D) Young PGE_2-exposed females spent the most time growing. Sex differences were seen in young PGE_2-exposed mice. Age differences were seen in WT males and PGE_2-exposed females. n represents the number of animals tested in each experimental group, originating from at least 3 different litters. Data are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

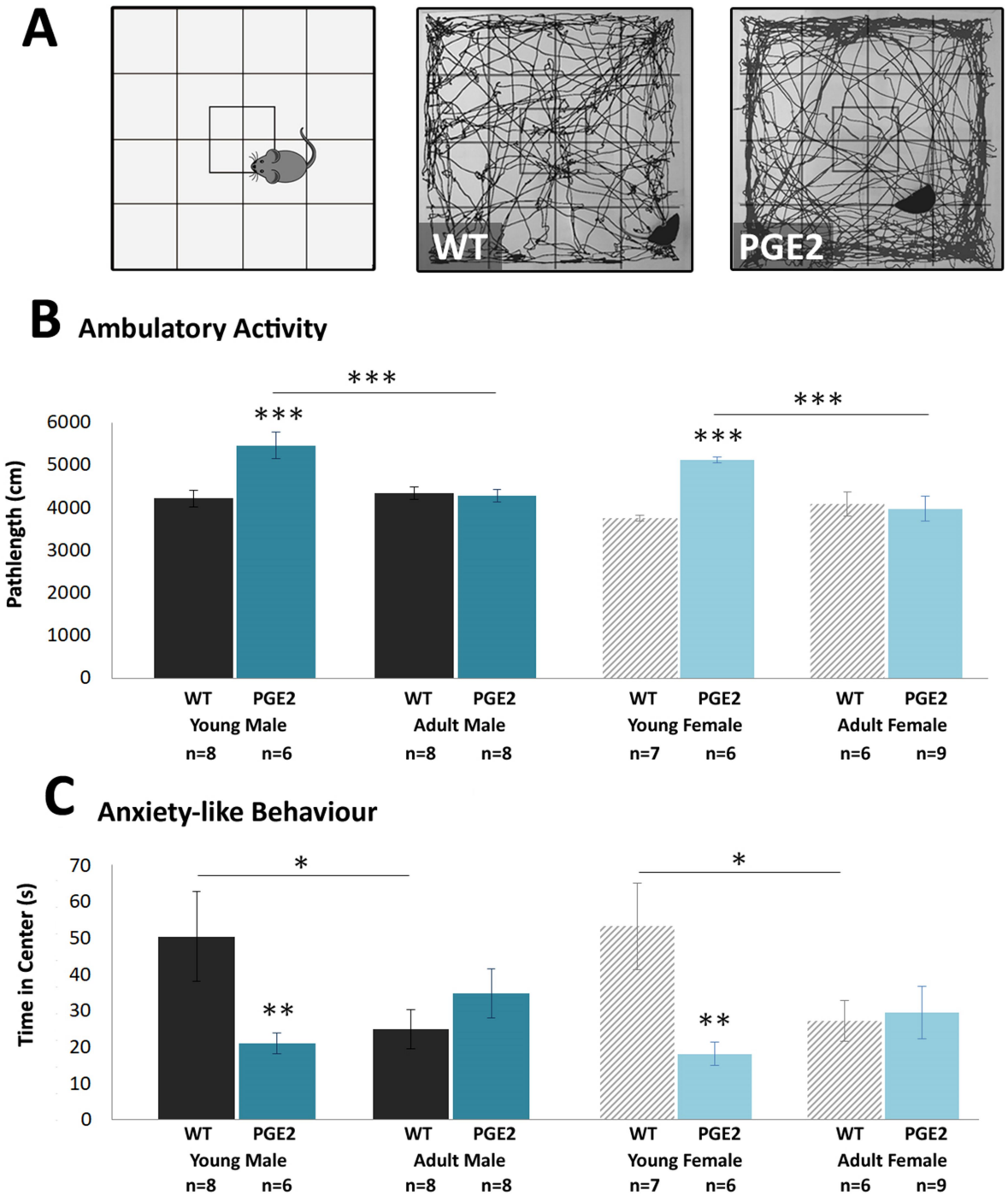

Fig. 8-3: Ambulatory activity and anxiety-linked behaviour in PGE_2-exposed mice in the open field test. (A) An automated tracking program determined the pathlength travelled. (B) Pathlength results showed that young PGE_2-exposed mice travelled more than WT controls in male and female groups. Age-dependent differences were only seen in PGE_2-exposed mice. (C) Measurements for anxiety-like behaviour revealed that young PGE_2-exposed mice spent less time in center than controls. Age-dependent differences in anxiety-related behaviour were found only in WT groups. n represents the number of animals tested in each experimental group.Data presented as mean±SEM, *p<0.05, **p<0.01, ***p<0.001.

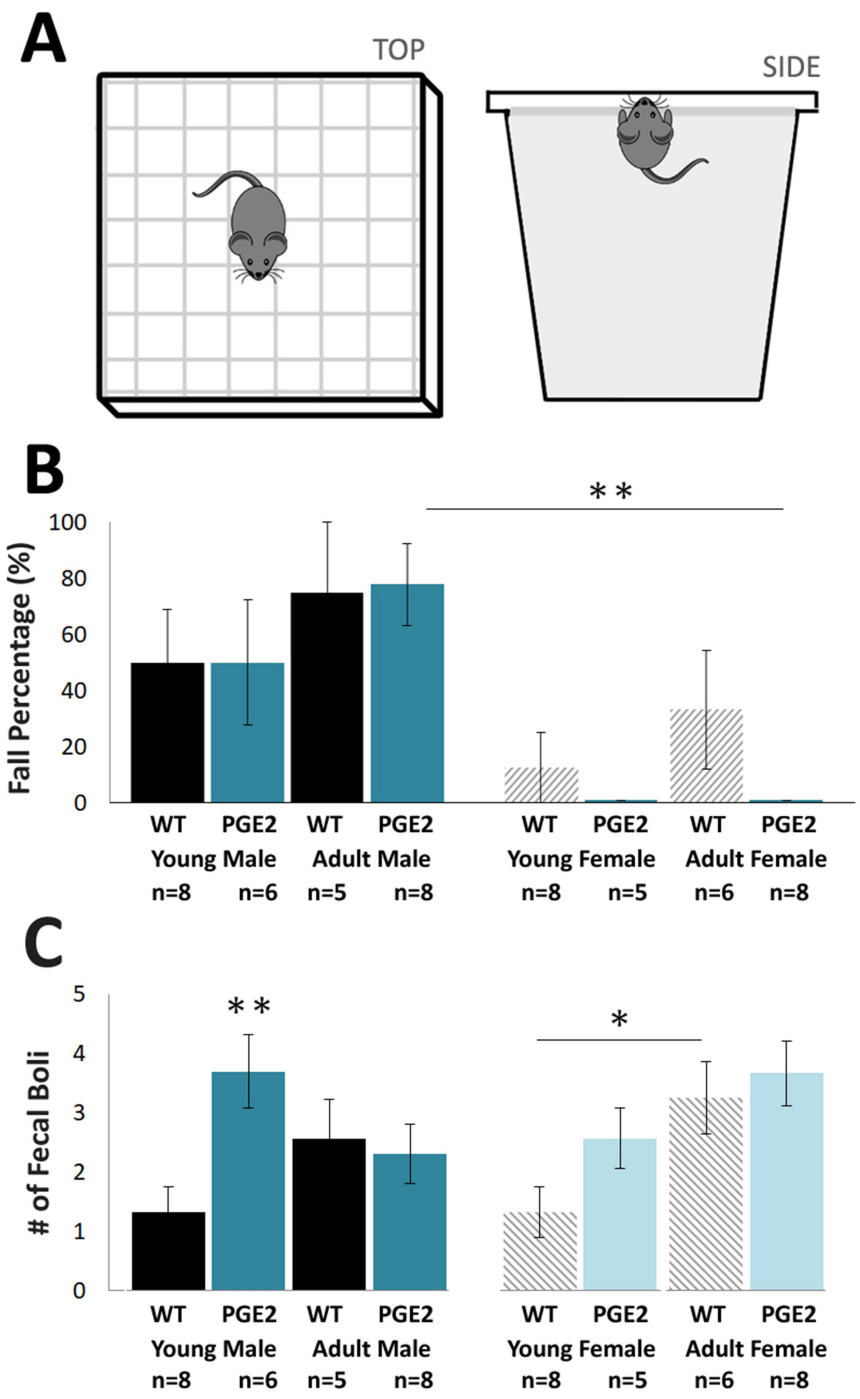

Fig. 8-4: Motor ability and anxiety-like behaviour was measured in PGE_2-exposed mice using the inverted screen test. (A) For the inverted screen test, whether or not the mouse subjects fell was recorded. (B) There was no significant difference in fall percentage between PGE_2-exposed mice and WT controls. Sex differences were seen between Adult PGE_2-exposed mice; males fell more frequently. (C) Young PGE_2-exposed males and females displayed greater anxiety-like behaviour by producing increased number of fecal boli. n represents the number of animals tested in each experimental group, originating from at least 3 different litters. Data are presented as mean ±SEM, *p<0.05, **p<0.01.

Table 8-2 : Expression of autism-linked genes in PGE_2 -exposed mice.

Gene expression analysis on autism-linked genes were completed on postnatal day 8 samples as shown as RQ units for male and female PGE_2 groups relative to respective controls (RQ=1). Values represent the mean of individuals from at least 3 independent litters. *p<0.05, **p<0.01, ***p<0.001

PGE_2 -exposed compared to WT controls : Autism-Linked Genes		
Males	**RQ Mean Values**	***p*-values**
Wnt2	**0.8859**	**0.02441***
Tcf4	**0.5781**	**0.00862****
Glo1	0.8618	0.15702
Grm5	0.8575	0.20863
Females	**RQ Mean Values**	***p*-values**
Wnt2	1.1072	0.33298
Tcf4	1.3067	0.17462
Glo1	**0.6259**	**0.00079*****
Grm5	1.2689	0.17402

Table 8-3. Summary of behavioural findings for PGE_2 -exposed mice.

Results represent the overall significant behavioural comparisons of PGE_2 -exposed mice to respective WT controls, where ↑ = Increased; ↓ = Decreased; - = not significant. Each finding is a representation of individuals from at least 3 independent litters.

PGE_2 -exposed mice compared to WT controls: Behavioural Results				
Behaviour	**PGE_2 Male**		**PGE_2 Female**	
	Young	**Adult**	**Young**	**Adult**
3 Chamber Test *Social-related behaviour* *(time spent in novel object chamber)*	-	↑	-	-
3 Chamber Test *Social-related behaviour* *(time spent in novel mouse chamber)*	-	↓	-	-
3 Chamber Test *Social-related behaviour* *(interaction with novel object)*	↑	↑	↑	↑
3 Chamber Test *Social-related behaviour* *(interaction with novel mouse)*	-	↓	↓	↓
Marble Burying Test *Anxiety-like & Repetitive Behaviour* *(marbles buried)*	↑	-	↑	↑
Marble Burying Test *Anxiety-like & Repetitive Behaviour* *(digging time)*	↑	-	-	↑
Marble Burying Test *Anxiety-like Behaviour* *(grooming time)*	-	-	↑	-
Open Field Test *Hyperactivity* *(pathlength)*	↑	-	↑	-
Open Field Test *Anxiety-like Behaviour* *(time in center)*	↓	-	↓	-
Inverted Screen Test *Motor Deficits* *(fall percentage)*	-	-	-	-
Inverted Screen Test *Stress-like Behaviour* *(defecation)*	↑	-	↑	-

8.8. References

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CHAPTER 9.

GENERAL DISCUSSION

9.1. Dissertation Objectives Revisited

Although clinical and epidemiological evidence have revealed a correlation between disrupted COX-2/PGE_2 lipid signalling and risk for the development of ASDs, literature surrounding the molecular mechanisms of this connection is sparse. Therefore, the global aim of this dissertation was: *To discover the molecular mechanisms by which abnormal signalling of lipid mediators, specifically prostaglandin E2 (PGE_2), may affect brain development and contribute to ASDs-related pathologies. Another important objective was to describe sex differences in mouse studies, which are often overlooked although critical for understanding disorders such as ASDs.* I hypothesized that abnormal COX-2/PGE_2 lipid signalling can disrupt healthy brain development and lead to molecular characteristics and behaviours associated with ASDs in a sex-dependent manner.

From our research studies, we first found in our cell model that PGE_2 treatment could affect important brain developmental processes including cell movement, proliferation and differentiation in neuroectodermal (NE-4C) stem cells, while also interfering with the Wnt signalling pathway by disrupting Wnt-target gene expression. Our investigations *in vivo* then determined that an increase or decrease of PGE_2 during development could also lead to changes in cell density, cell migration, microglial density and morphology, as well as expression of autism-linked genes and the manifestation of autism-like behaviours.

Sex-dependent differences are typically underrepresented in research. We observed sex-differences in all *in vivo* studies. Irregular COX-2/PGE_2 signalling via COX-2-deficiency or an increased level of PGE_2 led to sex-specific changes in cell density, neocortical cell migration,

microglial density, microglial morphology, expression of various developmental and autism-linked genes, and manifestation of autism-related behaviours.

Based on the results of the six presented research studies, we report that disturbances to normal COX-2/PGE_2 signalling could be detrimental to the developing brain due to the molecular, cellular, and behavioural conseuqences mentioned above. Our studies are the first to show that these changes are manifested differently in males and females at various developmental ages, which are consistent with clinical findings. We propose that the COX-2/PGE_2 signalling pathway may influence the pathogenesis of ASDs and is an autism-candidate pathway. Furthermore, PGE_2-injected and COX-2^- KI animals may serve as two new experimental model systems for studying specific types of ASDs.

9.1.1 Key Findings of Specific Objectives

In this section, the specific objectives outlined in this dissertation and respective results will be summarized. The implications of these major findings emerging from this collection of studies are discussed in further detail in the next section: "9.2. Discussion and Future Directions".

Objective 1) to study the effects of PGE_2 exposure on the proliferation and migration of early neuroectodermal (NE-4C) stem cells.

We showed that an elevated level of PGE_2 can influence the function of NE-4C cells by promoting cell proliferatory behaviour (Study 1 and 2) and increasing their final distance, pathlength, and average speed (Study 1).

Objective 2) to determine if an elevated level of PGE_2 influences the differentiation of NE-4C stem cells.

In Study 2, we found that increased levels of PGE_2 could increase the proliferation of NE-4C stem cells in a dose-dependent manner and could also promote their differentiation. Specifically, PGE_2 accelerated the progression of NE-4C cells into neurons through earlier expression of a neurosphere adhesion molecule (*Cdh2*) and modification of neurosphere characteristics (area, perimeter, roundness). Furthermore, PGE_2 increased the protein expression of β-catenin (signal transducer of Wnt canonical pathway) and altered the expression of Wnt-target genes *Wnt3*, *Tcf4*, and *Ccnd1* during differentiation.

Objective 3) to investigate the possible interaction between the PGE_2 pathway and a major developmental pathway of the nervous system called the Wnt signalling pathway.

In Study 1 and 2, we demonstrated for the first time that cross-talk exists between the PGE_2 and Wnt/β-catenin canonical signalling pathways, which was mediated through kinases, protein kinase A (PKA) and phosphatidylinositide 3-kinase (PI-3K). Furthermore, we also determined that in response to PGE_2, the levels of β-catenin as well as the expression of downstream Wnt-target genes previously associated with ASDs can be altered.

Objective 4) to verify our in vitro findings by studying the consequences of elevated maternal levels of PGE_2 on cell proliferation and neuronal migration in the developing brain of offspring mice.

In Study 3, a single subcutaneous maternal injection of PGE_2 during development at embryonic day 11 (E11) resulted in long-term changes at postnatal day 8 (P8); the densities of

cells originating from E11 and E16 were altered (increased in olfactory bulb and decreased in cerebellum and neocortex) and E11 and E16 cells displayed further neocortical migration. PGE_2 exposure also led to changes in the expression of cell growth and motility genes (*Spn*, *Actb*).

Objective 5) to describe if altered COX-2/PGE_2 signalling can disrupt microglial density and morphology.

In Study 4, prenatal PGE_2 exposure or COX-2-deficiency changed the density and morphology of microglia in P8 offspring. Both conditions led to higher microglial densities in the cerebellum, olfactory bulb, thalamus (PGE_2-exposed mice), and prefrontal cortex (COX-2-deficient mice). PGE_2-exposed mice had a greater percentage of amoeboid microglia and fewer ramified microglia, which was further demonstrated by decreased branching and process lengths. In contrast, COX-2-deficient mice displayed a lower percentage of amoeboid microglial cells and more ramified microglia. Greater branching and process lengths were also found in COX-2-deficient mice.

Objective 6) to examine the behavioural outcomes of COX-2-deficient and PGE_2-exposed mice.

In Study 5 and 6, autism-related behaviours were observed in both COX-2-deficient and PGE_2-exposed mice, respectively. COX-2-deficient mice displayed elevated hyperactive, repetitive, and anxiety-linked behaviours, motor deficits, and social abnormalities. PGE_2-exposed mice displayed distinct social abnormalities as well as increased hyperactive, repetitive, and anxiety-linked behaviours. At P8, COX-2-deficient mice displayed changes in the expression of ASD-associated genes: *Wnt2*, *Glo1*, *Grm5*, *Mmp9*. PGE_2-exposed mice at P8 also showed altered expression of ASD-associated genes: *Wnt2*, *Tcf4*, *Glo1*.

9.2. Discussion and Future Directions

Key topics emerging from our results that span across multiple *in vitro* and *in vivo* studies will be discussed further: the effect of PGE_2 on neurogenesis, the convergence of the PGE_2 and Wnt signalling pathways, the sex-dependent effects of COX-2/PGE_2, implications for increased or decreased levels of PGE_2, and their contributions to literature on Autism.

9.2.1. *The effect of PGE_2 on Neurogenesis*

Neurogenesis involves the stages of proliferation, migration, differentiation, and functional integration (Lazarov and Demars, 2012). In study 1 through 3, we show that PGE_2 can affect the proliferation, migration, and differentiation of neural cells.

Our investigations into the effect of PGE_2 on proliferation and cell density demonstrated that *in vitro* PGE_2 can promote the proliferation behaviour (increased percentage of cells splitting, Study 1, Fig. 3-4) and increase the total number of NE-4C stem cells (Study 2, Fig. 4-1). Interestingly, Study 3 showed that *in vivo* prenatal exposure to PGE_2 at E11 led to i) a decrease in the density of E11-born and E16-born cells at P8 in the cerebellum of both sexes, ii) a decrease in E16-born cells in the neocortex of females, and iii) an increase in E11-born and E16-born cells in the olfactory bulb of males. E11-born and E16-born cells likely represent neuronal cells since neurons are largely generated from E10 to E18 in rodents, while astrocytes and oligodendrocytes appear at later time points around E18 and postnatally, respectively (Reemst et al., 2016, Miller and Gauthier, 2007, Bayer and Altman, 1991). Although our *in vitro* studies revealed an increase in neuronal proliferation in response to PGE_2 treatment, our findings *in vivo* portrayed a more complex picture demonstrating that an elevated level of PGE_2 during prenatal development results in sex-dependent, region-specific, and time-sensitive changes. This

may be explained by the fact that neurogenetic processes are governed by both intrinsic and extrinsic factors (Calof, 1995). Extracellular signals that influence the microenvironmental conditions inside the brain (Navarro Quiroz et al., 2018), which are present *in vivo* (but are absent *in vitro*), are likely the cause for the distinct differences in how PGE_2 affects cell density in a region- and sex-dependent manner. We show that the COX-2/PGE_2 signalling pathway converges with the Wnt signalling pathway to regulate proliferation in NE-4C stem cells *in vitro*. Future studies are needed to further explore the potential cross-talk that happens between these two pathways that regulate cell proliferation *in vivo*. Additionally, subsequent investigations into whether a connection exists between the COX-2/PGE_2 pathway and other important cell growth and proliferation pathways such as the Notch and BMP signalling pathways (Jovanovic et al., 2018, Navarro Quiroz et al., 2018, Zhou et al., 2010) will help uncover the extent in which PGE_2 may impact key developmental processes.

An elevated level of PGE_2 can also alter the migration of cells *in vitro* and *in vivo,* and promote the differentiation of cells *in vitro*. Our *in vitro* study determined that an elevation in PGE_2 level can increase the final distance, pathlength, and speed of NE-4C stem cells (Study 1, Fig. 3-3, 3-4). Similarly, we also found that prenatal exposure to PGE_2 can also result in greater neocortical migration of cells originating from E11 and E16 (Study 3, Fig. 5-5). Alterations in the position of each neuron in the brain may have more significant consequences compared to other cells in the body since the functions of neurons rely on the precise connections made between neurons and target cells (Purves et al., 2011). The final location of a neuron will influence its differentiation and development, including the orderly organization of neural circuits that are responsible for processing information (Yuste, 2015). Furthermore, we showed that increased PGE_2 can also accelerate the differentiation of NE-4C stem cells into neurons

(Study 2, Fig. 4-2). Irregular migration or differentiation of neurons, resulting from an increase in PGE_2 level, could lead to disrupted connectivity of neural networks including those underlying complex social and emotional information processing (Zikopoulos and Barbas, 2013, Wegiel et al., 2010).

Overall, Study 1 through 3 demonstrated that PGE_2 can alter various stages of neurogenesis, including proliferation, migration, and differentiation. How PGE_2 affects the function of neurons, such as its influence on the formation of axons, synapses, and cell circuitry will need to be addressed in future experiments.

9.2.2. *Convergence of PGE_2 and Wnt Signalling Pathways*

Wnt molecules are morphogenic signals that are essential to the proper development of the nervous system (Wang et al., 2012). Anterior-posterior patterning, cell proliferation, neuronal maturation, and synaptogenesis are examples of some of the many processes regulated by Wnt signalling (Rosso and Inestrosa, 2013, Hirabayashi et al., 2004). PGE_2 signalling is also involved with brain development and maturation, including neurite outgrowth, synaptic plasticity, and synaptic transmission (Nango et al., 2017, Dean et al., 2012, Alix et al., 2008, Sang et al., 2005). Previous research identified that cross-talk between the PGE_2 and Wnt signalling pathways in diverse cell types including hematopoietic stem cells, gastric progenitor cells, and embryonic kidney cells (Goessling et al., 2009, Oshima et al., 2006, Fujino et al., 2002). However, the possible interaction between these two developmental pathways had not been investigated in neural cell types and the brain.

Our studies 1, 2, 5, and 6 demonstrated that altered levels of PGE_2 can influence Wnt signalling and its expression of gene targets. Our *in vitro* studies revealed that PGE_2 can alter

Wnt-induced proliferation and movement behaviour of NE-4C stem cells by increasing β-catenin protein expression through kinases (PKA and PI3K) downstream in the PGE_2 pathway (Study 1, Fig. 3-1, 3-2, 3-3). An elevated level of PGE_2 also increased the expression of Wnt target genes *Ctnnb1*, *Ccnd1*, *Mmp9*, *Wnt3*, and *Tcf4* but decreased expression of *Ptgs2* (Study 2, Fig. 4-4, 4-5). Interestingly *Ptgs2* encodes for the COX-2 enzyme, which is responsible for the production of PGE_2. Thus, we reported for the first time that cross-talk occurs between the PGE_2 and Wnt pathways in neural stem cells. Our results indicated that PGE_2 may promote the expression of Wnt-target genes, although feedback regulation likely also exists between these two pathways. This is in line with previous studies, which have predominately reported that PGE_2 activates the Wnt signalling pathway in various tissues (Nam et al., 2018, Goessling et al., 2009, Castellone et al., 2005), while a negative feedback loop between PGE_2 and the Wnt pathway has also been described (Gonzalez et al., 2010). Our investigations *in vivo* showed that prenatal exposure to PGE_2 led to a decrease in postnatal *Wnt2* and *Tcf4* expression (Table 8-2), while COX-2-deficiency resulted in an increase in *Wnt2* expression (Table 7-3). Our *in vivo* findings support a negative feedback loop whereby an increase in the level of PGE_2 may contribute to a future decrease in Wnt target gene expression. Similarly, this feedback regulation between the PGE_2 and Wnt pathways may be the reason that a decrease in PGE_2 level (via COX-2-deficiency) led to an increase in *Wnt2* expression at a later time point.

What remains to be determined are the mechanisms by which cross-talk occurs between the PGE_2 and Wnt pathways *in vivo*—perhaps through PKA, PI-3K, and/or β-catenin as seen *in vitro*. Furthermore, our studies focused on the canonical Wnt pathway since it is the most well-established of the Wnt pathways and because of its involvement in the control of gene expression and cell behaviour. Importantly, future investigations need to be made into the effect of PGE_2 on

non-canonical Wnt pathways, such as the Wnt/Ca2+ and Planar Cell Polarity (PCP) pathways. These pathways can act independently of β-catenin and also regulate key neurodevelopmental processes such as cell migration and cell polarization (McQuate et al., 2017, Gomez-Orte et al., 2013, De, 2011, Sugimura and Li, 2010, Komiya and Habas, 2008). A connection between PGE_2 and non-canonical Wnt signalling has been reported in osteoblasts and chondrocytes (Doroudi et al., 2014) but have yet to be examined in brain tissue cells.

9.2.3. *The Sex-dependent effects of COX-2/PGE_2*

Human diseases and disorders, including ASDs, can affect males and females differently. The sex of an individual can influence their susceptibility, development, and progression of a disorder (Pollitzer, 2013, Wald and Wu, 2010). Therefore, it was important for us to examine the sex-specific outcomes of abnormalities to the COX-2/PGE_2 signalling pathway. We identified various differences between males and females in our in vivo studies (Study 3, 4, 5, and 6): deviations to normal COX-2/PGE_2 levels (an increase or decrease) can lead to sex-dependent changes in cell density, neocortical cell migration, microglial density and morphology, and autism-related behaviours.

The sexually dimorphic physiology and behaviour observed in our studies perhaps could be explained by the interaction between sex hormones and PGE_2. The PGE_2-driven sex differences in the brain and in behaviours likely involved the molecular mechanisms behind the potent estrogen called estradiol. Estradiol is made from the androgen, testosterone, through the enzymatic activity of aromatase (Wright et al., 2010). In the rodent brain, estradiol has been shown to play a key role in physiological and behavioural masculinization (Wright et al., 2010, Wu et al., 2009). A burst of testosterone occurs in the male testes during early development, and

it is the subsequent conversion of testosterone, which enters the brain, into estradiol that drives the formation of male-specific neural circuitry (Wu et al., 2009). Perinatal up-regulation of COX-2 and consequent synthesis of PGE_2 can be induced by estradiol to mediate various functions including formation of dendritic spines, masculinization of microglial number and morphology, and development of male sex behaviour (Lenz et al., 2013, Wright et al., 2010, Amateau and McCarthy, 2004). Conversely, an increase in PGE_2 has also been found to cause an elevation of testosterone (Wade and Van der Kraak, 1993), while aromatase activity and estradiol level can also be regulated by PGE_2 (Pedersen and Saldanha, 2017, Subbaramaiah et al., 2011, Richards and Brueggemeier, 2003). Moreover, a positive feedback loop between estradiol and PGE_2 has been described in endometrial tissue (Waclawik et al., 2009). Interestingly, PGE_2 has been found to induce an increase in estradiol content following brain injury through a sex-dependent mechanism: PGE_2 acts through EP3 receptors in males, while PGE_2 binds to EP4 receptors in females (Pedersen and Saldanha, 2017). A similar sex-dependent mechanism between specific PGE_2 EP receptors and estradiol signalling may also exist during brain development and requires future attention.

Taken altogether, aberrations in the COX-2/PGE_2 pathway during early development, resulting in an increase or decrease in PGE_2 levels, could disrupt the careful coordination between estradiol and PGE_2 that orchestrates sex-specific development of the brain. Our research highlights the importance of analyzing males and females separately in order to uncover sex-specific differences, which may provide better insight into the neural underpinnings of the molecular mechanisms responsible for the effects of abnormal COX-2/PGE_2 signalling. Studying sex differences in mice models may provide clues into the origins of human brain disorders that

disproportionately affect one sex over the other, including ASDs which appears in more males than females (Baio et al., 2018).

9.2.4. *Increased and Decreased levels of PGE_2*

We studied the effect of increased and decreased levels of PGE_2 on autism-associated behaviours (Study 5 and 6) and on microglia (Study 4) and determined that either condition of excess or deficient levels of PGE_2 could disrupt normal neurodevelopmental outcomes. We found that mice prenatally exposed to PGE_2 through a single maternal injection displayed abnormal behaviours that resembled those observed in COX-2-deficient mice (Study 5 and 6). PGE_2-exposure and COX-2-deficiency led to social defects and an increase in repetitive, hyperactive, and anxious behaviours. Together, these two studies suggest that either an elevation or decrease in the level of PGE2 may contribute to behavioural pathologies related to ASDs. Our findings correspond with other research in mice and humans revealing that either an increase or decrease of PGE_2 during development could contribute to the manifestation of ASDs and their behavioural outcomes (Masarwa et al., 2018, Parker et al., 2017, Cortelazzo et al., 2016, Hoffman et al., 2016, Madore et al., 2016, Holzman, 2014, Shelton et al., 2014, Dean et al., 2012). This has been discussed in greater detail in Chapter 8. Interestingly, in our microglial study (Study 4), we found that microglial density was generally increased in both PGE_2-exposed and COX-2-deficient mice compared to respective controls. However, PGE_2-exposure and COX-2-deficiency led to contrasting results on microglial morphology: overall, PGE_2-exposed mice had a greater percentage of morphologically "active" amoeboid microglia, while COX-2-deficient mice had a greater percentage of morphologically "resting" ramified microglia from the norm. This indicates that the COX-2/PGE_2 signalling pathway can regulate divergent functions of microglia.

Considering our collective findings from our studies on microglia and autism-associated behaviours, a careful balance of COX-2/PGE_2 molecules is necessary for normal development. Deviations from the typical concentrations of COX-2/PGE_2 during sensitive periods of prenatal development can drive different cellular mechanisms that could contribute to similar autism-related behavioural consequences.

The versatile responses of increased or decreased levels of PGE_2 may be explained by investigating the four EP receptor subtypes that PGE_2 binds to, including EP1 through EP4. Each EP receptor activates a unique signal transduction pathway, which can yield activation of distinct second messenger systems including cAMP, Ca^{2+}, and inositol phosphate, and subsequent downstream kinases (Sugimoto and Narumiya, 2007). For example, we previously determined in Neuro-2A cells that PGE_2 can promote an elevation of intracellular calcium through EP2 receptor and downstream kinase, PKA, but PGE_2 can also be involved with the inhibition of intracellular calcium through EP4 receptor and PI-3K (Tamiji and Crawford, 2010). Furthermore, PGE_2 can induce differential desensitization and internalization of its EP receptors (Dey et al., 2006, Bilson et al., 2004, Desai et al., 2000, Nishigaki et al., 1996). This suggests that deviations to the level of PGE_2 could affect the EP receptors in which they interact with and their resulting functions. Future studies utilizing agonists and antagonists of specific EP receptors will elucidate the mechanisms for the diverse responses of PGE_2.

9.2.5. *Contribution to literature on ASDs*

Autism was historically believed to originate during life after birth, with the previous thought that the primary cause was poor parental nurturing (Evans, 2013, Kanner, 1954, Kanner, 1943). This drove autism research to adopt a postnatal-focused research approach for decades

(Cohmer, 2014). Although the debate into the etiology of autism has existed for some time (Stein, 1966), it was only recently that there has been true acknowledgement for the need of a greater understanding of the prenatal pathogenic processes that result in the highly heterogeneous phenotypes of ASDs (Courchesne et al., 2019). Evidence for the prenatal origins of ASDs has come from clinical research, which determined that of the 72 high-confidence genes identified as being recurrent and most penetrant in individuals with ASD, 94% of them are highly active during the prenatal stages of development (Courchesne et al., 2019, Kosmicki et al., 2017). These genes regulate cell proliferation, differentiation, migration and early organization, and may also impact synaptogenesis and early learning experiences that occur postnatally (Courchesne et al., 2019, Kosmicki et al., 2017). Moreover, a new retrospective study in humans utilizing fetal ultrasound parameters has confirmed that the pathogenesis of autism begins prenatally (Bonnet-Brilhault et al., 2018).

The series of studies presented in this dissertation was aimed at addressing this gap in knowledge and provide possible mechanisms by which autism pathogenesis may occur. Due to the clinical heterogeneity of ASDs, we believed it was important to approach their complex etiology from different angles and thus we investigated the influence COX-2/PGE_2 signalling may have from the molecular, cellular, and behavioural perspective. Our behavioural studies provided novel proof that the COX-2/PGE_2 signalling pathway can influence autism-associated behavioural outcomes, including social abnormalities, repetitive behaviours, hyperactivity, and anxiety (Study 5 and 6). Our other studies also showed that irregular levels of PGE_2 affect cellular processes involved with proliferation, migration, and microglial density and morphology (Study 1-4). This suggests that improper development of these particular biological processes may contribute to the autism-related behaviours displayed in our studies. Changes in cell density

and migration can have direct consequences on the structure, organization, and connectivity of the brain; abnormalities in these areas have been reported in autism patients (Stoner et al., 2014, Abrams et al., 2013, Ecker et al., 2013, Wegiel et al., 2010, Schumann and Amaral, 2006, Redcay and Courchesne, 2005, Belmonte et al., 2004, Herbert et al., 2003). Moreover, a number of autism-associated genes were affected in our model systems, providing further confirmation that the level of PGE_2 is likely involved in autism pathogenesis.

Our research in mice offers new information on the developmental roles of COX-2/PGE_2 signalling that could not otherwise be investigated in humans. Our findings provide insight into the potential mechanisms contributing to the pathologies of ASDs seen in human cases. Altogether, all our studies provide new evidence that COX-2/PGE_2 signalling can significantly impact neurodevelopment and support a relatively new hypothesis that the beginnings of ASDs occur prenatally (Courchesne et al., 2019, Donovan and Basson, 2017, Kaushik and Zarbalis, 2016).

9.3. Conclusions

This dissertation encompassed a compilation of molecular, cellular, and behavioural work that aimed to expand our understanding of how the endogenous lipid signalling pathway, namely the COX-2/PGE_2 pathway, may affect the developing brain.

Our *in vitro* studies provided the first evidence that PGE_2 can interfere with the signalling and function of key developmental morphogen, Wnt, by disrupting proliferation and differentiation while also causing irregular expression of Wnt-target autism-candidate genes. Our findings gave us confidence to pursue our investigations at the molecular, cellular, and behavioural level *in vivo*. The presented collection of *in vivo* studies determined that perturbations to this pathway, whether through increased PGE_2 exposure or decreased PGE_2

levels through a genetic defect, led to differences in cortical neuronal migration, cell density, microglial density and morphology, and autism-related behavioural outcomes. Moreover, our distinct sex-dependent findings indicate the etiological importance of studying both males and females, and also support the need for a personalized approach when treating ASDs. Althogether, our findings provide convincing evidence that the COX-2/PGE_2 signalling pathway is an autism candidate pathway that deserves further investigation. We propose that the PGE_2-injected and COX-2^- KI mice used in these studies could be considered as new experimental model systems for studying ASDs-related pathologies.

In closing, the COX-2/PGE_2 signalling pathway is important for crucial neurodevelopmental processes. Exposure to environmental risk factors that can disrupt the levels of PGE_2—such as immunological agents, air pollution, heavy metals, prolonged use of drugs like NSAIDs and acetaminophen, and particular consumer products—may be detrimental for the developing brain. Precautions taken to minimize exposure to these environmental risk factors during prenatal development are warranted.

9.4. References

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APPENDIX A

PUBLICATION LIST AND CONFERENCE PRESENTATIONS (DOCTORAL WORK)

Invited Review:

Wong C.*, Wais J., and Crawford DA. (2015). Prenatal exposure to common environmental factors affects brain lipids and increases risk of developing Autism Spectrum Disorders. *European Journal of Neuroscience.* 42(10):2742-2760 doi: 10.1111/ejn.13028.

Invited Book Chapter:

Wong C.* and Crawford DA. (2014). Lipid signalling in the Pathology of Autism. In: Patel V., Martin C., Preedy V. (Eds.) Comprehensive Guide to Autism: Springer Reference. Springer-Verlag Berlin Heidelberg.

Original Peer-reviewed Articles:

Wong, C.*, Bestard-Lorigados, I, Crawford, DA. (2019). Autism-related behaviors in the cyclooxygenase-2-deficient mouse model. *Genes, Brain and Behavior*. 2019; 18:e12506.

Bhogal-Rai B., **Wong. C.*,** Davidson J., Li H., Crawford D.A. (2018). Maternal exposure to prostaglandin E_2 affects expression of wnt-target genes in offspring – an autism connection. *Biochemistry and Biophysics Reports*, 14: 43–53. doi: 10.1016/j.bbrep.2018.03.012

Wong. C.*, Ussyshikin N., Ahmad E., Bhogal-Rai B., Li H., and Crawford DA. (2016). Prostaglandin E2 promotes neural proliferation and differentiation and regulates Wnt target gene expression., *Journal of Neuroscience Research.* 94(8):759-75. doi: 10.1002/jnr.23759

Davidson, J., **Wong C.*,** Li, H., Crawford, D.A. (2016)**.** Prostaglandin E2 facilitates subcellular translocation of the EP4 receptor in neuroectodermal NE-4C stem cells. *Biochemistry and Biophysics Reports*, 7:173-179. doi:10.1016/j.bbrep.2016.06.001

Davidson, J., **Wong C.*,** Rai-Bhogal, R., Li, H., Crawford, D.A. (2016). Prostaglandin E2 elevates calcium in differentiated neuroectodermal stem cells. *Molecular and Cellular Neuroscienc*e, 74:71-77. doi: 10.1016/j.mcn.2016.03.010.

Wong C.*, Ahmad E., Li H., and Crawford DA. (2014). Prostaglandin E2 alters Wnt-dependent migration and proliferation in neuroectodermal stem cells: implications for autism spectrum disorders. *Cell Communication and Signaling*, 12:19.

Articles submitted or in preparation:

Wong. C.*, Bestard Lorigados I., and Crawford DA. (2019). Maternal exposure to prostaglandin E2 leads to autism-like behaviours in C57/bl6 mouse offspring. (*submitted*)

Wong. C.*, Smith, J., and Crawford DA. (2019). Augmented microglial density and abnormal morphology in mice with defective cyclooxygenase-2/prostaglandin E2 signalling. (*In preparation*)

Wong. C.*, Bestard Lorigados I., and Crawford DA. (2019). Maternal exposure to prostaglandin E2 results in irregular cortical migration and cell densities in brains of mouse offspring. (*In preparation*)

Conference Presentations:

Wong, C.*, Bestard-Lorigados, I. and Crawford, D. A. (2019). Prenatal exposure to prostaglandin E2 leads to abnormal cell density and migration in the mouse brain – link to autism. #1-Cluster-240. *Canadian Association of Neuroscience Meeting*, Toronto, ON, Canada.

Bestard-Lorigados, I., **Wong, C.,** Rai-Bhogal, R. and Crawford, D. A. (2019). Prostaglandin E2 affects the expression of neuronal hemoglobin- link to autism spectrum disorders. #1-Cluster-238. *Canadian Association of Neuroscience Meeting*, Toronto, ON, Canada.

Wong C.*, Bestard Lorigados I., Rai-Bhogal, R., Crawford D.A. (2017). Abnormal prostaglandin E2 signalling results in autism-associated behaviours in novel mouse models. 283.01/B14, *Society for Neuroscience*, Washington DC, USA.

Wong C.*, Ahmad E., Li H., Crawford D.A. (2015). Collaborative regulation of developmental pathways: Prostaglandin E2 alters Wnt signalling in neural stem cells. Poster# 2-A-11, *Canadian Association of Neuroscience Meeting*, Vancouver, BC, CA.

Wong C.*, Ussyshikin N., Li H., Crawford D.A. (2014). Prostaglandin E2 promotes earlier differentiation of neuroectodermal stem cells: A link to Autism. 799.11/U5, *Society for Neuroscience*, Washington DC, USA.

Wong C.*, Crawford D.A. (2013). Prostaglandin E2 alters the differentiation of neuroectodermal stem cells. 244.17/T13, *Society for Neuroscience*, San Diego, CA, USA.

Bhogal R., **Wong C.**, Crawford D.A. (2013). Effects of PGE_2 on expression of wnt-target genes during critical period of mouse brain development. 244.19/T15, *Society for Neuroscience*, San Diego, CA, USA.

Wong C.*, Crawford D.A. (2013). PGE2 alters Wnt- regulated cell behaviour in neuroectodermal stem cells. Poster# 2-A-5, *Canadian Association of Neuroscience Meeting*, Toronto, ON, CA.

Bhogal R., **Wong C.**, (2013). Crawford D.A. Effects of Prostaglandin E2 Administration During Critical Period of Mouse Development on Expression of Wnt-Target Genes. Poster# 3-A-2, *Canadian Association of Neuroscience Meeting*, Toronto, ON, CA.

Wong C.*, Li H, Crawford D.A. (2012). Interaction between prostaglandin E2-Wnt signaling pathways in neural stem cells. Poster#244.01/E65, *Society for Neuroscience* 2012, New Orleans, LA, USA.

Wong C.*, Li H, Crawford D.A. (2012). The implications of PGE2-Wnt signaling pathway interaction in autism. Poster#9834, *International Meeting for Autism Research,* Toronto, ON, CA

Sin C., Li H, **Wong C**, Crawford D.A., Li H. (2012). Identification of genetic risk factors involved in ASD. Poster#11245, *International Meeting for Autism Research,* Toronto, ON, CA.

Wong C.*, Li H, Crawford D.A. (2011). The role of prostaglandin E2 signalling pathway in neuroectodermal stem cell function. Poster# 151.08/V6, *Society for Neuroscience* 2011, Washington, DC. USA

APPENDIX B

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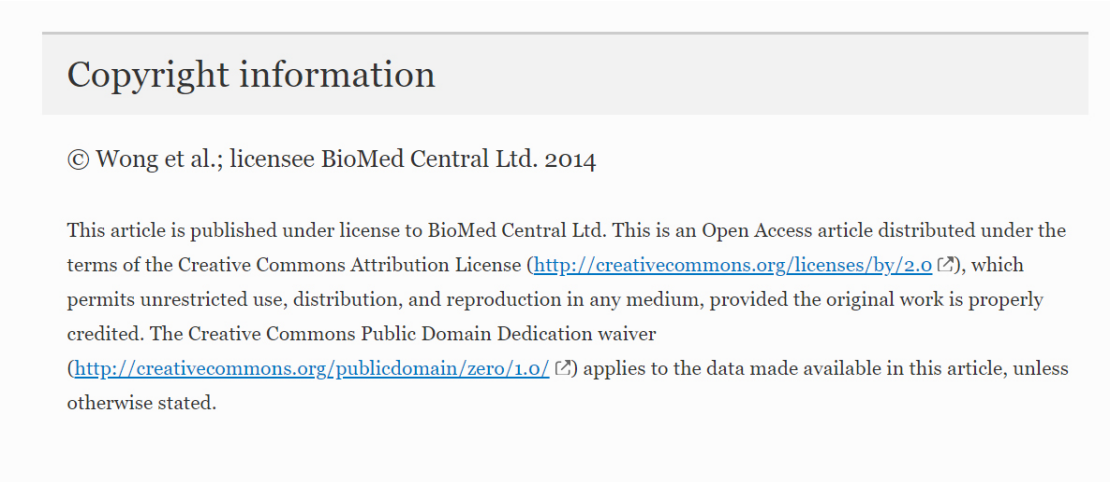

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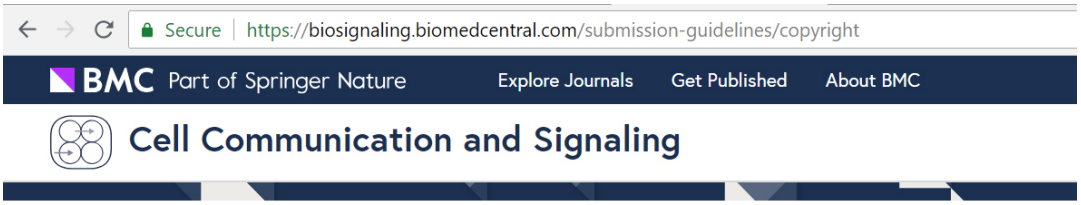

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APPENDIX C

FRONT PAGE OFFPRINTS OF RELEVANT PUBLISHED WORK

Lipid Signalling in the Pathology of Autism Spectrum Disorders

Christine Wong and Dorota Anna Crawford

Introduction to Lipids

Fatty acids are the simplest type of lipids that serve as building blocks for more complex lipids such as phospholipids, cholesterol, and vitamin E, which are integral to cell membranes (Fig. 1). Abnormal metabolism and imbalances of these three complex lipids have all been associated with autism spectrum disorders (ASDs) and will be outlined in following sections. Fatty acids can be saturated, monounsaturated, or polyunsaturated; the latter will be elaborated upon due to its link with ASDs. Essential fatty acids (EFAs), also called polyunsaturated fatty acids (PUFAs), play an important role in maintaining the structural and functional integrity of the central nervous system (CNS). They serve as major components of neural cell membrane phospholipids and are necessary for the modulation of ion channels, enzymes, and receptor activity (Boland et al. 2009; Guizy et al. 2008). PUFAs must be obtained through the diet since the ability to synthesize them is limited. The two major types of PUFAs are omega-6 linoleic acid (LA; 18:2n-6) and omega-3 α-linolenic acid (ALA; 18:3n-3), which are converted to arachidonic acid (AA, 20:4n-6) and to eicosapentaenoic acid (EPA, 20:5n-3) or docosahexaenoic acid (DHA, 22:6n-3), respectively (Haag 2003) (Figs. 1 and 2).

One of the most commonly derived PUFAs from cell membranes is AA. Through the action of phospholipase A_2 (PLA_2), an enzyme important for cell membrane maintenance, AA is cleaved from membrane phospholipids and further

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REVIEW

Prenatal exposure to common environmental factors affects brain lipids and increases risk of developing autism spectrum disorders

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Keywords: autism, blood brain barrier, chemicals, lipids, prostaglandin

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Abstract

The prevalence of autism spectrum disorders (ASDs) has been on the rise over recent years. The presence of diverse subsets of candidate genes in each individual with an ASD and the vast variability of phenotypical differences suggest that the interference of an exogenous environmental component may greatly contribute to the development of ASDs. The lipid mediator prostaglandin E2 (PGE_2) is released from phospholipids of cell membranes, and is important in brain development and function; PGE_2 is involved in differentiation, synaptic plasticity and calcium regulation. The previous review already described extrinsic factors, including deficient dietary supplementation, and exposure to oxidative stress, infections and inflammation that can disrupt signaling of the PGE_2 pathway and contribute to ASDs. In this review, the structure and establishment of two key protective barriers for the brain during early development are described: the blood–brain barrier; and the placental barrier. Then, the first comprehensive summary of other environmental factors, such as exposure to chemicals in air pollution, pesticides and consumer products, which can also disturb PGE_2 signaling and increase the risk for developing ASDs is provided. Also, how these exogenous agents are capable of crossing the protective barriers of the brain during critical developmental periods when barrier components are still being formed is described. This review underlines the importance of avoiding or limiting exposure to these factors during vulnerable periods in development.

Introduction

Autism is a neurodevelopmental disorder defined by impairments in communication, social interactions and language, and is associated with repetitive behaviors (Pelphrey *et al.*, 2014). Autism belongs to a spectrum known as autism spectrum disorders (ASDs), which also includes Asperger's syndrome, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified. Over recent years, there has been a dramatic increase in the prevalence of ASDs in children. The Centers for Disease Control and Prevention reported that one in 88 children had an ASD in 2008 (CDC, 2012), and in 2010 the prevalence increased to one in 68 children (CDC, 2014). Furthermore, school-aged boys were more than four times as likely to have an ASD compared with their female counterparts (Blumberg *et al.*, 2013). Although some argue that the increased prevalence is the result of changes in diagnostic criteria, this cannot fully explain the observed increases (Hertz-Picciotto & Delwiche, 2009). It is well established that the etiology of ASDs involves the interaction of genetic composition and exposure to environmental factors (Muhle *et al.*, 2004; Herbert, 2010; Meek *et al.*, 2013; Banerjee *et al.*, 2014; Hall & Kelley, 2014; Rossignol *et al.*, 2014; Tordjman *et al.*, 2014; Kim & Leventhal, 2015). Because genes do not evolve very rapidly in evolution, influence of environmental factors might contribute to the developmental differences in ASDs through modifications in gene expression.

There is sufficient research from twin and family studies demonstrating the involvement of genes in ASDs (Guo *et al.*, 2011; Frazier *et al.*, 2014). However, the most recent evidence suggests that in monozygotic twins (MTs) that share the same genetic material, the concordance rates range from 43 to 88% (Rosenberg *et al.*, 2009; Lichtenstein *et al.*, 2010; Stilp *et al.*, 2010; Hallmayer *et al.*, 2011; Ronald & Hoekstra, 2014). Additionally, MTs that are diagnosed with ASDs often display different subsets of autism symptoms (Kates *et al.*, 1998, 2004; Belmonte & Carper, 2006; Mitchell *et al.*, 2009). Furthermore, the concordance rates for dizygotic twins are about double that of non-twin siblings, suggesting that the uterine and maternal environment likely contribute to autism concordance rates (Bohm *et al.*, 2013). This suggests that investigations beyond heritable genetic differences should be taken to uncover the etiologies of ASDs. Various studies on ASDs using animal models and human samples have shown significant differences in gene expression during pre- and postnatal brain development (Garbett

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Prostaglandin E2 alters Wnt-dependent migration and proliferation in neuroectodermal stem cells: implications for autism spectrum disorders

Christine T Wong[1,2], Eizaaz Ahmad[3], Hongyan Li[1] and Dorota A Crawford[1,2,3*]

Abstract

Prostaglandin E2 (PGE_2) is a natural lipid-derived molecule that is involved in important physiological functions. Abnormal PGE_2 signalling has been associated with pathologies of the nervous system. Previous studies provide evidence for the interaction of PGE_2 and canonical Wnt signalling pathways in non-neuronal cells. Since the Wnt pathway is crucial in the development and organization of the brain, the main goal of this study is to determine whether collaboration between these pathways exists in neuronal cell types. We report that PGE_2 interacts with canonical Wnt signalling through PKA and PI-3K in neuroectodermal (NE-4C) stem cells. We used time-lapse microscopy to determine that PGE_2 increases the final distance from origin, path length travelled, and the average speed of migration in Wnt-activated cells. Furthermore, PGE_2 alters distinct cellular phenotypes that are characteristic of Wnt-induced NE-4C cells, which corresponds to the modified splitting behaviour of the cells. We also found that in Wnt-induced cells the level of β-catenin protein was increased and the expression levels of Wnt-target genes (*Ctnnb1*, *Ptgs2*, *Ccnd1*, *Mmp9*) was significantly upregulated in response to PGE_2 treatment. This confirms that PGE_2 activated the canonical Wnt signalling pathway. Furthermore, the upregulated genes have been previously associated with ASD. Our findings show, for the first time, evidence for cross-talk between PGE_2 and Wnt signalling in neuronal cells, where PKA and PI-3K might act as mediators between the two pathways. Given the importance of PGE_2 and Wnt signalling in prenatal development of the nervous system, our study provides insight into how interaction between these two pathways may influence neurodevelopment.

Keywords: Prostaglandin E2, Wnt signalling, Neuroectodermal stem cells, Cell motility, Proliferation, Autism

Background

The plasma membrane phospholipids play a fundamental role in the nervous system and act as a reservoir for second messenger molecules important for the development and normal functioning of the brain. Prostaglandin E2 (PGE_2) is a bioactive fatty acid that is derived from arachidonic acid, a major structural component of plasma membrane phospholipids, through the enzymatic metabolism of cyclooxygenases −1 and −2 (COX-1,-2) and then prostaglandin synthases [1]. Extracellular stimuli such as immunological and infectious agents [2-4], environmental toxins such as mercury and lead [5], and exposure to drugs including misoprostol and valproic acid [6] can trigger the local production of PGE_2 via specific biosynthetic pathways, resulting in altered cell signal transmission that modulates biological functions such as sleep, fever, inflammation, and pain [7].

The diverse action of PGE_2 is achieved through the activation of 4 different G-protein coupled E-prostanoid receptors (EP1 through 4) [8,9]. The divergent role of PGE_2 is amplified by the variety of different kinase-mediated signalling cascades that can be activated through its EP receptors, such as the protein kinase A (PKA), phosphatidylinositide 3-kinases (PI-3K), and protein kinase C (PKC) pathways [10].

During the early stages of pregnancy, there are elevated levels of COX-2 and PGE synthases, enzymes responsible for the production of PGE_2, which is indicative of the involvement of PGE_2 in prenatal development [11]. We

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Prostaglandin E_2 Promotes Neural Proliferation and Differentiation and Regulates Wnt Target Gene Expression

Christine T. Wong,[1,2] Netta Ussyshkin,[3] Eizaaz Ahmad,[2,3] Ravneet Rai-Bhogal,[2,3] Hongyan Li,[1] and Dorota A. Crawford[1,2,3]*

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Prostaglandin E_2 (PGE_2) is an endogenous lipid molecule that regulates important physiological functions, including calcium signaling, neuronal plasticity, and immune responses. Exogenous factors such as diet, exposure to immunological agents, toxic chemicals, and drugs can influence PGE_2 levels in the developing brain and have been associated with autism disorders. This study seeks to determine whether changes in PGE_2 level can alter the behavior of undifferentiated and differentiating neuroectodermal (NE-4C) stem cells and whether PGE_2 signaling impinges on the Wnt/β-catenin pathways. We show that PGE_2 increases proliferation of undifferentiated NE-4C stem cells. PGE_2 also promotes the progression of NE-4C stem cell differentiation into neuronal-lineage cells, which is apparent by accelerated appearance of neuronal clusters (neurospheres) and earlier expression of the neuronal marker microtubule-associated protein tau. Furthermore, PGE_2 alters the expression of downstream Wnt-regulated genes previously associated with neurodevelopmental disorders. In undifferentiated stem cells, PGE_2 downregulates *Ptgs2* expression and upregulates *Mmp9* and *Ccnd1* expression. In differentiating neuronal cells, PGE_2 causes upregulation of *Wnt3*, *Tcf4*, and *Ccnd1*. The convergence of the PGE_2 and the Wnt pathways is also apparent through increased expression of active β-catenin, a key signaling component of the Wnt/β-catenin pathways. This study provides novel evidence that PGE_2 influences progression of neuronal development and influences Wnt target gene expression. We discuss how these findings could have potential implications for neurodevelopmental disorders such as autism.

Key words: lipid signaling; prostaglandin; Wnt; neurodevelopment; neural stem cell; cell proliferation; cell differentiation; autism

The human brain is highly composed of lipids, and the availability and metabolism of these lipids are tightly integrated with healthy development, maintenance, and function of the nervous system (Lawrence, 2010). Prostaglandin E_2 (PGE_2) is a bioactive lipid molecule derived from plasma membrane phospholipids through the enzymatic activity of phospholipase A2 and cyclooxygenase-1 and -2 (COX-1, -2). PGE_2 binds to four E-prostanoid receptor subtypes (EP1, EP2, EP3, EP4) to regulate various functions in the developing nervous system, including memory formation and synaptic plasticity (Furuyashiki and Narumiya, 2011), thermoregulation and immune response modulation (Lazarus, 2006), and neurotransmitter release (Bezzi et al., 1998). Furthermore, various studies have linked abnormalities in the PGE_2 pathway to neurodevelopmental disorders, such as autism-spectrum disorder (ASD; Wong and Crawford, 2014). Altered PGE_2 signaling resulting from environmental factors has been reported in many cases of ASD (Landrigan, 2010; Tamiji and Crawford, 2010b). For example, exogenous stimuli that affect PGE_2 levels, such as immunological agents (Patterson, 2011), toxic chemicals (Schwartzer

SIGNIFICANCE

This study provides novel evidence that prostaglandin E2 (PGE_2) can influence the behavior of neural stem cells by increasing their proliferation and accelerating their differentiation into a neuronal lineage. In undifferentiated and differentiating neural stem cells, PGE_2 can also affect the expression of genes from the Wnt signaling pathway that is important for early development. PGE_2 decreases the expression of *Ptgs2* and increases the expression of *Wnt3*, *Tcf4*, *Mmp9*, and *Ccnd1*, which have been previously associated with neurodevelopmental disorders. We also show that PGE_2 increases the protein expression of active β-catenin. Because of the important processes regulated by PGE_2 and Wnt signaling in the brain as well as their link to autism disorders, we discuss the potential detrimental effects of these findings on early neurodevelopment.

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Genes, Brain and Behavior

ORIGINAL ARTICLE

Autism-related behaviors in the cyclooxygenase-2-deficient mouse model

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Prostaglandin E2 (PGE2) is an endogenous lipid molecule involved in normal brain development. Cyclooxygenase-2 (COX2) is the main regulator of PGE2 synthesis. Emerging clinical and molecular research provides compelling evidence that abnormal COX2/PGE2 signaling is associated with autism spectrum disorder (ASD). We previously found that COX2 knockout mice had dysregulated expression of many ASD genes belonging to important biological pathways for neurodevelopment. The present study is the first to show the connection between irregular COX2/PGE2 signaling and autism-related behaviors in male and female COX2-deficient knockin, (COX)-2^{-}, mice at young (4-6 weeks) or adult (8-11 weeks) ages. Autism-related behaviors were prominent in male (COX)-2^{-} mice for most behavioral tests. In the open field test, (COX)-2^{-} mice traveled more than controls and adult male (COX)-2^{-} mice spent less time in the center indicating elevated hyperactive and anxiety-linked behaviors. (COX)-2^{-} mice also buried more marbles, with males burying more than females, suggesting increased anxiety and repetitive behaviors. Young male (COX)-2^{-} mice fell more frequently in the inverted screen test revealing motor deficits. The three-chamber sociability test found that adult female (COX)-2^{-} mice spent less time in the novel mouse chamber indicative of social abnormalities. In addition, male (COX)-2^{-} mice showed altered expression of several autism-linked genes: *Wnt2*, *Glo1*, *Grm5* and *Mmp9*. Overall, our findings offer new insight into the involvement of disrupted COX2/PGE2 signaling in ASD pathology with age-related differences and greater impact on males. We propose that (COX)-2^{-} mice might serve as a novel model system to study specific types of autism.

KEYWORDS
age differences, autism, behavior, COX2, gene expression, inverted screen test, knockin model, lipid signaling, marble burying test, neurodevelopmental disorders, open field test, prostaglandin, sex differences, three-chamber sociability test

1 | INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopment disorder phenotypically characterized by difficulties in social interaction, abnormal communicatory behaviors and the presence of restricted and repetitive behaviors. Many individuals with ASD also experience motor difficulties,[1] sensory sensitivity[2] and anxiety.[3] Epidemiological data reveal that ASD is one of the most common neurodevelopment conditions in children, affecting 1 in 68.[4–7] The prevalence of ASD has a strong sex bias, with significantly higher diagnosis in males compared with females.[8] Sex also plays a role in the clinical presentation of ASD through varied symptomology.[9–11] For example, in males with ASD, attention deficit-hyperactivity disorder (ADHD) is more prevalent[12] and repetitive behaviors are more severe than females.[13–15] The manifestation of these deficient behaviors in ASD likely arises from complex interactions between genes and environment that result in improper brain development.[16–18]

Abbreviations: AA, arachidonic acid; ASD, Autism spectrum disorders; ADHD, attention deficit-hyperactivity disorder; COX1,2, cyclooxygenases-1 and -2; KI, knockin; PLA2, phospholipase A2; PGE2, prostaglandin E2; RQ, relative quantification; WT, wild-type