ABNORMAL COX2/PGE2 SIGNALLING IN THE DEVELOPING CEREBELLUM – A LINK TO AUTISM SPECTRUM DISORDERS

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Abstract

Autism spectrum disorders (ASDs) include a group of neurodevelopmental conditions that are characterized by deficits in social interaction and communication, increases in repetitive/restricted often stereotyped behaviour, and increases in anxiety. The heterogeneous nature of ASDs with regards to symptoms but also genetic profiles of ASD individuals, make understanding factors contributing to the disorder complex. However, literature suggests that ASDs arise from a combination of genetic and environmental factors. Clinical studies have suggested that abnormal lipid signalling, as a result of environmental insults can contribute to the etiology of ASDs. The phospholipid membrane of cells within the can be metabolized into lipid signalling molecules, including prostaglandins. Prostaglandin E2 (PGE2) is one of the most utilized lipid signalling molecules in the brain, involved in developmental processes such as synaptogenesis, migration, and differentiation of neuronal stem cells. Abnormal levels of PGE2, as well as COX-1 and COX-2, the rate-limiting enzymes in PGE2 synthesis have been linked to ASD. Furthermore, various environmental risk factors including exposure to heavy metals, infection/inflammation in pregnancy, exposure to pesticides, fragrances, and the use of over-the-counter medications such as aspirin and acetaminophen can affect PGE2 levels and are linked to ASD.

The exact mechanisms that link abnormal COX2/PGE2 signalling to ASD are still unclear. To help address the lack of information, in this dissertation we first examine the effect of exposure to PGE2 on differentiated neuroectodermal (NE-4C) stem cells. Further, Studies have demonstrated that the cerebellum may be important in the etiology of ASDs. Interestingly there is evidence that PGE2 can affect postnatal development of the cerebellum. We examine the effect of increases (in a maternal PGE2 injection model) and decreases (in a COX-2^{-}-KI model) in PGE2 levels on prenatal

neurodevelopment. We specifically examine the effects of these increases and decreases on cytoskeletal-dependent morphology through dendritic morphology within the cerebellum. Additionally, we examine the effect of prenatal PGE2-exposure on cerebellar-dependent motor function postnatally. Given the importance of sex as a factor in examining neurodevelopmental disorders such as ASD that have a large sex bias towards males, all of our *in vivo* studies address the modulation of the PGE2 effect by sex.

These studies demonstrate that abnormal COX2/PGE2 signalling can affect important neurodevelopmental processes *in vitro* and development of the cerebellum *in vivo*. We observed disruptions in cytoskeletal dynamics, and changes in the expression of cytoskeletal proteins corresponding to abnormal COX2/PGE2 signalling. In PGE2-exposed mice, the changes in dendritic morphology in the cerebellum, corresponded to deficits in cerebellar motor function. Further, we found that the disruption of COX2/PGE2 affected development in a sex-dependent manner. The findings strengthen the involvement of COX2/PGE2 signalling in normal development of the brain and further suggest that abnormal COX2/PGE2 signalling as a result of exposure to environmental factors can result in neuropathologies including those found in ASDs

Dedication

This dissertation is dedicated to all of the friends and family who have helped guide me through my graduate school journey:

My parents: Divya Kissoondoyal my mom who fostered my love of science and problem solving, and my dad Ash Kissoondoyal who made sure I knew that perseverance and hard work were key to reaching my goals.

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List of Abbreviations

AA	Arachidonic acid
ANOVA	Analysis of variance
ASDs	Autism spectrum disorders
Ca2+	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CldU 5-	Chloro-2′-deoxyuridine
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
$COX\text{-}2^{-}$	Cyclooxygenase-2 knockin
G1, G11, G16	Gestational day 1, 11, 16
EP	E-prostanoid
FC	Fold-Change
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
H89	H89 dihydrochloride hydrate
IdU	Iododeoxyuridine
KI	Knockin
KO	Knockout
MEM	Minimal essential media
NE-4C	Neuroectodermal stem cell line
NSAIDs	Nonsteroidal anti-inflammatory drugs
P8, P25, P30	Postnatal day 8, 25, 30
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehye
PGE2	Prostaglandin E2

PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PLA2	Phospholipase A2
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
RQ	Relative quantification
SFM	Serum-free media
SPN	Spinophilin
VPA	Valproic Acid
Wnt	Wingless-related MMTV integration site
WT	Wild-type

Chapter 1: Introduction

1.1 General Introduction

The metabolism of lipids in the brain results in a significant source of signalling molecules important for proper brain development. One of the major signalling lipids, Prostaglandin E2 (PGE2) is involved in developmental processes including synaptogenesis, differentiation, and proliferation (Chen and Bazan 2005, Tassoni, Kaur et al. 2008). The conversion of Arachidonic Acid through cyclooxygenase -1 and -2 is the rate limiting step in the production of PGE2.Given that the etiology of ASD is a result of a combination of genetic and environmental risk factors, PGE2 is a strong candidate as a molecule impacted by both genes and the environment (Tamiji and Crawford 2010, Wong, Wais et al. 2015). Environmental risk factors of ASD such as inflammation, oxidative stress, exposure to pollution and heavy metals, and exposure to pesticides and herbicides can all disrupt COX2/PGE2 signalling. The connection between PGE2 and ASD has been demonstrated in human studies. Significantly higher levels of serum PGE2 have been found in the plasma of male ASD individuals (El-Ansary and Al-Ayadhi 2012). Clinical examining the effect of prenatal exposure to Misoprostol, an analogue to PGE2, found increases in neuropathologies and an increase in the prevalence of Mobius Syndrome (Bandim, Ventura et al. 2003). Despite evidence of a COX2/PGE2 contribution to ASDs, information on the mechanism of this connection is limited. The studies in this dissertation provide novel evidence of the role of PGE2 and the effect of abnormal COX2/PGE2 signalling on neuronal development. These studies cover a range of cellular, molecular, and behavioural research conducted in both *in vitro* neuronal cell and *in vivo* mouse models. The findings reveal that abnormal COX2/PGE2 signalling can result in cytoskeletal disruptions and abnormal cerebellar morphology and cerebellar-related behaviours, all of which may contribute to the development of ASDs.

1.2 Overall Objectives and Hypothesis

The studies in this dissertation aim to address the overarching objective:

To identify the molecular mechanisms by which abnormal COX2/PGE2 lipid signalling can affect cerebellar development in a sex-dependent manner and contribute to ASD related pathologies.

Overall Hypothesis: Dysregulation of the COX2/PGE2 signalling pathway will disrupt normal cerebellar development resulting in molecular and behavioural ASD-associated changes in a sex-dependent manner.

To address the overarching objective, the following specific objectives were:

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

1) To study the effect of long-term PGE2 exposure on differentiation of neuroectodermal NE-4C stem cells
2) To examine cytoskeletal changes resulting from long-term PGE2 exposure during differentiation
3) To investigate the interaction between the PGE2 and the PKA signalling pathways

Using an *in vivo* mouse model (Studies 2 and 3)

4) To verify *in vitro* findings and to further study the consequences of disruptions in COX2/PGE2 signalling on cytoskeletal dynamics
5) To examine cerebellar changes in dendritic and dendritic spine morphology resulting from COX2/PGE2 signalling disruptions
6) To describe cerebellar-related motor dysfunction in mice prenatally exposed to PGE2.

1.3 Dissertation Layout

A general introduction to the dissertation (Chapter 1) is followed by a review of pertinent literature (Chapter 2) on lipid signalling within the brain focusing on the lipid signalling molecule PGE2 and its contribution to the etiology of ASDs. Addressing the listed objectives in 1.2, three studies were conducted and are presented in manuscript style in the chapters following (Chapters 3 through 5).

The study presented in Chapter 3 investigated changes in cytoskeletal morphology following PGE2 exposure in differentiating neuronal stem cells. The second study (Chapter 4) examined the dendritic and dendritic spine morphology within the cerebellums of COX-2^{-}-KI mice. Lastly, the third study (Chapter 5) examined cerebellar morphology via cell density and dendritic morphology and related cerebellar motor behaviours in an elevated PGE2 (PGE2-exposed) mouse model.

The concluding chapter of the dissertation (Chapter 6) describes key findings across all studies in the context of the current literature on ASDs. In this chapter the impact of abnormal COX2/PGE2 signalling on neurodevelopment, its disruption of normal cytoskeletal dynamics both *in vitro* and *in vivo* and the resulting distinct effects in the cerebellums of males and females will be discussed. Overall, our findings provide further evidence of the importance of COX2/PGE2 signalling during normal development and suggest that abnormal COX2/PGE2 signalling contributes to the etiology of ASDs.

1.4 Experimental Models

Mice are frequently used as to model processes in neurodevelopment and to better understand the basis of neurodevelopmental disorders. Mice not only have similar genomes, but undergo similar developmental processes, and exhibit analogous behaviours to humans. In this dissertation, both an *in vitro* murine cell line (Study 1), and an *in vivo* mouse animal model (Studies 2, and 3) are used.

In vitro model system for Study 1: Neuroectodermal (NE-4C) stem cells obtained from the American Tissue Culture Collection (ATCC) were obtained from primary brain cell cultures of prenatal day 9 mouse embryos. The entirety of the gestational period of mice is around 20 days (Lanman and Seidman 1977, Xu, Barnes et al. 2010) with cells becoming multipotent neural stem cells at around prenatal days 8-9 of development (Schlett and Madarász 1997). These cells undergo processes that occur in the developing brain including pathfinding, neurosphere formation, and differentiation (Kelava and Lancaster 2016). These similarities make NE-4C cells an appropriate model of neuronal differentiation.

In vivo model system for Studies 2 and 3: In this dissertation two mice models are used to study the molecular, cellular, and behavioral consequences of a disruption of COX2/PGE2 signaling during neuronal development. Increased PGE2 levels were modelled in mice offspring prenatally exposed to PGE2 and decreased PGE2 levels were modelled in mice deficient in the PGE2 producing enzyme COX-2.

PGE2-exposed mice were used in study 3. PGE2-exposed mice were generated through the subcutaneous injection of C57BL/6 pregnant dames on gestational day 11 (G11) with 16, 16-dimethyl prostaglandin E2 (dmPGE2). dmPGE2 was chosen as it is a stable analogue of PGE2 that is often used for *in vivo* models of COX2/PGE2 disruption (Ohno, Morikawa et al. 1978, Cruz Duarte, St-Jacques et al. 2012, Cook, Thomas et al. 2016) which mimics increases in PGE2 that were previously associated with ASDs. Pregnant dames were injected at G11 as this day marks the onset of neurogenesis in the mouse brain (Zhang and Jiao 2015). This time point corresponds to previous clinical studies in humans examining the misuse of misoprostol, a synthetic analogue to PGE2, during pregnancy which resulted in the manifestation of Mobius syndrome and ASD-related symptoms (Costa 1998, Gonzalez, Marques-Dias et al. 1998, Pastuszak, Schüler et al. 1998, Schüler, Pastuszak et al. 1999, Bandim, Ventura et al. 2003, Miller, Strömland et al. 2005, Bos-Thompson, Hillaire-Buys et al. 2008). Our lab

has previously shown ASD-related behaviours in PGE2-exposed mice including social deficits, restricted and repetitive behaviors, and anxious behaviours (ref PGE2 behaviour).

B6.129S6(FVB)-Ptgs2tm1.1Fun/J mice, also known as COX-2^{-}-KI mice are the mouse model used in study 2. They are a model of reduced PGE2 levels with endogenous levels of PGE2 reduced by half in COX-2^{-}-KI mice compared to control mice. COX-2^{-}-KI mice were generated by a targeted point mutation of the *Ptgs2* gene resulting in a substitution of the Y385F amino acid, leading to inactivation of cyclooxygenase activity but maintaining peroxidase activity (Yu, Fan et al. 2006). Founder COX-2^{-}-KI mice were backcrossed for at least 5 generations to wild-type 129S6/SvEvTac mice to generate the COX-2^{-}-KI mice. Our lab has previously shown that COX-2^{-}-KI mice exhibited ASD-related behaviours including deficits in social interaction, restrictive and repetitive behaviours, and anxious behaviours (Wong, Bestard-Lorigados et al. 2019).

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Chapter 2: Literature Review

Literature Review: Lipid signalling in Autism Spectrum Disorders

The following chapter provides an overview of Autism Spectrum Disorders (ASD) with a focus on the contribution of environmental risk factors to the etiology of autism. This chapter will discuss the connection between abnormal COX2/PGE2 signalling and ASD, with a focus on the development of the cerebellum, a region highly implicated in the development of ASDs.

2.1 Introduction to Autism Spectrum Disorders (ASDs)

The diagnosis of ASDs typically occur within the first 3 years of life and are defined primarily through deficits in social communication, restrictive or repetitive behaviours, and anxious behaviours (Do 2011, Association 2013). It is estimated that males are four times more likely to be diagnosed with ASD, demonstrating a large sex-bias (Rivet and Matson 2011, Christensen, Braun et al. 2018). Data from the Centers for Disease Control (CDC) in 2014 estimated a global annual cost of $230-260 billion associated with ASD. Further, families of children with ASD have reported significant impairments in both finances (Kogan, Strickland et al. 2008, Buescher, Cidav et al. 2014, Saunders, Tilford et al. 2015) and overall quality of life (Mugno, Ruta et al. 2007). There has been an alarming increase in the prevalence of ASD diagnosis over the past few decades (Brugha, McManus et al. 2011, Zablotsky, Black et al. 2015). While earlier estimates of ASD prevalence reported rates of 0.02-0.05% in the U.S, Europe, and Japan in the 1960s and 1970s, current estimates report rates to be at 1% (Figure 2-1) (Lotter 1966, Hoshino, Kumashiro et al. 1982, Fombonne 1999, Brugha, McManus et al. 2011). While some believe that the increases in ASD prevalence can attributed to increases in awareness of the disorders, earlier diagnosis, and changes in diagnostic criteria, studies have determined that these factors cannot fully drive the changes in prevalence (Hertz-Picciotto and Delwiche 2009, Weintraub 2011). As rates continue to increase it is not unexpected that there will be both a greater strain on

healthcare systems and public resources, but also on the families of children with ASDs. As such, it is becoming imperative to understand the factors that are contributing to the rise in ASD prevalence.

2.1.1 Pathogenesis of ASDs

One of the major concerns with ASD is that there is no current treatment for the core ASD behaviours, with all current pharmacological and behavioural interventions aiming to lessen symptoms or to treat comorbidities such as epilepsy, anxiety, and depression (Gillberg 1991, Ospina, Krebs Seida et al. 2008, Doyle and McDougle 2012). This is because, while categorized under the umbrella of Autism, ASDs are heterogeneous both in underlying causes and in the symptoms observed (Muhle, Trentacoste et al. 2004, Herbert 2010, Meek, Lemery-Chalfant et al. 2013, Banerjee, Bhat et al. 2014, Hall and Kelley 2014, Rossignol, Genuis et al. 2014, Tordjman, Somogyi et al. 2014, Kim and Leventhal 2015).

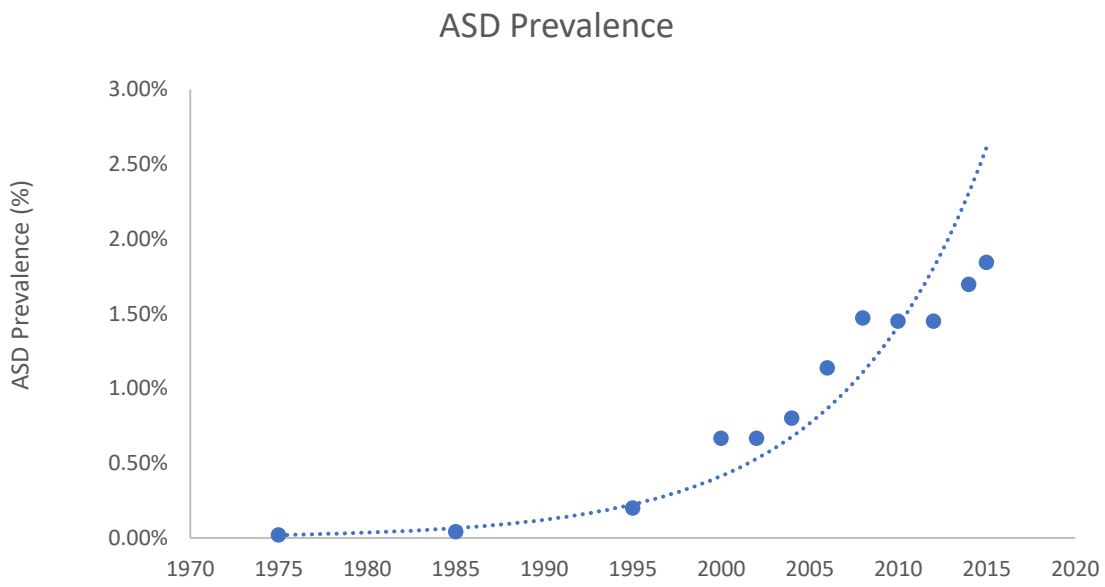

Figure 2-1: Percentage of children diagnosed with ASD from 1975 – 2016 in the USA as reported by the Center for Disease Control National Health Interview Survey. The graph depicts an exponential rise in ASD prevalence over a 40-year period. Figure created using data from (Maenner, Shaw et al. 2020)

While the exact pathology of ASDs are relatively unknown, current literature suggests that the pathogenesis of ASDs may arise during critical periods in prenatal development (Hyman, Arndt et al. 2005, Brown, Jones et al. 2008, Atladóttir, Thorsen et al. 2010, Froehlich-Santino, Tobon et al. 2014) Prenatal development is a result of important well-coordinated events including the proliferation, differentiation and migration of neural cells. (Stevens, Su et al. 2013, Le Belle, Sperry et al. 2014). The stringent timing and coordination of events make the brain particularly vulnerable to insults at this time. Examining the prenatal environment, studies have suggested a number of physiological mechanisms that may contribute to the development of ASD, including increased serotonin concentrations (Cook and Leventhal 1996, Chugani 2002, Williams, Brignell et al. 2013), low GABA concentrations (Fatemi, Reutiman et al. 2009, Coghlan, Horder et al. 2012), excitatory inhibitory signalling imbalances (Gao and Penzes 2015, Trakoshis, Rocchi et al. 2020), dysregulation of the immune system (Warren, Margaretten et al. 1986, Nardone and Elliott 2016, Ormstad, Bryn et al. 2018), oxidative stress and mitochondrial dysfunction (Frye and Rossignol 2011, Rossignol and Frye 2012, Pangrazzi, Balasco et al. 2020), and abnormal apoptosis and pruning through development (Koyama and Ikegaya 2015, Thomas, Davis et al. 2016, Hansel 2019).

2.1.2 Genetic and environmental Risk Factors of ASDs

Despite a large body of research dedicated towards understanding the pathogenesis of ASDs, the majority of ASD cases are classified as idiopathic and are believed to be the result of various environmental and genetic factors. The genetic basis of ASDs are fairly well documented. ASDs are known to be one of the most heritable of all neurological disorders with heritability rates estimated to be ~50% (Gaugler, Klei et al. 2014, Sandin, Lichtenstein et al. 2014). Genetic studies aimed at determining copy number variations and *de novo* mutations associated with ASD found changes in genes that ultimately regulate important neurodevelopmental pathways including neuronal development and synaptic transmission and function (Pinto, Delaby et al. 2014). The concordance

rates for ASD of monozygotic and dizygotic twins find that the rates of dizygotic twins are lower, providing strong evidence of the importance of genetic factors in the etiology of ASD (Muhle, Trentacoste et al. 2004, Hallmayer, Cleveland et al. 2011). Interestingly however, the concordance rate observed in siblings decreases the greater the interval between pregnancies is; there are likely risks associated with the maternal environment (Hallmayer, Cleveland et al. 2011, Bohm, Stewart et al. 2013). Further, monozygotic twins often display differences in ASD symptom severity and presence, providing further evidence that the etiology of ASDs are a result of the interaction between both genetic and environmental risk factors(Belmonte and Carper 2006, Mitchell, Reiss et al. 2009). Our lab has previously suggested that lipid signalling, being effected by both environmental and genetic risk factors, may contribute to the etiology of ASDs (Wong and Crawford 2014).

2.1.3 Sex differences in ASDs

The consensus on ASD prevalence indicates that there are 4 males to every 1 female diagnosed with ASD (Werling and Geschwind 2013). These findings are consistent, and were shown in different demographics, and regions of the world (Kim, Leventhal et al. 2011, Autism and Investigators 2012, Kohane, McMurry et al. 2012, Lai, Tseng et al. 2012). While the strong male bias indicates the influence of sex-dependent factors such as differences in gene expression, or hormonal factors, there is no definitive explanation for the male bias.

Interestingly, there are differences in the symptoms shown between males and females with ASD. For example, males with ASD tend to be over-represented among high-functioning cases of ASD, while in more severe cases of intellectual disability, the sex bias is not present (Volkmar, Szatmari et al. 1993, Fombonne 1999, Yeargin-Allsopp, Rice et al. 2003). In general, males with ASD show more externalizing behavioural symptoms including increases in aggressive behaviours, hyperactivity, and repetitive/restrictive behaviour, and decreases in prosocial behaviours (Hattier, Matson et al. 2011, Mandy, Chilvers et al. 2012, Szatmari, Liu et al. 2012). In contrast, females exhibit

more internalizing symptoms including anxiety and depression, with more emotional symptoms reported by caregivers (Solomon, Miller et al. 2012, Szatmari, Liu et al. 2012).

While there are clear differences in the symptoms of ASD in males and females, the mechanisms behind these differences are not as clear. The general theory behind the differences, and the increased prevalence in males is that females have a higher threshold for risk factors before they reach a level of affection that would result in ASD (Reich, Cloninger et al. 1975). This general theory is in line with the more specific extreme male brain theory of Autism which hypothesizes that ASD arises from hypermasculinization of the brain due to abnormal sex-hormone regulation during development (Baron-Cohen 2002). Fetal testosterone levels in ASD individuals were found to positively correlate with ASD-related symptoms (Knickmeyer, Baron-Cohen et al. 2005, Auyeung, Baron-Cohen et al. 2009). Evidence in postnatal rats suggests that hypermasculinization resulting in ASD-related behaviours can result from abnormal PGE2 signalling. Increases in PGE2, modelled by injection into the POA of newborn female rats masculinized the synaptic profiles within the POA and resulted in male sexual behaviour in the adult females. Showing the opposite effect, the same group also demonstrated that inhibiting the production of PGE2 stunted masculinization of the brain and resulted in an absence of male sexual behaviour in adulthood (Amateau and McCarthy 2002, Amateau and McCarthy 2004, Wright, Burks et al. 2008).

2.2 Lipid Signalling in ASDs

2.2.1 Lipid Signalling in the brain

Lipids make up the majority of the dry mass of the brain at around 60% of the total dry mass being made of lipids with the majority being phospholipids and polyunsaturated fatty acids (PUFAs). Lipid signalling in the brain begins with the metabolism of the phospholipid membranes (figure 2-2). The phospholipid membranes of neurons are composed of fatty acid derivatives which can be

metabolized into bioactive lipid molecules that serve as signalling molecules for a range of cellular functions (Bennett and Horrobin 2000, Boland, Drzewiecki et al. 2009). Fatty acids are primarily classified as monounsaturated or polyunsaturated. Polyunsaturated fatty acids can be further categorized as Omega-6 fatty acids which are metabolized into Arachidonic Acid (AA) and Linoleic Acid (LA), or Omega-3 fatty acids which are metabolized into α-Linoleic Acids (ALA), Eicosapentaenoic Acid (EPA), and Docosahexaenoic Acid (DHA)(Yehuda, Rabinovitz et al. 1999). Of particular interest in ASD is the Omega-6 derivative AA. The metabolism of Omega-6 linoleic is performed by phospholipase A (PLA2) which selectively hydrolyzes the sn-2 fatty acid ester bond of phospholipids to produce AA (Murakami and Kudo 2002). Through the cyclooxygenase activity of COX-1 and COX-2, AA is metabolised into PGH2, which is converted into prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2, and PGI_2) and thromboxane A2 (TXA_2) (Sang and Chen 2006, Andreasson 2010, López and Ballaz 2020). The metabolism of AA into PGH2 through COX-1 and COX-2 is the rate-limiting step in the formation of the prostaglandins and thromboxane A2. Among the prostaglandins, prostaglandin E2 (PGE2) is one of the most active derivatives, released both in response to inflammatory cues as well as during neural development (Carlson 2009). PGE2 acts on a specific set of four 7 transmembrane domain g-protein coupled receptors known as E-prostanoid (EP) receptors (EP 1-4; Figure 2-3). In development, PGE2 plays a role in the mediation of cell proliferation and differentiation, synaptogenesis, and learning and memory (Chen and Bazan 2005, Tassoni, Kaur et al. 2008, Andreasson 2010, Wong, Ahmad et al. 2014, Wong, Ussyshkin et al. 2016). While EP1 is involved in phosphotidyl inositol hydrolysis and increases intracellular Ca2+ levels, EP2 and EP4 are known to increase intracellular cAMP/PKA signaling. Though complex compensatory mechanisms have limited our understanding of the role of EP3, literature suggests EP3 can induce both Ca2+ and cAMP/PKA signaling pathways under different conditions (Bilson, Mitchell et al. 2004).

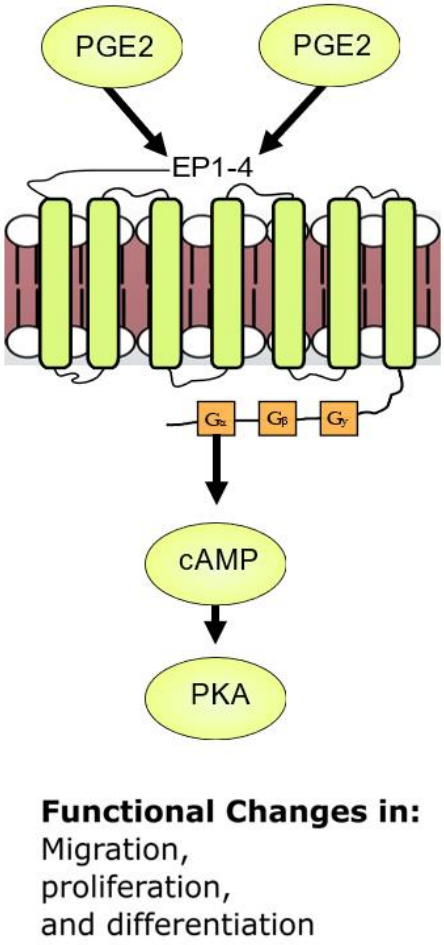

Figure 2-2 PGE2 and its synthetic analogue Misoprostol can stimulate EP1-4 and lead to the activation of PKA signalling cascades that impact cell function.

2.2.1 Polyunsaturated fatty acids in ASDs

Polyunsaturated fatty acids (PUFAs) are of special importance to the research of Autism Spectrum Disorders (ASDs). There are two major types of PUFAs, both of which have been implicated in development of the ASD phenotype: omega-6 linoleic, and omega-3α-linoleic acid. Imbalances between these two essential fatty acids have been implicated as an underlying factor of the ASD phenotype. Studies have indicated that a higher prevalence of omega-6 linoleic derivatives to omega-3α-linoleic acid derivatives seem to be present in the blood plasma of autistic individuals (Meguid, Atta et al. 2008, Gordon Bell, Miller et al. 2009). Alterations in the fatty acid composition of the

phospholipid bilayer has led to the appearance of ASD-related behaviours in rodent models (Shultz, MacFabe et al. 2009, Thomas, Foley et al. 2010). In humans the likelihood of infants for developing ASD is dramatically increased if they were not breastfed or given a PUFA supplemented diet (Schultz, Klonoff-Cohen et al. 2006). Studies have indicated that supplementation of omega-6 linoleic, and omega-3α-linoleic acid in children with ASD improve ASD-related behaviours including both social and motor skills (Politi, Cena et al. 2008, Doaei, Bourbour et al. 2021), as well as in anxiety and quality of life measures (Johnson and Hollander 2003).

Given that PUFAs are not produced by the body and obtained through food instead, one explanation for PUFA imbalances in ASD individuals is an insufficient maternal intake of PUFAs (Vancassel, Durand et al. 2001). An alternative is that there may be abnormalities in the enzymes required to metabolize omega-6 linoleic, and omega-3α-linoleic acid (Morales, Bustamante et al. 2011). It may be the case that both are involved with ASD individuals requiring an increased PUFA supply compared to the provided maternal amounts.

2.2.2 Phospholipase A2 in ASDs

The cleavage of AA from the phospholipid membrane by PLA2 is normally the result of binding by neurotransmitters including glutamate (Murakami and Kudo 2002), N -methyl- D -aspartic acid [93] , or α-amino-3-hydroxy-5-methyl-4-isoxazolee PPA (Williams and Glowinski 1996). However, this cleavage can also happen in response to environmental cues such as through cytokines during the inflammatory response (Linkous and Yazlovitskaya 2010, Casale, Kacimi et al. 2021). Two PLA2 isoforms, the human calcium independent PLA2 and secretory PLA2 have been mapped to chromosomal regions associated with ASD (Meyer, Schmuck et al. 1996, Lai, Fisher et al. 2000, Chen, Kono et al. 2006, Combi, Redaelli et al. 2010, Murakami, Nakatani et al. 2017). Children with ASD were shown to have a higher PLA2 concentration within their blood cells compared to matched

controls (Bell, MacKinlay et al. 2004). The expression of PLA2 was identified as a potential biomarker along with other molecules in the PGE2-synthesis pathway (El-Ansary, Hassan et al. 2016). A case study examining autistic individuals and individuals with Asperger's syndrome provided further evidence to support the effect of abnormal lipid signaling in development of ASDs. Belichenko, Oldfors et al. (1994) showed significantly higher PLA2 concentrations in the red blood cells of ASD individuals relative to healthy controls It has been hypothesized that increases in PLA2 activity contributes to imbalances in PUFA derivatives observed in ASD individuals. Altered levels of AA and a higher ratio of AA to DHA (an omega-3α-linoleic acid derivative) were reported in blood samples of ASD individuals compared to levels in a control population **(Vancassel, Durand et al. 2001, Wiest, German et al. 2009, Mostafa, El-Hadidi et al. 2010, Brigandi, Shao et al. 2015).**

2.2.3 COX enzymes in ASDs

As previously mentioned, the metabolism of AA into PGH2 through the COX enzymes is the rate-limiting step in the synthesis of prostaglandin molecules. Both COX-1 and COX-2 enzymes are involved in the production of prostanoids and can be inhibited by nonsteroidal anti-inflammatory drugs (Vane and Botting 2003). The COX enzymes themselves have been implicated in ASDs.

Typically believed to have a role in homeostasis COX-1 is constitutively expressed in the majority of human tissues and within microglial cells of the brain (Schwab, Nguyen et al. 2000, Hoozemans, Rozemuller et al. 2001). Beyond its housekeeping roles, COX-1 also plays a role in the inflammatory response and during development of the brain (Loftin, Tiano et al. 2002). In contrast, COX-2 expression is inducible throughout the body with the exception of the kidneys, the gastrointestinal tract, the female reproductive system, and within neurons of the brain where its expression is constitutive. Increases in COX-2 activity can result from stimuli such as growth factors (Hinz and Brune 2002), inflammatory cues such as infection (Ricciotti and FitzGerald 2011), oxidative stress (Kwiecien, Konturek et al. 2012), and synaptic activity (Yamagata, Andreasson et al. 1993).

Selective inhibition of COX-2 causes a reduction in long-term potentiation *in vitro* (Chen, Magee et al. 2002). Our lab has previously found altered expression of ASD-related genes and developmental pathways in a COX-2 KO mouse model (Rai-Bhogal, Ahmad et al. 2018). We have also shown that COX-2^{-}-KI mice have altered dendritic morphology (ref COX spines), and exhibit ASD-related behaviours including social deficits, repetitive/restricted behaviours, and anxious behaviours (Wong, Bestard-Lorigados et al. 2019). Evidence supports an important role of COX-2 during neurodevelopment and shows a high dendritic expression (Yamagata et al. 1993). Interestingly, there is an abnormal distribution of COX-2 within the dendrites of individuals with Rett's syndrome (Kaufman, Birmaher et al. 1997). A polymorphism in the gene encoding for the COX-2 protein *PTGS2* has been associated with the development of ASD (Yoo, Cho et al. 2008).

2.2.4 Prostaglandin E2 in ASDs

The abnormal expression of fatty acid derivatives such as PGE2 are implicated in ASDs. Exposure to external stimuli such as heavy metals, and infectious agents can cause abnormal stimulation of the PGE2 production pathway (Grandjean and Landrigan 2006). A study by El-Ansary and Al-Ayadhi (2012) showed significantly increased levels of PGE2 and other lipid mediators in ASD males. In addition, our research examining the expression patterns of EP receptors during mouse embryonic development have shown that the peak expression occurs during E11 which is when the height of neurogenesis occurs (Tamiji and Crawford 2010). This suggests an increased sensitivity to PGE2 at crucial stages of neurogenesis. We have also shown that the offspring of mice exposed to PGE2 during pregnancy exhibit ASD-related behaviours including social deficits, repetitive/restrictive behaviours, and anxious behaviours (ref PGE2 paper). Given the connections between ASD and PGE2 signaling, further investigation into the molecular effects of PGE2 on neuronal development may provide some insight into ASDs.

Clinical studies examining the effects of misuse of misoprostol provide a further link between abnormal PGE2 pathway signaling and ASDs (Bandim, Ventura et al. 2003). Misoprostol is a drug that is normally used in the treatment of stomach ulcers, in conjunction with other drugs for the termination of pregnancy or early induction of pregnancy in a controlled setting (Lin, Chien et al. 2011). However, use of this drug during the first trimester was shown to cause abnormal neural development of the children born including but not limited to ASDs and Mobius Sequence (Bandim, Ventura et al. 2003). Interestingly, misoprostol binds to and causes the activation of the EP receptors and the PGE2 pathway; misoprostol is a chemical analog of PGE2 (Tamiji and Crawford 2010). These results suggest that abnormal levels of PGE2 could contribute to abnormal neural embryonic development. It is therefore important to understand common factors that contribute to both abnormal COX2/PGE2 levels and the etiology of ASDs.

Despite being best known for its role in inflammatory pathway signalling, literature suggests that PGE2 also plays a role in brain masculinization. Males innately have higher PGE2 levels within the brain as a result of increased COX1 and COX2 levels due to elevated steroids in the male brain. Therefore, a reduction in PGE2 levels in males, stunts the masculinization process, and increases in PGE2 levels in females masculinizes the synaptic profile and sexual behaviour of females (Amateau and McCarthy 2002, Amateau and McCarthy 2004, Wright, Burks et al. 2008). In line with the extreme male brain theory of ASD, these findings further suggest that COX2/PGE2 signalling contributes to the etiology of ASD. A link between the PGE2 and the microglia provide additional evidence of sex-dependent contributions of PGE2 to ASDs. One study determined that there is a positive feedback loop between active microglia and PGE2 levels (Lenz, Nugent et al. 2013). Males have increased numbers of microglia with a greater amount of active microglia, increasing PGE2 levels. Interestingly, when females were treated with PGE2, the number of active microglia increased, further increasing PGE2 levels. Microglia are involved in the pruning of developing dendritic arbors and abnormal microglial

activation has been associated with E/I imbalances in ASD (Koyama and Ikegaya 2015, Andoh, Ikegaya et al. 2019, Andoh, Ikegaya et al. 2020).

2.3 Exposure to common environmental factors and ASDs

2.3.1 Air Pollution

A large case-study in the U.S (325 ASD individuals and 22,101 control individuals) found a significant linear correlation between perinatal exposure to heavy metals (lead, manganese, cadmium), and diesel particulates, and ASD (Roberts, Lyall et al. 2013). The children of mothers found living near a freeway at the time of delivery had an increase in the risk of being diagnosed with ASD (Volk, Hertz-Picciotto et al. 2011). The contribution of traffic-based air pollution to ASD have been further confirmed in a number of studies which found significant correlations between levels of diesel particulates (Roberts, Lyall et al. 2013, Talbott, Arena et al. 2015, Chiu, Hsu et al. 2016), and nitrogen dioxide (Kerin, Volk et al. 2018, Jo, Eckel et al. 2019, Pagalan, Bickford et al. 2019, Kim, Yan et al. 2021) and ASD diagnosis.

Prenatal exposure to diesel exhaust may also contribute to the excitatory inhibitory imbalances (E/I) observed in ASD individuals (Zikopoulos and Barbas 2013, Uzunova, Pallanti et al. 2016). There is a homeostatic E/I balance in in the brain (Haider, Duque et al. 2006, Kremkow, Aertsen et al. 2010), which is often permanently altered in ASD individuals (Cline 2005, Vogels and Abbott 2009). Literature suggests that the E/I imbalance in ASD is a result of reductions in GABA dependent signalling (Fatemi, Reutiman et al. 2009, Oblak, Gibbs et al. 2011) which is reduced in mouse (Gogolla, Leblanc et al. 2009) and human studies of ASD (Robertson, Ratai et al. 2016).

Exposure to diesel exhaust (the most significant source of air pollutants) results in microglial activation, and subsequent increases in oxidative stress (Gerlofs-Nijland, van Berlo et al. 2010, Levesque, Taetzsch et al. 2011, Cole, Coburn et al. 2016). The increases in oxidative stress can result

in peroxidation of the phospholipid membrane, resulting in increases in secondary lipid signalling molecules such as PGE2 (de Vries, Blom-Roosemalen et al. 1996, de Vries, Kuiper et al. 1997). The increase of PGE2 following treatment with air pollution particles has been demonstrated *in vitro* in neurons (Sang, Yun et al. 2011). As previously described, dysregulation of the maternal immune system of mice (Malkova, Yu et al. 2012), and primates (Bauman, Iosif et al. 2014) increased the ASD-related behaviours in offspring (Estes and McAllister 2016). Exposure to diesel exhaust has been found to induce this microglial response in rodent models (Gerlofs-Nijland, van Berlo et al. 2010, Levesque, Taetzsch et al. 2011, Cole, Coburn et al. 2016). As discussed, (section 2.2.4), increased levels of PGE2 as a result of microglial activation results in masculinization of the brain, and ASD-related behaviours.

2.3.2 Pesticides and Herbicides

With regards to ASDs, one of the most studied herbicides is glyphosate. Used in both agricultural rural areas but also commonly in urban settings, glyphosate is the most widely used herbicide globally (Samsel and Seneff 2015). A correlational study between ASD prevalence and glyphosate usage in soy and corn crops showed a strong positive temporal correlation between the two variables (Samsel and Seneff 2015). Further evidence of the connection between glyphosates and ASD comes from studies on glutamate/glutamine ratios. Glyphosates act as Manganese (Mn) chelators, which ultimately deplete the availability of Manganese. Manganese is a necessary cofactor in the conversion of glutamate to glutamine, and as such the reduction of Mn as a result of glyphosates results in an increase in the glutamate/glutamine ratio. Blood samples from children with ASD found increases in glutamate/glutamine ratios (Ghanizadeh 2013). Another study found that the ratio of glutamate/glutamine also affects the severity of ASD symptoms. High functioning ASD individuals had lower glutamate/glutamine ratios than the other ASD individuals (Shimmura, Suda et al. 2011). In cultured hippocampal cells in rats, glyphosate exposure delayed differentiation, reduced axonal

outgrowth and reduced dendritic branching (Coullery, Ferrari et al. 2016). Further studies found that glyphosate exposure down-regulated expression of Wnt family molecules involved in dendritic branching and outgrowth (Bodmer, Levine-Wilkinson et al. 2009), as well as neurite guidance in the cortex (Bodmer, Levine-Wilkinson et al. 2009). In the brains of postnatal rats, glyphosate exposure increased levels of PGE2, which our lab has previously implicated in the etiology of ASD (Wong and Crawford 2014, Wong, Wais et al. 2015).

Glyphosates may also contribute to the E/I imbalances observed in some ASD individuals. Both prenatal and postnatal exposure to roundup were found to impair glutamatergic signaling and suggested permanent E/I imbalances (Cattani, Cesconetto et al. 2017). Other studies have come to the similar conclusion that prenatal glyphosate exposure may contribute to the permanent E/I imbalances observed in ASD individuals (Rossignol and Frye 2012, Goines and Ashwood 2013)

2.3.3 Synthetic Fragrances

There is a strong correlation of the prevalence of ASD and use of perfumes and fragrances, with countries with high fragrance use like the USA and Japan having a higher ASD prevalence (Kawamura, Takahashi et al. 2008, Zablotsky, Black et al. 2015, Sealey, Hughes et al. 2016) than regions in the middle east where use of perfumes are banned (Al-Salehi, Al-Hifthy et al. 2009, Al-Farsi, Al-Sharbati et al. 2011). Unfortunately, the mechanisms by which synthetic fragrances may contribute to the etiology of ASD are not very well understood. A large factor limiting our understanding is the 1973 FDA Fair Packaging and labeling Act, which does not require the testing or labelling of ingredients within fragrances and cosmetics (Bagasra, Golkar et al. 2013). Research suggests that fragrances alter the expression of neuropeptides including oxytocin (OXT) and arginine vasopressin (AVP). Research *in vitro* in male and female fetal neuroblastoma cell lines has found a male specific loss of neurons containing OXT and AVP receptors following treatment with fragrances (Sealey, Hughes et al. 2016). Studies have reported significant reductions in plasma levels of OXT and AVP both in children with

ASD (Insel 2010, Meyer-Lindenberg, Domes et al. 2011, Xu, Shou et al. 2013) as well as the mothers of children with ASD (Xu, Shou et al. 2013).

2.3.4 Medications

The use of certain medications during pregnancy has been associated with increased risks of developing ASDs. Two major medications of interest are Valproic Acid (VPA) which is taken as an anticonvulsant or as a mood-stabilizer, and Acetaminophen/Paracetamol which is taken for pain relief. Studies in the 1990s discovered that use of VPA during pregnancy resulted in an increase in the occurrence of ASD in offspring (Roullet, Lai et al. 2013). Evidence has suggested that mechanistically, VPA-induced hyperacetylation of histones in the brain during development results in the abnormal formation of the brain and contributes to the development of ASD (Kataoka, Takuma et al. 2013). Further, VPA rodent models exhibit neuroimmune alterations in cytokines similar to those found in ASD individuals (Deckmann, Schwingel et al. 2018).

Acetaminophen is the recommended medicine for pain relief in pregnant women, and blocks the inflammatory pathway by inhibition of COX-2, subsequently reducing PGE2 levels (Werler, Mitchell et al. 2005, Lupattelli, Spigset et al. 2014).There is an association between exposure to acetaminophen prenatally (Avella-Garcia, Julvez et al. 2016, Liew, Ritz et al. 2016) and early postnatally (Schultz, Klonoff-Cohen et al. 2008, Bittker and Bell 2018) and the subsequent diagnosis of ASD. Several studies have also examined a dose-response relationship between acetaminophen use during pregnancy and the development of ASD symptoms and found a strong positive correlation between the two variables (Brandlistuen, Ystrom et al. 2013, Avella-Garcia, Julvez et al. 2016, Liew, Bach et al. 2016).

As previously discussed, (Section 2.2), lipid signalling during neurodevelopment is a tightly regulated process and disruptions have been implicated in neurodevelopmental disorders such as ASDs. Literature has also suggested that prenatal exposure to acetaminophen in humans can have pervasive

effects of PGE2 levels postnatally (Langhendries 2015). One explanation for the mechanism that acetaminophen may contribute to the etiology of ASD is by affecting the maternal immune activation. In rodent models and in humans, acetaminophen triggers immune and oxidative stress response pathways (Jetten et al., 2012, Da Silva et al., 2012), while suppressing COX-2 activity and subsequently PGE2 levels (Ghanem et al., 2016). Interestingly, fevers during pregnancy are associated with a higher risk of future ASD diagnosis (Zerbo et al., 2013; Brucato et al., 2017; Hornig et al., 2018). These findings further suggest that alterations in COX2/PGE2 signalling can occur through environmental factors and contribute to the etiology of ASD.

2.4 Neuropathologies in ASD

While the pathogenesis of ASD is not fully understood, some pathologies are frequently observed in the brains of ASD individuals. Early studies in ASD realized that head circumference and brain size was increased compared to age-matched controls, in a large subset of those with ASD (Sacco, Gabriele et al. 2015). Magnetic resonance imaging (MRI) of cortical areas between 6-12 months of age found that early enlargement of cortical surface area was a strong predictor of ASD diagnosis in infants, and that the rate of increase in cortical volume in the second year correlated with the severity of ASD symptoms in those diagnosed (Hazlett, Gu et al. 2017). One explanation for the increase in brain size is abnormalities in neurogenesis and neuronal migration, which is supported by the disorganization of both white and grey matter in ASD individuals (Wegiel, Kuchna et al. 2010, Stoner, Chow et al. 2014). Abnormal formation of minicolumns, which are functional structures comprised mostly of pyramidal projections within the neocortex are found in early onset ASD (Casanova, Buxhoeveden et al. 2002, Casanova 2006). For example, individuals with Asperger's syndrome have smaller, less dense minicolumns in the temporal region of the neocortex(Casanova, Buxhoeveden et al. 2002)

Perikaryal abnormalities, including differences in the volume and cytoplasmic contents of neurons are also common within the brains of ASD individuals. There is a large reduction in neuronal volumes across many brain regions of young children with ASD compared to age-matched control individuals (Wegiel, Flory et al. 2014, Wegiel, Flory et al. 2015). These differences are attributed to differences in developmental trajectories between ASD and control individuals; cell volume increases with age in ASD individuals but decreases with age in control populations (Wegiel, Flory et al. 2015). By adulthood perikaryal abnormalities are more similar (Wegiel, Flory et al. 2014) with the exception of some subsets of ASD including Rett syndrome (Wegiel, Flory et al. 2015). Cell density changes are unique between different subsets of ASD but also within different regions of the brain. For example, while cell density was unchanged in layers of the anterior superior temporal cortex (Kim, Camacho et al. 2015), lower cell densities were found in the septal subventricular zone in ASD individuals where epilepsy was not a comorbidity (Kotagiri, Chance et al. 2014).

Despite a large body of research dedicated to postnatal mechanisms of ASD, many of these changes in brain morphology suggest disruptions in mechanisms of prenatal development (Prem, Millonig et al. 2020). Understanding the contribution of prenatal developmental processes such as migration, dendritic outgrowth and synapse formation can provide novel information about the causes of ASDs.

2.4.1 Cell Migration in ASD

Morphological differences in the brains of ASD individuals can be attributed to abnormal migration during development. In humans, cells proliferate and migrate in an “inside-out” manner which produces the six neocortical layers with neurons that were created earlier migrating to the deepest cortical layers and those that were formed later migrating to more superficial layers (Sidman and Rakic 1973). Literature has provided evidence that chemokines, molecules involved in migration during neural development may serve as a potential biomarker for ASDs (Han, Cheung et al. 2017,

Heuer, Croen et al. 2019). Interestingly, chemokine levels in serum samples of ASD individuals are found in greater amounts than in control individuals or individuals with other neurodevelopmental disorders and are associated with ASD-associated behavioural deficits (Vargas, Nascimbene et al. 2005, Grigorenko, Han et al. 2008, Ashwood, Krakowiak et al. 2011, Suzuki, Matsuzaki et al. 2011). Loss of function of other genes important in neuronal migration including *Cullin3* (Morandell, Schwarz et al. 2021), *Cyfip1*(Haan, Westacott et al. 2021), and *Auts2* (Pang, Yi et al. 2021) are also associated with the development of ASD. These findings suggest that a hallmark of ASD individuals may be a disruption of normal migration during neurodevelopment. These findings are supported by post-mortem findings in the brains of ASD individuals, which have observed several structural abnormalities associated with abnormal migration including changes in region specific cell densities, a disorganization of neurons and 'inside-out' layering, and a general minicolumnar disorganization (Casanova, Buxhoeveden et al. 2002, Buxhoeveden, Semendeferi et al. 2006, Hutsler, Love et al. 2007, Wegiel, Kuchna et al. 2010, Stoner, Chow et al. 2014, Hashemi, Ariza et al. 2017).

2.4.2 Dendrite outgrowth in ASD

Another explanation for the morphological differences in the brains of ASD individuals is difficulties in dendritic pathfinding during development. Dendrites, exhibit a characteristic period of outgrowth in which there is an initial outgrowth of dendritic arborization and then pruning of inactive extensions (Knudsen 2004, Williams and Truman 2004). The formation and stabilization of the dendritic arbor is tightly regulated throughout development and involves the coordination of cytoskeletal elements including actin and microtubules (Skalecka, Liszewska et al. 2016). The increases in volume observed in the brains of ASD individuals may be explained by both overgrowth of extensions and a lack of activity-dependent pruning (Courchesne, Redcay et al. 2005). This hypothesis is supported by findings in both human and animal models of ASD. There are decreases in dendritic length in the visual cortex of *Nrxn1* knockout mice (Dudanova, Tabuchi et al. 2007), within the basal

ganglia of *Shank3* knockout mice (Peça, Feliciano et al. 2011), and within the hippocampus, the neocortex, and the primary motor cortex of *Mecp2* knockout mice (Jiang, Ash et al. 2013). Neurons within the hippocampus have reduced branching in ASD individuals relative to controls (Raymond, Bauman et al. 1996). There is reduced dendritic arborization in the neocortex (Belichenko, Oldfors et al. 1994) and within the hippocampus in CA1 (Chapleau, Calfa et al. 2009) of Rett's syndrome individuals. In post-mortem brain slices of ASD individuals, neurons show reduced dendritic branching in the hippocampus (Raymond, Bauman et al. 1996) and fewer dendrites within the prefrontal cortex (Mukaetova-Ladinska, Arnold et al. 2004) compared to control individuals.

Dendritic spine formation is also consistently affected in animal models of ASD and in ASD individuals. For example, the average volume of dendritic spines is decreased *Nlgn3* KI mice. While a deletion of *Shank3α* and *Shank3β,* was found to increase dendritic length, there was reduction in dendritic spine density in the hippocampus (Peça, Feliciano et al. 2011). In another model of *Shank3* dysfunction, with a c-terminus deletion, a decrease in dendritic spine density was observed. Observations in humans show increases in dendritic spine density in pyramidal cells of the frontal, temporal, and parietal cortex in ASD individuals relative to controls are observed (Hutsler and Zhang 2010).

2.5 The cerebellum in ASDs

2.5.1 Cerebellar Development

The anatomy and development of the cerebellum have been well documented across a range of mammalian models, including rodent models. The cerebellum is comprised of an assortment of neuron types including both excitatory glutamatergic neurons including granule, brush and eurydendriod cells and inhibitory GABAergic neurons including Purkinje, Golgi, stellate and Basket cells (Altman 1997, Butler and Hodos 2005). These neurons receive excitatory input from two types of afferent fibers:

climbing fibres which originate from the olivary nuclei and mossy fibers which originate from other precerebellar nuclei (Ito 2002). These neurons and afferent fibres are organized into three layers: Molecular layer (most superficial), Purkinje Cell layer (center), and Granule Cell layer (deepest layer). Within the molecular layer, Purkinje cell dendrites receive inputs from climbing fibers, and from granule and stellate cells. Within the granule cell layer, granule cells receive inputs from mossy fibers, and Golgi cells (Murakami and Morita 1987, Folgueira, Anadón et al. 2006, Ikenaga, Yoshida et al. 2006). The cerebellum can also be separated into three lobes with distinct functional representations(Makris, Hodge et al. 2003, D'Mello and Stoodley 2015). The floccular lobe, the most well conserved of the three lobes is often associated with postural and balance control. The anterior lobe is the second best conserved of the three lobes and is most prominently involved in muscle tone. The Anterior lobe is separated from the posterior lobe, the last lobe by the primary fissure. The posterior lobe is generally associated with coordination of movement. The cerebellum is formed through gestational and early postnatal development.

Neurogenesis within the cerebellum is cell type dependent, with the birth of different cell types being both spatially and temporally defined. Neurogenesis of the cerebellum typically commences from between G10.5-G13.5, with deep nuclear neurons and Purkinje cells being the first to form. (Miale and Sidman 1961, Pierce 1975, Altman and Bayer 1978). Purkinje cells migrate typically until around G13 and at G14 form a layer of cells several layers thick called the Purkinje Cell (PC) plate (Goffinet 1983, Yuasa, Kawamura et al. 1991). The external granular layer (EGL), which originates from the rostral half of the rhombic lip contains cells specified to become granule cell precursors. These begin to proliferate around G10-G11 and migrate to superficial regions of the cerebellum from G15 onward (Wingate and Hatten 1999, Lin, Cai et al. 2001). Golgi cells have a large period of neurogenesis ranging from G13.5 – postnatally but have a peak of neurogenesis between G14 – G16 (Sultan, Czubayko et al. 2003, Sudarov, Turnbull et al. 2011). Stellate and basket cells, both

GABAergic interneurons, originate from precursors in prospective what matter areas at later time points in development (G13 – PN5 with a peak around birth)(Leto, Bartolini et al. 2009, Fleming, He et al. 2013).

2.5.2 Cerebellar morphology in ASD

The cerebellum is one of the most frequently affected brain regions in individuals with autism (Courchesne, Redcay et al. 2005) and early disruption of cerebellar circuitry has been positively correlated with later diagnosis of ASD (Beversdorf, Manning et al. 2005, Limperopoulos, Bassan et al. 2007). There is a striking 36-fold increase in ASD diagnosis following the presence of cerebellar insults at birth (Figure 2-3) (Limperopoulos, Bassan et al. 2007, Wang, Kloth et al. 2014). Clinical cases have revealed that cerebellar lesions can lead to ASD (Schmahmann, Weilburg et al. 2007).

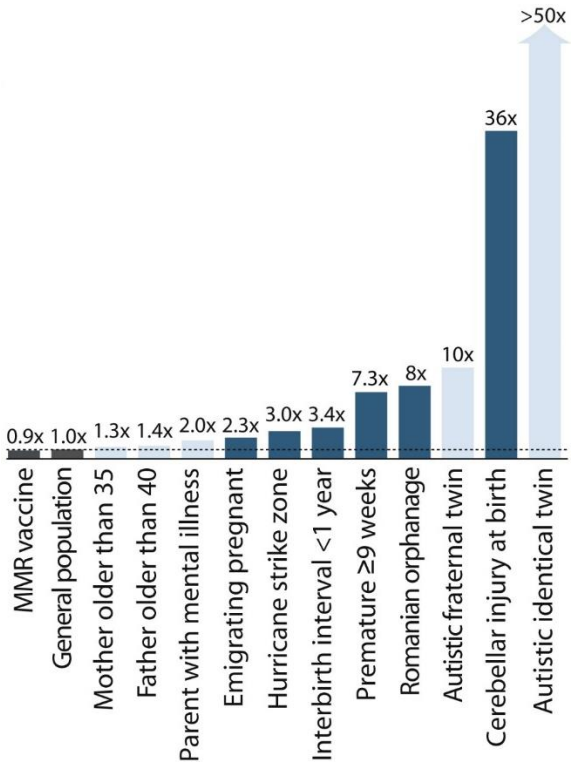

Figure 2-3: Risk ratios for ASD from environmental and genetic risk factors of ASD. Risk ratios were obtained from literature except for the highest four risk factors which were calculated based on the US population risk. Cerebellar injuries at birth present the highest risk not directly associated with genetic profiles. (Figure obtained from (Wang, Kloth et al. 2014))

Alterations in cerebellar morphology have been frequently described as a neuropathological feature of ASDs. There are consistent region-specific structural changes within the cerebellum across, various mouse models of ASD, including but not limited to fragile x, and Neurolgin-3 R541C knock-in mice (Ellegood, Pacey et al. 2010, Ellegood, Markx et al. 2014, Steadman, Ellegood et al. 2014). In humans, the mean size of Purkinje cells within the cerebellums of ASD individuals are smaller than in control populations. Subgroups of ASD individuals have reduced Purkinje cell density despite stable populations of other cells in the cerebellum . There is also a significant reduction in Purkinje cells, as well as abnormalities in cell orientation and organization of Purkinje cells in individuals with Fragile X syndrome (Greco, Navarro et al. 2011, Greco, Navarro et al. 2011). These results were confirmed in other ASD subjects, with findings of flocculondular dysplasia (Wegiel, Kuchna et al. 2013), cerebellar hypoplasia (Wegiel, Kuchna et al. 2010) and spheroids (Weidenheim, Goodman et al. 2001)

Changes in cerebellar volume are also associated with ASD. Literature suggests three distinct phases in which there are volumetric changes in the brains of ASD individuals postnatally (Courchesne, Redcay et al. 2005, Stigler, McDonald et al. 2011, Sacco, Gabriele et al. 2015). Despite normal brain volume at birth, there is an initial overgrowth phase within the first two years of life followed by an arrest of growth between years two and four. Studies have suggested that the initial phases in the cerebellum may be the consequence of insufficient pruning during development (Hoffman, Wright et al. 2016). Lastly, there is a consistent reduction in cerebellar volume of adolescent and adult ASD males (Bailey, Luthert et al. 1998, Courchesne, Karns et al. 2001, Webb, Sparks et al. 2009, Lange, Travers et al. 2015). Consistent with a reduction in cerebellar volume in ASD individuals, there is a reduction in gray matter and a smaller ratio of gray to white matter in children with autism compared to controls (Courchesne, Karns et al. 2001, Bolduc, du Plessis et al. 2012).

2.5.3 Cerebellar function in ASD

Initially known for its role in motor function, the cerebellum has become an established region of multimodal integration, allowing for the coordination of multisensory and sensorimotor brain regions (Ishikawa, Shimuta et al. 2015, Xiao and Scheiffele 2018). It has been suggested that during early developmental stages the role of the cerebellum is to process external sensory information and internal feedback to provide information to influence the direction of cortical maturation (Wang, Kloth et al. 2014). A study investigating functional connectivity between different brain regions in a large cohort of ASD and control individuals found that the cerebellums of ASD individuals exhibited hyperconnectivity to motor and sensory regions of the brain, despite these areas exhibiting underconnectivity to many other brain regions. In essence, this study as well as others have demonstrated that distal regions of the brain that are connected to the cerebellum may be also affected in cases of cerebellar dysfunction (Courchesne and Pierce 2005, Mosconi, Wang et al. 2015). The degree of hyperconnectivity to the cerebellum and the regions affected may explain some of the heterogeneity in ASD in both the penetrance and severity of symptoms.

Studies in both humans and animal models have shown ASD-related behaviours following disruption of the cerebellum. Bioinformatic analysis of data from the Mouse Genome Informatics bioinformatics resource, a database that captures data about experiments using mice to model human biology, have shown that many ASD-related genes are also associated with abnormal cerebellar motor behaviour (Meehan, Carr et al. 2011). Early disruption of cerebellar circuitry is positively correlated with the future development of ASD (Courchesne, Karns et al. 2001, Beversdorf, Manning et al. 2005, Limperopoulos, Bassan et al. 2007). Prenatal disruption of cerebellar circuitry has been found to result in ASD-related social deficits (Courchesne, Redcay et al. 2005, Chlebowski, Robins et al. 2013). (Boltshauser 2004, Geschwind and Levitt 2007). The effect of cerebellar insults has been modelled in rodents. Cerebellar lesions in rats result in abnormalities in social play, persisting behaviours, and

vocalization (Bobee, Mariette et al. 2000, Al-Afif, Staden et al. 2013). Tuberous Sclerosis 1 knock-out mice exhibit a loss in purkinje size and arborization and exhibit deficits in social interaction with other mice and in mother-pup interactions and increases in repetitive grooming behaviours (Tsai, Hull et al. 2012). The extent of the reduction in gray matter observed in the cerebellums of ASD children has been found to correlate with the severity of social interaction and communication deficits (Riva, Annunziata et al. 2013, D'Mello, Crocetti et al. 2015).

Interestingly, cortico-cerebellar, and cerebellar-spino tract disruptions have been observed in established genetic rodent ASD models. For example, in ASD mouse models lacking CAPS2, a protein involved in neurotrophin release, there are impairments in cerebellar development resulting in reductions in neuronal survival, differentiation and migration of postmitotic granule cells, and dendrite formation in Purkinje cells, gross structural abnormalities, and abnormal paired-pulse facilitation at Purkinje cell synapses (Sadakata, Kakegawa et al. 2007, Sadakata, Shinoda et al. 2012). Further, these mice also performed worse than controls in rotorod motor coordination and eye movement tests. In addition to ASD-related behaviours, mild cerebellar-related motor coordination deficits were also observed in the engrailed-2 (Brielmaier, Matteson et al. 2012), Fragile X (Koekkoek, Yamaguchi et al. 2005), *Mecp2* (Ben-Shachar, Chahrour et al. 2009), and neuroligin-3 (Baudouin, Gaudias et al. 2012) mouse models.

2.5.4 PGE2 and the Cerebellum

Studies have demonstrated that abnormal COX2/PGE2 signalling can affect proper cerebellar development and result in both changes in cerebellar morphology and function. Direct administration of PGE2 into the cerebellum of rats in the second week postnatally stunts the development of Purkinje cells including a reduction in arborization (Dean, Wright et al. 2012). In contrast the same group demonstrated that injection of COX-2 inhibitors into the cerebellums of rats during the same time point increases Purkinje cell arborization, dendritic spine density, and results in abnormal social behaviour in

males (Dean, Knutson et al. 2012). They suggested that the effect of PGE2 occurred only during a critical window during the second postnatal week, coinciding with the aromatase enzyme which is responsible for the conversion of androgen into estrogen. During this period, PGE2 levels were able to elevate estradiol concentrations and through a positive feedback loop the increases in estradiol impacted PGE2 levels similarly. In a recent study examining post-mortem human samples the same group demonstrated that a similar critical period of cerebellar development existed in humans (Wright, Hoffman et al. 2019). While findings in these studies demonstrate a postnatal critical window in which disruptions in PGE2 levels can result in abnormal cerebellar development, there is no information on the effect of prenatal disruption of PGE2.

2.6 Literature Review Summary

The prevalence of ASDs is increasing at an alarming rate, that cannot be explained by better diagnosis and testing alone. It is crucial that the mechanisms contributing to the development of ASDs are further investigated. Proper coordination of lipid signalling, including timing and levels of lipids is crucial during pre- and post-natal development for normal brain development. Throughout development lipid signalling is involved in processes including proliferation, migration, and synaptogenesis. Disruptions in lipid signalling can occur as a result of an assortment of genetic or environmental factors, that are associated with ASDs. As indicated above, clinical, and epidemiological studies provide sufficient evidence that disruptions in the COX2/PGE2 signalling during neurodevelopment contributes to the etiology of ASD. The increase in prevalence as well as the broad range of phenotypes associated with ASD may be explained by the impact of numerous environmental risk factors during different critical periods . Changes in cytoskeletal dynamics can provide new information on the mechanisms by which PGE2 signalling contributes to the neuronal pathologies such as changes in dendritic and dendritic spine morphology, which are common within the brains of ASD individuals. While literature provides evidence that disruptions in PGE2 signalling during development

may contribute to ASDs, more research is required to understand the underlying molecular mechanisms, and the direct consequences of lipid dysfunction on behaviour. A deeper understanding may help guide preventative measures, and provide tools for more efficient and earlier diagnoses, for ASD individuals.

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Chapter 3: Objectives and Hypotheses

3.1 Overall Objectives and Hypothesis

The studies in this dissertation aim to address the overarching objective:

To identify the molecular mechanisms by which abnormal COX2/PGE2 lipid signalling can affect cerebellar development in a sex-dependent manner and contribute to ASD related pathologies.

Overall Hypothesis: Dysregulation of the COX2/PGE2 signalling pathway disrupts normal cerebellar development resulting in molecular and behavioural ASD-associated changes in a sex-dependent manner.

To address the overarching objective, the following specific objectives were examined:

Using a neuroectodermal NE-4C *in vitro* cell model (Study 1):

1) To examine the effect of PGE2 exposure on cytoskeletal processes including neurite outgrowth and neurite pathfinding
2) To examine differences in the effect of PGE2 across days following the induction of differentiation to determine critical periods of PGE2 on differentiation.
3) To investigate whether PGE2-PKA signaling regulates the observed morphological changes

Using *in vivo* mouse models with high (PGE2-injected) and low (COX-2^{-}-knockin (KI)) levels of PGE2 (Studies 2 and 3):

4) To verify *in vitro* findings and to further study the consequences of disruptions in COX2/PGE2 signalling on cytoskeletal dynamics through an examination of dendritic morphology and the expression of cytoskeletal proteins
5) To describe cerebellar-related motor dysfunction in mice prenatally exposed to PGE2.
6) To determine sex-dependent differences in the effect of COX2/PGE2 disruptions in the observed morphological and behavioural changes.

3.2 Experimental Models

Mice are frequently used as to model processes in neurodevelopment and to better understand the basis of neurodevelopmental disorders. Mice not only have similar genomes, but undergo similar

developmental processes, and exhibit analogous behaviours to humans. In this dissertation, both an *in vitro* murine cell line (Study 1), and an *in vivo* mouse animal model (Studies 2, and 3) are used.

In vitro model system for Study 1: Neuroectodermal (NE-4C) stem cells obtained from the American Tissue Culture Collection (ATCC) were obtained from primary brain cell cultures of prenatal day 9 mouse embryos. The entirety of the gestational period of mice is around 20 days (Lanman and Seidman 1977, Xu, Barnes et al. 2010) with cells becoming multipotent neural stem cells at around prenatal days 8-9 of development (Schlett and Madarász 1997). These cells undergo processes that occur in the developing brain including pathfinding, neurosphere formation, and differentiation (Kelava and Lancaster 2016). These similarities make NE-4C cells an appropriate model of neuronal differentiation.

In vivo model system for Studies 2 and 3: In this dissertation two mice models are used to study the molecular, cellular, and behavioral consequences of a disruption of COX2/PGE2 signaling during neuronal development. Increased PGE2 levels were modelled in mice offspring prenatally exposed to PGE2 and decreased PGE2 levels were modelled in mice deficient in the PGE2 producing enzyme COX-2.

B6.129S6(FVB)-Ptgs2tm1.1Fun/J mice, also known as COX-2^{-}-KI mice are the mouse model used in study 2. They are a model of reduced PGE2 levels with endogenous levels of PGE2 reduced by half in COX-2^{-}-KI mice compared to control mice. COX-2^{-}-KI mice were generated by a targeted point mutation of the *Ptgs2* gene resulting in a substitution of the Y385F amino acid, leading to inactivation of cyclooxygenase activity but maintaining peroxidase activity (Yu, Fan et al. 2006). Founder COX-2^{-}-KI mice were backcrossed for at least 5 generations to wild-type 129S6/SvEvTac mice to generate the COX-2^{-}-KI mice. Our lab has previously shown that COX-2^{-}-KI mice exhibited ASD-related

behaviours including deficits in social interaction, restrictive and repetitive behaviours, and anxious behaviours (Wong, Bestard-Lorigados et al. 2019).

PGE2-exposed mice were used in study 3. PGE2-exposed mice were generated through the subcutaneous injection of C57BL/6 pregnant dames on gestational day 11 (G11) with 16, 16-dimethyl prostaglandin E2 (dmPGE2). dmPGE2 was chosen as it is a stable analogue of PGE2 that is often used for *in vivo* models of COX2/PGE2 disruption (Ohno, Morikawa et al. 1978, Cruz Duarte, St-Jacques et al. 2012, Cook, Thomas et al. 2016) which mimics increases in PGE2 that were previously associated with ASDs. Pregnant dames were injected at G11 as this day marks the onset of neurogenesis in the mouse brain (Zhang and Jiao 2015). This time point corresponds to previous clinical studies in humans examining the misuse of misoprostol, a synthetic analogue to PGE2, during pregnancy which resulted in the manifestation of Mobius syndrome and ASD-related symptoms (Costa 1998, Gonzalez, Marques-Dias et al. 1998, Pastuszak, Schüler et al. 1998, Schüler, Pastuszak et al. 1999, Bandim, Ventura et al. 2003, Miller, Strömland et al. 2005, Bos-Thompson, Hillaire-Buys et al. 2008). Our lab has previously shown ASD-related behaviours in PGE2-exposed mice including social deficits, restricted and repetitive behaviors, and anxious behaviours (ref PGE2 behaviour).

It should also be noted that for all *in vivo* studies, sex is considered a main effect when collecting and analyzing data. Given evidence for of the sexually dimorphic nature of neurodevelopmental disorders, we wished to examine whether any of the effects of PGE2 we observed were sex dependent. Thus, in study 2 we used male and female COX-2^{-}-KI and WT mice, and in study 3 we used both male and female PGE2-exposed male and female mice.

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Chapter 4: Study 1

Study 1: Prostaglandin E2 increases neurite length and the formation of axonal loops, and regulates cone turning in differentiating NE4C cells via PKA

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The dissertation was written in thesis format and the following is specific to the contributions in the chapter:

Contributions: Ashby Kissoondoyal designed, and performed all experimental work, analyzed the data, and prepared the manuscript. Dorota A. Crawford supervised the design of the study and was involved with editing the manuscript

4.1 Introduction

Membrane phospholipids are crucial sources of lipid signaling molecules necessary for healthy brain development. Prostaglandin E2 (PGE2) is one of the major bioactive lipids within the brain. PGE2 is synthesized from arachidonic acid (AA), which is released from the phospholipid bilayer through the action of phospholipase A2 (Sang and Chen 2006). AA is then converted into PGE2 through the activity of cyclooxygenase 1 or 2 (COX-1, -2) (Park, Pillinger et al. 2006, Rouzer and Marnett 2009). COX-1 and -2 are constitutively expressed within the brain primarily in microglial and neuronal cells, respectively (Maslinska, Kaliszek et al. 1999, Schwab, Nguyen et al. 2000, Hoozemans, Rozemuller et al. 2001, Norregaard, Kwon et al. 2015, Kirkby, Chan et al. 2016). PGE2 exerts its diverse role in the brain through four G-protein coupled E-prostanoid (EP 1-4) receptors (Khan, Bhagat et al. 2019). COX2/PGE2 signalling is involved in important processes during brain development, including proliferation and migration (Wright and McCarthy 2009), dendritic spine formation (Burks, Wright et al. 2007), and synaptogenesis and synaptic plasticity (Yang 2009).

Growing evidence from clinical studies provides a link between abnormal signaling of the COX2/PGE2 pathway and neurodevelopmental disorders such as Autism Spectrum Disorders (ASD). Various environmental insults including maternal infection, air pollution, or common over-the-counter medications such as acetaminophen and acetylsalicylic acid are all known to affect PGE2 levels during development(Schwab, Nguyen et al. 2000) (Tamiji and Crawford 2010, Wong and Crawford 2014) and have been associated with increased prevalence of ASD (Hodges, Fealko et al. 2020, Yoon, Choi et al. 2020). *In vitro* and *in vivo* research in our lab has already provided substantial evidence for potential molecular mechanisms by which changes in the PGE2 levels (increased or reduced) can lead to abnormal neuronal development. For example, in murine neuroectodermal (NE4C) stem cells and Neuro-2a cell lines we showed that PGE2 increased proliferation, migration, and differentiation of cells, elevated intracellular calcium levels within the cytosol and growth cones and affected the expression of various developmental genes (Tamiji and Crawford 2010, Davidson, Wong et al. 2016,

Wong, Ussyshkin et al. 2016). We also demonstrated that short-term exposure (up to 24 hours) to PGE2 in differentiating NE4C cells and prenatal exposure to PGE2 in mice resulted in a cross talk between PGE2 signaling and the Wnt canonical pathway through PKA and PI-3K (Wong, Ahmad et al. 2014, Rai-Bhogal, Wong et al. 2018). In addition, we have confirmed in mice that maternal exposure to PGE2 during pregnancy had significant changes in the expression of Wnt-target genes *Mmp7*, *Wnt2*, and *Wnt3a* in offspring during prenatal and early postnatal stages (Rai-Bhogal, Wong et al. 2018). Using microarray analysis, we also found that COX-2 deficient mice had abnormal regulation of many genes involved in proper neuronal development at embryonic day 16 and 19 (Rai-Bhogal, Ahmad et al. 2018). Further studies in the PGE2 injected and COX-2 deficient mice models also showed sex-dependent manifestation of autism-related behaviours (Wong, Bestard-Lorigados et al. 2017, Wong, Bestard-Lorigados et al. 2019).

Our previous study showed that PGE2 accelerates differentiation of NE4C cells, inducing earlier formation of neuronal clusters called neurospheres via a PKA-dependent mechanisms (Wong, Ahmad et al. 2014). This study further investigates whether PGE2/PKA signaling affects neurite outgrowth in differentiating NE4C cells. Here we demonstrate that PGE2 affects morphological features known to be influenced by actin cytoskeleton, including *neurite length*, *growth cone turning*, and the *formation of axonal loops* via PKA-dependent mechanisms. We also show that PGE2/PKA signaling increases the total level of actin-binding protein spinophilin, and its phosphorylation at ser-94. We propose that PGE2 can influence neuronal differentiation via PKA-dependent phosphorylation of actin bound proteins.

4.2 Methods

4.2.1 Cell Culture

Mouse neuroectodermal NE-4C stem cells were obtained from the American Tissue Culture Collection (CRL-2925,ATCC, Manassas, VA). Cells were plated on 0.01% poly-L-Lysine (Sigma) coated 60mm culture plates (BD Falcon). The cells were maintained in Minimum Essential Media (MEM) which was supplemented with 10% Fetal Bovine Serum (FBS), 2mM L-glutamine, and 1x penicillin – streptomycin (Invitrogen), which was changed every 2 days. Plates were kept in an incubator maintained at 5% [CO_2] with 95% humidity at 37˚C.

4.2.2 Cell Differentiation

NE-4C stem cells were passaged by dissociation using 0.05% trypsin-EDTA, followed by pelleting and resuspension in MEM+FBS (Minimum essential media and Fetal bovine serum; as described above), and then incubation overnight. Differentiation was induced with Serum-Free Media (SFM), which consisted of neurobasal media, supplemented with 1x B-27, 2mM L-Glutamine, and 1x penicillin-streptomycin as previously described in Wong et al (2016). Treatments were incubated in either SFM alone (Control), SFM supplemented with 1 µM PGE2 (Cayman Chemical, Ann Arbor MI), 10 µM Forskolin, 10 µM KT5720, a combination of 1 µM PGE2 and 10 µM KT5720 (PGE2 + KT5720) or a combination of 10 µM Forskolin and 10 µM KT5720 (Forskolin + KT5720).

4.2.3 Cell Morphology Imaging and Analysis

Images were taken using a Nikon Eclipse Ti-E microscope (Tokyo, Japan) on days 8, 10, and 12 from three separate biological replicates for each day. The images were used to quantify *neurite length, growth cone turning*, and *axonal looping* on each day of differentiation. *Neurite length* was defined as the distance from the base of an extension to the furthest reaching filopodia. Only the longest neurite was measured for any given cell. *Growth Cone Turning* was assessed through the shape of each growth cone. We use a novel approach to categorizing growth cones here. Growth cones were

classified as turning or not-turning, based on the symmetry of each growth cone. Firstly, the midpoint of the growth cone base was determined, and a line was drawn from the midpoint to the furthest reaching filopodia to bisect the cone. The area ratio of the larger portion of the bisected growth cone to the smaller was taken. Growth cones with a ratio > 1.4 were classified as asymmetrical and therefore turning, while those with a ratio < 1.4 were classified as *symmetrical*, and therefore not turning. *Axonal looping* was measured as the number of neurites forming axonal loops out of the total number of neurites examined. While normally categorized subjectively, we classified axonal loops based on the greatest exterior angle observed along the neurite. If this angle exceeded 270°, the neurite was categorized as forming an axonal loop.

4.2.4 Western Blot Analysis

Cell samples were collected using the NucleoSpin RNA/Protein isolation kit (Machery-Nagel), on cells after 8,10 and 12 days following the induction of differentiation from 3 biological replicates collected at different dates. For all analyses 25µg of protein was loaded into a 12% polyacrylamide gel (PAGE) and were electrophoresed at 100v for 1.5 hours. Gels were transferred to nitrocellulose membranes.

Total protein isolation was performed using the NucleoSpin RNA/Protein Kit (Macherey-Nagel), on cells after days 8, 10 and 12 following the induction of differentiation. PAGE gel electrophoresis was used to separate the isolated samples and then transferred to 0.45µM nitrocellulose membranes (Biorad). Membranes were blocked in 5% milk in 1X Tris buffer saline 0.05% Tween 20 (TBS-T) for 1h at room temperature and then probed with primary antibodies diluted in 2% milk to the same concentration across all runs. Protein samples were probed with anti -P - ser-94 spinophilin (RU499 ,1:1000; obtained from the Greengard laboratory; (Hsieh-Wilson, Benfenati et al. 2003)), mouse monoclonal anti-B-Actin (Abcam;1:10000; ab8245, Cambridge, MA,USA) and mouse monoclonal anti-GAPDH (Abcam,1:10000; ab8245, Cambridge, MA, USA) or anti-spinophilin (Cell

Signalling, 1:1000, mAb #14136, Danvers, MA, USA). Membranes were rinsed 5 times in 1x TBS-T before probing with appropriate HRP-tagged secondary antibodies. Goat-anti-rabbit(Abcam,1:10000, ab6276, Cambridge, MA, USA), was incubated overnight, and Goat-anti-mouse (1:10000; catalog No. ab6789) was incubated for 2h. Membranes were then washed 3 times for 5min in TBS-T and then incubated with ECL Prime Western Blotting Detection Reagent (BioRad). Membranes were imaged with the Geliance 600 Imaging System (Perkin Elmer). For quantification, protein signal intensity was first normalized to GAPDH signal intensity. The relative protein expression was then normalized to the control cell treatment (protein expression in control cells = 1).

4.2.5 Statistical Analysis

Statistical analyses were performed using the core open source software R (R Core Team 2013). All continuous data including neurite length, axonal loop formation, and western blot analysis was analyzed by use of a one or two-way ANOVA followed by TukeysHSD post-hoc comparisons to determine morphological differences and differences in protein expression levels and visually displayed using a violin plot to depict the probability distribution of data within a given treatment. Significance was determined as $p < 0.05$ for all tests. Numerical data in figures is presented as mean ± standard deviation (SD). Data for growth cone turning was analyzed through generalized logistical modelling and data in the tables is presented as Odds ratios (95% confidence interval). In all figures *n* represents the number of individual measurements made for a given treatment. All measurements were taken from 3 independent biological replicates. Total sample size was calculated based on morphology analysis using G*Power 3 software (Faul, Erdfelder et al. 2007). Effect size was set to 0.25 and a sample size of 269 was determined to reach a power of 0.8. Images were taken such that a minimum of 68 measurements could be performed for each test. However, all cells were measured for each image taken.

4.3 Results

4.3.1 Prostaglandin E2 increased neurite extension length via PKA-dependent mechanism

We previously established that neuroectodermal (NE-4C) stem cells go through specific stages of neuronal differentiation, including proliferation (Day 2), aggregation and inward migration (Day 4-6) and formation of neuronal clusters (neurospheres) followed by neurite outgrowth (Days 8-12) (Davidson, Wong et al. 2016, Wong, Ussyshkin et al. 2016). We showed that PGE2 increased proliferation of NE-4C stem cells and accelerated their differentiation into neuronal-lineage cells, including formation of neurospheres and expression of differentiation marker *Oct4* prior to day 8 of differentiation (Wong, Ussyshkin et al. 2016). In this study, we further examined the effect of PGE2 on neurite outgrowth on days 8-12 and investigated whether the effects were PKA-dependent. *Neurite length* was quantified by measuring the total distance between the base of the neurite, to the furthest filopodial extension (Figure 1A and methods).

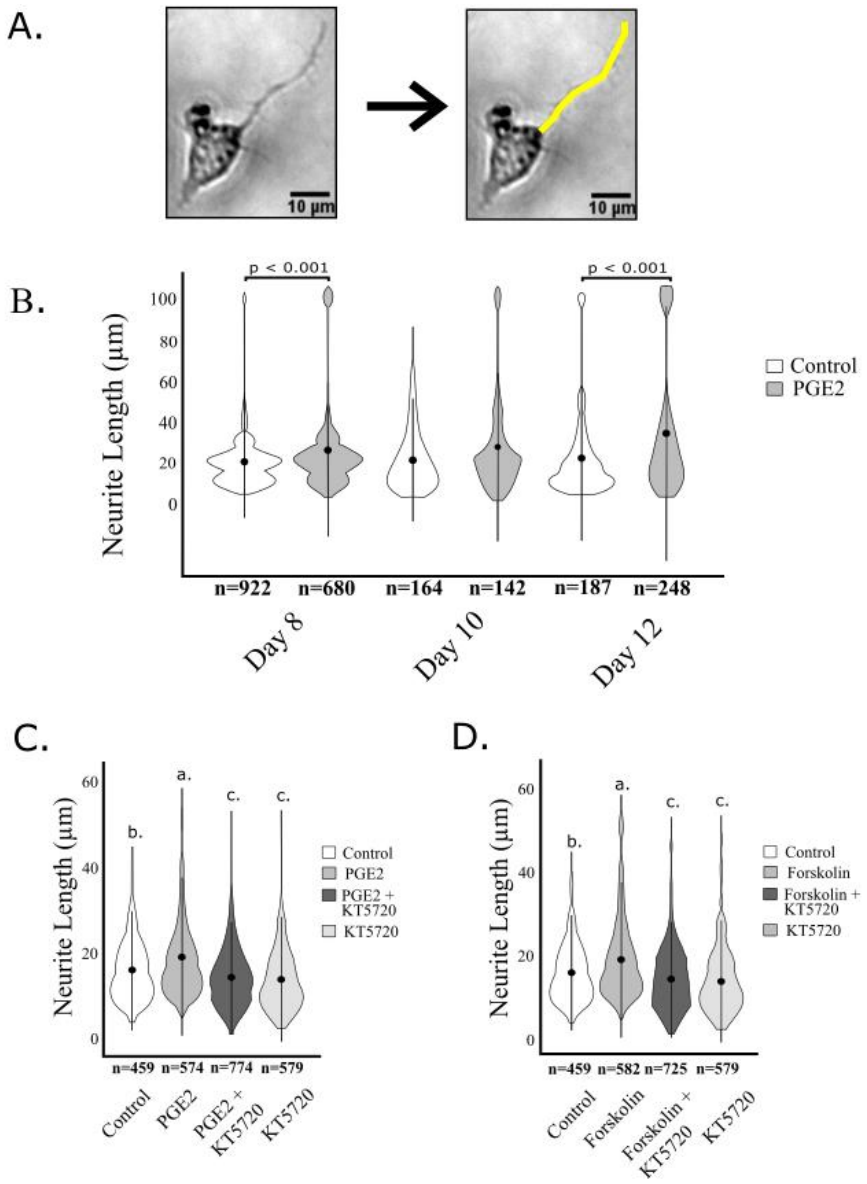

Figure 4-1. Average Neurite length of differentiated NE4C cells: NE4C cells were differentiated over 12 days and neurite length was quantified in µM (±SD). N-values shown represent the number of neurites measured for each treatment. (A) Representative images of neurite length measurement in a differentiated cell on day 12. (B) Treatment with PGE2 across days 8, 10 and 12 shows that PGE2 increases neurite length on days 8 and 12 compared to control cells. Significance is indicated as ***($p<0.001$) Cells treated with PGE2 (C) and Forskolin (D) were treated with KT5720 to determine PKA dependence. Significant differences are indicated with different letters. Cells treated with PGE2 showed similar trends to those treated with Forskolin on day 8. The effects of both treatments were attenuated by the addition of KT5720.

A two-way ANOVA was performed, comparing neurite length between treatments (Control and PGE2) across days (8,10, and 12). We found a significant interaction between day and condition ($F_{2,2437}$ = 4.056, n = 2343 $p < 0.05$). There were also significant differences for the day main effect ($F_{2,2437}$= 17.782, $p < 0.001$), with overall differences observed between day 8 and day 12 ($p < 0.001$) and between day 10 and day 12 ($p < 0.01$). However, no significant difference was found between days 8 and 10 ($p = 0.167$), suggesting a period of growth occurring predominantly between days 10 and 12. We also found differences for the main effect of treatment on neurite length ($F_{1,2437}$ = 48.635, $p <0.001$).

Given the significance of the interaction, as well as the significance of the treatment effect further pairwise comparisons within each day were performed. On day 8, neurites in cells treated with PGE2 were significantly longer than in control cells, with lengths of 23.42 µm and 18.92µm respectively (Figure 1B; $p < 0.001$). On day 10 of differentiation, cells treated with PGE2 had neurites 24.89 µm long, compared to control cells (19.81µm) but the difference did not reach significance ($p = 0.119$). By day 12, neurites of cells treated with PGE2 measured 31.82 µm long, which was significantly longer than the neurites in control cells at 21.15 µm ($p <0.001$). It appears that the effects of PGE2 on neurite length are dependent on the day of differentiation. Calculating rate of increase as Δ neurite length / Δ days., we observed that in the PGE2 treated cells the rate of neurite length increase per day (see methods) followed different trajectories as compared to the control cells. Between day 8 and 10 the rate of neurite length increase was 0.45µm per day in the control and it was 1.5 faster in the PGE2 cells at 0.74 µm per day. However, between days 10 and 12 the increase in neurite length rate was 0.67µm per day in the control cells whereas in PGE2 cells the rate of increase was 5 time greater at 3.47 µm per day (Figure 1B). Overall, we observed that the PGE2 treatment increased the neurite length across differentiation. Moreover, the neurite length in control cells increased at a relatively

constant rate during differentiation whereas in PGE2 treated cells the neurite length increases at a higher rate in later days of neuronal differentiation.

In this study we investigated whether PGE2-PKA mechanisms regulated neurite length extension using the PKA blocker KT5720 (Figure 1C). A one-way ANOVA was performed to identify differences between the treatments (Control, PGE2, PGE2+KT5720, and KT5720) on day 8 of differentiation and significant differences were found between treatments ($F_{3,2382}$= 52.95, n = 2386, p <0.001). Based on main effect significance, further pairwise comparisons between treatments were performed. We showed that neurite length was significantly higher in PGE2 treated cells at 19.05 µm compared to control cells at 15.89 µm ($p<0.001$). Moreover, as compared to the length in PGE2 treated cells, the addition of KT5720 significantly reduced the neurite length to 14.39 µm in PGE2+KT5720 treated cells ($p<0.001$), and KT5720 treated cells at 13.79 µm ($p<0.001$) suggesting a PGE2/PKA effect on neurite length. Both cells treated with KT5720 alone, or a combination of PGE2+KT5720 had neurite lengths that were significantly reduced relative to control cells ($p<0.001$)

To confirm that the neurite elongation observed in the PGE2 treated cells was PKA-dependent, we examined differentiated NE4C cells with the addition of forskolin (Figure 1D). A one-way ANOVA was performed comparing treatments (Control, Forskolin, Forskolin+KT5720, and KT5720) and a significant difference was found between treatments ($F_{3,2341}$= 29.85, n = 2345, $p<0.001$). We found that cells treated with Forskolin, had neurites that were 18.96 µm, which were significantly longer than neurites in control cells at 15.89 µm ($p <0.001$). Further cells treated with Forskolin had longer neurites than those treated with, Forskolin + KT5720 with neurites 14.79 µm long ($p<0.001$), or KT5720 alone with neurites 13.79 µm long($p<0.001$), mimicking results seen in PGE2 treated cells and showing the influence of PKA in neurite growth (Figure 1D). We also found that cells treated with KT5720 alone were significantly shorter that the control cells ($p<0.01$), and not significantly different than cells treated with Forsk.+KT5720 ($p = 0.277$) again suggesting a basal involvement of PKA in

neurite elongation. Additional experiments with another PKA inhibitor H89 show the same overall outcomes and are presented in the supplementary Figure S1.

4.3.2 Prostaglandin E2 affects growth cone symmetry

Growth cone turning is considered an essential behaviour for navigating axons (Gomez and Letourneau 2014). Growth cones often oscillate between symmetrical and asymmetrical shapes, which is an indicator of the navigational state and turning properties (Goodhill, Faville et al. 2015). In this study, we evaluated whether growth cone turning was affected by PGE2/PKA mechanisms during differentiation by examining the proportion of growth cones that were symmetrical vs. asymmetrical. *Asymmetrical* growth cones were assigned to a "turning" group and those that were *symmetrical* were assigned to a "non-turning" group. The proportion of growth cones turning was calculated as the number of growth cones turning to the total number of growth cones measured (Figure 2 and methods).

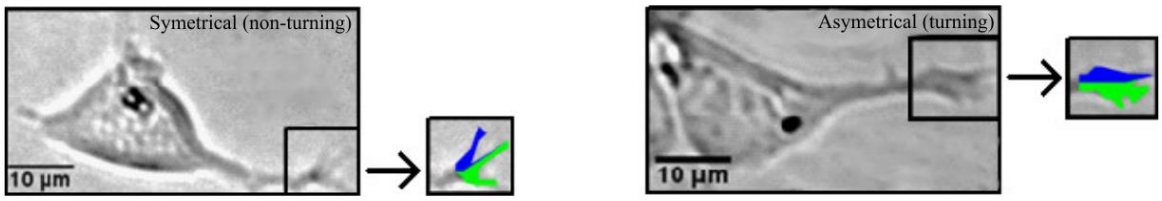

Figure 4-2: Measurement of turning growth cones. NE4C cells were imaged, and the symmetry of each growth cone was used to classify each as turning (asymmetrical) or non-turning (symmetrical).

Generalized logistic modelling was used to examine the odds of observing a turning (asymmetric) growth cone following treatment with PGE2 on days 8, 10 and 12 (see methods). Our model converged at an AIC of 629.4285 and our intercept (Control cells on day 8) was found to be significant with an odds ratio of 2.00 ($p < 0.05$) (Table 1A). PGE2 exposure significantly reduced the odds of observing turning growth cones with an odds ratio of 0.23 ($p < 0.001$). Examining the effect of day of differentiation, we saw no significant change in odds for control cells on day 10, and a decrease

in the odds of observing turning growth cones on day 12 with odds ratios of 2.21 ($p = 0.790$) and 0.68 ($p < 0.01$) respectively. Interestingly, in contrast to the decrease in the odds of observing turning growth cones in PGE2 exposed cells on day 8, the odds significantly increased in cells treated with PGE2 on days 10 and 12 with odds ratios of 10.13 ($p < 0.01$) and 64.34 ($p < 0.001$) respectively.

Table 4-1: Odds of observing turning growth cones between PGE2 and Control treated cells between days 8-12

Treatment - Day	OR (95% CI)	P-Value
Control – Day 8	2.00 (2.72 – 1.47)	**2.36E-02**
Control– Day 10	2.21 (3.24 – 1.51)	7.90E-01
Control – Day 12	0.68 (1.00 – 0.463)	**5.43E-03**
PGE2 – Day 8	0.23 (0.373 – 0.143)	**6.86E-06**
PGE2 – Day 10	10.13 (17.79 – 5.77)	**3.96E-03**
PGE2 – Day 12	36.32 (64.37 – 20.50)	**3.99E-07**

We further examined the contribution of PKA to the effects on growth cone turning on day 8, using KT5720. We used generalized logistic modelling to determine if any of our treatments (Control, PGE2, PGE2+KT5720, or KT5720) affected the odds of observing turning growth cones (Table 1B). Our model converged with an AIC of 485 and our intercept (Control cells) had a significant odds ratio of 2.18 ($p < 0.01$). As expected, PGE2 exposure reduced the odds of observing turning growth cones with an odds ratio of 0.55 ($p < 0.001$). The addition of KT5720 rescued growth cone turning. There was no significant effect of PGE2+KT5720 or KT5720 alone on the odds of observing turning growth cones, with odds ratios of 3.05 ($p = 0.27$) and 1.68 ($p = 0.47$) respectively. This suggests that growth cone turning is supressed by PGE2-PKA mechanisms.

Table 4-2: Odds of observing turning growth cones on day 8 for control cells and cells treated with PGE2, PGE2+KT570, or KT5720

Treatment	OR (95% CI)	P-Value
Control	**2.18 (2.82 – 1.69)**	**2.44E-03**
PGE2	**0.55 (0.78 – 0.39)**	**9.15E-05**
PGE2 + KT5720	3.05 (4.15 – 2.25)	2.74E-01
KT5720	1.68 (2.41 – 1.17)	4.69E-01

We used forskolin to further verify the PGE2/PKA effect on growth cone turning. We used generalized logistic modelling to compare the effect of our treatments (Control, Forskolin, Forskolin + KT5720, and KT5720) on the odds of observing turning growth cones (Table 1C). Our model converged with an AIC of 469 and our intercept (Control cells) had a significant odds ratio of 2.18 ($p < 0.01$). Similar to PGE2 exposed cells, there was a decrease in the odds of observing turning growth cones in forskolin treated cells with an odds ratio of 0.43 ($p < 0.001$). We again observed that the addition of KT5720 rescued growth cone turning. Treatment of cells with Forskolin + KT5720 or KT5720 alone did not significantly affect the odds of observing turning growth cones with odds ratios of 1.90 ($p = 0.66$) and 1.91 ($p = 0.72$) respectively. These findings further confirm that PGE2/PKA signaling can reduce growth cone turning in differentiating NE-4C cells.

Table 4-3: Odds of observing turning growth cones on day 8 for control cells, and cells treated with Forskolin, Forskolin + KT5720, or KT5720 alone

Treatment	OR (95% CI)	P-Value
Control	**2.18 (2.82 – 1.96)**	**2.44E-03**
Forskolin	**0.43 (0.62 – 0.30)**	**6.71E-06**
Forskolin + KT5720	1.90 (2.59 – 1.40)	6.59E-01
KT5720	1.68 (2.41 – 1.17)	4.69E-01

4.3.3 Prostaglandin E2 increases the incidence of axonal looping in differentiating cells

Axonal outgrowth and guidance are key to critical neurodevelopmental processes *in vivo* including early cell migration, and synapse formation (Raper and Mason 2010, Stoeckli 2018). Aberrations in axonal outgrowth and navigation can lead to self-fasciculation, in which axons turn inwards and adhere to themselves (Mondal, Black et al. 2014). This process can be visually observed through the presence of axonal loops and has been reported in culture cells (Davis, Merrison-Hort et al. 2017, Smit, Fouquet et al. 2017). In this study, we examined whether PGE2 can influence axonal loop formation during differentiation on days 8-12. We report the percentage of extensions forming loops out of the total number of neurites quantified. Axonal loops are defined as a neurite of any length that turned more than 270° regardless of if the neurite continued to elongate (Figure 3A).

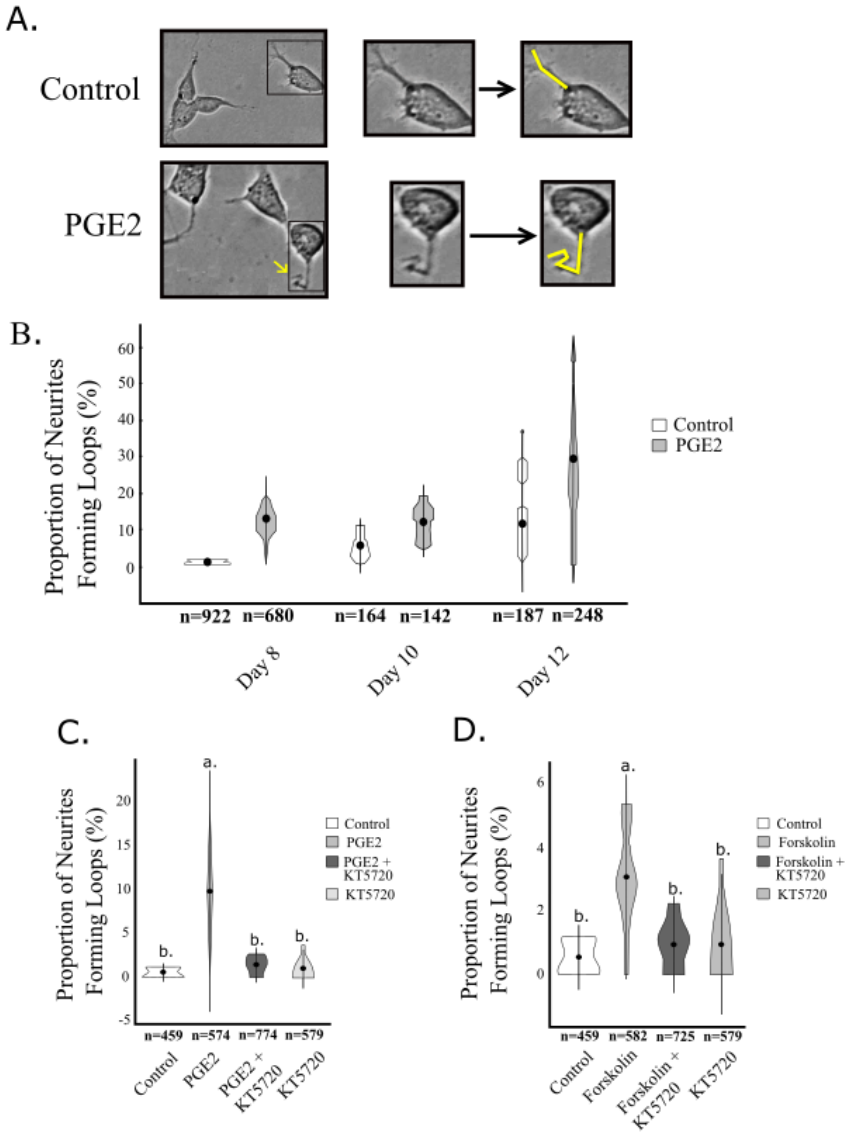

Figure 4-3: Percentage of axonal loops formed out of total neurites. NE4C cells were imaged during days 8, 10 and 12 during differentiation and the proportion of neurites forming loops of all cells measured was determined. Results are shown as a percentage of neurites forming loops out of the total number of neurites measured (±SD). N values, representing the total number of neurites measured for each condition are shown above the x-axis. (A) Representative images of control cells and PGE2 treated cells on day 8 of differentiation showing an example of typical neurites and neurites forming a loop, respectively. (B) Cells treated with PGE2 had a higher percentage of neurites form loops on day 12 compared to control cells as well as cells treated with PGE2 + H89 on both days 10 and 12. (C) Cells treated with PGE2+H89 formed significantly less loops than control cells on day 12. Between days, cells treated with PGE2 showed an increased in the percentage of neurites forming loops between days 10 and 12 but not between days 8 and 10. (D) Differences between cells treated with Forskolin and control cells were not observed. On day 12, cells treated with Forskolin had a higher percentage of neurites form loops than cells treated with a combination of Forskolin+H89.

We performed a two-way ANOVA comparing the mean percentage of loops observed turning between control and PGE2 treated cells, across days 8, 10, and 12 of differentiation (see methods). We observed no significant interaction between treatment and days ($F_{2,85}$= 1.699, n = 2343, p < 0.189). However, the main effect of day was significant ($F_{2,85}$= 18.366, p < 0.001), with no significant difference between days 8 and 10 (p = 0.982) but with significant differences between 10 and 12 (p <0.001) and 8 and 12 (p < 0.001) (Figure 3B). There was also a significant main effect of treatment observed ($F_{1,85}$ = 29.474, p <0.001), indicating that exposure to PGE2 can induce the formation of axonal loops *in vitro*.

To determine if the effect of PGE2 on the formation of axonal loops was PKA dependent we used KT5720. We performed a one-way ANOVA comparing the average proportion of axonal loops formed on day 8 between treatments (Control, PGE2, PGE2 + KT5720, KT5720) and found a significant effect between treatments ($F_{3,43}$ = 17.89, n= 2386 p < 0.001) (Figure 3C). The proportion of neurites forming loops in PGE2 treated cells was 9.7% compared to 0.5% in control cells (p < 0.001). Compared to PGE2 treated cells, the addition of KT5720 reduced the number of axonal loops to 1.4 % in PGE2 + KT5720 treated cells (p < 0.001) and 0.9% in cells treated with KT5720 alone (p < 0.001) indicating that the increased formation of axonal loops by PGE2 exposure was influenced by PKA. Additionally, there was no significant difference between control cells, and cells treated with PGE2 + KT5720 (p = 0.9328) or cells treated with KT5720 alone (p = 0.9912). These findings show that PGE2/PKA signaling can contribute to axonal loop formation.

We used forskolin to further confirm whether the increase in axonal loop formation seen with PGE2 was PKA dependent. The proportion of neurites forming axonal loops was compared between treatments (Control, Forskolin, Forskolin + KT5720, and KT5720) on day 8 using one-way ANOVA and a significant difference between treatments was found ($F_{3,44}$ = 12.85, n = 2345, p <0.001). The proportion of neurites forming axonal loops in forskolin treated cells was 3%, which was significantly

higher than 0.5% in control cells ($p < 0.001$), 0.9% in Forskolin + KT5720 treated cells ($p < 0.001$), and 0.9% in KT5720 treated cells ($p < 0.001$), producing a similar response to cells treated with PGE2 (Figure 3D). Additionally, we observed no significant difference in axonal loop formation between control cells, and Forskolin+KT5720 treated cells ($p = 0.7971$) or KT5720 treated cells ($p = 0.7883$), verifying that the increase in axonal looping we had observed was PKA dependent.

4.3.4 Prostaglandin E2 increases the level of phosphorylated spinophilin at ser94 through PKA

Many changes in neuronal morphology that occur during differentiation, including neurite outgrowth, and axonal pathfinding greatly rely on modification of actin cytoskeleton and actin stabilizing proteins such as spinophilin (Geraldo and Gordon-Weeks 2009, Davis, Merrison-Hort et al. 2017, Miller and Suter 2018). Previous *in vivo* studies have shown that the total expression of spinophilin within the preoptic areas young rats is increased by the direct injection of PGE2 (Burks, Wright et al. 2007). Other studies have also demonstrated that the phosphorylation of spinophilin by PKA at ser-94 reduces its binding affinity to actin (Hsieh-Wilson, Benfenati et al. 2003, Bielas, Serneo et al. 2007). In this study, we examined for the first time whether the phosphorylation of spinophilin at ser-94 can be regulated by a PGE2/PKA dependant mechanism during neuronal differentiation.

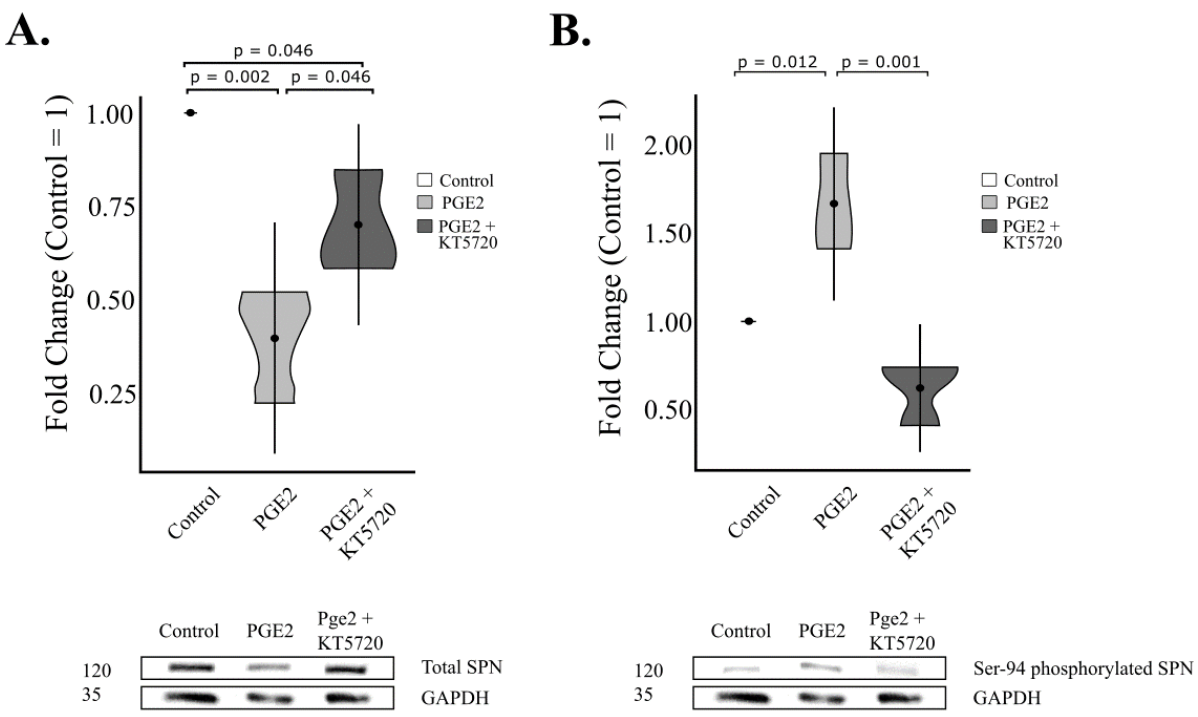

Figure 4-4: PGE$_2$-PKA effect on total and ser94 phosphorylated Spinophilin expression. Protein was isolated from cells on day 8 following the induction of differentiation after treatment with PGE2 or PGE2+KT5720 and the (A) total Spinophilin protein, and (B) P-Ser94 phosphorylated Spinophilin were quantified relative to control cells through western blotting. Results are shown as fold change to Control cells (±SD).

Western blot analysis was used to quantify relative amounts of total spinophilin between treatments (Control, PGE2, PGE2+KT5720) on day 8 of differentiation (Figure 4). A one-way ANOVA showed that there was a significant difference between treatments ($F_{2,6} = 19.47$, n = 9, $p < 0.01$). Compared to the basal level of total spinophilin in control cells (considered a fold change of 1.0), the expression level decreased to a fold change of 0.40 in PGE2 treated cells ($p < 0.01$) (Figure 4A). Treatment with PGE2+KT5720 resulted in a 0.70-fold change compared to the control cells ($p < 0.05$), which was significantly higher than cells treated with PGE2 alone ($p < 0.05$) indicating that the reduction of total spinophilin level was influenced by PGE2-PKA.

To further understand the potential regulation of spinophilin through PGE2, we quantified the expression of p-ser94 spinophilin using western blot (Figure 4B). A one-way ANOVA found a significant difference in p-ser94 spinophilin expression level between treatments (Control, PGE2, PGE2+KT5720) on day 8 of differentiation ($F_{2,6} = 23.43$, $n = 9$, $p < 0.05$). The expression of p-ser94 spinophilin was significantly higher in PGE2 treated cells showing a 1.66-fold increase compared to control cells ($p < 0.05$). The expression of p-ser94 spinophilin was lower in PGE2+KT5720 treated cells with a fold change of 0.62 relative to control cells, and significantly lower than in PGE2 treated cells ($p <0.001$), verifying that the increase in the p-ser94 spinophilin levels was PGE2/PKA dependent.

Overall, we show that in differentiating NE4C cells PGE2 treatment reduced expression level of total spinophilin and increased the expression of p-ser94 spinophilin both influenced by PKA-dependent mechanisms. The increased levels of p-ser94 spinophilin observed here likely affects it affinity to actin cytoskeleton, which was previously reported and will be further discussed.

4.4 Discussion

This study provides evidence for the first time that the PGE2/PKA signaling can influence actin-dependent neuronal morphology such as neurite length, and growth cone turning and looping as well as the level and phosphorylation of the actin-binding protein spinophilin. We used NE4C cells as a model system to show that as the differentiation progresses exposure to PGE2 promoted *neurite outgrowth*, increases *growth cone turning*, and promotes axonal *self-fasciculation*. We also show that PGE2 decreases the level of total actin-bound spinophilin and increases the level of its unbound form phosphorylated at ser 94.

4.4.1 The effect of PGE2 on cytoskeletal morphology in differentiated NE-4C cells

We found a PKA-dependent increase in *neurite length* in cells treated with PGE2 throughout differentiation. The presence of neurites is one of the defining characteristics that neuronal cells have arrested proliferation and become differentiated cells (Craig and Banker 1994). The process of neurite elongation is driven by the polymerisation of the actin cytoskeleton and the coordination of the actin and microtubule cytoskeletons to drive the leading edge of the neurite's growth cone (Dent and Gertler 2003). Neurite length *in vitro* can be a good representation of abnormalities with dendrite and axonal outgrowth *in vivo* (Radio and Mundy 2008). We have previously shown that 24h exposure to PGE2 on day 12 of differentiation in NE4C stem cells increased neurite length (Davidson, Wong et al. 2016). Increased neurite length was also observed in neuronal motor cell lines following 48h exposure to PGE2 as well as in sensory-like neuronal cells exposed to PGE2 for 24h (Mitani, Sekiguchi et al. 2016, Nango, Kosuge et al. 2017). In this study, we provide additional evidence that exposure of NE4C cells to PGE2 from the initiation of differentiation significantly increased neurite length during neuronal differentiation in a PKA-dependent manner. We observed that the rate of neurite length was greatly influenced by PGE2 during later differentiation stages after day 10 when compared to the control cells. This may speak to a critical period of the effect of PGE2 during differentiation. A study by (Park, Ma et al. 2015) found that levels of PGE2 increased throughout neuronal differentiation of primary rat neural stem cells. The same group also found that through PKA, PGE2 affected the phosphorylation of developmental proteins p38 MAPK, CREB and Bcl-2. A separate group found that PGE2 induced neuritogenesis was abolished by the inhibition of PKA, providing further evidence of a PGE2/PKA effect (Mitani, Sekiguchi et al. 2016). It is feasible to speculate that prenatal exposure to environmental risk factors known to influence PGE2 levels (Tamiji and Crawford 2010, Wong and Crawford 2014, Wong, Wais et al. 2015) may also affect progression of neuronal development during the critical times.

We also observed decreases in the proportion of *growth cones turning*, as classified by the symmetry of each growth cone, which is indicative of abnormal axonal pathfinding. The establishment of proper neuronal circuitry throughout development depends on precise axonal pathfinding. The motility of growth cones enables them to respond to attractive and repulsive cues as neurites navigate to their intended targets (Gasperini, Pavez et al. 2017, Craig 2018). The structure of a growth cone can be separated into a central region filled with organelles and microtubules and a peripheral region or the leading edge which consists primarily of actin fibers (Craig 2018, Miller and Suter 2018). While growth cone turning is dependent on the spatio-temporal coordination of the assembly and disassembly of actin and microtubule cytoskeletons the shape of a growth cone is primarily determined by actin at the leading edge of a growth cone (Gordon-Weeks 2004, Lowery and Van Vactor 2009, Dent, Gupton et al. 2011). Both attractive and repulsive growth cone turning affect the shape of a growth cone. Prior to attractive turning, there is a rapid accumulation of F-actin at the site where the growth cone will turn (Lin and Forscher 1993, Gallo and Letourneau 2000). In contrast, localized increases in retrograde actin flow, leading to localized growth cone collapses occur during repulsive growth cone turning (Luo, Raible et al. 1993, Fan and Raper 1995). While overall disruptions in actin have been found to disrupt neurite outgrowth, actin disruption localized to the growth cone increased growth cone collapse, decreasing overall growth cone area.(Zhou and Cohan 2001). We postulate that the marked decline in in the asymmetry (turning) of growth cones as a result of exposure to PGE2 is a strong indicator that there was a dysregulation in actin polymerisation dynamics. Moreover, we determined that these morphological changes are driven by PKA. Our findings show that during the process of neuronal differentiation PGE2/PKA can not only increase the neurite length but also affect axonal turning, both likely via dysregulation of actin dynamics.

In this study we also determined that PGE2-PKA signaling affected *axonal loop formation* during differentiation of NE4C cells. The formation of axonal loops is indicative of the inability of dendrites to self-avoid, a key aspect of axonal pathfinding (Amthor and Oyster 1995, Sdrulla and Linden 2006). Issues with self-avoidance can ultimately result in nervous system circuit abnormalities as dendrites cannot form connections properly. Such circuit abnormalities are believed to be related to neurodevelopmental disorders including retinal dysplasia (Weiner, Koo et al. 2004), tuberous sclerosis (Choi, Di Nardo et al. 2008), and most commonly in ASD (Minshew and Williams 2007). Abnormal calcium levels may help to explain the higher proportion of axonal loops observed here in PGE2 treated cells. In fact, we have previously shown that exposure to PGE2 increases baseline and amplitude of intracellular calcium levels within growth cones of differentiating NE4C cells (Davidson, Wong et al. 2016). Calcium signals are well-established mediators of growth cone turning and outgrowth, which were both dysregulated following PGE2 exposure in this study (Cohan, Connor et al. 1987, Zheng 2000). In addition to the contribution of calcium to abnormal growth cone turning, which we observed in this study, intracellular calcium levels have a key role in neurite adhesion, with many cell adhesion proteins inducing increases in calcium (Doherty, Williams et al. 2000, Sheng, Leshchyns'ka et al. 2013). Previous research suggests that cross-talk between PKA and calcium is important for processes in cell migration including the restructuring of the actin cytoskeleton (Howe 2011). The increase in axonal loop formation in PGE2 treated cells may speak to a dysregulation of both PKA and calcium dependent migration mechanisms ultimately leading to a dysregulation of actin. Literature provides evidence that increases in axonal loop formation can result in abnormalities in neuronal projections through cortical layers , affecting the organization of neurons within minicolumns of the cortex (DeFelipe 2005). We speculate that there may be abnormalities in neuronal projection and minicolumns organization in increased PGE2 models *in vivo*.

4.4.2 Abnormal expression and phosphorylation of Spinophilin

We also observed a decline in the total levels of the actin-binding protein spinophilin in cells exposed to PGE2 during differentiation. Spinophilin was independently isolated from rat brain by two groups characterizing a strong binding affinity to both F-actin filaments (Nakanishi, Obaishi et al. 1997) as well as the dendritic spine enriched protein phosphatase 1 (PP1) (Allen, Ouimet et al. 1997). Spinophilin functions as a link between excitatory synaptic activity and changes in dendritic spine morphology and density, with early studies demonstrating that spinophilin is involved in the stabilization of the actin cytoskeleton in both dendritic spines and filopodia (Satoh, Nakanishi et al. 1998, Yan, Hsieh–Wilson et al. 1999). Studies in spinophilin knock-out mice found an increase in spine density and a retraction of filopodia (Feng, Yan et al. 2000). The connection of PGE2 and total spinophilin expression has already been shown in rodent *in vivo* models. There was a reduction in the expression of total spinophilin at postnatal day 14 (PN14) in cerebellum of rats injected daily with COX2 inhibitors between PN7 and PN13 (Dean, Knutson et al. 2012, Dean, Wright et al. 2012). The same group also found an increase in total spinophilin expression following postnatal injection of PGE2 into the preoptic area (Burks, Wright et al. 2007, Wright and McCarthy 2009). Using the anti-ser94-spinophilin antibody provided by Hsieh-Wilson, Benfenati et al. (2003) our current study is the first to show that despite the decrease in the total expression of spinophilin due to PGE2/PKA signaling there was also increased levels of ser94 phosphorylated spinophilin. Spinophilin contains several sequences for phosphorylation by PKA with the two major sites identified being Ser94 and Ser177 (Hsieh-Wilson, Benfenati et al. 2003). Phosphorylation of spinophilin at ser94 decreases the stoichiometry of the spinophilin to F-actin interaction by as much as 53% (Hsieh-Wilson, Benfenati et al. 2003). Higher proportions of bound (unphosphorylated) spinophilin are associated with greater actin stability, which is thought to be the result of the actin-capping activity of bound spinophilin in which spinophilin binds to the barbed ends of polymerizing actin, thereby preventing its polymerization (Schuler and Peti 2008). Proper regulation of actin-capping is necessary for normal neurite elongation,

and axonal pathfinding, both of which were affected in our increased PGE2 model. Interestingly, Hsieh-Wilson, Benfenati et al. (2003) showed that unphosphorylated actin-bound spinophilin was localized to the post-synaptic density (PSD) whereas phosphorylated Ser94 and Ser177 unbound spinophilin was found in the cytosol. This shift in the subcellular localization of spinophilin was influenced by PKA. Our findings show for the first time that the PGE2-PKA signaling can upregulate the levels of Ser94 unbound spinophilin and actin dynamics such as axonal looping. We suggest that abnormal PGE2 level during prenatal stages of development due to environmental influences can contribute to modification of dendritic spines function through change actin dynamics resulting from the phosphorylation of spinophilin at ser94.

Examining our results in their entirety, we propose a model in which PGE2 can regulate actin-dependent cellular morphology through PKA dependent signalling (Figure 5). Arachidonic acid is normally released from the cell membrane in response to environmental cues and is metabolized into PGE2. Through its EP receptors PGE2 can regulate the activity of PKA (Regan 2003, Jiang and Dingledine 2013). It has been shown that the phosphorylation of spinophilin at ser94 reduces the affinity of spinophilin to actin, causing a reduction in the stability of actin and actin-microtubule crosslinking (Uematsu, Futter et al. 2005, Xu, Chen et al. 2008). The PGE2-regulated changes in cytoskeletal stability in turn caused the abnormal actin-dependant morphologies observed in this study, including increases in neurite length and the formation of axonal loops, as well as decreases in the proportion of growth cones categorized as turning.

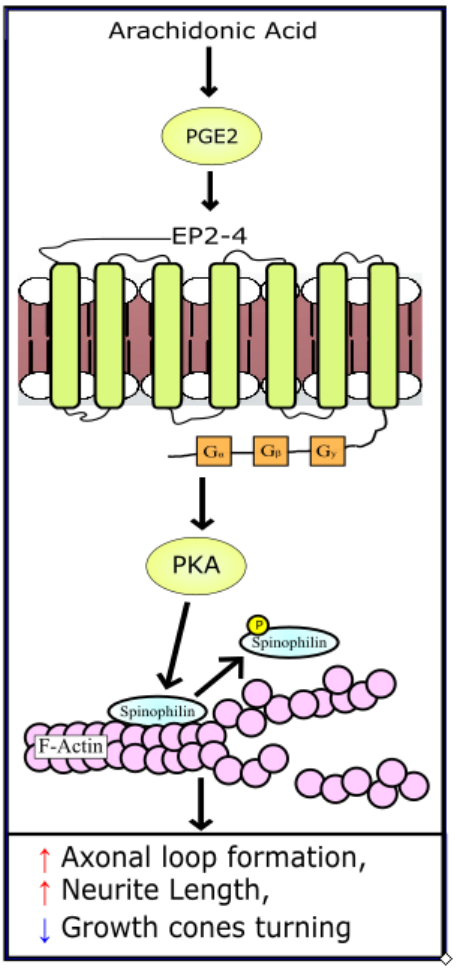

Figure 4-5: Proposed model of PGE2 effect on Spinophilin. PGE2 is synthesized from Arachidonic Acid which is released from the cell membrane in response to various environmental factors. Literature has demonstrated that PGE2 can increase the activity of PKA through E-Prostanoid receptors. Here we have demonstrated that through PGE2, PKA can phosphorylate Spinophilin. We propose that this results in dysregulation of Actin stability, leading to morphologies including increased axonal loop formation and neurite length, and a decrease in growth cone turning.

As PGE2 levels can be influenced by various environmental risk factors during prenatal development, this model may help to elucidate the connection between abnormal lipid signaling and the etiology of some neuropathologies (Wong and Crawford 2014, Wong, Wais et al. 2015). The regulation of cytoskeletal dynamics is important throughout the course of development. We have provided first evidence that increases in the levels of lipid signalling molecule PGE2 can affect these dynamics *in vitro*. We also provide evidence that it is PGE2-PKA signaling that contribute to certain

neuronal defects due to abnormalities in cytoskeletal dynamics. We speculate that changes in PGE2 level during critical time in development may contribute to some neuronal pathologies. These findings provide a first step in examining these changes *in vivo*, currently ongoing in our lab in mouse models of ASD.

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Chapter 5: Study 2
Study 2: Abnormal dendritic morphology in the cerebellum of Cyclooxygenase-2- Knockin mice

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The dissertation was written in thesis format and the following is specific to the contributions in the chapter:

Contributions: Ashby Kissoondoyal imaged Golgi-COX-stained slides and analyzed the data, created figures and tables, and wrote the manuscript. Ravneet Rai-Bhogal collected samples and performed Golgi-Cox staining and conducted the western blots. Dorota A. Crawford supervised the design and coordination of the study and was involved with writing the manuscript.

5.1 Introduction

Prostaglandin E2 (PGE2), the major bioactive lipid mediator in the brain, plays an essential role in the development of the nervous system. Growing evidence from clinical and epidemiological research shows a link between abnormal PGE2 signalling and neurodevelopmental disorders (Tamiji and Crawford 2010, Wong and Crawford 2014). PGE2 synthesis begins with the release of arachidonic acid (AA) from the phospholipid cell membrane through the action of phospholipase A2 (PLA2) (Sang and Chen 2006). AA is further converted into PGE2 by cyclooxygenase-1 or -2 (COX-1 or COX-2) (Park, Pillinger et al. 2006, Rouzer and Marnett 2009). COX-1 is constitutively expressed throughout most of the body, and within microglial cells of the brain (Schwab, Nguyen et al. 2000, Hoozemans, Rozemuller et al. 2001). In contrast, the expression of COX-2 is inducible throughout the body as part of the normal inflammatory response, but is constitutively expressed within the kidneys, the gastrointestal tract, the female reproductive system, and within neurons of the brain (Maslinska, Kaliszek et al. 1999, Kirkby, Chan et al. 2016).

PGE2 exerts its action through the activation of the E-prostanoid receptors 1-4 (EP1-4) (Khan, Bhagat et al. 2019). COX-2/PGE2 signalling is involved in key processes in neuronal development including proliferation and migration (Wright and McCarthy 2009), synaptogenesis and (Yang 2009), and the formation and plasticity of dendritic spines (Burks, Wright et al. 2007). Disruptions in COX-2/PGE2 signalling, resulting in abnormal PGE2 levels during prenatal development are linked to the etiology of Autism Spectrum Disorders (ASD) (Tamiji and Crawford 2010, Wong and Crawford 2014). Interestingly, PGE2 levels can be affected by various environmental risk factors throughout prenatal development including air pollution (Volk, Hertz-Picciotto et al. 2011, Costa, Cole et al. 2017), herbicides such as glyphosates (Suzuki, Matsuzaki et al. 2011, Samsel and Seneff 2015), phthalate molecules used commonly in consumer products (Larsson, Weiss et al. 2009, Miodovnik, Engel et al. 2011, Testa, Nuti et al. 2012), and common over the counter drugs such as acetylsalicylic acid or

acetaminophen (Brandlistuen, Ystrom et al. 2013, Avella-Garcia, Julvez et al. 2016, Liew, Ritz et al. 2016); all previously shown to be linked to ASD (Tamiji and Crawford 2010, Wong and Crawford 2014).

The molecular mechanisms by which abnormal PGE2 levels affect the developing brain are still largely unknown. Research in our lab and others have already provided molecular evidence of how impaired COX2/PGE2 signalling effects neuronal pathologies and leads to manifestation of autism-related behaviours. In neuroectodermal (NE-4C) stem cells and Neuro-2a cell lines we found that exposure to PGE2 increased intracellular calcium within the cytosol and growth cones (Davidson, Wong et al. 2016), increased proliferation, migration and differentiation of cells (Wong, Ussyshkin et al. 2016). In NE-4C cells exposed to PGE2 during differentiation, we observed increases in neurite length and self-fasciculation, decreases in growth cone turning and abnormal expression levels of total actin-bound spinophilin and unbound PKA- phosphorylated ser-94 spinophilin (Kissoondoyal and Crawford 2021). We have found crosstalk between PGE2 and canonical Wnt signalling pathways through PKA and PI-3K in differentiating NE-4C stem cells, as well as in mice offspring exposed to PGE2 during pregnancy (Wong, Ahmad et al. 2014, Wong, Ussyshkin et al. 2016, Rai-Bhogal, Wong et al. 2018). Reduced PGE2 levels, as seen in our COX-$2^{-/-}$ mouse model (Ayoub, Botting et al. 2004, Bosetti, Langenbach et al. 2004, Li, Wu et al. 2010), resulted in differential expression of many developmental genes, affecting biological pathways important in synaptic transmission, long-term potentiation, neurite navigation, and dendritic spine formation (Rai-Bhogal, Ahmad et al. 2018). Other studies in rats have also shown that abnormal increases or reductions in PGE2 levels during critical postnatal developmental periods alter dendritic arborization within the cerebellum (Dean, Wright et al. 2012) and the hippocampus (Burks, Wright et al. 2007). We have also demonstrated that COX-2^{-}-KI and maternal exposure to PGE2 during early prenatal development (PGE2-injected) result in sex-

dependent autism-related behaviours postnatally (Ackermann, Wildgruber et al. 1998, Steinlin 2007, Wong, Bestard-Lorigados et al. 2017, Wong and Crawford 2020).

There is strong evidence that PGE2 signalling can disrupt normal cerebellar development. Another study found that incubation of rat cerebellar granule neurons *in vitro* with PGE2, resulted in an EP1 dependant potentiation of GABAA currents (Yang, Dong et al. 2015). Another study found that local injection of PGE2 into the cerebellums of young postnatal rats increased local estriol production (Dean, Wright et al. 2012). In the same study, there was a stunting of dendritic arborization within the cerebellums of the PGE2-injected rats as well as a reduction in excitatory synaptic density. The observed reduction of excitatory synaptic density correlated to impairments in play behaviour (Dean, Wright et al. 2012, Hoffman, Wright et al. 2016). In contrast, inhibition of PGE2 production with by means of local injection of COX-2 inhibitors into the cerebellums of young postnatal rats increased arborization of the dendritic tree and increased excitatory synaptic density (Dean, Knutson et al. 2012). Pathology of the cerebellum is one of the most consistent brain regions implicated in ASD (Wang, Kloth et al. 2014). ASD subjects were found to have increased Purkinje cell size (Fatemi, Halt et al. 2002, Wegiel, Kuchna et al. 2013). Developmental abnormalities of the cerebellum including flocculondular dysplasia (Wegiel, Kuchna et al. 2013), cerebellar hypoplasia (Wegiel, Kuchna et al. 2010) as well as the presence of cerebellar spheroids (Weidenheim, Goodman et al. 2001) have been found in humans with ASD.

In our present study we use COX-2^{-}KI male and female mice to examine a range of actin-regulated dendritic morphologies including arborization, length and thickness, self-fasciculation, as well as dendritic spine density and morphology within the cerebellums on postnatal day 25 (PN25). We found that the COX-2^{-}KI increases dendritic arborization, and dendritic looping in both males and females, and has sex-dependent effects on dendritic thickness, and on dendritic spine density and

morphology. We also show that the COX-2^{-}KI has a sex-dependent effect on the expression level of β-Actin, and the total level of spinophilin, a dendritic spine protein involved in actin stabilization. We discuss how these molecular changes in the cerebellum may contribute to pathologies that are often linked to neurodevelopmental disorders such as ASD.

5.2 Methods

5.2.1 Animals

Founder 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) and backcrossed for a minimum of 5 generations to wild-type 129S6/SvEvTac (WT) mice acquired from Taconic Laboratory. *Ptgs2*^{Y385F} mice are a genetic mouse model created by a targeted point mutation of the *Ptgs2* gene resulting in a Y385F amino acid substitution of the COX2 protein. The resultant COX2 protein has no cyclooxygenase activity but maintains peroxidase activity (Yu, Fan et al. 2006). In COX-2^{-} - KI mice, endogenous levels PGE2 within the brain are reduced by half when compared to WT mice (Ayoub, Botting et al. 2004, Bosetti, Langenbach et al. 2004, Li, Wu et al. 2010). As female mice homozygous for the *Ptgs2*^{Y385F} mutation are infertile(Lim, Paria et al. 1997, Wang, Zhao et al. 2010), homozygous offspring were generated by crossing heterozygous (COX)-2^{-} KI females with homozygous males. Mice were housed under the same conditions at York University in group housing on a 12-hours light/dark cycle. All experiments and protocols were performed in accordance with the York University Animal Care Committee ethics guidelines and were approved by the Research Ethics Board of York University.

5.2.2 Genotyping

DNA was extracted from individual ear punch samples, which were first denatured in alkaline lysis buffer (25 mM NaOH) at 95°C for 30minutes, followed by addition of neutralization buffer (40mM Tris-HCL). Genotyping of mice was performed using polymerase chain reaction (PCR) on primer sequences provided by Jackson Laboratory according to the Jackson Laboratory Ptgs2$^{tm1.1Fun}$ protocol.

5.2.3 Western Blotting

Whole brain tissue samples were collected from WT and COX-2^{-} KI male and female mice at P25. Brain tissue was homogenized in 1µL of TRIzol using a Polytron power homogenizer. Total protein was isolated through the standard phase separation TRIzol method (Sigma). From each sample, 30-40ug of protein was loaded into a 12% polyacrylamide gel (PAGE) as we have previously described (Rai-Bhogal, Wong et al. 2018). Wild-type protein samples were pooled from mice from at least 3 separate litters, while protein samples from each COX-2^{-} - KI mice were loaded individually. PAGE gel electrophoresis was used to separate isolated samples before being transferred to a 0.45µM nitrocellulose membranes (Biorad). Before probing with primary antibodies, all membranes were washed in TBS and then blocked in 5% milk in 1X Tris buffer saline 0.05% Tween 20 (TBS-T) for 1h at room temperature. Protein samples were probed with monoclonal rabbit anti-spinophilin (Cell Signalling, 1:1000, mAb #14136, Danvers, MA, USA) in 5% milk in TBS-T overnight at 4°C, followed by mouse monoclonal anti-β-Actin (Abcam;1:10000; ab8245, Cambridge, MA,USA) in 2% milk in TBS-T overnight at 4°C and then with mouse monoclonal anti-GAPDH (Abcam,1:10000; ab8245, Cambridge, MA, USA) for 1 hour at room temperature. After each primary antibody, membranes were washed 5 times in 1x TBS-T before probing with appropriate HRP-tagged secondary antibodies. Goat-anti-rabbit (Abcam,1:10000, ab6276, Cambridge, MA, USA), and Goat-anti-mouse (1:10000; catalog No. ab6789) were each incubated in 2% milk in TBS-T for 2h at RT. Probed

membranes were then imaged with the Geliance 600 Imaging System (Perkin Elmer). For quantification, protein signal intensity was first normalized to GAPDH signal intensity. The relative protein expression was then normalized to wild-type males (protein expression in WT-M = 1).

5.2.4 Golgi COX staining

Whole brain tissues were collected at postnatal day 25 (P25). Samples were extracted from 3 males and females from both WT and (COX)-2^{-} KI mice. Golgi staining was performed according to (Zaqout and Kaindl 2016). Brains were dissected in half, rinsed in cold 0.1M PBS solution before immediately being placed in a 4% paraformaldehyde solution for an hour. Following fixation, brain samples were washed with ddH20 and then placed in Golgi-COX solution consisting of 5% w/v potassium dichromate, 5% w/v mercuric chloride, and 5% w/v potassium chromate, dissolved in ddH20. After 24h the brain tissues were placed in a fresh Golgi-COX solution and incubated for additional 14 days at room temperature in the dark. The brains were then transferred into tissue protectant solution which consisted of 30% w/v sucrose, 20% w/v ethylene glycol, and 1% w/v polyvinylpyrrolidone and kept in this solution for up to 7 days.

Cryostat sectioning of the brain samples was performed by the research histology lab at the University Health Network (Toronto, Ontario; www.uhnresearch.ca). Samples were sliced along the sagittal plane at approximately 100 µm thickness and mounted onto gelatin-coated slides. A 3:1 ammonia to H20 solution followed by a solution of 5% w/v sodium thiosulfate in H20 was used to develop the slices. Slides were dehydrated through serial dehydration in 70%, 95% and 100% ethanol before immersion in xylene and being cover-slipped.

5.2.5 Microscopy

Images were acquired using brightfield microscopy on the Zeiss Laser Scanning Confocal Microscope (LMS 700; Advanced Light and Electron Microscopy at York University). The full cerebellum of each animal was imaged using 10x magnification with z stacks taken every 1 to 2.5µm

(50-100 stacks on average), while individual cells within the cerebellum were imaged using 100x magnification with z-stacks taken every 0.01 to 0.02 µm (1000-2000 stacks on average). Images were quantified by 10 observers per image (to account for observer bias) using Fiji ImageJ software (Schindelin, Arganda-Carreras et al. 2012).

5.2.6 Sholl Analysis and dendritic loop quantification

From 10x magnification images taken, a total of 5 neuronal cells within the cerebellum were selected for each animal resulting in a total N=60 cells and an n of 15 per condition. Cells were selected from within the cerebellum. Sholl analysis was performed using the open source Fiji ImageJ software(Schindelin, Arganda-Carreras et al. 2012, Rueden, Schindelin et al. 2017), with the Simple Neurite Tracer plugin (Longair, Baker et al. 2011). Concentric circles were generated by the software every 10µm from a point made at the center of the soma of each cell and the number of intersecting neurites were computed (Binley, Ng et al. 2014). While normally quantified subjectively, we used a quantitative method to determine self-fasciculating or looping dendrites as described in (Kissoondoyal and Crawford 2021). Using the traced neurites from the Simple Neurite Tracer plugin, the maximum angle from each neurite was used to classify each neurite as looping or non-looping. If the angle for a given neurite exceeded 270°, the neurite was classified as a loop as we previously described (Kissoondoyal and Crawford 2021).

5.2.7 Dendrite length and thickness quantification

From 100x images taken, the *dendrite length* and *dendrite thickness* were measured. *Dendrite length* was defined as the distance from the base of a dendrite to the furthest reaching point, drawing a line along the dendrite. *Dendrite thickness* was measured by taking 8 equidistant measurements drawing perpendicular lines to the *dendrite length* line and taking the average of these 8 measurements to account for any variations along the length of the dendrite.

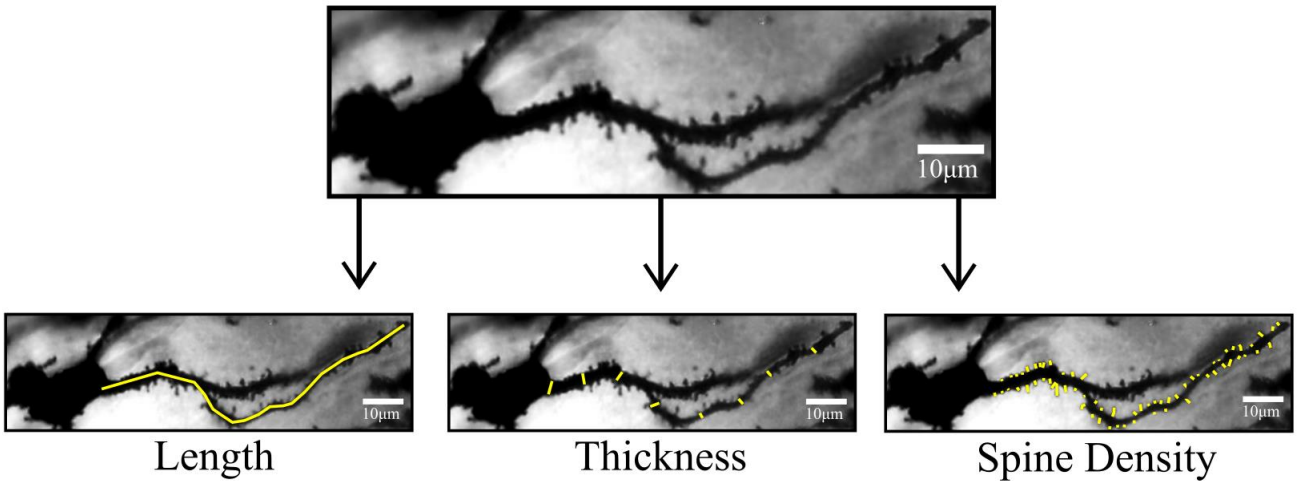

Figure 5-1: Measurement of Dendritic morphology in the mouse cerebellum. Morphology was measured on the longest dendrite of each cell measured. Length was measured from the furthest point of the dendrite until the outside of the cell soma, thickness was measured by taking the average of 8 equidistant points along the length of the dendrite and spine density was measured as the total number of spines per length of dendrite.

5.2.8 Dendritic spine density and shape quantification

Dendritic spines were measured as any extension coming off of the dendrite which was not a branch. The *dendritic spine density* was defined as the total number of measured spines divided by *dendrite length* (Bae, Sung et al. 2012, Briones, Tang et al. 2018). Once identified, the length and width of each spine was measured. The length of each spine was the distance from the base of the spine to the furthest reaching point, and the spine width was determined by the longest line that could be drawn perpendicular to the spine length. Using the length and the width of each spine, the dendritic spines

were classified into shapes using a top-down approach which was adjusted from classification criteria from literature (Ghani, Mesadi et al. 2017, Urban, Rezaei et al. 2019). First, spines with a width greater than the length will be classified as stubby. Of those remaining, those with a width greater than 0.6µm will be classified as mushroom. Finally, those with a length less than 2µm will be classified as thin, and those with a length greater than 2µm will be classified as filopodia.

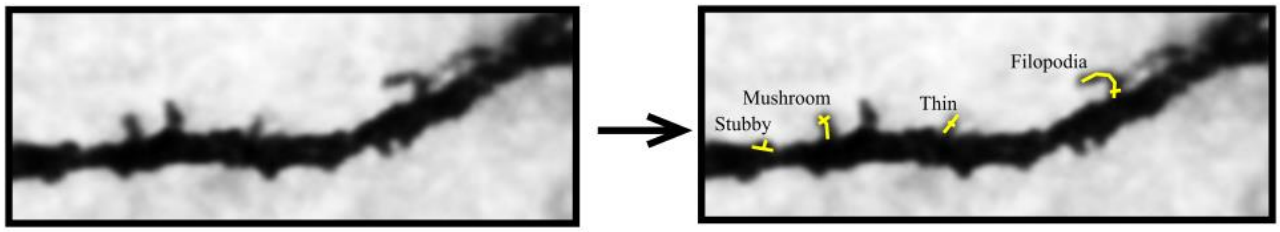

Figure 5-2: Classification of dendritic spine shapes in the mouse cerebellum. Dendritic spines were classified using a top-down approach. All spines with a width greater than their length were classified as stubby shaped. Of those remaining, those with a width greater than 0.6 µm were classified as mushroom shaped. Of those remaining, those with a length greater than 2 µm were classified as Filopodia shaped and those with a length less than 2 µm were classified as thin shaped.

5.2.9 Statistics

Statistical analyses were performed using the core open source software R (Team 2013). All categorical data including loop formation and dendritic spine shape classification was analyzed using multinomial logistic regression with data presented in tables as odds ratio (95% confidence interval) (Venables 2002). All remaining data including sholl analysis, dendrite length, dendrite thickness, dendritic spine density, and all qRT-PCR and western blot analysis were analyzed using linear mixed effect modeling to account for random effects in our experiment (Bates, Mächler et al. 2015). Any variable of interest was treated as a main effect and potential confounds were assigned as random effects. Random effects in this study included, litter and image observer, to account for litter and observer bias, respectively. Linear mixed models were fit by maximum likelihood and t-tests were

performed using Satterwaite's adjustment method and the Akaike Information Criterion (AIC) was used to determine the model of best fit for each test. Linear mixed model data was visually displayed using a violin plot to depict the probability distribution of the data within a given treatment. Significance was determined as $p < 0.05$ for all tests. All measurements were taken from 3 independent biological replicates per treatment. Total sample size was calculated based on literature using G*Power 3 software (Faul, Erdfelder et al. 2007) using an estimated effect size of 0.25.

5.3 Results

5.3.1 Dendrite length

We have previously demonstrated that PGE2 can increase neurite length of differentiating NE4C stem cells through PKA-dependent mechanisms (Kissoondoyal and Crawford 2021). Findings from other groups showed that local injection of PGE2 into the rat cerebellum at postnatal day 10 (PN10) and PN12 reduced dendritic length in both adult males and females (Dean, Wright et al. 2012). Another study demonstrated that subcutaneous injection of the COX-2 inhibitor nimesulide into postnatal rats for 3-7 consecutive days starting on PN7 increased Purkinje cells dendrite length (Dean, Wright et al. 2012). In this study we use COX-2^{-}-KI male and female mice to assess whether reduced PGE2 levels (Ayoub, Botting et al. 2004, Bosetti, Langenbach et al. 2004, Li, Wu et al. 2010) throughout prenatal and early postnatal development affected *dendrite length* within the cerebellum at PN25. Dendrite length was defined as the total distance between the base of each dendrite to the end of the longest extending branch (figure 1).

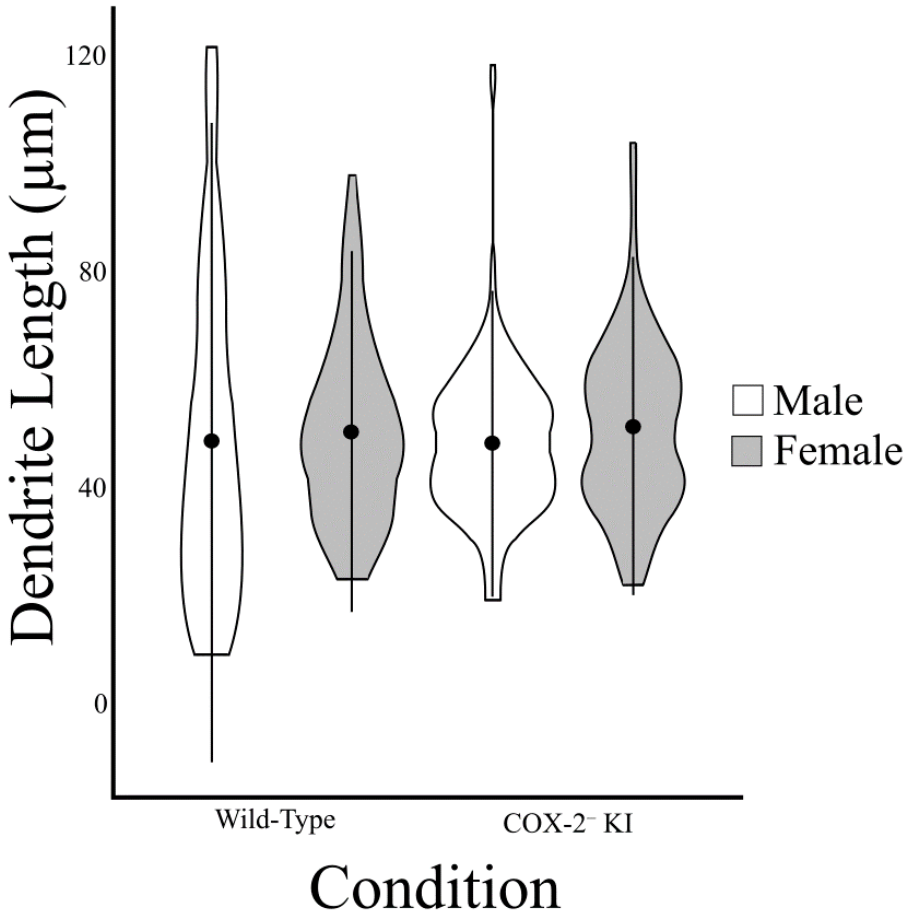

Figure 5-3: Average Dendrite Length in the cerebellum of P25 Mice. A. Dendrite length was measured from cells within the cerebellums of WT and COX2^{-} - KI male and female mice at P25. B. Length data are presented as mean ± SD. *n* values are recorded in the methods text.

A linear mixed effects model was used to fit the dendritic length measurements. Observers and litter were assigned as random effect controls and we examined the fixed effects condition (Wild-Type or WT vs COX-2^{-}KI) and sex, as well as the interaction between condition and sex. We did not obtain a significant interaction between condition and sex ($t(584) = 0.132$, $p= 0.895$). Further, no significant main effects in condition ($t(6.17) = -0.006$, $p = 0.996$) or sex ($t(584) = -1.807$, $p = 0.071$) were observed. We concluded that there was no effect of the COX-2^{-}KI on dendrite length within the cerebellum at PN25

5.3.2 Dendrite Thickness

Dendrite thickness can influence postsynaptic potential along the dendrite (Gansert, Golowasch et al. 2007). We investigated whether *dendrite thickness* (Figure 1 and methods) is affected in the cells of COX-2$^-$ - KI mice cerebellum. We used linear mixed effects modelling to fit the thickness measurements to compare our fixed effects of condition and sex as well as the interaction between the two factors (figure 3). We again assigned litter and observer as random effects to account for bias from each factor. We saw a significant interaction between condition and sex ($t(584) = -4.809$, $p < 0.001$). While there was no significant effect between our conditions ($t(7.42) = 0.214$, $p = 0.836$), we did see a significant effect of sex on dendrite thickness ($t(584) = 4.131$, $p < 0.001$).

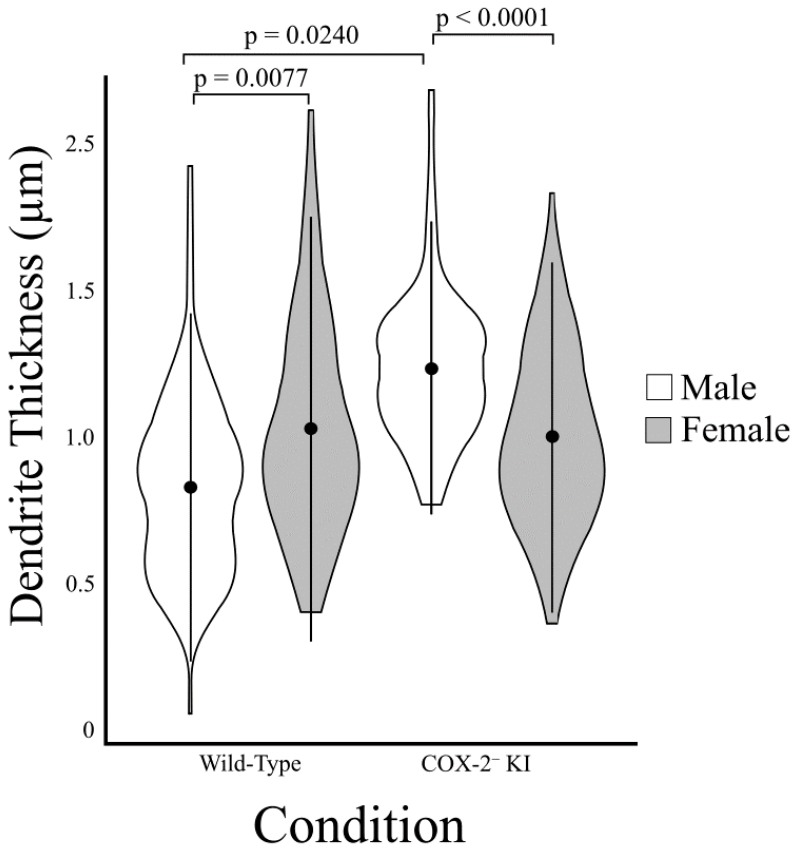

Figure 5-4: Average Dendrite Thickness in the cerebellum of P25 Mice. A. Dendrite thickness was measured from cells within the cerebellums of WT and COX2$^-$ - KI male and female mice at P25 by taking the average of 8 equidistant measurements along the dendrite. B. Thickness data are presented as mean ± SD. *n* values are recorded in the methods text.

Following the significant interaction term, we performed further pairwise comparisons. WT males (WTM) had an average dendrite thickness of 0.826µm which was significantly thinner than WT females (WTF) with an average dendrite thickness of 1.030µm, ($t(584) = 2.673$, $p = 0.0077$). Interestingly, we saw the opposite trend in COX-2^- - KI mice, in which COX-2^- - KI males (COXM) had significantly thicker dendrites with an average thickness of 1.306µm compared to COX-2^- - KI Females (COXF) with an average thickness of 0.994µm ($t(584) = -4.131$, $p < 0.0001$). We also saw a significant increase in dendrite thickness in COXM compared to WTM ($t(7.4) = 2.831$, $p = 0.0240$), but no significant difference between COXF and WTF mice ($t(7.4) = -0.214$, $p = 0.837$). These results show that dendrite thickness is sex dependent with WT males exhibiting thinner dendrites than WT females whereas COX males have thicker dendrites than COX females. Overall, it appears that the COX-2^- - KI males are most affected.

5.3.3 Dendritic Arborization

The structure and arborization of the dendritic tree of a neuron can provide some information about how that neuron receives and transmits synaptic inputs (Bird and Cuntz 2019). In this study, the degree of branching was estimated using sholl analysis to determine the number of intersections made with concentric circles every 10µm (Binley, Ng et al. 2014) (Figure 3A). Sholl analysis has been widely established as a method of examining neuronal morphology to represent arbors of a neuron (Rajković, Marić et al. 2016, Chittajallu, Wester et al. 2017, Keil, Sethi et al. 2017, Wilson, Sethi et al. 2017, Bird and Cuntz 2019). A linear mixed effects model was used to fit the number of intersections (figure 3B and methods). Litter was assigned as a random effect to control for potential litter bias, and we examined the fixed effects of condition, distance from the soma, sex, and the interaction between the condition and sex terms, as well as the condition and distance terms. While we observed no significant interaction between the condition and sex factors ($t(654) = -0.467$, $p = 0.641$), there was a significant effect of condition on distance at 20µm ($t(654) = -6.16$, $p < 0.001$), 30µm ($t(654) = -8.49$, p

< 0.001), 40µm (t(654) = -3.33, p < 0.001), and 60µm (t(654) = 2.05, p = 0.040). The main effects of condition (t(31.66) = 0.11, p = 0.92), and sex (t(654) = 0.25, p < 0.80) were not significant.

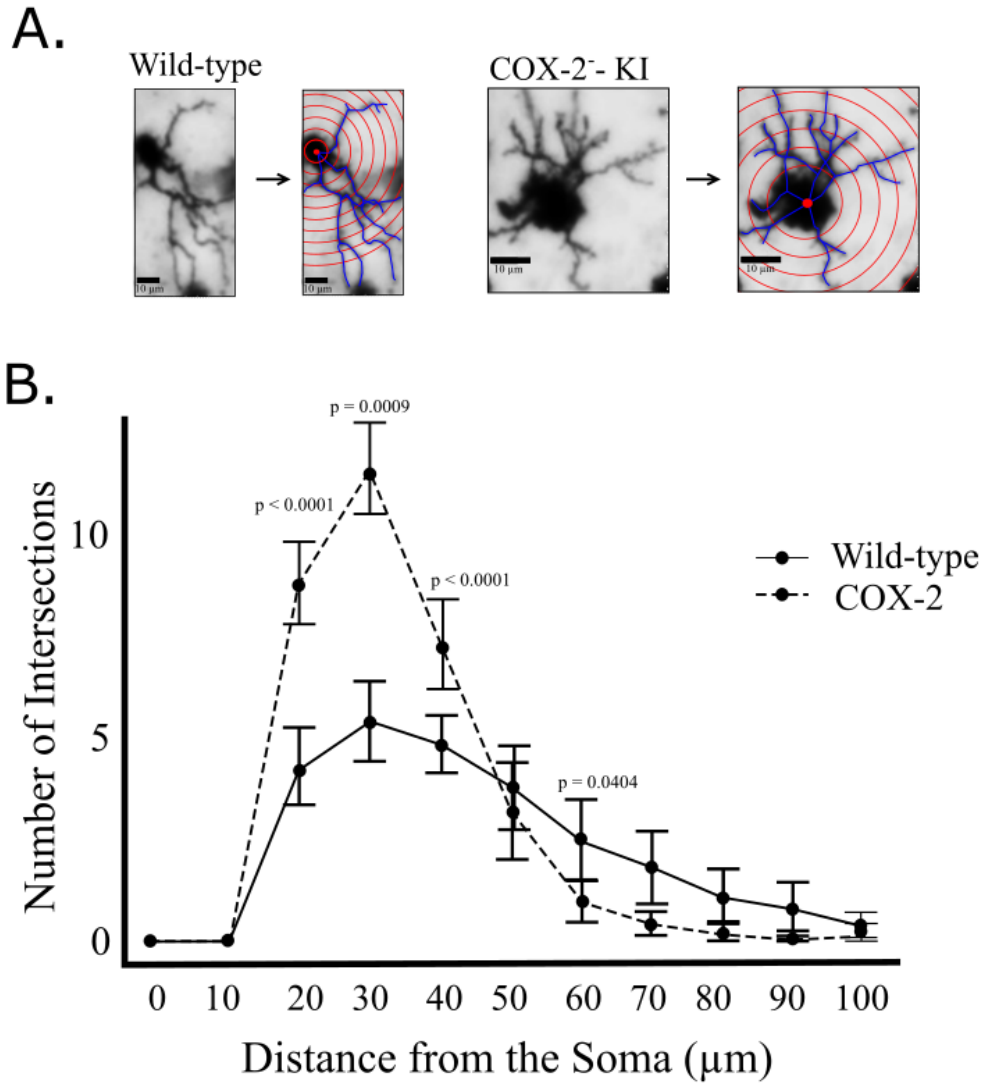

Figure 5-5: Sholl analysis in the cerebellum of P25 mice. A.The average number of intersections of dendrites with concentric circles drawn at 10 µm intervals from the cell soma shown in a representative Wild-type and COX-2^{-} - KI cell. B. Data are presented as mean as mean ± SD. *n* values are recorded in the methods text.

Given the significance of the condition on distance, we examined comparisons between conditions (WT and COX2$^-$ - KI) at each distance (figure 3B). As there was no significant effect of the condition-sex interaction, or of the main effect of sex, we did not examine pair-wise comparisons involving sex. At distances 0 and 10, we observed no significant differences in the number of intersections with the number of intersections being 0 for all conditions ($t(654) = 0$, $p = 1$). At 20µm, COX2 mice had 8.73 intersections which was significantly higher than, cells in WT mice which had an average of 4.23 intersections ($t(654) = 16.91$, $p < 0.0001$). At 30µm from the soma, COX-2$^-$- KI mice still had a greater number of intersections with an average of 11.57 compared to WT mice with only 5.37 ($t(654) = 22.40$, $p <0.0009$). A similar trend continued at 40µm, with 7.23 intersections in the COX-2$^-$ - KI mice and 4.80 in the WT mice ($t(654) = 14.01$, $p = 0.0001$). At 50µm there was no significant difference between conditions with COX-2$^-$ - KI mice and WT mice having 3.17 and 3.77 intersections respectively ($t(654) = 0.822$, $p = 0.4116$). However, at 60µm we report the opposite effect compared to the one observed at the 20-40µm range. The number of intersections was lower in COX-2$^-$ - KI mice with 0.93 compared to WT mice with 2.43 ($t(654) = 2.054$, $p = 0.0404$). No significant differences between COX-2$^-$ - KI mice and WT mice were observed at further distances with 0.4 and 1.8 intersections at 70µm, respectively ($t(654) = 1.917$, $p = 0.0556$), 0.17 and 1.07 intersections, respectively at 80µm ($t(654) = 1.233$, $p = 0.2182$), 90µm with COX-2$^-$ - KI mice and WT mice having 0.03 and 0.77 intersections respectively ($t(654) = 1.004$, $p = 0.3156$), or 100µm in which COX-2$^-$ - KI mice and WT mice having 0.13 and 0.33 intersections respectively ($t(654) = 0.274$, $p = 0.7843$). These results suggest that there may be a shift of branch dense regions of the dendritic arbor in COX-2$^-$ - KI mice closer to the cell soma. Despite no significant differences in dendritic length (figure 2), we see greater arborization in the 20-40µm range in COX-2$^-$-KI mice. In summary, compared to WT mice, dendritic arborization in COX-2$^-$-KI mice was greater closer to the soma but reduced further away from the soma.

5.3.4 Dendritic Loop Formation

We have previously demonstrated *in vitro* that exposure to PGE2 throughout differentiation of NE-4C stem cells significantly increased the proportion of neurites that formed loops (Kissoondoyal and Crawford 2021). Here, we examine for the first time the proportion of neurites forming dendritic loops in an *in vivo* model. We examined the odds that any given neurite would form a dendritic loop. The greatest exterior angle of all neurites measured was determined, and any neurite with an angle above 270° was classified as forming a dendritic loop as we have previously described (Kissoondoyal and Crawford 2021) (methods; figure 5). When this distinction was made, there were visible differences in the distribution of neurites forming dendritic loops between WT and COX-2^- - KI mice (males and females combined) with a denser distribution of loops in COX-2^-- KI mice (Figure 5). Given the differences in dendritic loop distribution, and the categorical nature of these measurements, a binomial logistic regression was performed to determine whether the odds that any given neurite would self-fasciculate would be affected by condition (WT or COX-2^- - KI), and sex (Table 1).

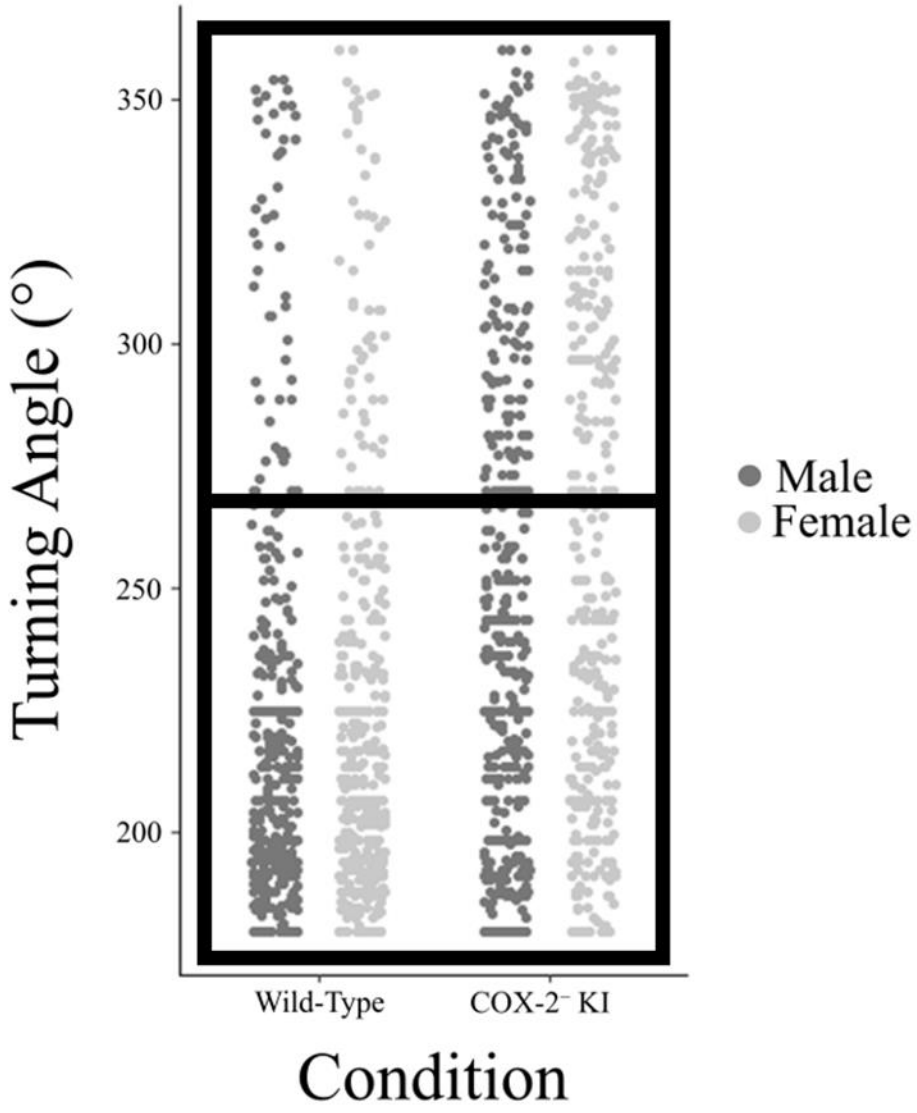

Figure 5-6: Distribution of dendrite exteripr turning angles in the cerebelum of P25 mice. The greatest exterior turning angle of dendrites in COX2^{-}-KI male and female mice at P25 were measured. Dendrites were classified as looping if the greatest exterior angle of the dendrite exceeded 270° and nonlooping otherwise.

We used multinomial logistic regression to determine first the odds that our baseline intercept (WTM) would form dendritic loops. We found an odds ratio of 0.133 in our WTM intercept ($p< 0.001$) indicating that any given dendrite in WTM mice was more likely to be a non-looping dendrite than a looping dendrite (Table 1). Following this, we examined the factors of sex and condition to determine

if changing either of these two factors would affect the odds of observing looping neurites. When compared to the baseline of males (WTM), the sex factor (if the animal was a female) was not significant, indicating there was no effect of the sex of the mouse on the likelihood of loop formation irrespective of the condition (OR = 1.261, p = 0.0595). Interestingly, if a mouse belonged to the COX-2^- - KI treatment group, there was a significant increase in the odds of observing dendritic loops (OR = 3.352, $p < 0.001$). This indicates that the odds of observing dendritic loops was largely increased in COX-2^- - KI mice regardless of sex.

Table 5-1: Odds of observing dendritic loops
The likelihood of a given dendrite forming an axonal loop was determined for Golgi-COX-stained cerebellar neurons of mice at postnatal day 25. Odds ratios (95% confidence intervals) are given for the baseline (Wild-type male) as well as the factors of sex and the COX-2—KI. 3 animals per condition from 3 separate litters were used.

Factor	OR (95% CI)	P-Value
Baseline (WTM)	0.133 (0.152 – 0.117)	**1.76E-55**
Sex (Female)	1.261 (1.4262 - 1.115)	5.95E-02
COX2 KI	3.352 (3.820 - 2.942)	**2.01E-20**

5.3.5 Dendritic Spine Density

Previous research demonstrated that abnormal PGE2 levels may affect dendritic spine density. For example, PGE2 treated cultured preoptic area cells showed an increase in spine-like processes (Amateau and McCarthy 2002). Also, cerebellar injection with PGE2 at PN10 and PN12 inhibited dendritic development, reducing the total number of dendritic spines per neuron (Dean, Wright et al. 2012). Our previous study using the whole genome microarray analysis conducted in COX-$2^{-/-}$ mice showed an impairment in protein networks involved in dendritic spine formation in males [26] but not in females [data not shown]. In this study, we use the COX-2^- - KI mice to examine the dendritic spine density and morphology in the cerebellum (Figure 1 and methods).

A linear mixed effects model was used to fit the dendritic spine density measurements (Figure 6). Observer and litter were assigned as random controls and we examined the fixed effects condition and sex, as well as the interaction between condition and sex. We observed a significant interaction between condition and sex ($t(589) = 4.122$, $p < 0.0001$), but no significant main effects for either condition ($t(20.9) = -1.422$, $p = 0.155$) or sex ($t(579) = -0.789$, $p = 0.430$). Given the significance of the interaction we performed further pairwise comparisons.

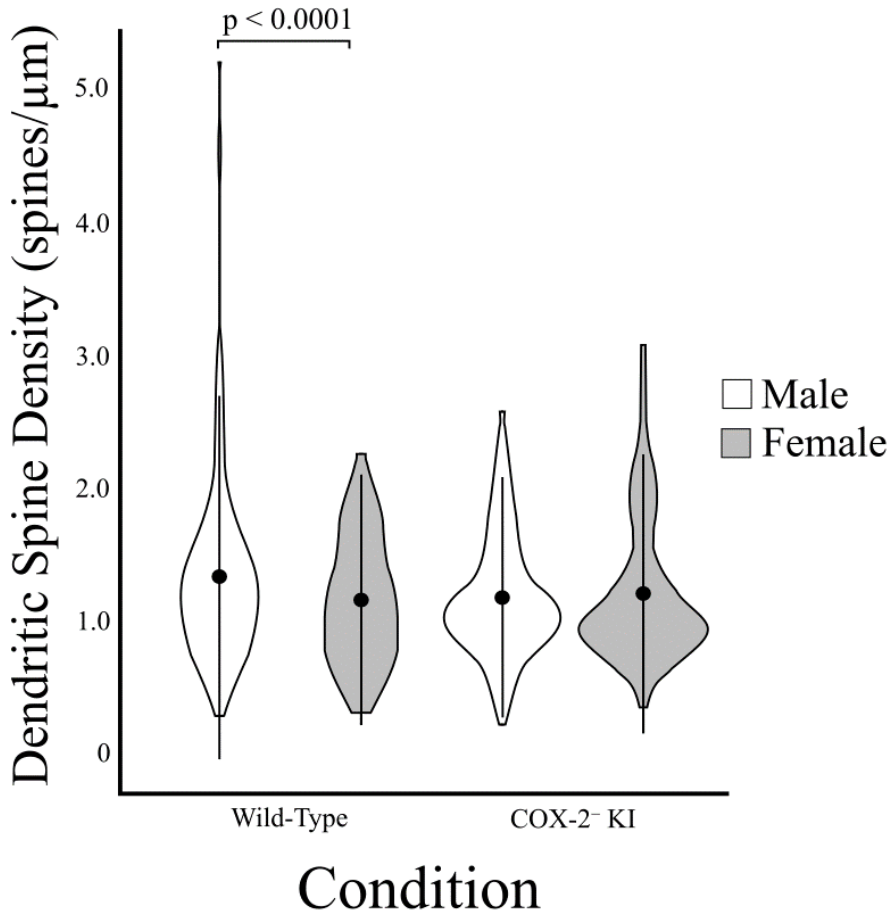

Figure 5-7: Dendritic spine density in the cerebellum of P25 Mice. The number of spines per µm of dendrite were measured from cerebellums of WT and WT and $COX2^-$ - KI male and female mice at P25. Data are presented as mean ± SD. *n* values are recorded in the methods text.

We observed a dendritic spine density of 1.33 spines/µm in WT males. We observed no significant difference between spine density in WT males and COX-2$^-$ - KI males, which had a dendritic spine density of 1.15 spines/µm ($t(49) = -1.995$, $p = 0.0516$). There was also no significant difference between WT females with a dendritic spine density of 1.15 spines/µm and COX-2$^-$ - KI females which had a dendritic spine density of 1.20 spines/µm ($t(54) = 1.442$, $p = 0.155$). Examining sex differences within conditions we see that WT males have a greater spine density than WT females ($t(589) = -5.224$, $p < 0.0001$). Interestingly, there is no significant sex difference between COX-2$^-$ - KI males and females ($t(579) = 0.789$, $p = 0.4302$). We did not observe any significant differences between WT females and COX-2$^-$ - KI males ($t(56) = -0.952$, $p = 0.3452$). These findings show that while there is a sex difference in spine density between WT male and female mice, the COX-2$^-$ - KI males and females no longer show this difference ultimately suggesting a sex-dependent effect of the COX-2 KI on dendritic spine density.

5.3.6 Dendritic Spine Morphology

Changes in the morphology of dendritic spines allow complex regulation of short- and long-term plasticity within the brain (Mel, Schiller et al. 2017). Spine morphology is often classified as mushroom, stubby, or thin with mushroom spines considered as more mature (Figure 7 and methods) (Tashiro and Yuste 2003). Here we examined the relative odds that thin or stubby shaped spines would be observed compared to a mushroom shaped spine in each condition. Multinomial logistic regression was used to determine the likelihood that any given spine was mushroom shaped or was thin or stubby shaped spine within WT or COX-2$^-$ - KI males or females and is represented as an odds ratio (OR Table 2 and methods). An OR above 1 for any given shape within a condition indicates that within that condition, there is a higher likelihood of observing that shape compared to mushroom shaped spines. To control for potential liter and observer bias, both variables were assigned as random effects.

Table 5-2: Odds of observing dendritic spine shapes

The likelihood of a given dendritic spine being a mushroom shaped, or thin, or stubby shaped was determined for dendritic spines of Golgi-COX-stained cerebellar neurons of male and female COX-2^{-}-KI and Wild-type mice at postnatal day 25. Odds ratios (95% confidence intervals) are given for the likelihood of each shape for each condition. Dendritic spines were measured from 3 animals per condition from 3 separate litters.

Factor	Shape	OR (95% CI)	P-Value	Likelihood of observing mature spines
Wild-type Male	Thin	0.998 (1.024– 0.972)	0.928	Increased
	Stubby	0.723 (0.743– 0.703)	**<0.001**	
Wild-type Female	**Thin**	1.855 (1.930 – 1.782)	**<0.001**	Decreased
	Stubby	2.368 (1.382 – 0.906)	**<0.001**	
COX 2 Male	**Thin**	3.057 (3.190 – 2.929)	**< 0.001**	Decreased
	Stubby	3.823 (3.997– 3.657)	**< 0.001**	
COX2 Female	**Thin**	0.346 (0.368 – 0.324)	**< 0.001**	Increased
	Stubby	0.568 (0.604 – 0.535)	**<0.001**	

WT males were less likely to have stubby shaped spines compared to mushroom shaped spines with an odds ratio of 0.723 ($z = -11.54$, $p < 0.001$). However, the odds of observing thin shaped spines compared to mushroom did not differ significantly in WT males with an odds ratio of 0.998 ($z = -0.09$, $p = 0.9281$). Overall, it appears that there is an increased likelihood of observing mature (mushroom) shaped spines compared to immature (stubby) shaped spines in WT males. However, in WT females, there was an increased likelihood of observing both thin and stubby shaped spines compared to mushroom shaped spines, with odds ratios of 2.368 ($z = 20.53$, $p <0.001$) and 1.855($z =15.73$, $p <0.001$) respectively. In contrast to WT males, there was an increase in the odds of observing immature (stubby and thin) shaped spines compared to mature (mushroom) shaped spines.

Compared to mushroom shaped spines, there was an increased likelihood of observing thin and stubby shaped spines in COX-2^{-} - KI males with odds ratios of 3.823 ($z = 30.20$, $p < 0.001$) and 3.057 ($z = 26.22$, $p < 0.001$) respectively. These results indicate that there is an increased likelihood of observing immature (stubby and thin) spines compared to more mature (mushroom) spines in COX-2^{-} - KI males, which is the opposite of what was observed in WT Males. In contrast with COX-2^{-} - KI males, COX-2^{-} - KI females were less likely to observe thin and stubby shaped spines compared to mushroom spines with odds ratios of 0.346 ($z = -16.80$, $p <0.001$) and 0.568 ($z = -9.26$, $p < 0.001$) respectively. These results indicate that there is an increased likelihood of observing mature (mushroom) shaped spines, compared to immature (stubby and thin) shaped spines in COX-2^{-} - KI females. These results contrast the findings in WT females in which there was increased odds of observing immature spines.

Together these results indicate that COX-2^{-} - KI mice have an altered profile of mature and immature spine shapes relative to sex-matched controls with COX-2^{-} - KI males more likely to have immature spines and COX-2^{-}-KI Females more likely to have mature spines than their sex-matched WT controls.

5.3.7 Actin and Spinophilin Protein Expression

The above findings show changes in dendrite thickness, dendritic arborization, the formation of dendritic loops, as well as dendritic spine density and shape within the cerebellum of the COX2^{-} - KI mice. These dendritic dynamics are primarily driven through actin-regulated outgrowth (Harris 1999, Nakahata and Yasuda 2018). Our previous whole genome microarray study in the COX-2$^{-/-}$ mice showed an increased expression level of *β-Actin* (Rai-Bhogal, Ahmad et al. 2018). We previously also showed that exposure of differentiating NE-4C cells to PGE2 affected the actin-dependent neuronal morphology as well as the levels of the actin-binding protein spinophilin [25]. In this study, we examined the expression level of β-Actin and spinophilin in COX-2^{-} - KI mice.

Linear mixed effects modelling was used to fit the fold change of β-Actin relative to the expression observed in WT males across 3 separate western blot runs (figure 8). We tested the fixed effects of condition and sex and the interaction between condition and sex. We assigned technical replicates as a random effect to account for any technical variation as well as the litter of each sample to account for litter bias. We found a significant interaction between condition and sex ($t(20) = 4.083$, $p = 0.0006$) as well as significance for our main effects of condition ($t(11) = -3.592$, $p = 0.002$), and sex ($t(18) = -2.240$, $p = 0.038$).

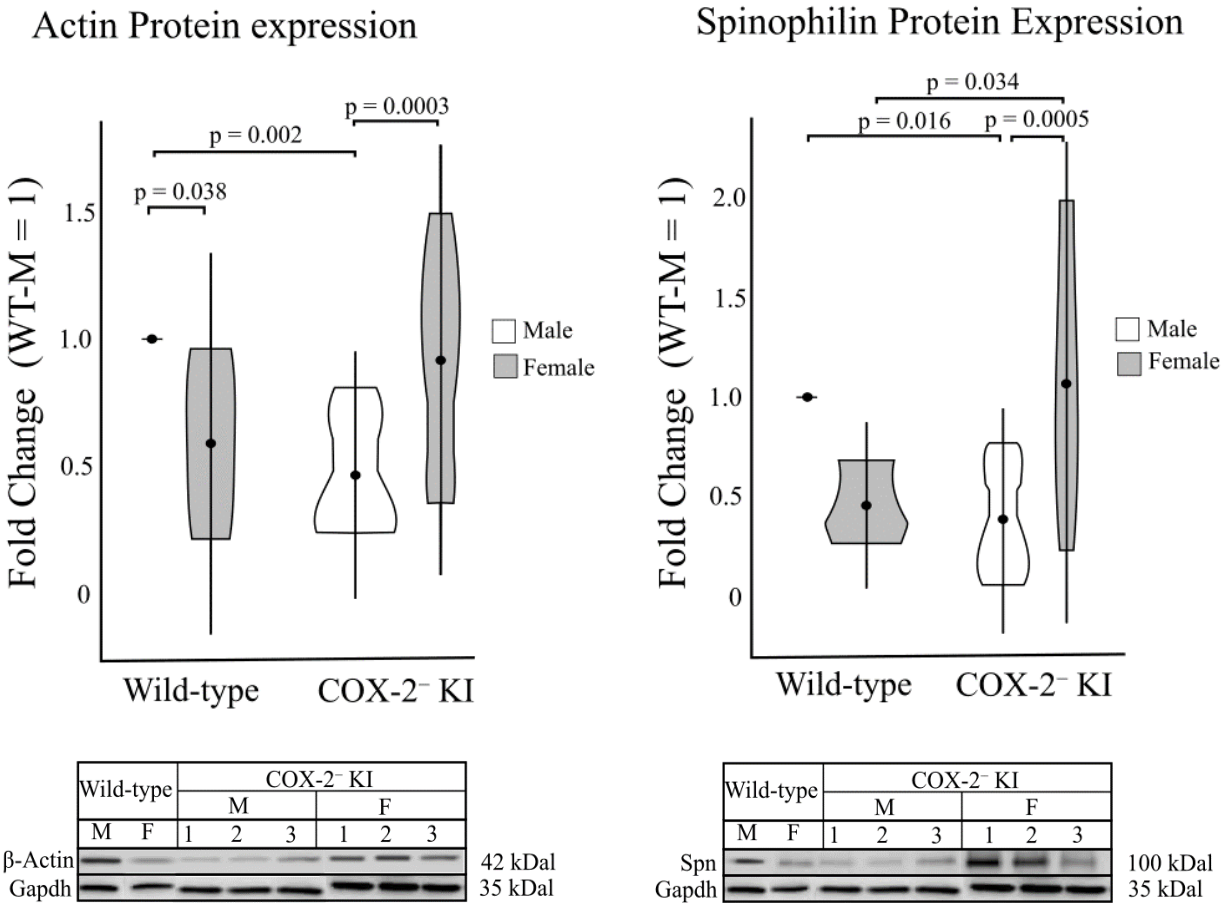

Figure 5-8: Protein expression of Actin and Spinophilin within P25 Mice. Whole brain isolates of Wild-type and COX-2^{-} - KI male and female P25 mice were probed for the expression of β-Actin and Spinophilin. Protein expression was normalized to the house keeping gene GAPDH and then quantified relative to the expression in WT-Males. WT-M and WT-F represent average of 3 animals. Individual COX-2^{-}KI-M and COX-2^{-}KI-F bands are shown on the WB but are plotted as mean fold change ± SD.

Given significant interaction and main effects, we performed pairwise comparisons between treatments. Compared to the WT Males (FC = 1.0) we found a lower expression of β-actin in WT females with a fold-change of 0.487 ($t(18) = 2.402$, $p = 0.038$). However, we saw the opposite effect in the expression of β-actin between COX-2^- - KI males (FC= 0.3302) and COX-2^- - KI females (FC= 0.8743) ($t(17) = -4.5086$, $p = 0.0003$). We observed a decrease in β-actin expression in COX-2^- - KI males compared to WT males ($t(18) = 3.5198$, $p = 0.002$). In contrast, we saw no significant difference in β-actin expression between WT Females and COX-2^- - KI females ($t(18) = - 0.8398$, $p = 0.4118$). These results suggest that there is a sex-dependent effect of the COX-2 KI on β-actin expression. The sex-dependent difference in β-actin expression observed was opposite to what was observed in COX-2^- -KI mice.

To examine the expression of Spinophilin, we used linear mixed effects modeling to fit the fold change relative to the expression in WT males across 3 separate western blot runs. Condition and sex as well as the interaction between condition and sex were examined as fixed effects, and run number and litter were used as random factors. We saw a significant interaction between condition and sex ($t(20) = 3.568$, $p = 0.002$) as well as the main effect of condition ($t(19) = -2.652$, $p = 0.016$), however the effect of sex was non-significant ($t(19) = -1.818$, $p = 0.085$).

Given the significance of the interaction term we performed pairwise comparisons between treatments. Using WT males as a reference (FC = 1) we found no significant difference in spinophilin expression between WT males and females (FC =0.456) ($t(19) = 1.8184$, $p = 0.085$). In contrast the expression of spinophilin was lower in COX-2^- - KI males (FC = 0.353) compared to COX-2^- - KI females (FC = 1.022) ($t(19) = 04.1469$, $p = 0.0005$). We saw a significant reduction in spinophilin expression in COX-2^- - KI males compared to WT males ($t(18) = -4.5086$, $p < 0.001$).but a significant increase in spinophilin expression in COX-2^- - KI females compared to WT females ($t(18) = -2.2847$, p

= 0.034). These results show that the COX-2$^-$ - KI affects the expression of spinophilin in males and females differently, with the expression in COX-2$^-$ - KI males being lower than WT males, and the opposite in females.

In summary, we found sex-dependent differences in expression of β-actin and actin-bound spinophilin in both the WT and COX-2$^-$ - KI. We saw that the expression of β-actin was reduced by the COX-2$^-$-KI in females specifically and that the expression of spinophilin was reduced in COX-2$^-$-KI males specifically.

5.4 Discussion

5.4.1 Abnormal Dendrite outgrowth in COX-2$^-$-KI mice

The primary objective of this study was to understand how the lack of COX-2 function in the COX-2 KI offspring affected dendritic morphology within the cerebellum. We demonstrate for the first time *in vivo* that abnormal COX-2/PGE2 signaling in COX-2 KI offspring affects actin-dependent morphology of dendritic extensions and dendritic spines in a sex-dependent manner. Within the cerebellum of COX-2 KI male and female offspring cells exhibited increased dendritic arborization at distances closer to the soma and increased the odds of dendritic loop formation (Table 3). COX-2 KI males specifically had increased dendrite thickness, and lower odds of seeing mature spines compare to the age matched controls. Finally, the COX-2 KI had divergent effects on the expression of β-actin and the actin-binding protein spinophilin, resulting in sex differences opposite to WT mice.

Table 5-3: Summary of COX-2^{-}-KI effect on dendritic morphology

Dendritic morphology findings are summarized. The effect and direction of the effect between COX-2^{-}-KI male and females are shown compared to sex matched WT controls.

Measure	COX-2 Male	COX-2 Female
Dendrite Length	-	-
Dendrite Thickness	↑	-
Dendritic Arborization	↑	↑
Dendritic Looping	↑	↑
Spine Density	-	-
Odds of Mature Spines	↓	↑
Expression of β-Actin	↓	
Expression of Spinophilin	↓	↑

Various clinical and epidemiological studies have reported that abnormal PGE2 levels during prenatal development due to genetic or environmental risk factors are linked to neurodevelopmental disorders including ASD (Wong and Crawford 2014, Wong, Wais et al. 2015). We have already shown that the COX-2^{-} - KI mice exhibit autism-related symptoms such as impaired social interaction, hyperactivity, and anxiety (Wong, Bestard-Lorigados et al. 2019). In addition, our previous whole-genome microarray study in the COX-2 deficient mouse model showed impairments in protein networks involved in dendritic spines and synaptic development in males specifically (Rai-Bhogal, Ahmad et al. 2018). This study provides further molecular evidence that the lack of PGE2 producing enzyme COX-2 leads to sex-dependent morphological changes in dendrite extension and dendritic spines in the cerebellum.

Our lab previously observed an increase in the neurite length of differentiated cells neuroectodermal (NE-4C) cells exposed to PGE2 (Kissoondoyal and Crawford 2021). While there was not a significant effect of COX-2$^-$ - KI on dendrite length, dendritic length is often examined in conjunction with dendritic arborization. We found significant changes in dendritic arborization in COX-2$^-$ - KI mice based on the distance from the cell soma, irrespective of sex. Cells in the COX-2$^-$ - KI mouse cerebellum had increased arborization at 20-40µm and decreased arborization at 60µm from the cell soma compared to WT mice. Another study also found an increase in branch density as a function of distance in postnatal rats as a result of local injection of COX-2 inhibitors into the cerebellums between PN7-PN13. Maternal stress, which can elevate PGE2 levels during pregnancy, between gestational days 14 and 20 also increased the dendritic arbor area of Purkinje cells in rats postnatally (Pascual, Ebner et al. 2010). There is a strong positive correlation between the branching as determined from Sholl analysis with the expected number of synaptic contacts a given cell will make; greater branching is associated with a greater number of synaptic contacts (Liley and Wright 1994). Further, branching points are a major determinant of the direction of signals and ultimately integration of incoming signals by a neuron (Koch, Poggio et al. 1982).Within the cerebellum it appears that COX-2$^-$-KI mice likely form more synaptic contacts than WT controls, particularly in regions closer to the soma.

Though we found that in the healthy brain WT females had thicker dendrites than WT males, we observed a sex-specific opposite trend in COX-2$^-$-KI mice with males having thicker dendrites than females. Dendritic thickness affects the transmission of postsynaptic potentials, by affecting electrical resistance, with thicker dendrites propagating signals more easily than thin dendrites (Koch, Poggio et al. 1982, Loopuijt, Silva Filho et al. 2007). Dendritic diameter was previously positively correlated with total Post Synaptic Density (PSD) area within the cerebellums of 12-week-old C57BL/6 male mice; a greater diameter corresponded to greater Post Synaptic Density area (Parajuli, Urakubo et al.

2020). The increase in dendritic diameter in COX-2^{-}-KI males observed in our study could result in excitatory/inhibitory (E/I) signalling imbalances within the cerebellum that are observed in other ASD mouse models (Rubenstein and Merzenich 2003, Lee, Lee et al. 2017, Sohal and Rubenstein 2019). E/I signalling imbalances have been observed in the Scn1a$^{+/-}$ mouse model of autism in which inhibitory dysfunction was connected to social deficits and repetitive behaviours (Han, Tai et al. 2012, Han, Tai et al. 2014). In both studies, rescue of the inhibitory dysfunction reduced social behaviour abnormalities. Studies in Rett syndrome mouse models have found reduction in the function and development of interneurons (Mierau, Patrizi et al. 2016). The male specific COX-2^{-}-KI effect in dendritic thickness could explain the suggested E/I imbalance in ASD may affect males and females differently (Lai, Lombardo et al. 2015, Lai, Lerch et al. 2017, Trakoshis, Martínez-Cañada et al. 2020).

Self-fasciculation of dendrites is characteristic of a dysregulation in actin cytoskeletal dynamics, either in the polymerization/depolymerization rate, or in the direction of polymerization and can result in dendritic loops (Amthor and Oyster 1995, Sdrulla and Linden 2006). We observed an increase in the odds of dendritic loop formation in COX-2^{-} KI mice, suggesting abnormal pathfinding and potential disruptions in actin cytoskeletal dynamics. We previously demonstrated that increased PGE2 levels during differentiation of neuroectodermal stem cells increased the proportion of neurites forming loops (Kissoondoyal and Crawford 2021). In this study we demonstrate for the first time *in vivo* that COX-2^{-} KI can also increase dendritic looping in both males and females. Dendritic circuit abnormalities, resulting from disruptions in self-avoidance have been linked to neurodevelopmental disorders including retinal dysplasia (Weiner, Koo et al. 2004) and ASD (Minshew and Williams 2007). Similar to the above models, we expect that some cerebellar neurons of COX-2^{-}-KI mice to exhibit difficulties in the formation of synaptic connections, ultimately affecting circuit organization within the cerebellum.

5.4.2 Dendritic spine formation in COX-2$^-$-KI mice

For the first time to our knowledge, we demonstrate innate sex differences in dendritic spine density of adolescent C57BL/6 mice, with males having a greater spine density than females. Interestingly, in the COX-2$^-$ KI mice, the expected sex differences in density of dendritic spines were no longer observed. While we did not find statistically significant differences between our WT and COX-2$^-$ KI male mice, there was a strong trend towards a significant decrease in density in COX-2$^-$ KI males compared to WT males. Reduction in dendritic spine density were previously observed within the cerebellums of other ASD animal models including prenatal VPA-exposed rat models (Mychasiuk, Richards et al. 2012), as well as the genetic Shank3 and Mecp2 deficient rat models (Hering and Sheng 2001, Penzes, Cahill et al. 2011). Within post-mortem human cerebellums of ASD individuals with the UBE3A mutation reduced spine densities were also observed (Yi, Berrios et al. 2015). Interestingly, many of these studies have only examined males, and while their results match the trend we observed in our COX-2$^-$-KI males results, we saw no differences in females, further emphasizing the need for examining sex as a factor in analysis.

Beyond spine density, trends in the shapes of dendritic spines can give us insights into the contribution of the dendrite to overall neuron synaptic plasticity (Lai and Ip 2013). Mushroom spines are typically thought of as more mature spines, are often associated with spine long term potentiation (LTD) (Kasai, Matsuzaki et al. 2003, Bourne and Harris 2007), while thin and stubby spines are often considered immature spines, transitioning from thin, to stubby, to mushroom (Bourne and Harris 2008). Here we use the likelihood of observing a particular spine shape (thin, stubby, and mushroom) to provide information about relative amounts. Our WT males had a greater density of spines that were more likely to be mature (mushroom) shaped compared to WT females having more immature spines (stubby and thin). However, in the COX-2$^-$ KI mice, the expected sex differences in density of dendritic spines were no longer observed, with males were more likely to have immature (stubby and thin) and females mature (mushroom) shaped spines, in contrast to WT mice. Dendritic spine profiles, which

include information about spine density as well as the relative amount of mature to immature spines are affected in autism related disorders (Penzes, Cahill et al. 2011, Joensuu, Lanoue et al. 2018). In Rett syndrome for example there is a lower density of spines in early phases of development, with a lower proportion of mushroom spines, and in Fragile X syndrome in which there is a higher density of spines in early stages of development with a higher proportion of immature spines (Phillips and Pozzo-Miller 2015). Changes in dendritic spine profiles including density and morphology differences in our COX-2 deficient mice show strong evidence of disruption in cerebellar development.

5.4.3 Abnormal β-Actin and Spinophilin expression in COX-2^{-}-KI mice

Throughout this study we observed changes in dendritic morphology as resulting from the COX-2 KI, often observed in a sex-dependent manner. Actin is the primary cytoskeletal element of dendritic spines providing stability and support (Bosch and Hayashi 2012, Penzes and Rafalovich 2012, Koleske 2013). The rate of actin polymerization and depolymerization influences the shape and growth of dendritic spines during development and learning (Pollard and Cooper 2009, Blanchoin, Boujemaa-Paterski et al. 2014). To further understand the differences in dendritic morphology in our COX-2^{-}-KI mice we examined the expression of β-actin as well as the actin-binding protein spinophilin highly enriched in dendritic spines. We found sex-differences in the expression of β-actin in the WT mice with lower expression in females and the opposite effect in the COX-2^{-}-KI mice. Moreover, while there was a decrease in β-actin expression in COX-2^{-}-KI males compared to WT males, no difference was observed between females, suggesting that the effect was COX-2^{-}-KI male specific (table 3). With regards to spinophilin expression, we observed a male decrease but an increase in females in our COX-2^{-}-KI mice compared to our WT controls. Dendritic morphologies examined here including dendrite outgrowth and dendritic spine morphology are dependent on actin cytoskeletal structure (Matus 2000, Pelucchi, Stringhi et al. 2020). Decreases in β-actin and actin stabilizing molecules underly the transition from mature to immature spine shapes that are seen during long-term depression (LTD) with

the opposite being true for LTP (Hotulainen and Hoogenraad 2010, Pelucchi, Stringhi et al. 2020). It is likely that the increase and decrease in β-actin in COX-2^{-}-KI males and females respectively, may partially explain the decrease and increase in the odds of observing mature spines in COX-2^{-}-KI males and females, respectively.

Spinophilin functions as a mediator between excitatory synaptic activity and actin driven changes in dendritic and dendritic spine morphology and function through its role in stabilizing the actin cytoskeleton (Satoh, Nakanishi et al. 1998, Yan, Hsieh–Wilson et al. 1999). One study found spinophilin expression was increased in the cerebellums of PN14 mixed sex rat pups injected postnatally with the COX-2 inhibitor nimesulide (Dean, Knutson et al. 2012). Spinophilin interacts with actin and plays a key role in dendritic spine morphology (Satoh, Nakanishi et al. 1998, Grossman, Hsieh-Wilson et al. 2002, Ryan, Alldritt et al. 2005). Spinophilin knock-out mice were found to have an increase in dendritic spine density, and a retraction of filopodial extensions (Feng, Yan et al. 2000). Those findings are dissimilar to our findings in which we did not see differences in dendritic spine density or in dendrite length. However, the changes in spinophilin expression agree with our spine morphology findings. LTD, which is normally associated with the transition from mature spines (mushroom) with to immature spine shapes (thin, and stubby), was reduced in spinophilin knock-out mice (Zhou, Homma et al. 2004, Hill and Zito 2013). The decrease in spinophilin levels in COX-2^{-}-KI males could correspond to a decrease in mature spines, agreeing with our findings. In contrast, the increase in spinophilin in COX-2^{-}-KI females, would correspond to an increase in mature spines, again agreeing with our findings. The morphological changes in dendritic thickness, arborization or dendritic loop formation observed in the cerebellums of COX-2-KI mice along with the abnormal expression levels of β-Actin and spinophilin suggest that the impaired PGE2 signaling in the COX-2 KI offspring may affect synaptogenesis and synaptic signalling within the cerebellum.

To summarize, COX-2^{-} KI mice showed disruptions in dendrite extensions and dendritic spine morphology, with effects on spine morphology having distinct sex-dependent effects. We demonstrated changes in dendritic architecture within the cerebellums of COX-2-KI mice that suggest abnormal neurite pathfinding, and formation of synaptic connections throughout development. These findings provide an important first step in examining the importance of PGE2 signalling in cerebellar development.

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Chapter 6: Study 3

Study 3: Maternal exposure to prostaglandin e2 results in abnormal dendritic morphology in the cerebellum and related motor behaviour in offspring

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The dissertation was written in thesis format and the following is specific to the contributions in the chapter.

Contributions: Ashby Kissoondoyal designed all behavioural, protein, and Golgi-COX experiments, analyzed all data, prepared all figures, and prepared the manuscript. Kelly Ho performed all behavioural experiments. Christine Wong performed cerebellar migration experiments. Sakina Hussein analyzed dendritic arborization and axonal looping and Dorota A. Crawford supervised the design of the study and was involved with editing the manuscript

6.1 Introduction

Prostaglandin E2 (PGE2) is a bioactive lipid signalling molecule, that plays a vital role in nervous system development including mediating cell proliferation and differentiation, dendritic spine formation, synaptogenesis, and learning and memory (C. Chen & Bazan, 2005; Tassoni et al., 2008). Synthesis of PGE2 begins with the release of arachidonic acid (AA) from the cell membrane through the enzymatic activity of phospholipase A2 (PLA2) (Sang & Chen, 2006). PGE2 is then metabolized from AA through cyclooxygenase -1 or -2 (COX-1 or COX-2) (J. Y. Park et al., 2006; Rouzer & Marnett, 2009). BCOX-1 and COX-2 are constitutively expressed in the brain with COX-1 primarily within microglial cells (Hoozemans et al., 2001; Schwab et al., 2000) and COX-2 mainly in neuronal cells (Kirkby et al., 2016; Maslinska et al., 1999).

Abnormal levels of PGE2 during prenatal development as a result of disruptions in COX-2/PGE2 signalling are linked to the etiology of Autism Spectrum Disorders (ASDs) (Tamiji & Crawford, 2010b; C. Wong & D. A. Crawford, 2014). During prenatal development PGE2 levels can be affected by various environmental risk factors including air pollution (L. G. Costa et al., 2017; Volk et al., 2011), glyphosates and other herbicides (A. Samsel & S. Seneff, 2015; K. Suzuki et al., 2011), phthalate molecules commonly used in consumer products, and common over the counter drugs such as acetylsalicylic acid and acetaminophen (Alemany et al., 2021). Misuse of the drug misoprostol, a synthetic analogue to PGE2, for a termination of pregnancy has been linked to ASD and Moebius syndrome (Bandim et al., 2003). These environmental risk factors are all linked to ASDs (Tamiji & Crawford, 2010b; Christine T Wong et al., 2015).

ASDs include a group of neurodevelopmental disorders which are primarily characterized through behavioural deficits including those in social interaction, repetitive behaviour, and increased anxiety (Battle, 2013). ASD individuals also display motor deficits including delays in developmental

milestones including lying, righting, sitting and crawling (Teitelbaum, Teitelbaum, Nye, Fryman, & Maurer, 1998), deficits in both fine and gross motor movements (Nancy J Minshew, Sung, Jones, & Furman, 2004), difficulties in postural control (Kohen-Raz, Volkman, & Cohen, 1992; Nancy J Minshew et al., 2004), and a general lack of motor coordination appearing as clumsiness (Ghaziuddin & Butler, 1998; Mari, Castiello, Marks, Marraffa, & Prior, 2003; Miyahara et al., 1997). These atypical behaviours correspond to molecular and morphological changes within associated brain regions (Courchesne et al., 2019). Within the post-mortem brains of ASD individuals, studies have found impairment in cell layering within the cortex, atypical minicolumns organization and overall underconnectivity across the brain (Abrams et al., 2013; Ecker et al., 2013; M. R. Herbert et al., 2003; Redcay & Courchesne, 2005; Stoner et al., 2014; Wegiel et al., 2010). We have previously demonstrated that mice prenatally exposed to PGE2 at the onset of neurogenesis (PGE2-exposed) and mice lacking COX-2 (COX-2^{-}-KI) exhibit autism-related behaviours such as deficits in social communication, repetitive behaviours, hyperactivity, and anxiety (Kissoondoyal et al.; C. T. Wong et al., 2019; C. T. Wong, I. Bestard-Lorigados, R. Rai-Bhogal, & D. A. Crawford, 2017).

The cerebellum is often disrupted in ASD individuals (E. Courchesne et al., 2005; D'Mello et al., 2015; Mosconi et al., 2015; Palmen, van Engeland, Hof, & Schmitz, 2004). For example, post-mortem samples from ASD individuals were found to have increases in Purkinje cell sizes (S. H. Fatemi et al., 2002; Wegiel et al., 2013; Whitney, Kemper, Bauman, Rosene, & Blatt, 2008). Additionally, developmental disorders associated with the cerebellum including flocculondular dysplasia (Wegiel et al., 2013), cerebellar hypoplasia (Wegiel et al., 2010), and the presence of cerebellar spheroids (Weidenheim et al., 2001), have been found in humans with ASD. Early pre- and postnatal disruptions of cerebellar development are positively correlated to the development of ASD (D. Q. Beversdorf et al., 2005; E. Courchesne et al., 2001; Hashimoto et al., 1995; C. Limperopoulos et al., 2007). We have previously found evidence that impaired PGE2 signalling can disrupt cerebellar

development. We observed sex-dependent changes in the morphology of dendrites and dendritic spines within the cerebellums of COX-2^{-}-KI mice including increases in dendritic thickness, arborization, and the incidence of dendritic loops as well as changes in the likelihood of observing mature relative to immature dendritic spines (Kissoondoyal et al., 2021). Another study found that a local postnatal injection of PGE2 into the rat cerebellums increased local estriol production (S. L. Dean, Wright, et al., 2012) that subsequently resulted in stunted dendritic arbors and a reduction in cerebellar synaptic density [46]. These rats also exhibited impairments in social play behaviour associated with the cerebellum.

In this study we examine the sex-dependent effect of a single maternal exposure to PGE2 (PGE2-exposed mice) at gestational day 11 (G11) in offspring on cell density from cells originating from G11 and G16, and on dendritic and dendritic spine morphology within the cerebellum at postnatal day 30 (PN30). We then examine the expression levels of cytoskeletal proteins spinophilin, □-actin, and N-cadherin. We show for the first time *in vivo* that a single exposure to PGE2 during a critical time in pregnancy has significant molecular and morphological consequences within the cerebellum, that result in sex-dependent manifestations of relevant behaviours in offspring.

6.2 Methods

6.2.1 Animals

Male and female C57BL/6 mice were purchased from Jackson Laboratories. All animals were housed via group housing at the York University animal facility. Male and female C57BL/6 mice were housed together overnight for breeding and each morning females were checked for the presence of a vaginal plug. Gestation day 1 (G1) was marked as the day that a vaginal plug was observed and from that point on the females were housed individually for the remainder of their pregnancy. On G11, females were injected with a single subcutaneous injection of 0.25µg/g 16,16 dimethyl prostaglandin E2 (dmPGE2; Cayman Chemical) diluted in saline to a final volume of 300µL. While considered an analogue to

PGE2, dmPGE2 is metabolized at a slower rate than PGE2 and as such remains active for a longer time frame (T. Ohno, H. Ohtsuki, & S. Okabe, 1985; S. Steffenrud, 1980). Control animals were injected with 300µL saline. Injections of dmPGE2 were performed on G11 to coincide with the onset of neurogenesis in mice (B. D. Semple, K. Blomgren, K. Gimlin, D. M. Ferriero, & L. J. Noble-Haeusslein, 2013). In the mouse, the neurogenic interval extends from gestation day 11 (G11) through early G17 in most brain regions (Clancy, Darlington, & Finlay, 2001; Clancy, Finlay, Darlington, & Anand, 2007; Rodier, 1980; Takahashi, Nowakowski, & Caviness, 1995). It also corresponds to the time the drug misoprostol was misused by pregnant women to terminate pregnancy that resulted in Moebius syndrome and autism (Bandim et al., 2003; Pastuszak et al., 1998). Mouse offspring subjected to dmPGE2 exposure will be referred to as "PGE2-exposed mice" and those injected with saline will be referred to as "Saline control mice".

6.2.2 CldU and IdU labelling

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 saline control and PGE2-exposed mice. CldU and IdU are different thymidine analogues that are incorporated into the dual helix of any cell actively synthesizing DNA during at the time that the thymidine marker is injected (Pastuszak et al., 1998; Alex H. Tuttle et al., 2010) With this technique, we were able to detect differences in cell proliferation in the cerebellum at two unique time-points. An injection of CldU at G11 and IdU at G16 were given, to capture both early and late phases of this interval, and animals were sacrificed at Postnatal Day 8 (PN8). For PGE2-exposed mice, pregnant dames were given a single co-injection of CldU and PGE2 at G11 at described concentrations.

6.2.3 Immunohistochemistry (IHC)

Left hemisphere brain samples were extracted and immediately fixed at4°C for 48 hrs in 4% paraformaldehyde (in PBS). Paraffin-embedding and serial slicing from the mid-sagittal plane outwards in slices of 4μm in thickness were completed by The Centre for Phenogenomics (Toronto, Canada). Immunohistochemistry was performed as previously described (A. H. Tuttle et al., 2010). Xylene incubation was performed to remove paraffin from samples. This was followed by rehydration through serial ethanol incubation and followed by cell permeabilization with 0.2% Triton X-100 in PBS. We then performed antigen retrieval using 0.01M pH 6.0 sodium citrate buffer followed by a 1.5N HCl incubation. Sections were circled using a liquid blocking super PAP pen (Cedarlane) and sections were then treated at 37°C for 3 min with 0.25% trypsin EDTA in a pre-warmed hydration chamber. The remaining steps were then performed in a hydration chamber. For primary antibody staining, samples were first blocked in 5% goat serum diluted in PBS, followed by 4°C overnight incubation of the Cldu antibody, Rat anti-BrdU (1:100, ab6326, Abcam), diluted in 5% goat serum in PBS. Samples were then incubated at 37°C with agitation for 20 min at 225 rpm in a high stringency wash of low salt TBST buffer (36 mM Tris, 50mM NaCl, 0.5% tween-20; pH 8.0) (Alex H. Tuttle et al., 2010). For the Idu primary antibody (Mouse anti-IdU;1:100, ab181664, Abcam), samples were incubated overnight at 4°C overnight. 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).

CldU and IdU staining was visualized and captured using an Eclipse 80i upright fluorescent microscope with DS-5MC camera (Nikon). To correct for variations between brain slices, estimated cell density measurements in the cerebellum were calculated by dividing the total number of cells counted by the area.

6.2.4 Golgi COX Staining

Whole brain tissues were collected at PN30 from 3 males and 3 females from 3 separate litters from both Saline Control and PGE2-exposed mice. Golgi staining was performed as we have previously described (Zaqout & Kaindl, 2016). After extraction, brains were dissected in half, rinsed in cold 0.1M PBS solution and were immediately placed in a 4% paraformaldehyde solution for a day. Following fixation brain samples were washed with ddH2O and placed in Golgi-COX solution (5% w/v potassium dichromate, 5% w/v mercuric chloride, and 5% w/v potassium chromate) dissolved in ddH2O. After 24h the brain tissues were transferred into fresh Golgi-COX solution and incubated for an additional 7 days at room temperature in the dark. The brains were then transferred into tissue protectant solution (30% w/v sucrose, 20% w/v ethylene glycol, and 1% w/v polyvinylpyrrolidone) in ddH2O and kept in this solution for up to 7 days.

Brains were sliced along the sagittal in slices of 100µm in thickness using cryostat sectioning by the research histology lab at the University Health network (Toronto, Ontario; www.uhnresearch.ca). Brain slices were mounted onto gelatin-coated slides and staining was developed with a 3:1 ammonia to H2O solution followed by a 5% w/v sodium thiosulfate in H2O solution. Serial dehydration in 70%, 95% and 100% ethanol and then immersion in xylene was performed before the slides were cover-slipped.

Cerebellar G11 and G19 immunolabelled slides were imaged on Eclipse 80i upright fluorescent microscope with DS-5MC camera (Nikon). Cerebellar Golgi-cox-stained slides were imaged on the Zeiss Laser Scanning Confocal Microscope (LMS 700; Advanced Light and Electron Microscopy at York University), using brightfield microscopy. The full cerebellum of each animal was imaged at 10x magnification with z-stacks taken every 1-2.5µm (50-100 stacks on average). Individual cell within the cerebellum were imaged using 100x magnification with z-stacks taken every 0.01 – 0.02µm (1000-2000 stacks on average).

6.2.5 Analysis of cell density and dendritic arborization, length, and loop quantification

Cerebellar G11 and G16 were counted, and cell density was calculated by dividing the total number of CldU and IdU cells by the area of interest as described in (Kissoondoyal et al.; C. T. Wong et al., 2017). Image analysis was completed using NIS-Elements software (Nikon). Cell selection, sholl analysis and quantification of axonal loop formation was performed as previously described in (Kissoondoyal et al., 2021). From the 10x magnification images taken, 5 neuronal cells per animal within the cerebellum were chosen for a total of N=60 cells with 15 cells per condition. The Simple Neurite Tracer (SNT) plugin within the open-source Fiji ImageJ software was used to conduct sholl analysis (Longair et al., 2011; Rueden et al., 2017; Schindelin et al., 2012). Concentric circles were generated by the software every 10µm from a point defined in the center of the soma of each neuron examined and the number of intersections cell extensions made with these circles was measured. Dendritic length measurements were taken from SNT established arbors. The average length of all primary dendrites was taken for each cell used for sholl analysis. We previously demonstrated a method for the quantitative quantification of self-fasciculation in neurites (Ashby Kissoondoyal & Dorota A. Crawford, 2021; Kissoondoyal et al., 2021). Using the traced neurites from the SNT plugin, the maximum angle of each neurite was used to classify each neurite as looping or non-looping; neurites with a turning angle greater than 270° were classified as looping as previously described (Kissoondoyal et al., 2021).

6.2.6 Dendritic Spine analysis

Individual cell images were used to quantify dendritic spine density and morphology. Identification and classification of dendritic spines was performed using Nikon NsAi software (Nikon). Training of the SegmentAI software was performed on a previously quantified image set with a training loss off less than 0.02 used as a cut-off for successful training. DenoiseAI was used to reduce background detection. After SegmentAI detection of dendritic spines, false positives were filtered out by length (> 0.2µm), width (> 0.2µm), circularity (< 0.88), and area (> 0.6µm^2). Dendritic spine

density was calculated as the number of dendritic spines per the length of the dendrite. Each dendritic spine was classified as a shape through a sequential top-down approach we have previously described (Kissoondoyal et al., 2021). Spines with a width > length were classified as stubby, of the remainder those with a width > 0.6μm were classified as mushroom, and those that remained were classified as thin.

6.2.7 Motor Behaviour

Mice were kept on a 12-hour light/dark cycle with behavioural testing administered from the start of the light phase. Mice were separated by condition into PGE2-exposed (see maternal injections in methods) and vehicle, and by sex (male and female) on the day of weaning. From each condition 10 males and 10 females were selected from at least 3 litters. To ensure that litter could be assigned as a random effect in statistical analyses, 10 litters were used, with at least 2 animals used per litter. Between behavioural testing, all equipment was disinfected and deodorized with antiseptic clinicide and wiped clean. All behavioural tests were administered by the same female researcher to avoid increases in stress levels reported in behavioural tests administered by male experimenters (Sorge et al., 2014). To facilitate more accurate comparisons of overall motor behaviour of each mouse, each mouse was tested in each of the adhesive sticker, grid walking and cylinder tests. All behavioural tests were measured on PN30 with training days occurring the two days prior (PN28 and PN29) and recorded. The recorded videos were analyzed by 5 trained individuals.

6.2.8 Adhesive Sticker Test

The adhesive sticker test was used to assess sensory motor limb coordination (S. M. Fleming et al., 2004; H. A. Lam et al., 2011; Richter, Gerstenberger, Bauer, Liang, & Richter, 2017). A red sticker with a diameter of 0.5cm was placed just above the nose of a restrained mouse. The mouse was released into an empty cage with bedding and the mouse was recorded from above. Each mouse was tested at PN30 for 3 trials with at least 2 minutes of rest between each trial. The average of their

measurements for the *first contact* with the adhesive sticker, and the number of *swipes per second* to remove the sticker were recorded. Time until first contact was recorded as the time from when each mouse was released until either forelimb contacted the adhesive sticker, and number of swipes per second were calculated as the number of attempts at removing each sticker divided by the time from *first contact* until the sticker was removed.

6.2.9Grid Walking Test

The grid walking test was used to assess sensory motor coordination of the limbs and balance (Feather-Schussler & Ferguson, 2016; Heyser, 2004). We used an apparatus consisting of an elevated metal grid, enclosed with wooden walls and an open top. An opening in the mesh grid on one end allowed the mouse to escape into a home cage with bedding and space to hide. The grid walking test spanned 3 days (2 training and one testing) starting on PN28 (testing day occurred on PN30). Training days consisted of two sets of 5 runs in which the mice were placed inside the grid walking apparatus (60cm x 13cm x 7.5cm) with a mesh grid of 1cm x 1cm to acclimatize to the test. Testing day included 1 warmup run followed by three trials with at least 2 minutes of rest between each trial. The testing apparatus (60cm x 20cm x 7cm) had a mesh grid of 2.5cm x 2.5cm. To record limb slips, a mirror was placed such that the bottom of the mesh grid could be recorded (Fig 7A). The recorded videos were manually analyzed by 5 trained individuals and the average of their measurements for number of slips through the grid and the total time to escape were recorded.

6.2.10 Cylinder Test

Exploratory behaviour and spatial motor behaviour were assessed using the cylinder test (Rattka, Fluri, Krstić, Asan, & Volkmann, 2016; Schönfeld, Dooley, Jahanshahi, Temel, & Hendrix, 2017; Schönfeld, Jahanshahi, et al., 2017). Mice were placed into a long glass cylinder (8.5cm diameter x 21.5 cm) with an open top. The glass cylinder was placed on top of a pane of glass and the bottom view was recorded. The test spanned 3 days (2 training and 1 testing) starting on PN28 (with testing

occurring on PN30). Training days consisted of 3, 90 second runs with at least 5 minutes between each run. On testing days, each mouse performed 1 warmup run of 3 minutes, followed by 3, 10-minute trials, with at least 5 minutes rest between each trial. Recorded videos were manually analyzed by 5 trained individuals and the average of their measurements for number of rears, steps, and forelimb glass touches were recorded.

6.2.11 Protein isolation and Western Blotting

Cerebellar brain tissue samples were collected from Saline and PGE2-exposed male and female mice offspring from animals that underwent behavioral testing at PN30. Following homogenization of tissue samples using a polytron power homogenizer, total protein isolation was performed through standard RIPA method (Abcam). From each sample 30-40µg of protein was loaded into a 12% polyacrylamide gel as previously described (Ashby Kissoondoyal & Dorota A. Crawford, 2021; R. Rai-Bhogal, C. Wong, et al., 2018b). Protein samples from Saline Control and PGE2-exposed male and female were pooled for the analysis. In addition, protein samples from select PGE2-exposed male mice which performed exceptionally low or high on behavioral tests were loaded individually. Protein samples were separated using PAGE gel electrophoresis before being transferred to a 0.45µM nitrocellulose membrane (BioRad). Before probing with primary antibodies, membranes were washed in TBS and blocked in 5% milk in TBS-T (1X Tris buffer saline 0.05% Tween 20) for 1h at room temperature. Protein samples were probed with polyclonal rabbit anti-spinophilin (Abcam, 1:1000, ab18561, Cambridge, MA, USA) overnight at 4°C, and anti-β-Actin (Abcam, 1:5000, ab6276, Cambridge, MA, USA), anti-cofilin (Abcam; 1:1000, ab124979, Cambridge, MA, USA), anti-n-cadherin(Abcam; 1:10000, ab76011, Cambridge, MA, USA), and anti-GAPDH (Abcam; 1:5000, ab8245, Cambridge, MA, USA) for 1h at room temperature. After each primary antibody, membranes were washed 5 times in 1x TBS-T before probing with appropriate HRP-tagged secondary antibodies. Goat-anti-rabbit (Abcam, 1:10000, ab6276, Cambridge, MA, USA), and Goat-anti-mouse (1:10000,

ab9789, Cambridge, MA, USA) were each incubated in 2% milk in TBS-T for 2h at RT. Probed membranes were then imaged with the Geliance 600 imaging system (Perkin Elmer). For quantification, protein signal intensity was first normalized to GAPDH signal intensity, and then relative protein expression was normalized to the expression of that protein in Wild-type males (protein expression in Saline-M = 1).

6.2.12 Statistics

All data collection and image and behavioural analyses were performed blind to condition and blinding was removed prior to statistical analyses. Statistical analyses were performed using the core open-source software R (Team, 2013). Categorical data (loop formation and dendritic spine shapes) were analyzed using multinomial logistic regression with all data presented as odd ratios (95% confidence intervals) in tables (Croissant, 2020). All remaining data including the remainder of dendritic morphology, all behavioral data, and all protein analysis were analyzed using linear mixed effect modeling to account for confounding variables in our data (Bates et al., 2015). Variables of interest were assigned as main effects and confounds were assigned as random effects. Random effects in this study included litter, trial number, and technical replicates. Linear mixed models were fit by maximum likelihood and the Akaike Information criteria was used to determine the model of best fit for each data set. T-tests were performed with Satterwaite's adjustment with significance determined as $p < 0.05$ for all tests. Data was displayed using a violin plot to depict the probability distribution of the data within a given treatment. All measurements were taken from a minimum of 3 independent biological replicates (litters) per treatment. Total sample sizes for each test were calculated based on literature using G*Power 3 software (Faul et al., 2007) using an estimated effect size of 0.25.

6.3 Results

6.3.1 Cerebellar Cell Density

We have previously demonstrated that maternal exposure of dmPGE2 at G11 caused altered expression of autism-linked genes and resulted in autism related behaviours in mice(Kissoondoyal et al.; C. T. Wong et al., 2017). Here we observed a distinct localization pattern of CldU-labelled (G11) and IdU-labelled (G16) cells within the developing cerebellum at PN8. We separately counted cells originating from G11 and G16 (Fig 1A).

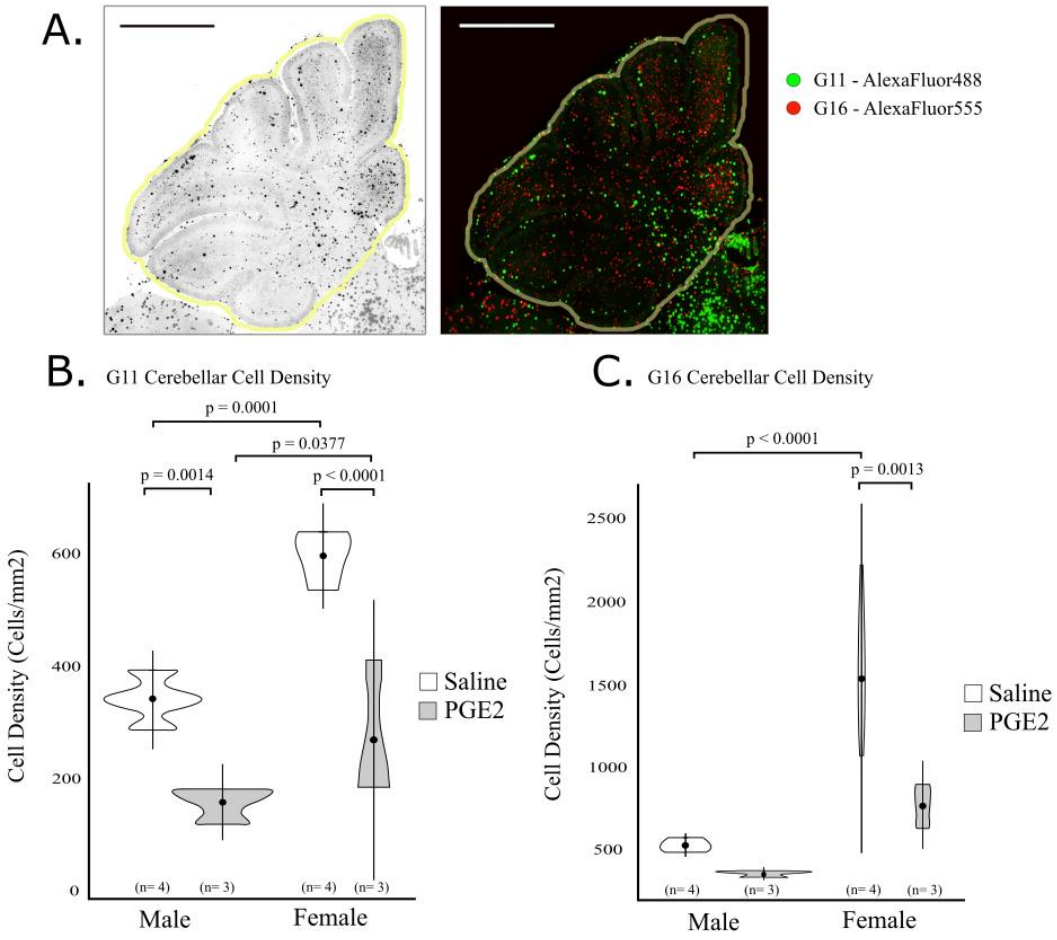

Figure 6-1: G11 and G16 Cohort-labelled cell densities in the cerebellum of PN30 PGE2-exposed mice. (A) CldU and IdU immunolabelled G11 (red) and G16 (green) cells in the cerebellum at PN8. (B) Decreased cell density of G11-labelled cells in males and females. In the Saline controls and PGE2-exposed groups, cerebellar G11 cell density was greater in females than males. (C) Increased cell density of G16-labelled cells observed in females but not males. Cerebellar G16 cell density was greater in Saline females than males. Means represent at least 3 independent animals (shown as n) for each experimental group from 3 separate litters. Data are presented as mean ±SD, with significant differences shown above.

A linear mixed effects model was used to fit G11 cell density (cells/mm^2). Litter was assigned as a random control, and the fixed effects of condition and sex, as well as the interaction between condition and sex were examined. The interaction between condition and sex ($t(7.3) = -2.558$, $p = 0.0363$), condition ($t(12.2) = -4.112$, $p = 0.0014$), and sex ($t(8.0) = 6.804$, $p = 0.0001$) all had a significant effect on G11 cell density. Given the significance of the interaction term and the main effects we performed further pairwise comparisons. PGE2-exposed mice had a lower cell density than Saline Control mice in males (Fig 1B, $p = 0.0014$, Saline =339.588, PGE2 = 156.256), and females (Fig 1B, $p < 0.001$, Saline = 595.015, PGE2 = 267.592). Within conditions, males had a lower cell density than females in Saline control mice (Fig 1B, $p = 0.0001$, M = 339.588, F = 595.015) as well as PGE2-exposed mice (Fig 1B, $p = 0.0377$, M = 156.256, F = 267.592).

A linear mixed model was also used to fit G16 cell density within the cerebellum. Litter was assigned as a random control, and the fixed effects of condition and sex, as well as the interaction between condition and sex were examined. While the interaction between condition and sex ($t(14.0) = -2.208$, $p = 0.0444$) and the main effect of sex ($t(14.0) = 5.693$, $p < 0.001$) were significant, no significance of the condition main effect was found ($t(14.0) = -0.892$, $p = 0.3875$). Given the significance of the interaction between condition and sex and sex alone, we performed further pairwise comparisons. No significant difference was found in G16 cell density between PGE2-exposed males and Saline males (Fig 1C, $p = 0.3875$, Saline = 526.633, PGE2 = 356.428). However, a significantly lower cell density was found within the cerebellums of PGE2-exposed females than in Saline females (Fig 1C, $p = 0.0013$, Saline = 1532.498, PGE2 = 766.310). While the cell density of Saline males was significantly lower than in Saline females (Fig 1C, $p < 0.001$, M = 526.633, F = 1532.498), there was no significant difference between PGE2-exposed males and females (Fig 1C, $p = 0.0642$, M = 356.428, F = 766.310).

To summarize, we observed a significant decrease in cell density within the cerebellum of PGE2-exposed mice. At G11, PGE2 reduced cell density in both males and females, and at G16 there was a female-specific reduction in cell density within the cerebellum.

6.3.2 Dendrite Length

Average length of primary dendrites was measured from cells within the mature cerebellum at PN30 (Fig 2A and methods) and compared between Saline control and PGE2-exposed male and female mice. Dendrite length was fit using a linear mixed effects model, with litter assigned as a random control. The fixed effects of condition and sex, and the interaction between condition and sex were examined (Fig 2B). We observed no significant effect of the interaction between condition and sex ($t(60.0) = -0.708$, $p = 0.4820$), or condition ($t(60.0) = 1.474$, $p = 0.1458$), but a significant effect of sex ($t(60.0) = 2.160$, $p = 0.0348$) on dendrite length. Given the significant effect of sex, we performed further pairwise comparisons to examine sex-differences within conditions. Cells within the cerebellums of Saline females, were significantly shorter dendrites than in Saline males (Fig 2B, $p = 0.0107$, $M = 23.346$, $F = 38.017$). Interestingly, there was no significant difference between PGE2-exposed male and female mice (Fig 2B, $p = 0.2508$, $M = 33.355$, $F = 41.230$). PGE2-exposure results in a loss of the innate sex difference seen in Saline mice.

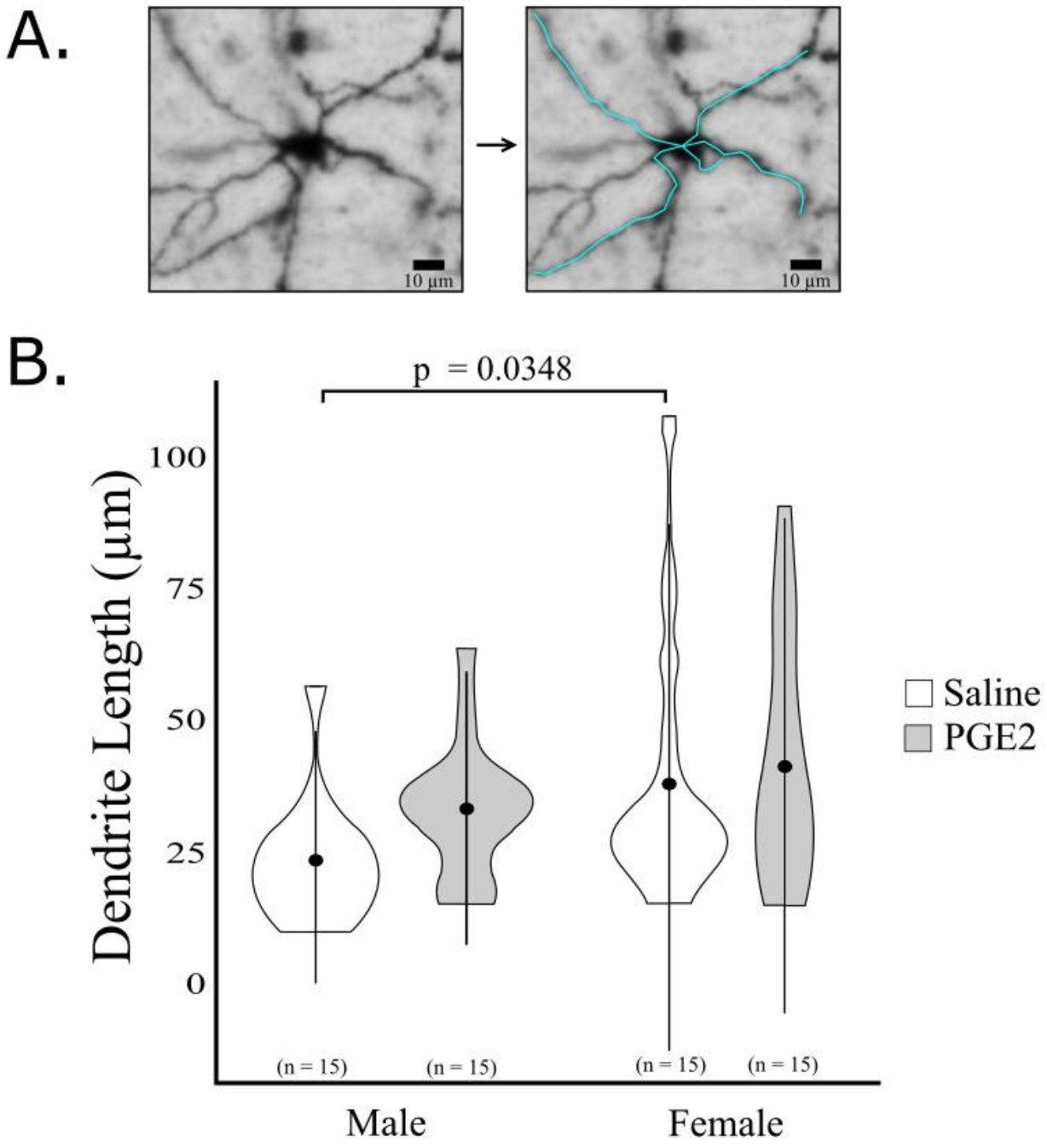

Figure 6-2: Average dendrite length of cerebellar cells inPN30 Mice. Average length of primary dendrites was measured from cells of Saline controls and PGE2-exposed male and female mice at PN30 from at least 3 separate litters pr condition. Length data are presented as mean ± SD. *n* = 15 cells per condition.

6.3.3 Dendritic Arborization

Dendritic arborization is used to provide information about the ability of a neuron to integrate synaptic potentials (Bird & Cuntz, 2019). The degree of branching was obtained using sholl analysis to determine the number of intersections made with concentric circles every 10µm from the center of the cell soma (Fig 3A) (Binley et al., 2014). We have previously found an increase in dendritic arborization in COX-2^{-}-KI mice closer to soma (Kissoondoyal et al., 2021). Here we examined the number of intersections in the PGE2-exposed mice using linear mixed modelling (Fig 3B). The model of *best fit* was determined to exclude sex as a factor and as such sex was removed from dendritic arborization analysis. Litter was assigned as random control and we examined the fixed effects of condition, and the distance from the soma, as well as the interaction between condition and distance. The significant overall effect of condition ($t(715) = -6.289$, $p < 0.001$), prompted us to further investigate pair-wise comparisons at each distance between Saline controls and PGE2-exposed mice.

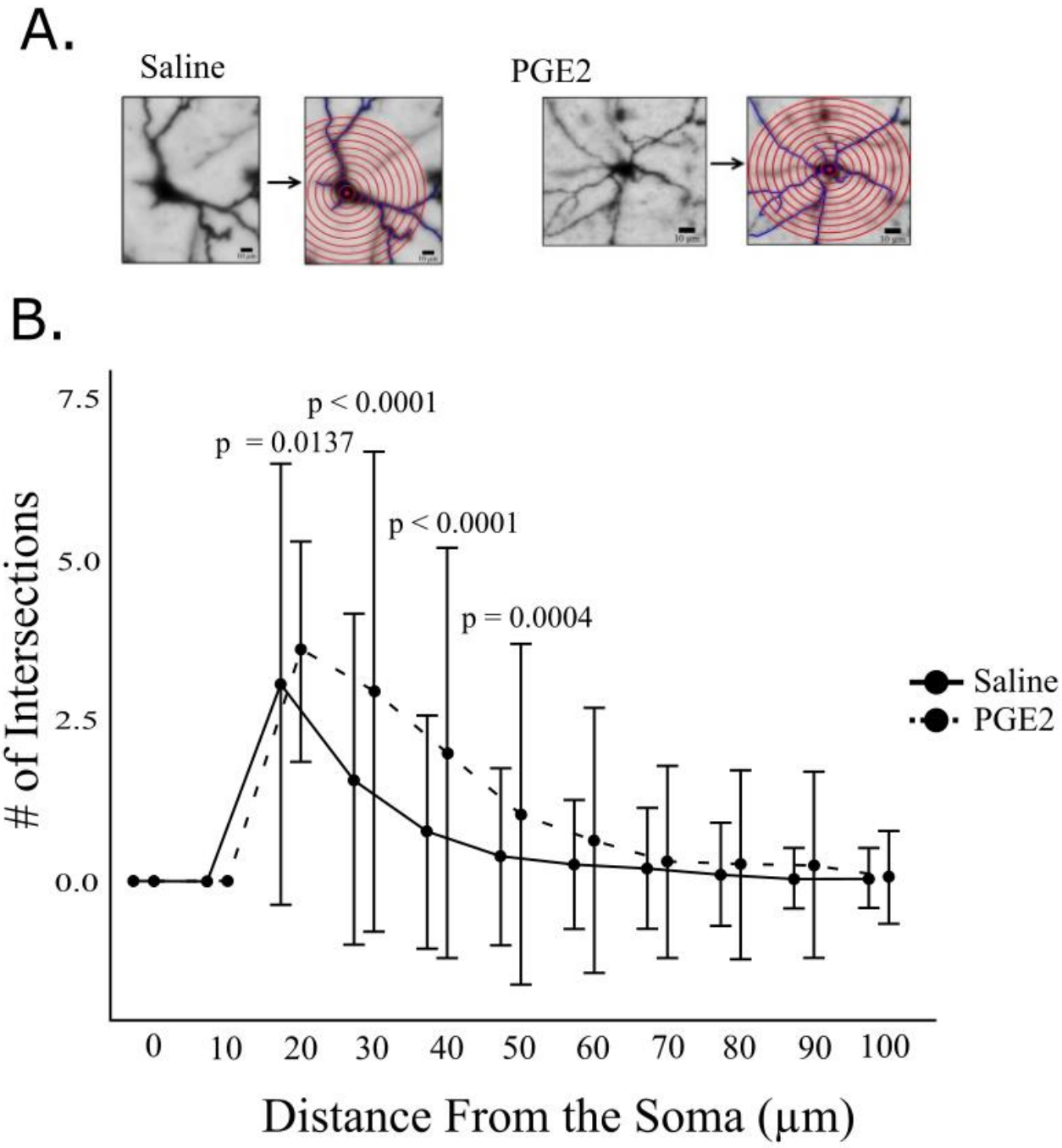

Figure 6-3: Sholl analysis in the cerebellum of PN30 mice. A. The average number of intersections of dendrites with concentric circles drawn at 10 μm intervals from the cell soma shown here in representative cells from Saline controls and PGE2-exposed. B. Data are presented as mean as mean ± SD (males and female combined). *n* = 30 cells per condition from male and female mice derived from at least 3 separate litters per condition.

Dendritic arborization was compared between Saline controls and PGE2-exposed mice at 10 µm increments from 0 µm to 100 µm (Fig 3B). There were no significant differences in dendritic arborization between Saline controls and PGE2-exposed mice at 0 ($t(715) = 0$, $p = 1.000$, Saline = 0.000, PGE2 = 0.000), and 10 µm ($t(715) = 0$, $p = 1.000$, Saline = 0.000, PGE2 = 0.000) distances. However, we observed a significantly increased number of intersections in PGE2-exposed mice at 20 µm ($t(715) = -2.472$, $p = 0.0137$, Saline = 3.057, PGE2 = 3.600), 30 µm ($t(715) = -6.202$, $p < 0.001$, Saline = 1.571, PGE2 = 2.933), , 40 µm ($t(715) = -5.594$, $p < 0.001$, Saline = 0.771, PGE2 = 2.000), and 50 µm ($t(715) = -2.884$, $p = 0.0040$, Saline = 0.400, PGE2 = 1.033) distances. No significant differences between the conditions were observed at 60 µm ($t(715) = -1.713$, $p = 0.0871$, Saline = 0.257, PGE2 = 0.633), 70 µm ($t(715) = -4.554$, $p = 0.6490$, Saline = 0.200, PGE2 = 0.300), 80 µm ($t(715) = -0.6939$, $p = 0.4870$, Saline = 0.114, PGE2 = 0.267), 90 µm ($t(715) = -0.802$, $p = 0.4226$, Saline = 0.057, PGE2 = 0.233), and 100 µm ($t(715) = -0.043$, $p = 0.9654$, Saline = 0.057, PGE2 = 0.067) distances from the center of the cell soma.

In summary, there was greater dendritic arborization within the cells of the cerebellums in PGE2-exposed mice at distances closer to the some particularly between the 20-50µm range.

6.3.4 Dendritic Looping

We have previously demonstrated *in vitro* that exposure to PGE2 during the differentiation of NE-4C stem cell significantly increased the proportion of neurites that formed loops (Ashby Kissoondoyal & Dorota A. Crawford, 2021). We have also found an increase in the odds of observing dendritic loops in the cerebellum of COX-2^{-}- KI mice (Kissoondoyal et al., 2021). Here we investigate the odds of observing dendritic loops in the cerebellum of PGE2-exposed mice. The greatest exterior angle of all neurites found was measured and dendrites with an exterior angle exceeding 270° were classified as loop forming. (Figure 4, methods). With this distinction made, there were clear differences in the distribution of dendrites forming loops with higher proportion of looping dendrites exceeding

270° in PGE2-exposed mice compared to Saline control mice. To further investigate these differences, and given the categorical nature of the loop classification, we fit loop classifications using a binomial logistic regression. We examined the odds that any given neurite would form a dendritic loop given its condition (Saline controls or PGE2-exposed) and sex .

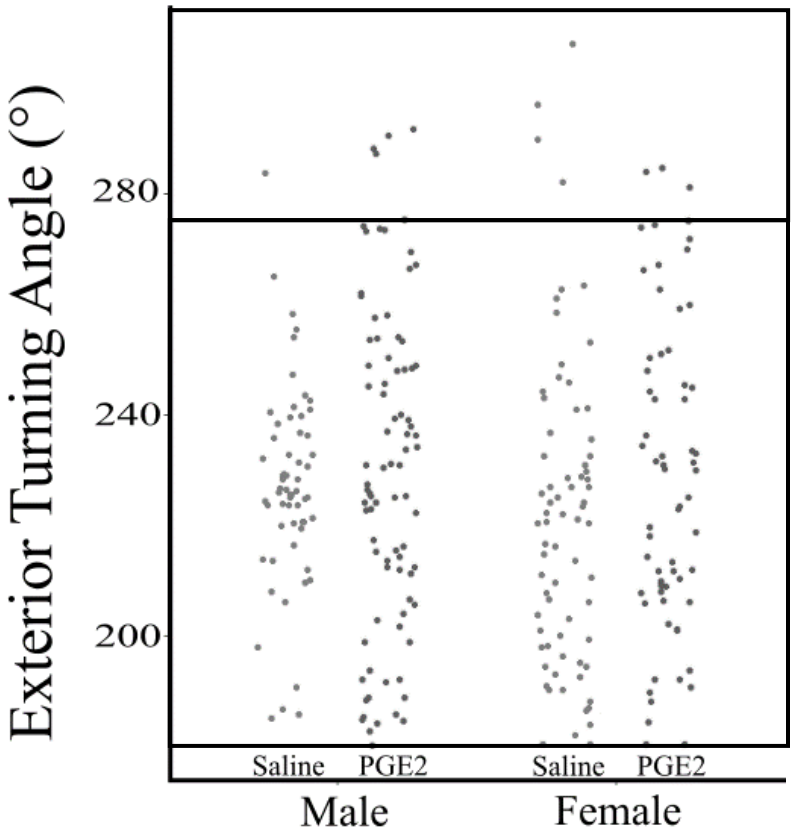

Figure 6- 4: Distribution of turning angles of dendrites in the cerebellum. The greatest exterior angle was measured for dendrites within the PN30 cerebellum of Saline control and PGE2-exposed male and female mice from at least 3 separate litters per condition. Dendrites with a turning angle greater than 270° were classified as axonal loops. Each dot represents individual dendrite

We used multinomial logistic regression to determine the odds that our baseline intercept (Saline Male control) would form dendritic loops (Methods). We found a significant odds ratio (OR) of 0.142 in the Saline male controls intercept ($p < 0.001$), signifying that it was less likely to observe a looping dendrite than a non-looping dendrite in these control males (Table 1). Examining the sex factor (if the

animal was female), we determined that sex did not affect the likelihood of observing dendritic loops from what was observed in Saline male controls (OR = 0.747, p = 0.5270). However, the condition factor (if the animal was exposed to PGE2) significantly increased the odds of observing a dendritic loop (OR = 0.312, p = 0.0279).

Table 6-1: Odds of observing dendritic loops in the cerebellum of PGE2-exposed offspring
Likelihood of a given dendrite being classified as an axonal loop was determined for Golgi-COX-stained cerebellar neurons of mice at PN30. Odds ratios (95% confidence intervals) are given for the baseline (Saline control male) as well as the factors of sex and PGE2-exposure. At least 3 animals per condition from 3 separate litters were used.

Factor	OR (95% CI)	P-Value
Baseline (Saline Control Male)	0.142 (0.110-0.183)	**1.76E-55**
Sex (Female)	0.747 (0.471-1.18)	5.27E-01
PGE2-exposed	0.312 (0.183-0.530)	**2.79E-02**

Overall, we found that while sex did not have a significant effect on the odds of observing dendritic loops, there was a significant effect of condition for both males and females.

6.3.5 Dendritic Spine Density

Previous studies have shown that exposure to PGE2 during critical time points in postnatal development affects dendritic spine density. For example, injection of PGE2 at PN10 and PN12 into the cerebellum of rats inhibited dendritic spine formation (S. L. Dean, Wright, et al., 2012). In this study we examine the effect of prenatal exposure to PGE2 at G11 on dendritic spine density and morphology within the cerebellum in PN30 mice offspring (Fig 5A).

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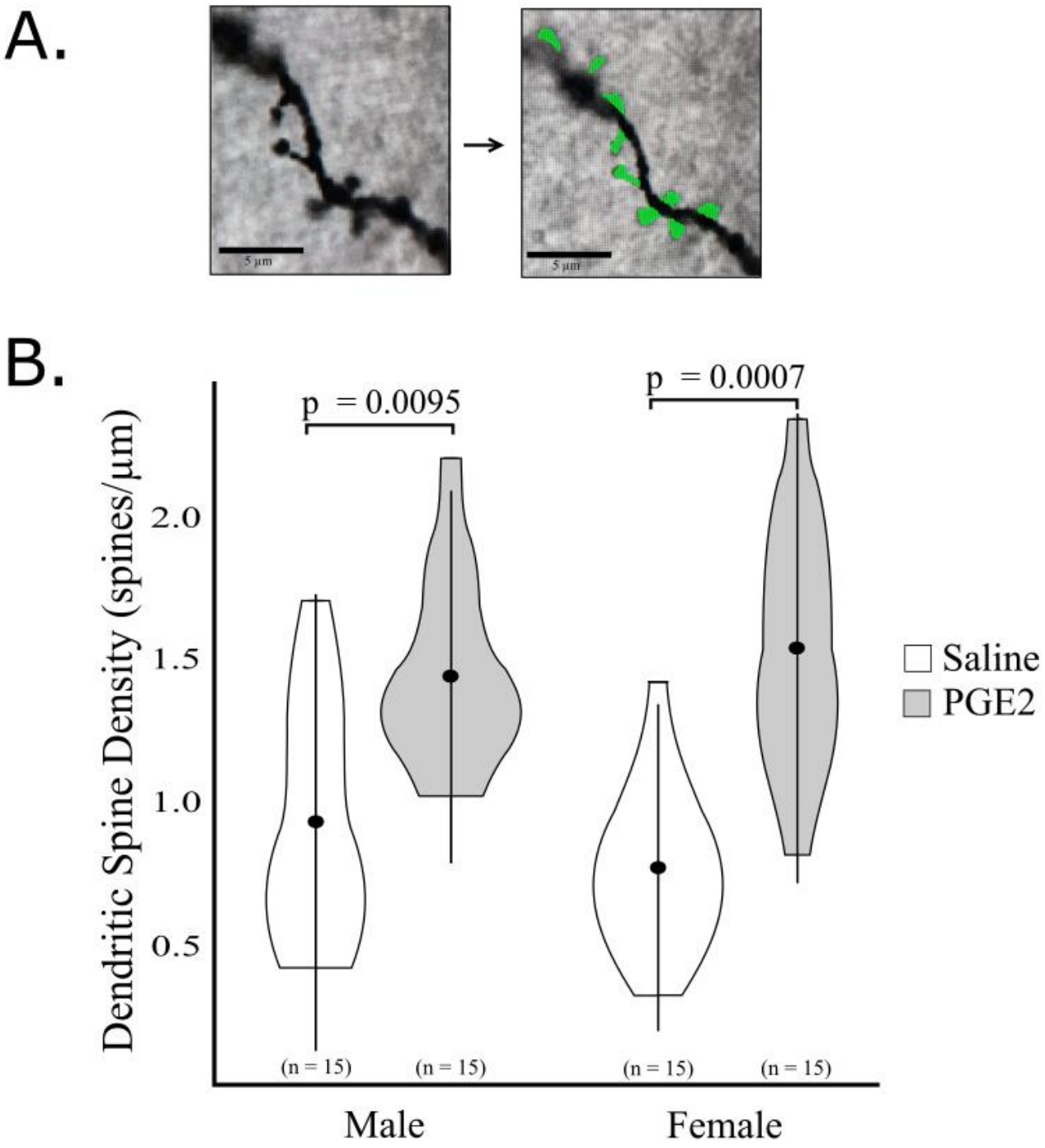

Figure 6-5: Dendritic spine density in the cerebellum of P30 Mice. Dendritic spines were detected by AI Software (Nikon). (A) A representative Golgi-Cox staining of dendrites in cells of the cerebellum with detected spine shapes shown in green (*right* image). (B) The number of spines per µm of dendrite length were measured from cerebellums of Saline control and PGE2-exposed male and female offspring from at least 3 different litters per condition. Data are presented as mean ± SD. *n* = 15 number of dendrites per condition.

First, dendritic spine density (spines/µm) was fit using a linear mixed effects model, with litter assigned as a random control. The fixed effects of condition and sex, and the interaction between condition and sex were examined (Fig 5B). While we saw no significant effect of the interaction between condition and sex ($t(54.0) = 1.572$, $p = 0.1218$), or the main effect of sex ($t(54.0) = -1.360$, $p = 0.1794$), there was a significant effect of condition ($t(10.7) = 3.151$, $p = 0.0095$) on dendritic spine density. Given the significant effect of condition, we examined pairwise comparisons between PGE2-exposed mice and their sex-matched Saline controls. Exposure to PGE2 significantly increased dendritic spine density in both males (Fig 5B, $p = 0.0095$, Saline = 0.9249, PGE2 = 1.4368), and females (Fig 5B, $p = 0.0007$, Saline = 0.7666, PGE2 = 1.537). In summary, PGE2-exposed mice regardless of gender, showed an increase in dendritic spine density compared to Saline control mice.

6.3.6 Dendritic Spine Morphology

Dendritic spines change shape as they mature, progressing from thin, to stubby to mushroom (J. Bourne & Harris, 2007; K. O. Lai & Ip, 2013; Tashiro & Yuste, 2003). These changes in dendritic spine morphology correspond to the function of the spines and influence short-term and long-term plasticity of the neuron, and ultimately the brain as a whole (Mel et al., 2017; Sardi et al., 2018). We have previously shown that COX-2$^-$-KI male mice were less likely and COX-2$^-$-KI females were more likely to have mature spines than the control counterparts (Kissoondoyal et al., 2021). Here, we investigate the effect of prenatal PGE2 exposure on dendritic spine morphology in male and female offspring at PN30. We investigated the relative odds that thin or stubby shaped spines would be observed compared to mushroom shaped spines in each condition. A multinomial logistic regression was used to fit the data and the likelihood that any given spine was mushroom shaped or was thin or stubby shaped was determined by condition. Litter was assigned as a random effect to control for potential litter-bias. An odds-ratio (OR) above 1 for a shape within a condition signified that there was a higher likelihood of observing that shape compared to a mushroom shaped spine.

Table 6-2: Odds of observing dendritic spine shapes in the cerebellum of PGE2-exposed offspring
The likelihood of a given dendritic spine being mushroom shaped, or thin or stubby shaped was determined for dendritic spines of Golgi-COX-stained cerebellar neurons of male and female PGE2-exposed and Saline control mice at PN30. Odds ratios (95% confidence intervals) are given for the likelihood of each shape in each condition. Dendritic spines were measured from at least 3 animals per condition from 3 separate litters.

Factor	Shape	OR (95% CI)	P-Value	Odds of Mature spines (Compared to Saline Control Male)
Saline Control Male	**Stubby**	0.782 (0.730– 0.838)	**<0.001**	-
	Thin	0.121 (0.105– 0.139)	**<0.001**	
Saline Control Female	**Stubby**	1.427(1.295 – 1.574)	**<0.001**	Decreased
	Thin	1.400 (1.156 – 1.697)	0.0795	
PGE2-exposed Male	**Stubby**	0.810 (0.738 – 0.889)	**0.0145**	Increased
	Thin	0.596 (0.486 – 0.732)	**0.0116**	
PGE2-exposed Female	**Stubby**	0.646 (0.568 – 0.735)	**< 0.001**	Increased
	Thin	1.346 (1.023 – 1.773)	0.2794	

We assigned Saline male controls as our baseline intercept for comparisons. First, we established that in Saline male controls, there was a higher likelihood of observing mushroom shaped spines compared to both stubby (Table 2, OR = 0.782, p <0.001), and thin (OR = 0.121, p < 0.001) shaped spines. Next, we found that compared to Saline control males, Saline female controls were more likely to see stubby shaped spines than mushroom shaped spines (OR = 1.427, p < 0.001) but there was no difference for the likelihood of observing thin shaped spines (OR = 1.400, p < 0.001).

In PGE2-exposed males we found an increased likelihood of observing mushroom-shaped spines compared to stubby (OR = 0.810, p = 0.0145), or thin (OR = 0.596, p = 0.0116) shaped spines relative to the likelihood in Saline male mice. Similarly, in PGE2-exposed females had an increased

likelihood of observing mushroom-shaped spines compared to stubby shaped spines (OR = 0.646, p <0.001) but there was no difference in the likelihood of observing thin shaped spines (OR = 1.346, p = 0.2794) compared to the likelihood in Saline male controls.

In summary, we observed an innate sex difference in dendritic spine morphology between Saline male and female control mice, with females being more likely to observe immature stubby shaped spines and males with more mature mushroom spines. However, PGE2-exposure increases the odds of observing mature mushroom shaped spines in males and females.

6.3.7 Adhesive sticker test

Our previous study showed that the single maternal exposure to PGE2results in autism-related behaviours in offspring postnatally including deficits in social interaction, anxious behaviours, and repetitive or restrictive behaviours (Kissoondoyal et al.; C. T. Wong et al., 2017). The goal of this study is to evaluate whether there is a potential consequence of the morphological changes described above and specific cerebellum-related behaviours. First, we use the *adhesive sticker test* to measure sensory motor coordination in mice as they attempted to remove a small adhesive sticker from just above their noses (S. M. Fleming et al., 2004; H. A. Lam et al., 2011; Richter et al., 2017) (Fig 6A). First, to verify that there was not a confounding effect of anxiety on differences in motor coordination in our mice, we measured the time until the mouse made first contact with the adhesive sticker. A linear mixed effect model was used to fit the time until mice made first contact with the adhesive sticker (Fig 6B). Litter was assigned as a random control and the fixed effects of condition and sex, as well as the interaction between condition and sex were examined. We observed no significance in sex, condition, or the interaction between sex and condition indicating that any differences in the adhesive sticker test would not be due to anxious behaviour.

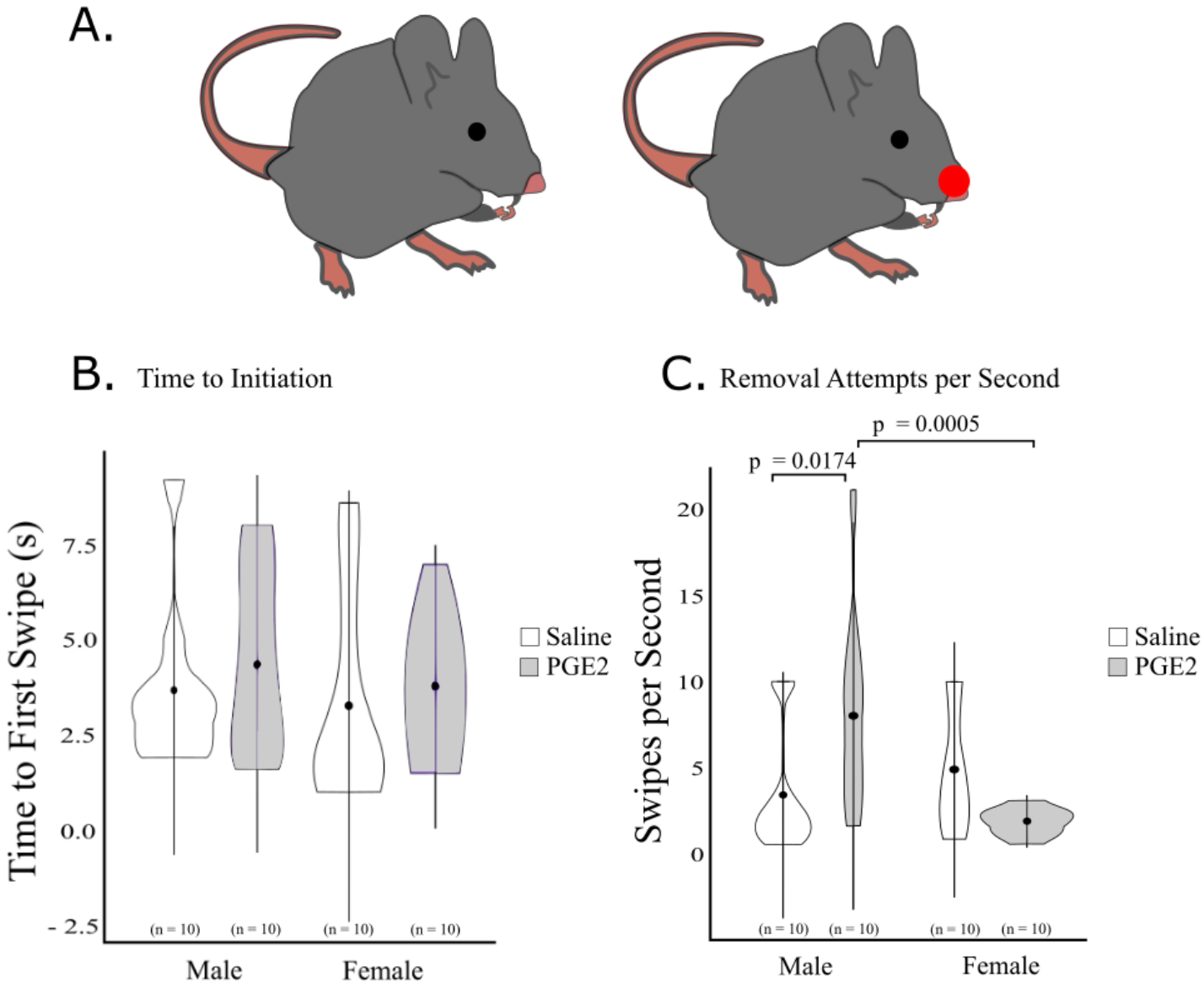

Figure 6-6: Adhesive Sticker Test of motor coordination. A. A small adhesive sticker was placed just above the nose of Saline control and PGE2-exposed mice on PN30. The time until the mouse made first contact with the adhesive sticker and the number of attempts to remove the sticker were per second were recorded. B. No significant differences were observed between conditions in the time until the first removal attempt was made. C. PGE2 exposed males made significantly more attempts per second than both C57 controls and PGE2 exposed females. *n* = 10 mice per condition, derived from at least 4 separate litters per condition Data are presented as mean ±SD, with significant differences shown above.

Next, we measured performance in the *adhesive sticker test* as the number of swipes each mouse made, which was corrected with the time taken by the mouse to remove the sticker. Swipes per second was fit using a linear mixed effects model, with litter assigned as a random control. The fixed effects of condition and sex, and the interaction between condition and sex were examined (Fig 5C). We observed a significant interaction between condition and sex ($t(38.4) = -3.297$, $p = 0.0021$) and the main effect of sex ($t(37.6) = 3.764$, $p = 0.0006$), but no significant effect of condition alone ($t(11.2) = 1.79$, $p = 0.1010$). Given the significance of the interaction and the main effect of sex, we performed further pairwise comparisons. There was a statistically significant increase in the number of swipes per second in PGE2-exposed males compared to Saline male controls (Fig 6C, $p = 0.0174$, Saline = 3.421, PGE2 = 8.029). However, there was no significant difference in swipes per second between PGE2-exposed and Saline control female mice (Fig 6C, $p =0.1010$, Saline = 4.876, PGE2 = 1.913). While there was no significant difference in swipes per second in Saline male and female control mice (Fig 6C, $p = 0.3822$, M = 3.421, F = 4.876), PGE2-exposed males had a significantly higher number of swipes per second than PGE2-matched females (Fig 6C, $p < 0.001$, M = 8.029, F = 1.913).

In summary, we observed an PGE2-male specific increase in swipes per second, demonstrating reduced motor coordination in these mice.

6.3.8 Grid walking test

The grid walking test further tests sensory motor coordination of mice as they attempted to cross a mesh grid. It measures the number of limb slips each mouse made through the grid and the time taken by each mouse to cross the grid (Feather-Schussler & Ferguson, 2016; Heyser, 2004) (Fig 7A). We first examined the number of limb slips made by each mouse as they crossed the grid (Fig 7B). Number of slips through the grid was fit using a linear mixed effects model, with litter assigned as a random control. The fixed effects of condition and sex, and the interaction between condition and sex were examined (Fig 7B). While the interaction between condition and sex was significant ($t(95.5) = -$

3.016, p = 0.0033), we saw no significant effect of the condition (t(11.2) = 0.872, p = 0.4017), or sex (t(90.6) = -0.065, p = 0.9484) main effects on number of slips. Given the significance of the interaction between condition and sex, further pairwise comparisons were performed. We saw no significant difference between PGE2-exposed mice and sex matched Saline controls in males (Fig 7B, p = 0.4017, Saline = 4.1, PGE2 = 4.718), or females (Fig 7B, p = 0.3236, Saline = 4.289, PGE2 = 3.317). While there was no difference in the number of slips through the grid between Saline male and female controls (Fig 7B, p = 0.3878, M = 4.1, F = 4.289), PGE2-exposed males made significantly more slips through the grid than PGE2-exposed females (Fig 7B, p = 0.0001, M = 4.718, F = 4.289).

The time taken by each mouse to escape the grid was also examined (Fig 7C). Time to escape was fit using a linear mixed effects model, with litter assigned as a random control. The fixed effects of condition and sex, and the interaction between condition and sex were examined. We observed a significant effect of the interaction between condition and sex (t(95.0) = -3.872, p = 0.0002), and the main effect of sex (t(90.8) = 2.200, p = 0.0303), but no significant effect of the condition main effect (t(11.1) = 1.679, p = 0.1211). Given the significant interaction between condition and effect and the significant effect of sex, we performed further pairwise comparisons. We saw no significant differences between PGE2-exposed and Saline control males (Fig 7C, p = 0.1211, Saline = 24.642, PGE2 = 35.605), or females (Fig 7C, p = 0.5487, Saline = 29.963, PGE2 = 31.179). We observed opposite effects between sexes within Saline controls and PGE2-exposed mice. Though Saline male controls escaped significantly faster than Saline female controls (Fig 7C, p = 0.0303, M = 24.642, F = 29.963), PGE2-exposed males were slower to escape than PGE2-exposed females (Fig 7C, p = 0.0018, M = 35.605, F = 31.179).

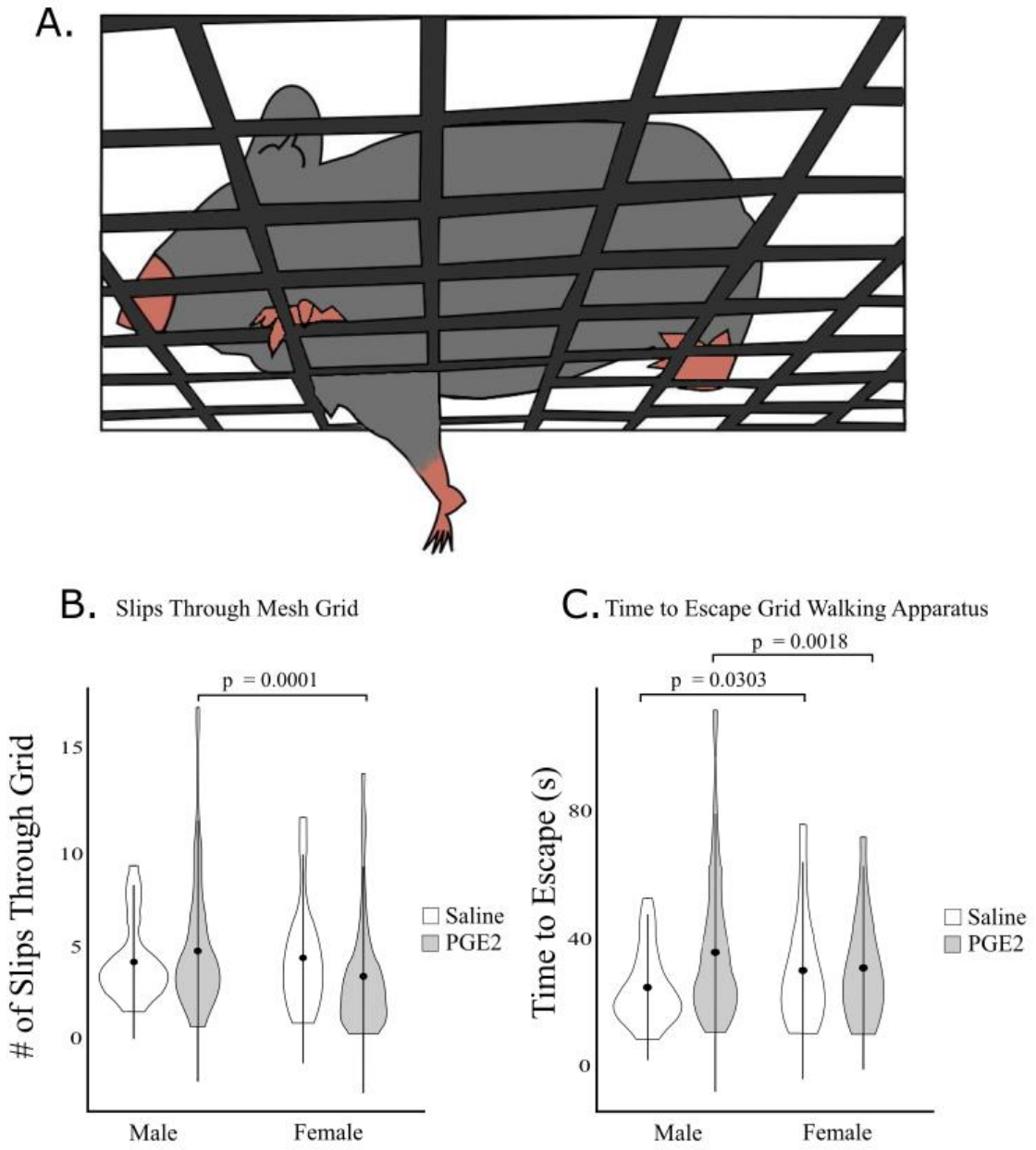

Figure 6-7: Grid Walking Test of motor coordination. A. Mice were allowed to cross an enclosed mesh grid on PN30. The number of limb slips of each mouse moving through the grid as well as the time it took each mouse to escape the grid were recorded. B. PGE2 males slipped significantly more than PGE2 females. C. Saline male controls were quicker to escape the grid than Saline female controls, but PGE2-exposed males were slower to escape than PGE2-exposed females. Means represent 10 animals for each experimental group taken from at least 4 separate litters per condition. Data are presented as mean ±SD, with significant differences shown above. Means represent 10 animals for each experimental group from at least 4 separate litters. Data are presented as mean ±SD, with significant differences shown above.

In summary it appears that there is a sex-dependent effect of PGE2 on motor coordination in the grid walking test. In number of slips a sex difference was present in the PGE2-exposed mice that was not seen in Saline control mice. In time to escape there were sex differences within Saline controls with female being slower and within PGE2-exposed mice with male being slower.

6.3.9 Cylinder test

The cylinder test measures exploratory motor behaviour as mice explore a tall glass cylinder (Rattka et al., 2016; Schönfeld, Dooley, et al., 2017; Schönfeld, Jahanshahi, et al., 2017). In this study, we use the cylinder test to measure the number of forelimb touches to the side of the cylinder, the total number of rears, and the total number of steps (Fig 8A). Forelimb touches were quantified as the number of times the mouse would touch the side of the cylinder with either of their forelimbs. Forelimb touches were fit using a linear mixed effects model, with litter assigned as a random control. The fixed effects of condition and sex, and the interaction between condition and sex were examined (Fig 8B). While we observed no significant effects of the interaction between condition and sex ($t(110.1) = -1.138$, $p = 0.2578$), or the condition main effect ($t(0.6) = 1.708$, $p = 0.5414$), there was a significant effect of sex ($t(97.7) = 2.662$, $p = 0.0091$). Given the significant effect of the sex condition, we examined pairwise comparisons between sex within each condition. There were significantly fewer forelimb touches by Saline male control mice than Saline female control mice (Fig 8B, $p = 0.0091$, $M = 5.236$, $F = 9.008$). However, there were no sex-differences in forelimb touches between PGE2-exposed males and females (Fig 8B, $p = 0.285$, $M = 5.592$, $F = 7.110$).

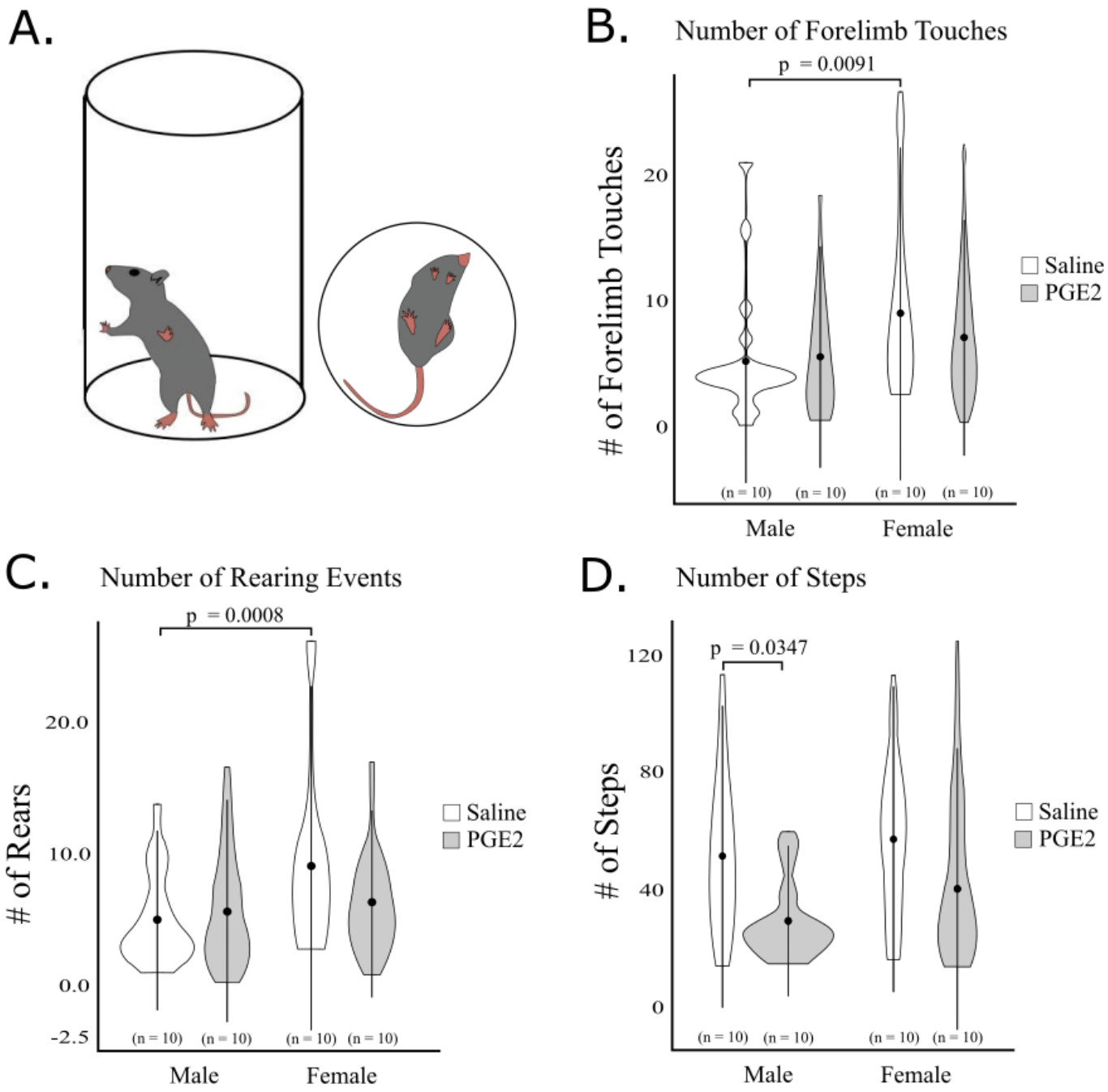

Figure 6-8: Cylinder Test of motor coordination. A. Saline control and PGE2-exposed mice on PN30 were placed into a long clear cylinder (*side* and *top* view). The number of forelimb touches to the cylinder, the number of rears, and the number of steps were recorded. B. Saline female controls made significantly more touches than Saline male controls with no difference between PGE2-exposed mice. C. Saline female controls reared more than Saline male controls again with no difference between PGE2-exposed mice. D. PGE2-exposed males made significantly less steps than Saline male controls.

Rears were measured whenever the mouse would rise up of its forelimbs onto its hind limbs. Rears were fit using a linear mixed effects model, with litter assigned as a random control. The fixed effects of condition and sex, and the interaction between condition and sex were examined (Fig 8C). We observed a significant effect of the interaction between condition and sex ($t(104.0) = -2.082$, $p = 0.0398$), and sex ($t(97.4) = 3.476$, $p = 0.0008$), but no significant effect of condition was observed ($t(14.9) = 0.900$, $p = 0.3825$). Given the significant effects of the interaction and the sex main effect, we performed further pairwise comparisons. We observed no significant difference in the number of rears between Saline and PGE2-exposed males (Fig 8C, $p = 0.3825$, Saline = 4.952, PGE2 = 5.675), or between Saline and PGE2-exposed females ($p = 0.2798$, Saline = 8.942, PGE2 = 6.196). There were sex-differences between conditions. Saline male controls reared significantly less than Saline female controls (Fig 8C, $p = 0.0008$, M = 4.952, F = 8.942). However, this sex-difference was no longer observed between PGE2-exposed males and females (Fig 8C, $p = 0.6008$, M = 5.675, F = 6.196).

We also measured the number of steps taken by each mouse while in the cylinder. Steps were fit using a linear mixed effects model, with litter assigned as a random control. The fixed effects of condition and sex, and the interaction between condition and sex were examined (Fig 8D). While there was no significant interaction between condition and sex ($t(100.4) = 1.559$, $p = 0.1221$), or a significant effect of sex ($t(93.0) = 0.076$, $p = 0.9396$), we observed a significant effect of condition ($t(13.6) = -2.346$, $p = 0.0347$) on number of steps. Given the significant effect of condition, we performed further pairwise analysis on differences based on condition. We saw a significant reduction in the number of steps taken by PGE2-exposed males compared to Saline male controls (Fig 8D, $p = 0.0347$, Saline = 51.138, PGE2 = 29.233). However, there was no significant difference in number of steps between PGE2-exposed and Saline female controls (Fig 8D, $p = 0.3607$, Saline = 57.013, PGE2 = 40.082).

In summary, we found changes in motor function in PGE2-exposed mice including loss of the innate sex difference observed in Saline control mice for forelimb touches and number of rears, and a reduction in the number of steps taken, which was specific to males.

6.3.10 Cerebellar Protein Expression

In this study we have found that prenatal exposure to PGE2 affects density of migrating cells and the morphology of dendrites and dendritic spines within the cerebellum of offspring in a sex-dependent manner. Normally, dendritic morphology is regulated by cytoskeletal dynamics (Dent et al., 2011; Joensuu et al., 2018; Peter Penzes & Igor Rafalovich, 2012). Our previous studies in differentiating NE-4C stem cells exposed to PGE2 [66] and COX-2^{-}-KI mice (Kissoondoyal, unpublished) showed abnormal expression of □-actin and the actin-binding protein spinophilin highly abundant in dendritic spines

We have already demonstrated *in vitro* that exposure to PGE2 during differentiation of NE-4C cells affects actin-dependent neurite morphology as well as the expression of the actin-binding protein spinophilin highly abundant in dendritic spines (Ashby Kissoondoyal & Dorota A. Crawford, 2021). We have recently found sex-dependent changes in the expression of □-actin and spinophilin in COX-2^{-}-KI mice.

Here, we examine whether the expression levels of spinophilin, and □-actin is affected in the cerebellum of PGE2-exposed offspring at P30. In addition, we also analyse the level of N-Cadherin, a protein that promotes dendritic outgrowth as well as establishment and stabilization of mature synapses (Hirano & Takeichi, 2012; Mendez, De Roo, Poglia, Klauser, & Muller, 2010; Suzuki & Takeichi, 2008; Takeichi & Abe, 2005; Togashi et al., 2002). For each protein linear mixed modelling was used to fit the fold change relative to the expression of in Saline male controls for each protein across 3 separate western blot runs (Fig 9). To avoid confounding by differences between technical replicates, we assigned technical replicates as a random effect.

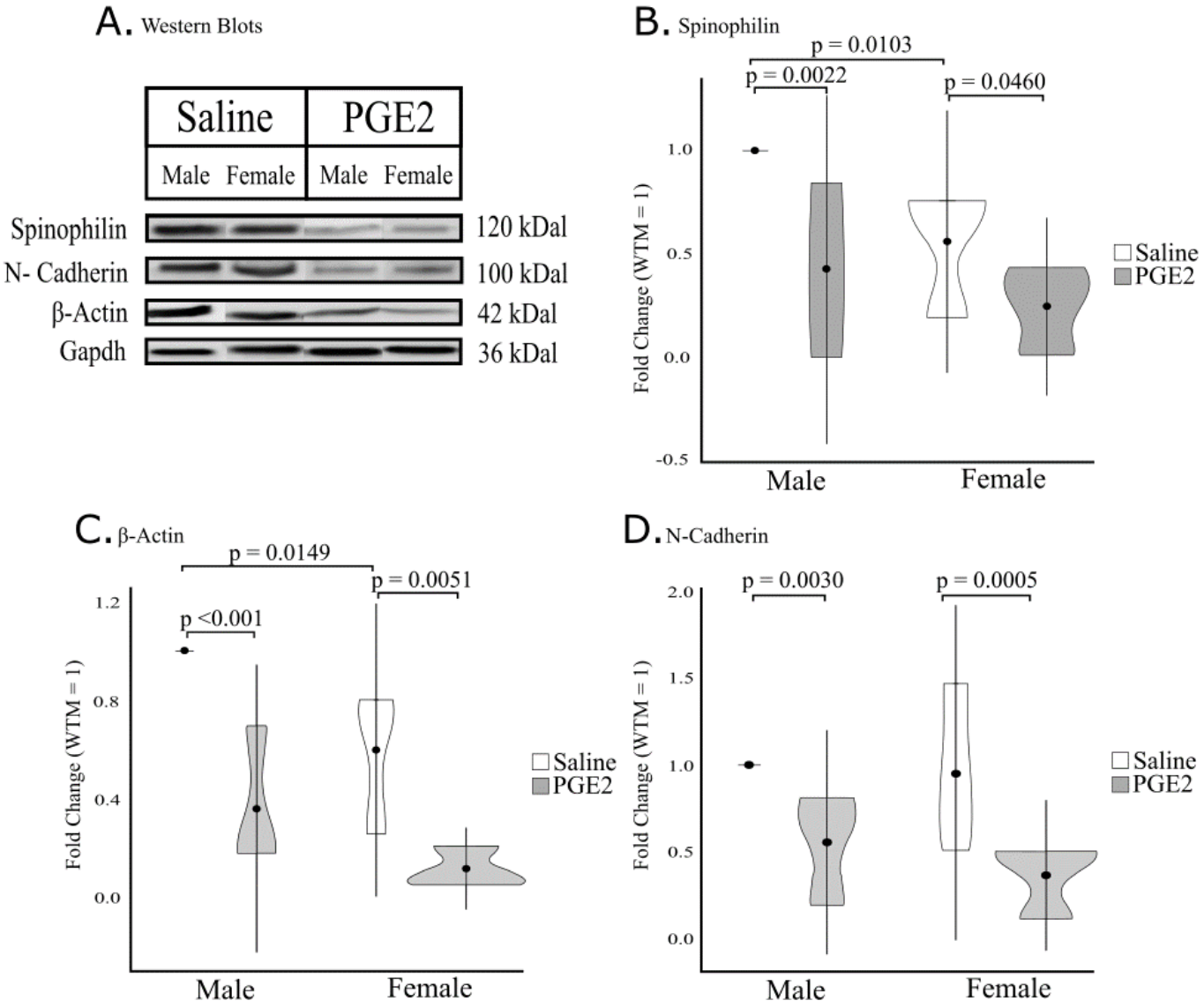

Figure 6-9: Protein expression of Spinophilin, □-Actin and N-Cadherin within P30 mice. A. Cerebellar isolates of Saline control and PGE2-exposed male and female mice were quantified for the expression levels of Spinophilin (B), □-Actin (B) and N-Cadherin (D). Protein expression was normalized to the house keeping gene GAPDH and then quantified relative to the expression in Saline male controls. All samples are pooled from 10 animals derived from at least 4 separate litters. Data are presented as mean fold change ± SD. Each graph represents is an average of 3 individual Western blot runs per condition.

Condition, sex and the interaction between condition and sex were tested for spinophilin expression level. While there was no effect of the interaction between condition and sex ($t(9.0) = 1.355$, $p = 0.2084$), we found significant effects of both condition ($t(9.0) = -4.230$, $p = 0.0022$), and sex ($t(9.0) = -3232$, $p = 0.0103$) alone. Given the significant effects of condition, and sex we performed further pairwise comparisons to examine the effects of each. Compared to sex-matched Saline controls, there was a significant reduction in the expression of spinophilin in PGE2-exposed males (Fig 9A and B, p= 0.0022, Saline = 1.000, PGE2 = 0.4241) and females (p = 0.0460, Saline = 0.5600, PGE2 = 0.2449). Within conditions we observed that while the expression of spinophilin was higher in Saline male controls than in female controls (p = 0.0103, M = 1.000, F= 0.5600), there was no significant difference between PGE2-exposed males and females (p = 0.2209, M = 0.4241, F= 0.2449).

Again condition, sex and the interaction between condition and sex were tested for □-actin expression levels. We found no significant effect of the interaction between condition and sex ($t(12.0) = 0.807$, $p = 0.4353$), but found significant effects of both condition ($t(12.0) = 0.0007$, p =), and sex (t(12.0) = , p = 0.0149). Given that the effects of both condition and sex were significant, pairwise comparisons involving condition and sex were further investigated. Similar to spinophilin, the expression of □-actin was significantly lower in PGE2-exposed males (Fig 9A and B, p =0.0007, Saline = 1.000, PGE2 = 0.3556) and females (Fig 9B, p = 0.0051, Saline = 0.5985, PGE2 = 0.1155), compared to sex-matched Saline controls. Moreover, the expression of □-actin was higher in Saline male than female controls (p = 0.0149, M = 1.000, F = 0.5985), but no significant difference was found between PGE2-exposed males and females (Fig 9B, p = 0.1150, M = 0.3556, F = 0.1155).

Lastly, we also tested condition, sex and the interaction between condition and sex were tested, with technical replicate assigned as a random effect. While we found no significant effect of the interaction between condition and sex ($t(9.0) = -0.556$, $p = 0.5915$), or sex ($t(9.0) = -0.277$, $p = 0.7881$), the condition effect was found to be significant ($t(9.0) = -2.512$, $p = 0.0332$). As the effect of condition

was significant, we examined further pairwise comparisons between conditions within each sex. There was a reduction in N-Cadherin expression in both PGE2-exposed male (Fig 9C, p = 0.0332, Saline = 1.000, PGE2 = 0.5519), and female (Fig 9C, p = 0.0092, Saline = 0.9506, PGE2 = 0.3622) mice compared to sex-matched Saline controls.

In summary, PGE2-exposure resulted in reduced expression levels of □-actin, spinophilin and N-cadherin within the male and female cerebellum, proteins involved in dendritic outgrowth and migration of neuronal cells.

6.3.11 Behaviour Matched Protein-expression

Our final aim was to assess a potential correlation between the observed behaviour in PGE2-exposed offspring and the expression level of the proteins analysed. Within our behavioural experiments, PGE2 males consistently performed the worst on motor tasks. Thus, from the PGE2-exposed males tested (Male Pooled in Fig 9) we selected 3 males from independent litters that performed the best and worst overall across the behavioural tests. Male 1 had the best performance (similar to Saline male controls) among all the PGE2 males across tests, Male 2 had the worst scores on the adhesive sticker and cylinder test, and Male 3 had the worst scores on the grid walking test. We compared the cerebellar expression levels of spinophilin, □-actin, and N-Cadherin within these individual males to pooled Saline control and pooled PGE2-exposed male samples (Fig 10A). As above, we used linear mixed modelling to fit the fold-change of each protein expression level relative to Saline male controls across three separate western blot runs (Fig 10B-D).

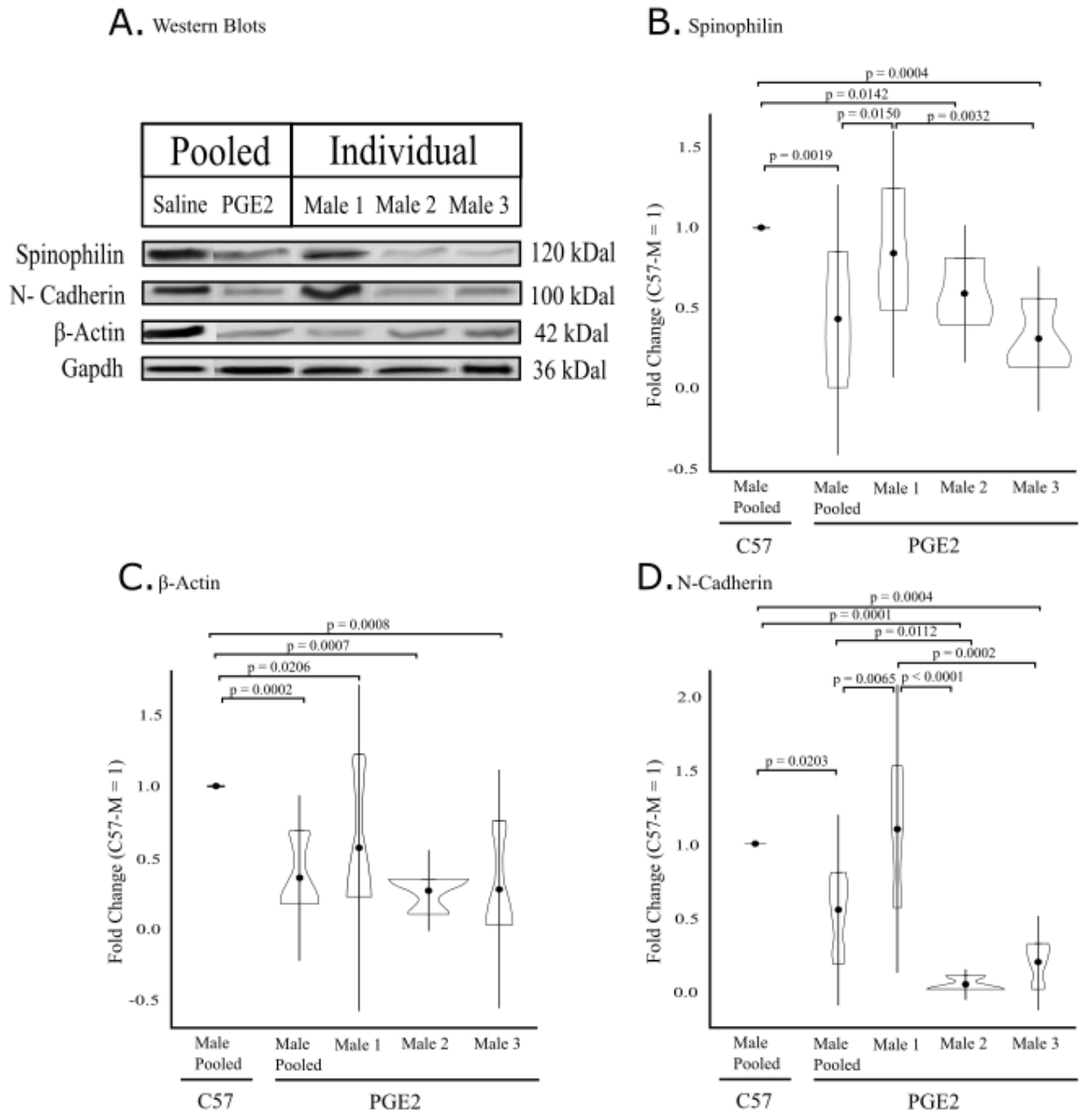

Figure 6-10: Protein Expression of Spinophilin, □-Actin and N-Cadherin in Saline and PGE2 pooled and individual male mice. Cerebellar samples of pooled Saline controls and PGE2-exposed male P30 mice and selected PGE2-exposed male mice were analysed for the expression of Spinophilin, □-Actin and N-Cadherin. Protein expression was normalized to the house keeping gene GAPDH and then quantified relative to the expression in the pooled Saline Male controls. Pooled samples derive from 10 animals from at least4 separate litters (as in Figure 9). Male 1 had the best performance in cerebellar behaviour across all PGE2 males, while Males 2 and 3 had the worst performance across all PGE2 males. Data are presented as mean fold change ± SD. Graphs for each protein are representative of 3 individual runs per condition.

First, we compared samples (Saline-Males Pooled, PGE2-males Pooled, Male 1, Male 2, Male 3) to each other using technical replicate as a random effect (Fig 10B). As reported in Figure 9, we found a significant reduction in spinophilin expression in PGE2-males compared to Saline male controls ($p = 0.0019$, Saline = 1.000, PGE2 = 0.4241). Interestingly, the expression of spinophilin in Male 1 was not significantly different than in Saline male controls ($p = 0.2811$, Saline = 1.000, Male 1 = 0.8361), but was significantly greater than in PGE2-males ($p = 0.0150$, PGE2 = 0.4241, Male 1 = 0.8361). In contrast, the expression of spinophilin for Male 2, was significantly lower than Saline male controls ($p = 0.0142$, Saline = 1.000, Male 2 = 0.5839), but not significantly different than in PGE2-males ($p = 0.2927$, PGE2 = 0.4241, Male 2 = 0.5839). Similarly, spinophilin expression was lower in Male 3 than Saline male controls ($p = 0.0043$, Saline = 1.000, Male 3 = 0.3032), but not different than in PGE2-males ($p = 0.4217$, PGE2 = 0.4241, Male 3 = 0.3032). While we found no differences in spinophilin expression between Male 1 and Male 2 ($p = 0.1081$, Male 1 = 0.8361, Male 2 = 0.5839), or Male 2 and Male 3 ($p = 0.0773$, Male 2 = 0.5839, Male 3 = 0.3032), spinophilin expression in Male 1 was significantly higher than in Male 3 ($p = 0.0032$, Male 1 = 0.8361, Male 3 = 0.3032).

We compared our samples (Saline-males, PGE2-males, Male 1, Male 2, Male 3) to one another for in □-actin expression levels (Fig 10C). Technical replicates were assigned as a random effect. Again, we found a significant reduction in □-actin expression levels in PGE2-exposed males compared to Saline male controls ($p = 0.0020$, Saline = 1.000, PGE2 = 0.3556). Compared to the Saline male controls pooled the expression of □-actin was reduced in Male 1 ($p = 0.0206$, Saline = 1.000, Male 1 = 0.5644), Male 2 ($p = 0.0007$, Saline = 1.000, Male 2 = 0.2661), and Male 3 ($p = 0.0008$, Saline = 1.000, Male 3 = 0.2749). However, we found no significant difference between PGE2-males pooled and Male 1 ($p = 0.2256$, PGE2 = 0.3556, Male 1 = 0.5644), Male 2 ($p = 0.5939$, PGE2 = 0.3556, Male 2 = 0.2661), or Male 3 ($p = 0.6304$, PGE2 = 0.3556, Male 3= 0.2749). . We also found no significant differences in □-actin expression between Males 1 and 2 ($p = 0.0929$, Male 1 = 0.5644, Male 2 =

0.2661), Males 2 and 3 (p = 0.9578, Male 2 = 0.2661, Male 3 = 0.2749) or between Males 1 and 3 (p = 0.1019, Male 1 = 0.5644, Male 3 = 0.2749).

Lastly, we compared expression of N-Cadherin between the samples (Saline-males, PGE2-males, Male 1, Male 2, Male 3), assigning technical replicates as a random effect (Fig 10D). As expected, we found a significant reduction in N-Cadherin expression levels in PGE2-exposed males pooled compared to Saline male controls (p = 0.0203, Saline = 1.000, PGE2 = 0.5519). The expression of N-Cadherin in Male 1 was not significantly different from the Saline male controls pooled (p = 0.5504, Saline = 1.000, Male 1 = 1.1030) but unexpectedly higher than in the PGE2-male pool (p = 0.0065, PGE2 = 0.5519, Male 1 = 1.1030). The expression of N-cadherin in Male 3 was significantly lower than the Saline male controls pooled (p = 0.0001. Saline = 1.000, Male 2 = 0.0499), Male 1 (p < 0.001, Male 1 = 1.1030, Male 2 = 0.0499), but not significantly different from Male 3 (p = 0.4024, Male 2 = 0.0499, Male 3 = 0.1954). Similarly, the expression level in Male 3 was significantly lower than the Saline male controls pooled (p = 0.0001. Saline = 1.000, Male 3 = 0.1954) and Male 1 (p < 0.001, Male 1 = 1.1030, Male 2 = 0.0499). Surprisingly, compared to the PGE2-exposed males pooled, there was a reduction in N-Cadherin expression in Male 2 (p = 0.0111, PGE2 = 0.5519, Male 2 = 0.0499).

In summary, we showed that even though PGE2-exposed male mice on average had reduced expressions of spinophilin, □-actin, and N-Cadherin, the levels of these proteins vary depending on individual behavioural outcomes. The PGE2-exposed Male 1 which performed the best across cerebellar motor tests had expression levels of spinophilin and N-Cadherin similar to Saline control mice, while those that performed the worst across cerebellar motor tests (Males 2 and 3) had expression levels of spinophilin and N-Cadherin more similar to the PGE2-exposed mice.

6.4 Discussion

Clinical and epidemiological studies have documented that numerous environmental risk factors can affect the levels of PGE2 at various points of prenatal development and result in ASDs (Tamiji & Crawford, 2010b; Wong & D. A. Crawford, 2014; Christine T Wong et al., 2015). The molecular mechanisms by which changes in PGE2 levels affect neuronal development prenatally and lead to ASD are still largely unclear. In this study we demonstrated for the first time that a single maternal injection of PGE2 in mice during a critical time point in pregnancy significantly affects the developing cerebellum including cell density, actin-dependent morphology of dendrites and dendritic spines, the expression of cytoskeletal proteins and relevant behavioural outcomes postnatally. While literature provides some insight into the consequences of altered COX2/PGE2 signalling during perinatal development, our unique approach investigates the effects of prenatal exposure to PGE2 during neuronal development of the offspring and resulting relevant behaviours. Importantly, many of the molecular and behavioural changes observed were sex dependent.

Table 6-3: Summary of PGE2 effects on cerebellar dendritic morphology, protein expression and behaviours

Dendritic morphology findings are summarized. The effect in PGE2-exposed male and females are shown compared to sex matched Saline controls. Sex differences within PGE2 and Saline mice are also indicated by up and down arrows. Sex differences in PGE2-exposed animals that were different from the Saline controls are indicated in **bold**.

Measure	PGE2 Male	PGE2 Female	Sex differences M=Male; F=Female; ↑=higher; ↓=lower
Cell Density			
G11 cells	↓	↓	Saline: M↓; F↑ PGE2: M↓; F↑
G16 cells	-	↓	Saline: M↓; F↑ **PGE2: No sex-difference**
Dendritic Morphology			
Dendrite Length	-	-	Saline: M↓; F↑ **PGE2: No sex-difference**
Dendritic Arborization	↑	↑	Saline and PGE2: No sex-difference
Dendritic Looping	↑	↑	Saline and PGE2: No sex-difference
Spine Density	↑	↑	Saline and PGE2: No sex-difference
Odds of Mature Spines	↑	↑	Saline: M↑; F↓ **PGE2: No sex-difference**
Motor Behaviour			
Adhesive Sticker Test: Time to first Swipe	-	-	Saline and PGE2: No sex-difference
Adhesive Sticker Test: Swipes per Second	↑	-	C57: No sex-difference **PGE2: M↑; F↓**
Grid Walking Test: # of Slips	-	-	C57: No sex-difference **PGE2: M↑; F↓**
Grid Walking Test: Time to Escape	-	-	C57: M↓; F↑ **PGE2: M↑; F↓**
Cylinder Test: # of Forelimb Touches	-	-	C57: M↓; F↑ **PGE2: No sex-difference**
Cylinder Test: # of Rears	-	-	C57: M↓; F↑ **PGE2: No sex-difference**
Cylinder Test: # of Steps	↓	-	Saline and PGE2: No sex-difference
Cytoskeletal Protein Expression			
Spinophilin	↓	↓	C57: M↑; F↓ **PGE2: No sex-difference**
β-Actin	↓	↓	C57: M↑; F↓ **PGE2: No sex-difference**
N-Cadherin	↓	↓	Saline and PGE2: No sex-difference

The cerebellum has been highly implicated in the etiology of ASD, with early disruption of cerebellar circuitry having a strong positive correlation to later development of ASD symptoms (E.

Courchesne et al., 2005). Alterations in cerebellar cell morphology including reduced Purkinje cell density (Elizabeth R. Whitney, Thomas L. Kemper, Douglas L. Rosene, Margaret L. Bauman, & Gene J. Blatt, 2009), and abnormalities in cell orientation (Claudia M. Greco et al., 2011) have been reported in ASD individuals. Our findings here provide evidence that PGE2 can disrupt normal cerebellar development, Overall, we observed that the cerebellum of offspring exposed to PGE2 during pregnancy exhibit (1) reduced density of cells originating at G11 and G16, (2) increased dendritic arborization, looping, spine density and odds of observing mature spines, (3) impaired related motor coordination, and (4) decreased expression level of cytoskeletal proteins (Table 3).

Within the postnatal cerebellum of PGE2-exposed mice we observed decreased density of cells originating at G11 (in both males and females) and G16 (in females alone), and increased dendritic arborization, the odds of observing dendritic loops, dendritic spine density, and the odds of observing mature spines. PGE2-mice also exhibit abnormal cerebellar motor function including increased number of swipes in the adhesive sticker test, changes in innate sex differences seen in Saline mice in the grid walking test, and a decrease in steps and changes in innate sex differences seen in Saline mice in the cylinder test. We saw a decrease in the expression of spinophilin, □-actin, and N-cadherin in PGE2 exposed mice. Finally, we saw similarities between performance on cerebellar motor function tests in PGE2-exposed males and the expression of spinophilin and N-cadherin. We discuss these deficits and sex differences below.

6.4.1 The effect of PGE2 on cell density and dendritic morphology in the cerebellum.

In this study we observed that the maternal exposure to PGE2 during the critical time in development at G11 causes a reduction of cell density of two cohorts of cells originating at G11 and G16 in the cerebellum as well as significant changes on the morphology of dendrites and dendritic spines. More specifically, we found that PGE2-exposed males and females had a lower density of G11-labelled cells compared to sex-matched controls. PGE2-exposed females also exhibited a lower density of G16-labelled cerebellar cells than controls. One of the most consistently reported abnormalities of the brain in ASD cases is the decreased number of Purkinje cells in the cerebellum (M. L. Bauman & Kemper, 2005; S. H. Fatemi et al., 2002). Moreover, cerebellar dysplasia (Wegiel et al., 2010) and a reduction in total cerebellar volume in children and (Carper & Courchesne, 2000) adults (Hallahan et al., 2009) with ASD have been reported. The decreases in cerebellar cell density found in this study complement findings in ASD models (M. L. Bauman & Kemper, 2005; S. H. Fatemi et al., 2002). Perhaps decreased cerebellar volume found in ASD may be attributed to diminished cell numbers as a result of alterations in cell proliferation that may occur prenatally.

Our previous *in vitro* study has demonstrated that PGE2 exposure of neuroectodermal (NE-4C) cells during differentiation increases neurite length. (Ashby Kissoondoyal & Dorota A. Crawford, 2021). Here we did not observe any difference in dendrite length in the cerebellum with PGE2-exposure. We observed an innate sex-difference between Saline male and female control mice with females having significantly longer dendrites than males (Table 3). This is in line with other studies showing that dendritic length in female rats is longer within certain areas of the brain including the locus coeruleus (Bangasser, Zhang, Garachh, Hanhauser, & Valentino, 2011), and CA3 within the hippocampus (McEwen et al., 1997) compared to males. Interestingly, the innate male-female sex difference in dendrite length found in the healthy brain was not observed in PGE2-exposed mice suggesting that sex differences are disrupted as a result of PGE2-exposure.

Although dendrite length was not changed in PGE2-exposed mice, we observed an increase in dendritic arborization in PGE2-exposed males and females closer to the soma at 20-50µm compared to Saline control mice. A positive feedback loop between the sex-hormone estradiol, and PGE2 has been demonstrated in endometrial tissue (Waclawik, Jabbour, Blitek, & Ziecik, 2009) with findings in the brains of postnatal rats providing further support of the loop (S. L. Dean, Wright, et al., 2012; Hoffman et al., 2016). Interestingly, the increase in estradiol within the medial prefrontal cortex (mPFC) of postnatal rats, was shown to correspond to an increase in dendritic arborization (Garrett & Wellman, 2009; Radley et al., 2004). There have been similar findings in the cerebellum, with increases in PGE2 corresponding to an increase in dendritic arborization. However, another study found that in the cerebellum of rats exposed to PGE2 during the second week of postnatal development there was a reduction in dendritic tree arborization within the cerebellum (S. L. Dean, Knutson, et al., 2012). This suggests that the dysregulation of COX2/PGE2 signalling may likely have different effects throughout critical times of development.

We have previously observed an increase in neurite looping in differentiating NE-4C cells treated with PGE2 (Ashby Kissoondoyal & Dorota A. Crawford, 2021) and also increased odds of finding looping dendrites in the cerebellums of COX-2^{-}-KI mice (Kissoondoyal et al., 2021). Here we observed that PGE2-exposed male and female offspring also exhibit increased the odds of observing dendritic looping. The formation of dendritic loops is often associated with dysregulation of the polymerization rate or in the direction of polymerization of the actin cytoskeletal dynamics, leading to disruptions in self-avoidance mechanisms and resulting in abnormal axonal pathfinding (F. R. Amthor & C. W. Oyster, 1995; A. D. Sdrulla & D. J. Linden, 2006). The increase in neurite loop formation is often a sign of disruptions in neurite outgrowth and pathfinding which are linked to neurodevelopmental disorders including retinal dysplasia (Weiner et al., 2004), and ASD (N. J. Minshew & Williams, 2007).

We also observed that dendritic spine density was increased in both PGE2-exposed male and female mice compared to sex-matched Saline controls (Table 3). Higher dendritic spine density typically corresponds to an increase in connectivity between neurons (Fiala, Spacek, & Harris, 2002). Our findings are dissimilar from spine density findings in the cerebellum in the Shank3+/ΔC and $Mecp2^{R308/Y}$ genetic (Kloth et al., 2015) and the VPA (Mychasiuk et al., 2012) environmental ASD rodent models, but similar to findings in humans with fragile X syndrome (Hinton, Brown, Wisniewski, & Rudelli, 1991; Irwin et al., 2001; Wisniewski, Segan, Miezejeski, Sersen, & Rudelli, 1991). As previously discussed, increases in estradiol, increase PGE2 levels through a positive feedback loop. Increases in estradiol were found to increase spine density within the hippocampus (Woolley, Wenzel, & Schwartzkroin, 1996). Excitation Inhibition (E/I) imbalances refer to global states within the brain where the normal homeostasis of excitatory and inhibitory signals result in proper development and maintenance of the brain. These imbalances are common in the brains of ASD individuals, and in many genetic ASD *in vitro* and *in vivo* rodent models which also exhibit changes in dendritic spine density (S. Han et al., 2014; Lee et al., 2017; Rubenstein & Merzenich, 2003). For example, in cultured hippocampal neurons from mice with an upregulation of CYF1P1, there was an increase in excitatory synapse numbers, and the frequency of miniature excitatory postsynaptic currents (Sohal & Rubenstein, 2019). Interestingly, the increase in excitatory signalling corresponded to a decrease in dendritic spine density. The cooccurrence E/I imbalances and changes in dendritic spine density are consistent in other mouse models of ASD (Belichenko et al., 2009; Chao, Zoghbi, & Rosenmund, 2007; Tropea et al., 2009), including in the Rett syndrome (*Mecp2* KO), and *mTOR* KO mice (Tang et al., 2014). These findings are unsurprising given the contribution of dendritic spines to the strength and plasticity of excitatory synapses (Luebke et al., 2010), and suggest that prenatal PGE2-exposure creates an E/I imbalance in the cerebellum of offspring mice.

Besides the increased spine density in PGE2-exposed mice we also observed increased odds of seeing mature spines. Normally dendritic spines can be found in different shapes which are related to their function, and are categorized as thin, stubby (less mature) and mushroom (mature) shaped (J. Bourne & Harris, 2007; J. N. Bourne & Harris, 2008; Kasai et al., 2003). We reported dendritic morphology by examining the likelihood of observing a mature spine (mushroom) compared to an immature spine (thin or stubby). First, we observed that in Saline controls there was a decrease in the odds of observing mature (mushroom shaped) spines in females compared to males. Estrus-cycle dependant variations in dendritic spine morphology have been reported in adult female rats, with spines being predominantly mushroom-shaped in proestrus, and thin in estrus (de Lacalle, 2006; González-Burgos, Alejandre-Gómez, & Cervantes, 2005). Being the intermediary shape between thin and mushroom shaped spines, the increase in stubby shaped spines in Saline control females compared to males may speak to the increase in shifts between thin and mushroom spines. Interestingly, we also observed the greatest variation in the odds of observing spines in Saline control females. This may also speak to the changes in spine shape associated with the estrus cycle. PGE2-exposed males and females were both more likely to have more mature (mushroom shaped) spines compared to Saline controls with sex-differences no longer present. The loss of the sex difference indicates that there may be a reduction in estrus-dependent changes in spine shape in PGE2-exposed females.

Within the cerebellum, abnormal increases in estradiol have been shown to increase the relative quantities of mushroom shaped spines (Rubenstein & Merzenich, 2003). Our observations are in line with these findings given the positive feedback loop between estradiol and PGE2 levels. Increases in mature mushroom spines have been observed in other ASD *in vitro* and *in vivo* models including cultured neurons from syngap1 and syngap2 loss of function mice and in the PTEN knockout mouse model (Cupolillo et al., 2016; Haws et al., 2014).

6.4.2 Abnormal motor behaviour in PGE2-exposed offspring

Abnormal morphology of neurons in the cerebellum have been associated with ASDs. Moreover, early disruption of cerebellar circuitry is associated with future diagnosis of ASD (David Q Beversdorf et al., 2005; Eric Courchesne et al., 2001; Catherine Limperopoulos et al., 2007). Rodent models have also shown that disruptions in cerebellar morphology including increases or decreases in: cell density and volume, dendritic arborization, dendritic spine density, and changes in dendritic spine morphology have resulted in ASD related behaviours including social deficits, difficulties in task-switching and anxious grooming behaviours (Al-Afif et al., 2013; Bobee et al., 2000; Tsai et al., 2012), with some studies showing that greater disruptions cerebellar morphology corresponded to greater severity of ASD-related behaviours (D'Mello et al., 2015; Riva et al., 2013). We previously demonstrated that PGE2-exposed mice exhibit autism-related behaviours (Kissoondoyal et al.; C. T. Wong et al., 2017). To build on those findings we further investigated whether PGE2-exposed mice exhibited any cerebellar-related motor dysfunction. Overall, we observed a male specific effect on sensorimotor coordination in the adhesive sticker test and a reduction in locomotion in the cylinder test. In addition, we found clear sex differences within PGE2-exposed animals which were distinct from the controls.

In the *adhesive sticker test*, which is a sensitive test for sensorimotor dysfunction (Sheila M Fleming et al., 2004; Hoa A Lam et al., 2011; Richter et al., 2017) we found a male-specific increase in the number of swipes per second, indicating that PGE2-exposed males had difficulties in sensory-motor integration. Basic motor dysfunction in ASD is often attributed to deficits in sensorimotor integration in which individuals are unable to extract and integrate sensory information into an executable motor plan (Khoury et al., 2020; Mosconi & Sweeney, 2015; Sokhadze, Tasman, Sokhadze, El-Baz, & Casanova, 2016). Literature suggests that these deficits in motor coordination arise from disruptions in cortico-cerebellar networks (Khoury et al., 2020; Unruh et al., 2019). Our findings suggest that PGE2

exposure may make males more inefficient in the execution of motor planning, resulting in an excess of swipes before the adhesive sticker is removed.

The *grid walking test* (or foot-fault test) is an established measure of sensorimotor coordination of the four limbs in rodent models (Feather-Schussler & Ferguson, 2016; Heyser, 2004). However, to our knowledge, this is the first instance of the grid walking test used to measure sensorimotor coordination of an ASD mouse model. Our findings suggest that PGE2-exposure may affect sensorimotor coordination differently in males and females. We saw a non-significant increase in the number of slips (number-of-faults) and the time to escape in PGE2-exposed males, and a decrease in number of slips and the time to escape in PGE2-exposed females, compared to Saline controls. The opposite effect of PGE2-exposure on males and females resulted in a significant difference between PGE2 males and females, with males slipping more and escaping more quickly than females. Taken together with our findings from the *adhesive sticker test* the increase in slips in PGE2-exposed males suggest that cerebellar motor performance is more disrupted in males than females. Interestingly, despite making more slips, females spent more time in the grid walking apparatus than males. We previously demonstrated a female-specific increase in anxious behaviour in PGE2-exposed mice shown in grooming behaviours and time spent in the center of the *open* field test. We suspect the increased time to escape in PE2-exposed female mice may be impacted by differences in anxious behaviour.

The findings in the grid walking test also demonstrate a divergence in sex effect trends from Saline controls to PGE2-exposed mice. Interestingly, sex-differences in cerebellar function and morphology that are not observed in the general population can be found in ASD individuals. For example, ASD females exhibit hyperconnectivity between cerebellar and cortical regions, whereas males exhibit hypoconnectivity (Smith et al., 2019). Additionally, there are regional differences in grey matter quantity and the ratio of grey to white matter between the cerebellums of ASD males and females. Interestingly, this sex difference in ASD males and females is not present in the general

population (Supekar & Menon, 2015). Our findings here further highlight the importance of examining sex differences in the study of neurodevelopmental disorders.

We also conducted the *cylinder test* to measure cerebellar-dependent spontaneous forelimb and hindlimb use as well as the postural control necessary for rearing in mice (Rattka et al., 2016; Schönfeld, Dooley, et al., 2017; Schönfeld, Jahanshahi, et al., 2017). We found innate sex-differences in forelimb touches and rearing and between male and female control mice with males making less forelimb touches and rearing less frequently than females (Table 3). This is consistent with other studies showing that male mice inherently rear less than females (König et al., 2020). However, the expected sex differences in forelimb touches and rearing were no longer observed in PGE2-exposed mice. In addition, we observed a male specific decrease in number of steps in PGE2-exposed mice compared to Saline controls. Despite locomotion being often affected in ASD individuals (J. Cook, 2016; Kindregan, Gallagher, & Gormley, 2015; Shetreat-Klein, Shinnar, & Rapin, 2014; Valagussa, Trentin, Signori, & Grossi, 2018), information on locomotion in ASD rodent models is limited. Examining cerebellar motor function in other rodent models may provide useful information in understanding the etiology of ASD.

6.4.3 Abnormal levels of essential cytoskeletal proteins

As discussed above the PGE2-exposed mice offspring exhibit abnormal cell density and dendritic morphology within the cerebellums, which could potentially arise from a dysregulation of the cytoskeleton or neurite pathfinding mechanisms during development. In fact, we have previously demonstrated that similar abnormalities of neurite and dendritic morphology in PGE2-exposed differentiating NE-4C stem cells *in vitro* (Kissoondoyal Crawford, 2021) and COX-2^{-}-KI mice (Kissoondoyal et al., 2021) are associated with abnormal expression levels of □-actin (decreased in COX-2^{-}-KI mice) and actin-binding protein spinophilin (increased in NE-4C stem cells and COX-2^{-}-KI mice). In this study, we detected decreased expression levels of □-actin, spinophilin, and N-cadherin

in PGE2-exposed mice relative to sex-matched controls. Within both Saline control mice and PGE2-exposed mice, we observed a higher expression of both □-actin and spinophilin in males compared to females. We did not observe any sex-differences in N-Cadherins in PGE2-exposed or Saline control mice.

□-actin and spinophilin play key roles in the maintenance of the actin-cytoskeleton. The rate of actin-polymerisation/depolymerization affects the shape and growth of dendritic spines and is a determinant of neurite pathfinding (Blanchoin, Boujemaa-Paterski, Sykes, & Plastino, 2014; Borovac, Bosch, & Okamoto, 2018; Koleske, 2013; Penzes & Rafalovich, 2012; T. D. Pollard & J. A. Cooper, 2009). The primary function of spinophilin is in the stabilization of the actin cytoskeleton and is involved in actin driven changes in dendrite and dendritic spine morphology as result of excitatory synaptic activity (A. Satoh et al., 1998; Z. Yan et al., 1999). Here we observed and increase in density of more mature spines (or less immature spines) in PGE2-exposed mice that coincided with a reduction in the expression level of □-actin and spinophilin. This is in line with a previous study in rats injected with PGE2 postnatally which found decreased spinophilin protein in the cerebellums (Dean, Wright, et al., 2012). In line with our findings, another study also reported an increase in dendritic spine density in spinophilin-knockout mice (Feng et al., 2000). The reduction in □-actin and spinophilin protein content indicate cytoskeletal dysfunction within the cerebellums of PGE2-exposed mice, which might subsequently contribute to the abnormal cerebellar motor behaviour we observed in the PGE2-exposed mice.

Lastly, we also examined the expression levels of N-Cadherin, a well-known cell adhesion molecule with important roles throughout neural development (Arikkath & Reichardt, 2008; Basu, Taylor, & Williams, 2015; Seong, Yuan, & Arikkath, 2015; Suzuki & Takeichi, 2008). N-Cadherins have also known roles in neurite outgrowth and guidance, and synaptogenesis and synaptic plasticity (Hirano & Takeichi, 2012; Takeichi & Abe, 2005). Mutations in N-Cadherin including copy number

variations and single nucleotide polymorphisms are associated with ASD (Wang et al., 2009; (Lin, et al. 2016). We observed a decrease in N-cadherin protein expression in PGE2-exposed male and female mice. Interestingly, previous findings in cultured neurons showed that an inhibition f N-Cadherin by use of a dominant-negative N-Cadherin (cN390Δ), resulted in a filopodia-like elongation of dendritic spines, and a disruption of the distribution of proteins on the postsynaptic membrane (Mendez et al., 2010; Togashi et al., 2002). We observed no sex-differences between either Saline control, or PGE2-exposed males or females.

We further examined the expression of these three proteins in PGE2-exposed males as we found them to be the most affected group in behavioural testing manifested in a range of outcomes. Interestingly, we determined that the expression levels of spinophilin and N-cadherin in Male 1 (which performed the best among the PGE2 males) was close to the average seen in the Saline control males whereas Males 2 and 3 (worst scoring PGE2-exposed males) had expression levels similar to PGE2-exposed males. This demonstrates a connection between the expression of spinophilin and N-cadherin and performance in tests which measure cerebellar-motor function. We have previously shown that maternal exposure to PGE2 can differentially affect the expression of ASD genes in genetically identical offspring (Rai-Bhogal, Wong, et al., 2018b). Our findings here provide further evidence that maternal exposure to PGE2 at during prenatal development may result in different behavioural outcomes in genetically identical pups. One of the difficulties in the treatment of ASDs is the heterogeneity of the disorder. Clinical studies have demonstrated that abnormal changes in PGE2 levels through development can result from maternal exposure to environmental risk factors that are linked to ASD. Taken together, we suggest that the variability in the initial exposure to environmental risk factors combined with the variability in the consequences of abnormal PGE2 levels during development contribute to the heterogeneity observed in ASD-related symptoms.

6.4.4 Concluding remarks

To summarize, we show for the first time that a one-time maternal exposure to PGE2 results in disruptions in cell density, and dendritic and dendritic spine morphology postnatally in males and females. These changes in cell density, the cytoarchitectural differences in the dendritic and dendritic spine morphology in PGE2-exposed mice corresponded to changes in the expression of □-actin, spinophilin, and N-cadherin Additionally, PGE2-exposed mice performed abnormally on cerebellar motor function tests, with innate sex-differences seen in Saline mice not being found in PGE2 mice. Examining PGE2 mice we found that performance on cerebellar motor function tests corresponded to the expression of spinophilin and N-cadherin. These findings build on previous clinical examples that exposure to common environmental factors that affect lipid signalling during development have consequences for important events in neuronal development. Given the information obtained in this study by examining sex-differences, we emphasize the importance of examining sex-differences which are underrepresented in the literature.

6.5 References

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Chapter 7: General Discussion

7.1 Dissertation Objectives Revisited

Previous findings have shown a connection between disruptions in COX2/PGE2 signalling as a risk factor for the development of ASDs. To add to the growing body of evidence supporting this connection, and to explore the molecular basis of this connection further, this overall aim of this dissertation was: *To identify the molecular mechanisms by which abnormal COX2/PGE2 lipid signalling can affect cerebellar development in a sex-dependent manner and contribute to ASD related pathologies.*

7.1.1 Key findings for Specific Objectives

Objective 1) To examine the effect of PGE2 exposure on cytoskeletal processes including neurite outgrowth and neurite pathfinding (Study 1)

Long-term exposure to PGE2 from the induction of differentiation increased neurite length, and the proportion of neurites that formed neurite loops and we saw a decrease in the proportion of turning (asymmetrical) growth cones. We saw a decrease in the total (actin-bound) form of the actin stabilizing protein spinophilin.

Objective 2) To examine differences in the effect of PGE2 across days following the induction of differentiation to determine critical periods of PGE2 on differentiation. (Study 1)

We found that exposure to PGE2 on days 2,4,6,8, 10 and 12 during the differentiation of NE-4C stem cells affected cell morphology related to differentiation including neurite length, and the formation of axonal loops. We found that the effect of PGE2 was dependent on the day of differentiation, suggesting critical periods of the effect of PGE2.

Objective 3) To investigate PGE2-PKA signaling regulation of the observed morphological changes (Study 1)

We observed a PKA-dependant effect of PGE2 on neurite length and the formation of neurite loops. We also observed an increase in the levels of PKA-phosphorylated spinophilin, which has a reduced binding affinity for Actin.

Objective 4) To verify in vitro findings and to further study the consequences of disruptions in COX2/PGE2 signalling on cytoskeletal dynamics through an examination of dendritic morphology and the expression of cytoskeletal proteins (Study 2 and 3)

COX-2^{-}-KI mice had an increase in dendritic arborization, and dendritic looping compared to WT mice. We saw sex-dependent effects of the COX-2^{-}-KI on dendrite thickness (male specific increase), and the odds of observing mature (mushroom-shaped) spines (Study 2).

We saw abnormalities in cell density in cell populations originating from G11 and G16, an increase in dendritic arborization, the formation of dendritic loops, dendritic spine density and the odds of observing mature (mushroom-shaped) spines in mice prenatally exposed to PGE2 (Study 3). Additionally, we observed a decrease in the expression of cytoskeletal proteins β-actin, spinophilin, and N-Cadherin in PGE2-exposed mice compared to C57 controls.

Objective 5) To describe cerebellar-related motor dysfunction in mice prenatally exposed to PGE2. (Study 3)

PGE2-exposed mice exhibited abnormal cerebellar-related motor behaviours in the adhesive sticker test, the grid walking test, and the cylinder test. There was a strong positive correlation between the expression of Spinophilin and N-Cadherin and performance on the behaviour tests.

Objective 6) To determine sex-dependent differences in the effect of COX2/PGE2 disruptions on the observed morphological and behavioural changes. (Study 2 and 3)

The innate sex differences found in our WT mice were changed in our COX-2^{-}-KI mice. While dendrite length was longer in WT females compared to males, the length was shorter in COX-2^{-}-KI females compared to males. There was a greater spine density in WT males compared to females, and this difference was lost in our COX-2^{-}-KI mice. Spine morphology was affected in a sex-dependent manner. Compared to WT males, females were less likely to have mature (mushroom-shaped spines). In contrast COX-2^{-}-KI females were more likely to have these spines than COX-2^{-}-KI males. Further, while the expression of β-actin and spinophilin was higher in WT males than females, the expression of each was lower in COX-2^{-}-KI males compared to females.

While there were sex-differences in Sal control mice, we did not observe any sex differences in G16 cell density, dendrite length and the odds of observing mature (mushroom-shaped) spines in PGE2-exposed mice. Similarly, there were innate sex-differences in the expression of Spinophilin and β-actin in Sal control mice that were not observed in PGE2-exposed mice.

There were differences between the sex differences in Sal and PGE2-exposed mice across many of the cerebellar-related motor behavioral tests. While no sex-differences were found in Sal control mice in the number of swipes per second in the adhesive sticker test, and the number of slips through the grid in the grid walking test we saw an increase in both in PGE2-exposed males compared to females. There were innate differences in time to escape in the grid walking test, number of forelimb touches and number of rears in the cylinder test between Sal male and female mice with decreases in males compared to females. These sex-differences were lost in the number of forelimb touches and number of rears between PGE2-males and females. Further, the opposite trend was observed in PGE2-exposed males and females in time to escape in the grid walking test with PGE2-exposed males taking longer to escape than females.

7.2 Discussion and Future Directions

In this section the following key themes emerging from results across the studies in this dissertation will be discussed: The role of COX2/PGE2 signaling in neuronal development, the effect of abnormal COX2/PGE2 signaling on cytoskeletal dynamics, abnormal PGE2 signalling in the cerebellum, sex dependent effects of PGE2, and the novel contributions of these findings to the literature on Autism Spectrum Disorders.

7.2.1 Disruption in organization and timing of COX2/PGE2 signalling

Development of the brain is a complex highly coordinated process relying on the proper sequencing of different signals and events. Disruption of these events, including the disruption of lipid signalling during prenatal development can impact the proper formation of the brain.

We have previously demonstrated that *in vitro* PGE2 can increase proliferation, cell viability, and accelerate progression of cells through differentiation (Wong, Ussyshkin et al. 2016). Further, *in vivo*, we have observed that prenatal exposure to PGE2 affected cell density in the olfactory bulb and in the neocortex in cells from two different cohorts (G11 and G16 derived). However, the findings *in vivo* show a more complex picture of the effect of PGE2. In the olfactory bulb there was a male-specific increase in cell density at both G11 and G16, and in the neocortex there was a female specific decrease in cell density at G16 in PGE2-exposed mice relative to Sal control mice (ref PGE2 paper). In study 3 we further observed that there was a reduction in cerebellar cell density at both G11 and G16. These findings indicate that there are regional and sex-dependent differences on the proliferation of cells. Future investigations may wish to further examine cell density in other brain regions and to examine the sex- and brain region-specific effect of PGE2 on proliferation and viability in primary cell cultures.

Beyond regional and sex-specific differences, our findings in study 1 indicated that there was a critical period of the effect of PGE2 on the differentiation of neuronal cells. For example, we saw an effect of PGE2 on neurite length on days 8 and 12 but not 10. A previous study *in vivo* in rats found a

critical period of PGE2-estradiol pathway activity, with a dysregulation of the pathway during the second week postnatally resulting in reduced dendritic arbor complexity of Purkinje cells and impaired juvenile social play behaviour in males (Hoffman, Wright et al. 2016). More evidence for critical windows of the PGE2 effect come from molecular analyses. For example, gene expression of the PGE2 receptor EP1 increases during critical periods of masculinization in the preoptic area of rats (Burks, Wright et al. 2007). Downstream of PGE2, Park, Ma et al. (2015) found PGE2 levels and PGE2-PKA phosphorylation of p38 MAPK and CREB. increase throughout neural differentiation. We would speculate that similarly to the postnatal critical window of PGE2 activity established by (Hoffman, Wright et al. 2016) that there exists a prenatal window based on the effect of PGE2 on early neuronal differentiation and migration. To further explore this idea further, future studies could examine the consequences prenatal PGE2 exposure at different prenatal dates. While our lab has established an effect of PGE2 exposure at G11 it is imperative to determine the window of the PGE2 effect to understand the prenatal processes affected by PGE2 exposure.

7.2.2 The effect of abnormal COX2/PGE2 signaling on cytoskeletal dynamics

Cytoskeletal dynamics are a tightly regulated process during neuronal development. Processes including neurite outgrowth and pathfinding, and the formation and maintenance of dendritic spines are primarily driven by the polymerization/depolymerization of the actin-cytoskeleton (Koleske 2013, Blanchoin, Boujemaa-Paterski et al. 2014, Borovac, Bosch et al. 2018).

Our findings from study 1 suggested that PGE2 exposure increased the disorganization of the actin-cytoskeleton resulting neurite length, and the proportion of neurites that formed neurite loops and a decrease in the proportion of turning (asymmetrical) growth cones. Given the decrease in total (actin-bound) spinophilin and the increase in PKA-phosphorylated (unbound from Actin) spinophilin that we observed, we proposed a model in which PGE2 increased the PKA phosphorylation of spinophilin, resulting in its dissociation from Actin, and resulted in a reduction in actin-stability. Our *in vivo*

findings in Study 2 and 3 mice supported our findings from cells, with increases in dendritic arborization, and spine density (only in PGE2-exposed mice), but also increases in dendritic loops. Interestingly, we observed changes not only in the density of dendritic spines, but also changes in dendritic spine morphology in both studies 2 and 3.

Our lab has previously demonstrated that exposure of NE-4C cells to PGE2 *in vitro* can increase neurite length (Davidson, Wong et al. 2016) and decrease migration (Wong, Ahmad et al. 2014). In *vivo* in mice PGE2 exposure decreased migration in males and increased migration in females, of G11 originating cells, and increased migration in males of G16 originating cells (ref PGE2 paper). In a microarray study of COX-2 KO mice, we found dendritic spine modelling and dendrite morphogenesis among the top affected protein networks, and β-actin among the most dysregulated genes (Rai-Bhogal, Ahmad et al. 2018). Other studies have also demonstrated that abnormal COX2/PGE2 signalling can affect cytoskeletal dynamics *in vivo*. Changes in dendritic length and arborization, and in dendritic spine density (Song, Feng et al. 2019) are found in PGE2-exposed, and in COX-2 deficient rodent models.

We observed that changes in PGE2 (high or low), affected the expression of the cytoskeletal proteins spinophilin, β-actin, and N-Cadherin. N-Cadherin, one of the cadherin family cell adhesion molecules has roles in neurodevelopment including in neurite outgrowth and pathfinding, in synaptogenesis, and synaptic plasticity(Takeichi and Abe 2005). The expression of N-Cadherin was only examined in study 3 in PGE2-exposed mice, in which we observed a decrease in N-cadherin expression in PGE2-exposed mice. β-actin and spinophilin are key in regulating the stability of the actin-cytoskeleton. Spinophilin functions as an intermediary between incoming excitatory signals, and resultant changes in dendritic spine morphology and density (Satoh, Nakanishi et al. 1998). Bound spinophilin is associated with an increase in actin stability, with the opposite being found in cells with more unbound spinophilin (Yan, Hsieh-Wilson et al. 1999). In NE-4C cells exposed to PGE2 we saw a

PKA-dependent decrease in the expression of total (actin-bound) spinophilin and a PKA-dependent increase in PKA-phosphorylated (unbound from actin) spinophilin (Study 1). We hypothesized that due to an increase in PKA-phosphorylated (unbound) spinophilin, that we would see dysregulation of the actin cytoskeleton and ultimately changes in dendritic and dendritic spine morphology *in vivo*. In study 2 in our COX-2^{-}-KI mouse model we observed sex-dependent changes in the expression of spinophilin (decreased in COX-2^{-}-KI males and increased in COX-2^{-}-KI females), while in PGE2-exposed mice we saw a decrease in the expression of spinophilin in both male and female mice. Previous studies have found a decrease in spinophilin expression in the cerebellums of rats given postnatal injections of estradiol (Dean, Knutson et al. 2012) which is in line with our findings. Interestingly, the same study found that co-injection of estradiol and the COX2 inhibitor nimesulide still resulted in a decrease in spinophilin expression. We saw a similar effect of COX-2^{-}-KI on the expression of β-actin with a male-specific decrease in the expression of β-actin compared to WT male controls (Study 2). In study 3 in PGE2-exposed mice we saw a decrease in the expression of β-actin in both males and females compared to sex-matched controls. Our findings show that both increases and decreases in prenatal levels of PGE2 result in similar reductions in the expression of both spinophilin and β-actin. The similarity in increased and decreased PGE2 levels may be a result of changes in the sensitivity of the EP receptors to PGE2. We have previously demonstrated that EP receptors can elicit different responses within Neuro-2A cells (Tamiji and Crawford 2010). Given that the sensitivity of the EP receptors differentially adjusts based on PGE2 levels, differences in EP receptor activation in low or high PGE2 models may differ(Dey, Lejeune et al. 2006). We speculate that the resulting differences in EP receptor signalling cascades result in similar responses between our COX-2^{-}-KI and PGE2-exposed mice.

In Study 3, by using samples from the same mice that were tested for cerebellar motor-dysfunction, we were able to demonstrate a connection between the expression of spinophilin and N-

Cadherin to performance on the motor tests. We saw that PGE2-exposed males that performed worse on the motor tests, also had a lower expression of these proteins that was similar to the average expression of the PGE2-male pooled sample. Beyond this, the best performing male among PGE2-exposed males had spinophilin and N-cadherin expression levels that were more similar to the Sal control pooled sample than the PGE2-exposed pooled sample. These findings provide a connection between changes in cerebellar structure, and the disruptions in cerebellar-motor behaviour observed in PGE2-exposed mice. Previous studies have found that postnatal injection of PGE2 into the cerebellums of rats resulted in a decrease of spinophilin content (Dean, Knutson et al. 2012). The same lab found an increase in male-sexual behaviour in female rats given the postnatal injection of PGE2 (Wright and McCarthy 2009). However, this is the first study providing a direct connection between PGE2 induced changes in cytoskeletal proteins Spinophilin and N-cadherin prenatally, and cerebellar-motor function. Future studies could further investigate the effects of PGE2 on cytoskeletal formation through use of primary cell models or induced pluripotent stem cell models. Live imaging of cytoskeletal dynamics would also help to further understand the mechanisms at play. Future studies should continue to examine the relationship between protein expression and the degree that neurodevelopmental disorder associated behaviours are present. Especially given the heterogeneity of ASD symptoms, it would be helpful to understand other complex behaviours such as social interaction across a variety of ASD rodent models.

7.2.3 Abnormal PGE2 signalling in the cerebellum

The cerebellum is one of the most frequently implicated regions in ASD with early disruption of cerebellar circuitry strongly linked to subsequent ASD diagnosis. The effect of COX2/PGE2 signalling on cerebellar development was addressed in study 2 and study 3. We saw structural abnormalities in the cerebellum in cell density, and dendritic and dendritic spine morphology in both high (PGE2-exposed) and low (COX-2^{-}-KI) mouse models. Postnatal effects of PGE2 exposure appear

to differ from our findings. While in rats given a cerebellar injection of PGE2 postnatally, there was a stunting of dendritic arbor length, there was an increase in arborization and complexity in rats injected with the COX-2 inhibitor nimesulide (Dean, Wright et al. 2012). These may speak to the critical windows of the effect of PGE2 on development. However, our findings are in line with the structural abnormalities of the cerebellum observed in ASD individuals. For example, in line with our observed reduction in cell density, there is commonly a reduction in Purkinje cell density in ASD individuals. Studies have also suggested that there may be insufficient pruning of the dendritic arbor of cerebellar neurons, which was in line with our findings of increased dendritic arborization in both PGE2-exposed and COX-2^{-}-KI mice.

To determine if the changes in cerebellar morphology could be connected to the behavioural outcomes, we examined cerebellar-related motor deficits in PGE2-exposed mice in the adhesive sticker test, the grid walking test, and the cylinder test (study 3). Our findings provided further support for the idea that prenatal PGE2-exposure disrupted normal neurodevelopment of the cerebellum. While there was only a direct effect of PGE2-exposure on the number of swipes per second in the adhesive sticker test, and the number of steps in the cylinder test in males, we saw a striking effect of PGE2 on the innate sex differences in control animals. Structural and functional abnormalities have been commonly found in the cerebellums of ASD individuals (Fatemi, Aldinger et al. 2012, Becker and Stoodley 2013). In high functioning ASD individuals, fMRI studies have found a decrease in cerebellar activation and cerebro-cerebellar connectivity (Mostofsky, Powell et al. 2009). Further, the decrease in connectivity corresponds to reduced motor performance (Floris, Barber et al. 2016, Jack, Keifer et al. 2017). A recent study examining fMRI analysis of resting-state potential in ASD individuals found sex-specific patterns in global functional connectivity of the cerebellum in ASD individuals that were not present in control individuals (Smith, Avery et al. 2019). It is becoming increasingly evident that there is a sexual

dimorphism of cerebellar development in ASD individuals, and our findings suggest that a disruption in COX2/PGE2 signalling may contribute to this divergence.

A limiting factor in the treatment of ASD symptoms is the age of diagnosis. Autism is typically characterized clinically by impairments in social interactions, exhibiting persistent or repetitive behaviours, and exhibiting anxious behaviours, which are normally apparent at around 3 years of age (Lai, Lombardo et al. 2014). However, literature suggests that differences may be present earlier and there is a large body of research dedicated to finding biomarkers of ASDs(Nelson, Grether et al. 2001, Waligóra, Waligóra et al. 2019, Shen, Liu et al. 2020). While many current approaches are aimed at identifying molecular biomarkers, characterizing cerebellar motor function in ASD individuals may provide a more accessible approach in a clinical setting. The cerebellar tests performed in study 3 were performed on postnatal day 30, well before many common tests of memory and socialization are sensitive enough to detect differences. For example, the three-chamber test, commonly used to assess sociability in ASD mouse models is performed on mice usually no younger than 6-8 weeks old (Kaidanovich-Beilin, Lipina et al. 2011, Yang, Silverman et al. 2011, Rein, Ma et al. 2020). The Morris water maze, a test of spatial memory for rodents, is usually performed on mice no younger than 3 months old (Provenzano, Pangrazzi et al. 2015). Given that the ability to diagnose cerebellar motor function earlier than social deficits also translates into humans, understanding the cerebellar-dependent motor deficits found in ASDs would allow for earlier diagnosis and subsequently earlier treatment of ASD symptoms. Future studies may wish to study the effects of changes in prenatal exposure to PGE2 on cerebellar-dependent motor coordination in humans to verify our findings here.

Overall, our findings here build on previous findings showing that PGE2-exposed mice which have previously demonstrated ASD-related behaviours including social deficits, restrictive and repetitive behaviours, and anxious behaviours (ref PGE2 paper), also exhibit cerebellar-motor deficits that were detectable through behavioural testing.

7.2.4 Sex dependent effects of PGE2

There is an increasing movement to push for the study of sex differences in developmental disorders including ASDs. Particularly with neurodevelopmental disorders such as ASD, the rate of male diagnosis is higher than females (May, Adesina et al. 2019). Further, evidence suggests that there are differences in the pathogenesis of ASDs between males and females (Banach, Thompson et al. 2009). Given that there seems to be no inherent genetic sex bias in ASD risk genes (Voineagu, Wang et al. 2011, Gupta, Ellis et al. 2014, Iossifov, O'Roak et al. 2014), the sex difference in ASD likely is a result of environmental risk factors. Our lab has previously suggested a connection between environmental risk factors associated with ASDs and changes in the levels of PGE2 in the brain (Tamiji and Crawford 2010, Wong, Wais et al. 2015). We have previously demonstrated sex-differences in ASD-related behaviours in COX-2^{-}-KI mice with abnormally low levels of PGE2 (Wong, Bestard-Lorigados et al. 2019), and mice prenatally exposed to high levels of PGE2 (ref PGE2 paper). Across the *in vivo* studies in this dissertation, several sex-dependent differences were found in models of abnormal COX2/PGE2 signaling. Within the cerebellum, disruptions in PGE2 signalling resulted in sex-dependent changes in cell density, dendritic morphology, cytoskeletal protein expression, and cerebellar motor function (Study 2 and 3).

The sexually dimorphic effect of PGE2 may be related to the masculinization theory of ASD which postulates that while ASD gene expression may be similar between males and females, ASD risk factors are involved in male development, and changes in ASD risk factors therefore affect male development (Werling, Parikshak et al. 2016). Sex-specific steroids are involved in neurogenesis, synaptogenesis, and cell differentiation during development, which are all affected in ASDs (Mccarthy, De Vries et al. 2009). Previous studies have examined the role of PGE2 in masculinization of the brain postnatally. A single injection of PGE2 into the mPOA of newborn female rats resulted in a change of hormonal synaptic profiles and adult sexual behaviour to resemble that of male rats (Wright, Burks et

al. 2008). In contrast, the injection of nimesulide, a COX inhibitor, into the mPOA of newborn male rats blocked masculinization and impaired adult male sexual behaviour (Amateau and McCarthy 2004). These studies have indicated that PGE2 can function as a mediator of steroid-dependent masculinization of the brain which may explain the sex-dependent effect of PGE2 (McCarthy and Wright 2017).

Within the cerebellum, increases in PGE2 levels increases aromatase enzyme activity and local production of the sex-hormone estradiol. A positive feedback loop has been described between estradiol and PGE2 in endometrial tissue (Waclawik, Jabbour et al. 2009). There is a normal increase in PGE2 levels increase during the second postnatal week of development and both increases and decreases in PGE2 levels during this time affect normal Purkinje cell formation (Dean, Wright et al. 2012). Further, a study examining the induction of estradiol production via PGE2 found in the brain found that the induction occurred primarily through EP3 in males and EP4 in females (Pedersen and Saldanha 2017). These findings, in addition to the findings in this dissertation, suggest that while not primarily considered a sexually dimorphic region, COX2/PGE2 can elicit sex-dependent changes in cerebellar development. The sex-dependent differences PGE2-exposed males and females that we observed in the cerebellum are in line findings in other studies. In a study examining structural imaging data from a matched group of ASD boys and girls, grey matter patterns within the cerebellum were reliably able to discern ASD boys from girls (Supekar and Menon 2015). As previously described, resting-state fMRI scans of the cerebellum show differences between male and female ASD individuals in global connectivity, and cortico-cerebellar connectivity (Smith, Avery et al. 2019). While we do not fully understand the underlying causes for the inherent sex differences and the changes in these differences in PGE2 disrupted mice, we have seen that cytoskeletal dynamics are disrupted in these models. Future studies may wish to examine other important processes during prenatal development such as metabolic and inflammatory pathways.

Overall, the sex-differences we observed further highlight the importance of examining sex as a factor in the study of neurodevelopmental processes. Given that many neurodevelopmental disorders have a disproportionately high incidence in males, understanding the sex-differences may provide insight into the mechanisms of these disorders.

7.2.5 Novel contributions to ASD literature

The prevalence of ASD is increasing at an exponential rate with the prevalence rate being as high as 1% (Brugha, McManus et al. 2011, Zablotsky, Black et al. 2015, Maenner, Shaw et al. 2020). The economic contribution of government organizations in supporting treatment of individuals with ASDs as well as the financial (Kogan, Strickland et al. 2008, Buescher, Cidav et al. 2014, Saunders, Tilford et al. 2015), and quality impairments (Mugno, Ruta et al. 2007) of the families of children with ASDs continue to increase with the prevalence of ASDs. Despite the increasing need to address the increasing prevalence of ASDs, our understanding of the etiology of ASDs is limited.

The studies in this dissertation were aimed at increasing the body of literature on the mechanisms contributing to the etiology of ASDs and serve as a first step in addressing the continual increase in prevalence. Despite the heterogeneity in ASDs, we aimed our studies at factors that have a high prevalence amongst ASD individuals, namely cytoskeletal dysfunction, and cerebellar abnormalities. Our studies *in vitro* and *in vivo* demonstrated that abnormal COX2/PGE2 signalling resulting in increased or decreased levels of PGE2 affected processes involving the actin cytoskeleton including neurite elongation and pathfinding, and the formation and morphology of dendritic spines. In study 3 we demonstrated that irregular PGE2 levels resulted in abnormalities in cerebellar structure but also affected performance on cerebellar-related motor tasks. These findings build on previous studies showing ASD-related behaviours in PGE2-exposed mice and add to the growing body of literature examining the link between the cerebellum and ASDs.

Our research ultimately provides novel information on the role of COX2/PGE2 signalling during neuronal development. Given that these studies would not be feasible in humans, our findings contribute to the greater understanding of the potential mechanisms contributing to the etiology of ASDs in humans. We continue to emphasize the examination of environmental risk factors, particularly those linked to COX2/PGE2 signalling. Our findings provide additional evidence of the importance of examining sex as a factor in neurodevelopmental disorders. Altogether, we provide evidence that disruptions in COX2/PGE2 signalling can impact important neurodevelopmental processes that persist into adulthood.

7.3 Study limitations

In study 1 NE-4C stem cells were examined as a model of neuronal differentiation. Given the progression of the studies, the use of primary cultures taken from our PGE2-exposed and COX-2^{-}-KI models would have provided a better connection from cell to animal studies. Additionally, these cells lack the p53 protein. The p53 protein has been implicated in the regulation of the cytoskeleton, which was a focus of study 1. It may be worth repeating these studies in other Neuronal cell lines. For study 2 the COX-2^{-}-KI mouse was used as a model of low PGE2 levels. While being the major prostanoid, and being primarily regulated by the COX enzymes, other downstream metabolites may have contributed to the effects observed. In study 1 the quantification of cellular morphology was performed by one student. Improving on this, in study 2, dendritic morphology analysis was performed by a minimum of 10 students and the measurements were averaged, with statistical controls introduced to remove outliers. For study 3, while behavioural analysis was performed with 5 students each examining the same set of videos similarly to study 2, we transitioned to an AI based analysis of dendritic morphology. Ideally, all analysis would be performed by use of a trained AI to reduce observer bias of any kind. Expanding the time-points examined in studies 2 and 3 would have provided a greater understanding of the processes occurring in dendritic spine morphology. To add to these studies,

examining an earlier postnatal day such as P8, as well as a late gestational day such as G19 would have allowed us to examine changes in the cerebellum through its late development.

7.4 Conclusions

This dissertation examined the role of COX2/PGE2 signalling in neurodevelopment across both *in vitro* and *in vivo* models. We showed the importance COX2/PGE2 signalling in cytoskeletal dynamics and that disruptions in this pathway affected the normal development of the cerebellum and associated cerebellar motor behaviours *in vivo*. Further for many of the changes we observed, the effect of high or low PGE2 on the development of the cerebellum was sex dependent.

Examined together, our findings provide further evidence of the consequences of abnormal COX2/PGE2 signalling during prenatal development. Given that abnormal COX2/PGE2 signalling can arise from exposure to common environmental factors (chapter 1), continuing to understand the consequences of this signalling pathway on neuronal development is essential. We demonstrate the abundance of disruptions in cerebellar development that arise from high or low levels of PGE2 and show that these disruptions can affect cerebellar-motor function. The cerebellum is becoming increasingly known as an area of interest in the study of ASDs. The findings in this dissertation add further evidence of the COX2/PGE2 pathway as a candidate model for studying ASD. Future studies can further build on these findings in order to further examine the mechanisms of COX2/PGE2 signalling but also to characterize the full extent of the consequences that disruptions to this pathway have on neuronal development.

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Appendices

Appendix A: Additional Manuscript

Additional Manuscript: Maternal exposure to prostaglandin E2 alters cell density and migration in the brain of C57bl/6 mouse offspring and results in autism-like behaviours

Manuscript Citation (In preparation):

Kissoondoyal A, Wong C, Bestard-Lorigados I, Crawford DA. (*in preparation, 2021*) Maternal exposure to prostaglandin E2 alters cell density and migration in the brain of C57bl/6 mouse offspring and results in autism-like behaviours. *Submitted to Genes, Brain and Behaviour*

The dissertation was written in thesis format and the following is specific to the contributions in the chapter:

Contributions: Christine T. Wong designed and conducted experiments, collected samples. Ashby Kissoondoyal analyzed the data, created figures and tables, and wrote the manuscript Isabel Bestard-Lorigados performed selected gene expression experiments. Dorota A. Crawford supervised the design and coordination of the study and was involved with writing the manuscript.

A.1 Introduction

Prostaglandin E2 (PGE2), the major bioactive lipid mediator in the brain, plays an essential role in the development of the nervous system, including cell proliferation, synaptogenesis, and learning and memory (C. Chen & Bazan, 2005; Tassoni et al., 2008). PGE2 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 PGE2 by cyclooxygenase-1 or -2 (COX-1 or COX-2), enzymes constitutively expressed in the brain mainly in microglia or neuronal cells, respectively.

The PGE2 signaling pathway can be affected by genetic and environmental factors, including exposure to chemicals in pesticides, air pollution, consumer products, and drugs such as acetylsalicylic acid, acetaminophen, or misoprostol. These same factors have been associated with an increased risk for autism spectrum disorders (ASD)(Tamiji & Crawford, 2010b; C. Wong & D. A. Crawford, 2014; C. T. Wong et al., 2015). For instance, maternal exposure to misoprostol—a drug structurally analogous to PGE2 and misused to terminate pregnancy—has been linked to the neurodevelopmental conditions such as ASD and Moebius syndrome (Bandim et al., 2003; Bos-Thompson et al., 2008; Pastuszak et al., 1998). ASDs are a group of neurodevelopmental disorders characterized by behavioral difficulties involving social interaction, communication, and repetitive or restricted behaviors (Association, 2013). Current research has also established that atypical behaviors observed in individuals with ASD are associated with specific molecular and morphological changes in relevant brain regions. For example, structural abnormalities including reduced Purkinje cell numbers within the cerebellum, organizational abnormalities including impaired neuronal layering and minicolumns organization, and overall underconnectivity between neurons have been found in the post-mortem brains of autism patients (Abrams et al., 2013; Belmonte et al., 2004; Courchesne et al., 2019; Ecker et al., 2013; M. R. Herbert et al., 2003; Redcay & Courchesne, 2005; Stoner et al., 2014; Wegiel et al., 2010). Although behavioral phenotypes of ASDs generally become apparent during toddlerhood, the manifestation of ASD-related

brain pathologies are believed to result from abnormalities in prenatal biological processes, such as irregular proliferation and migration of neural cells (Courchesne et al., 2019).

Studies in our lab in cell lines and mice models already provided molecular and behavioural evidence for a connection between the abnormal COX2/PGE2 signalling and neuronal pathology. For example, exposure of various neuronal cell cultures to PGE2 resulted in increased calcium levels in the cytosol and growth cones, and affected neural stem cell proliferation, migration, and differentiation (Davidson et al., 2016; A. Kissoondoyal & D. A. Crawford, 2021; Tamiji & Crawford, 2010c; C. T. Wong et al., 2014; C. T. Wong et al., 2016). Increased PGE2 levels also altered the expression of autism-linked genes, including *Ptgs2*, *Mmp9*, *Ctnnb1*, and *Wnt3*, in neural stem cells and differentiating neurons(C. T. Wong et al., 2016). Moreover, maternal exposure to PGE2 during early pregnancy changed the expression of developmental genes associated with autism, including *Wnt2* and *Wnt3a*, in mice offspring across crucial prenatal developmental time-points (R. Rai-Bhogal, C. Wong, et al., 2018b). Similarly, whole genome microarray analysis in male mice lacking COX-2 (COX-$2^{-/-}$) found differential expression of numerous ASD-linked genes and defects in biological processes influencing neurodevelopment, including axonal pathfinding and synaptic function (R. Rai-Bhogal, E. Ahmad, et al., 2018b). Other groups have reported that an early postnatal increase or decrease in PGE2 levels resulted in irregular cerebellar development and decreased reciprocal social behaviour in male rats (S. L. Dean, Knutson, et al., 2012; Hoffman et al., 2016). Our recent behavioural study already revealed that mice offspring lacking expressed COX-2 (COX-2^{-}-KI) exhibit sex- and age-specific autism-related behaviours, including increased hyperactivity, anxiety, and repetitive behaviour, and defects in motor ability and social interaction (C. T. Wong et al., 2019).

In the current study, we examine the sex-dependent effect of a single maternal exposure to PGE2 (PGE2-exposed mice) at embryonic day 11 (E11) on gene expression, density, and migration of

cells in the brain and resulting autism-related behaviours. Density of cells originating from two separate cohorts (E11 and E16) was quantified at postnatal day 8 (P8) in the cerebral cortex and olfactory bulb, regions previously implicated in ASDs (Becker & Stoodley, 2013; Brang & Ramachandran, 2010; Courchesne et al., 2011; Courchesne et al., 2019; Dager et al., 2007; Raymond et al., 1996; Wegiel et al., 2010). We also examined the effect of PGE2 on neocortical cell migration. Further, we investigated autism-like behaviour in 4-6 weeks old offspring using the three-chamber sociability, marble burying, open field, and inverted screen tests. Finally, we analyzed the expression of autism-related developmental genes, including *Wnt2, Tcf4, Glo1, Spn* and *Actb*. This study shows for the first time that a single time point exposure to PGE2 during pregnancy significantly affects the expression of essential genes in offspring, alters density and migration of cells in the brain resulting in manifestations of sex-specific abnormal social, repetitive, anxiety-related, and hyperactive behaviours.

A.2 Materials and Methods

A.2.1 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. Behavioural testing was administered during the light phase. Mice were housed with siblings. 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 [23]. For gene quantification analysis and experiments involving immuno-stained tissues, brain samples were collected at postnatal day 8 (P8), where birth was considered postnatal day 0. P8 in mice is of particular interest since it is analogous to infanthood in humans (Auvin & Pressler, 2013; Bridgette D Semple, Klas Blomgren, Kayleen Gimlin, Donna M Ferriero, & Linda J Noble-Haeusslein, 2013), where symptoms of ASD 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.

A.2.2 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 gestation day 1 (G1) and females were then housed individually for the duration of their pregnancies. On G11, 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 (dmPGE2; Cayman Chemical) diluted in saline as used in previous studies (Ma, 2010; Okamoto, Saito, Tabata, & Uemoto, 2011; Ravneet Rai-Bhogal et al., 2018; Tessner, Muhale, Riehl, Anant, & Stenson, 2004). The metabolic rate of $dmPGE_2$ is slower and thus considered a stable analogue of PGE_2 (Tomochika Ohno, Hiroshi Ohtsuki, & Susumu Okabe, 1985; S Steffenrud, 1980). Control animals were given saline-only injections. Administration of dmPGE2 exposure was conducted on G11 since this time-point marks the onset of neurogenesis in embryonic mice (B. D. Semple et al., 2013). G11 also corresponds to the time that the drug misoprostol was taken in human cases resulting in Moebius syndrome and autism characteristics (Bandim et al., 2003; Pastuszak et al., 1998). Maternal exposure of dmPGE2 at G11 was also previously shown to result in altered expression of autism-linked genes during prenatal development (Ravneet Rai-Bhogal et al., 2018; C. T. Wong, I. Bestard-Lorigados, R. Rai-Bhogal, & D. A. Crawford, 2017). Herein, mouse offspring subjected to maternal exposure of dmPGE2 as described above are referred to as “PGE2-exposed mice”.

A.2.3 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 (Alex H. Tuttle et al., 2010). Through this technique, the later fate of a specific cohort of cells can be studied. 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 neurogenic interval extends from gestation day 11 (G11) through early G17 (Takahashi et al., 1995). For dual labelling of early- and late-born cohorts of neural cells, an injection of CldU at G11 and IdU at G16 were given, and animals were sacrificed at P8. For the PGE2-exposed group, pregnant mice were given a single co-injection of CldU and PGE2 at G11 at described concentrations.

A.2.4 Immunohistochemistry

Left hemisphere brain samples were carefully extracted and fixed in 4% paraformaldehyde (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 (Alex H. 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 then 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).

A.2.5 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 by the area of interest. Neocortical cell migration distances from the subplate to the labelled cells were measured in the cortex. The migration percentage was then calculated by taking the migration distance of each cell and dividing it by the total distance between the subplate and cortical plate. Image analysis was completed using NIS-Elements software (Nikon). All analyses were completed blind to the condition.

A.2.6 Three-Chamber Sociability Test

Sociability was evaluated using the three-chamber test as previously described (Oksana Kaidanovich-Beilin, Tatiana Lipina, Igor Vukobradovic, John Roder, & James R. Woodgett, 2011; Silverman, Yang, Lord, & Crawley, 2010; C. T. Wong et al., 2019). 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 begun 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 during the light-cycle.

A.2.7 Marble Burying Test

Anxiety and repetitive behaviour were investigated using the marble burying test as previously described (Angoa-Pérez, Kane, Briggs, Francescutti, & Kuhn, 2013; Deacon, 2006; C. Wong et al., 2017). 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 behaviours 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 during the light-cycle. 56 animals were recorded for analysis of digging time behaviour.

A.2.8 Open Field Test

Ambulatory activity and anxiety-like behaviour was determined using standard open field test methods as previously described (Seibenhener & Wooten, 2015; C. T. Wong et al., 2019). 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 cm2 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 during the light-cycle.

A.2.9 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 (R. Rai-Bhogal, C. Wong, et al., 2018b; C. T. Wong et al., 2019). 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 2). 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 A-1. qRT-PCR Primers

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
Spn	Forward	CAAGGACTACCAGCAAAAGGAGAT	24
	Reverse	CCTGGCTAGCTCCGACTCTTC	21
Actb	Forward	GCTTCTTTGCAGCTCCTTCGT	21
	Reverse	AGCGCAGCGATATCGTCAT	19
Ccnd1	Forward	GCACTTTCTTTCCAGAGTCATCAA	24
	Reverse	CTCCAGAAGGGCTTCAATCTGT	22

A.2.10 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. Statistical analyses were performed using the core open-source software R (Team, 2013). Two-way ANOVA followed by post-hoc Bonferroni comparisons were conducted to determine if there were significance differences between PGE2-exposed and wildtype (WT) control groups and between males and females. Data is presented as violin plots which show the probability distribution of the data as well as the mean ±SD for each condition. Significance was determined for p values less than 0.05.

A.3 Results

A.3.1 Olfactory Bulb Cell Density

The localization of CldU-labelled (E11) and IdU-labelled (E16) cells in the olfactory bulb at P8 shows a very distinct distribution pattern. Cells originating from E11 and E16 were counted separately per area at P8 (Fig.1A). Two-way ANOVA analysis on G11 cell density values (cells/mm2) comparing the main effects of condition and sex as well as the interaction between condition and sex was completed (Fig. 1B). While we did not find a significant effect of the interaction between our condition and sex ($F(1,14) = 4.061$, $p = 0.0634$) or our condition ($F(3,14) = 3.485$, $p = 0.0830$), we did see a significant effect of sex ($F(3,14) = 12.015$, $p = 0.0038$) on G11 cell density. Given the significance of the sex main effect and the trend towards significance in the condition main effect and the interaction between condition and sex we further investigated pairwise comparisons. PGE2-exposed males had significantly higher G11 cell density compared to WT males (Fig. 1B, p=0.0432; WT=1475.8, PGE2=3342.4). There was no statistical difference in densities between females (Fig. 1B, p=1.00; WT=725.1, PGE2=723.5). Sex differences were only seen between PGE2-exposed mice, where males had a greater cell density than females (Fig. 1B, p=0.0089; M=3342.4, F=723.5).

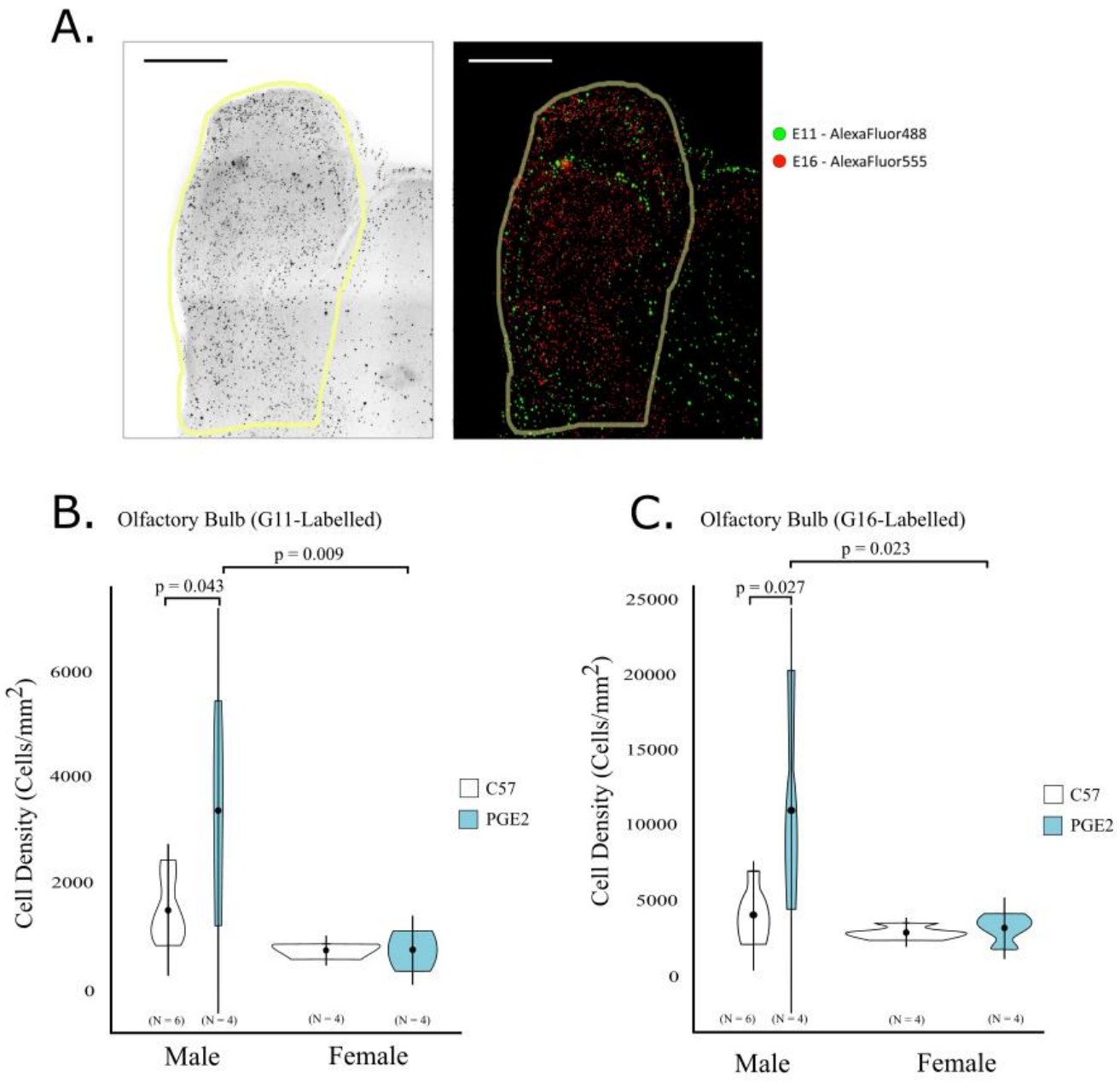

Figure A-1: G11 and G16 Cohort-labelled cell densities in the Olfactory Bulb. (A) Cell density of E11 and E16 labelled cell populations were counted in the olfactory bulb (B) Increased cell density of G11-labelled cells in the olfactory bulb of PGE2-exposed males. In the PGE2-exposed group, olfactory bulb G11 cell density was greater in males than females and greater than in control males. (C) Similarly, there was increased cell density of G16-labelled cells in the olfactory bulb of PGE2-exposed males. Olfactory bulb G16 cell density was greater in PGE2-exposed males than females. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SD, with significant differences shown above.

Two-way ANOVA analysis on G16 cell density values were conducted again comparing the main effects of condition and sex as well as the interaction between condition and sex (Fig. 1C). While we did not find a significant effect of the interaction between condition and sex ($F(3,14) = 4.377$, $p = 0.0551$), we found both main effects of condition ($F(3,14) = 4.921$, $p = 0.0436$) and sex ($F(3,14) = 6.871$, $p = 0.0201$) to be significant. Given the trend towards significance in the interaction between condition and sex and significant effects of both the condition and sex main effects, we examined pairwise comparisons. Similarly, to G11 cell density, PGE2-exposed males also had significantly higher G16 cell density compared to WT males (Fig. 1C, p=0.0272; WT=3630.6, PGE2=10963.6). There was no difference in G16 olfactory bulb cell densities between females (Fig. 1C, p=0.9994; WT=2887.5, PGE2=3169.2). Sex-dependent differences were only seen between PGE2-exposed mice, where males had a greater cell density than females (Fig. 1C, p=0.0235; M=10963.6, F=3169.2).

In short, PGE2-exposed males had greater olfactory bulb cell densities for G11 and G16 cohorts compared to WT controls. Olfactory bulb cell densities were not affected in PGE2-exposed females compared to female controls. Sex-dependent differences were seen in PGE2-exposed mice for G11 and G16 olfactory bulb cell densities; males had greater cell densities compared to females.

A.3.2 Neocortical Cell Density

Cells originating from G11 and G16 were also counted in all layers of the neocortex (Fig. 2A). Two-way ANOVA analysis on G11 cell density values (cells/mm2) examining, condition, sex and the interaction between condition and sex were performed (Fig. 2B). There was no significant interaction between condition and sex ($F(3,14) = 0.132$, $p = 0.7214$), or the condition main effect ($F(3,14) = 1.359$, $p = 0.2632$). However, we saw a significant effect of sex on G11 neocortical cell density ($F(3,14) = 18.627$, $p = 0.0007$). Given the significant effect of our sex condition, we further examined sex differences within our conditions. Sex differences were seen between WT mice, where males had lower neocortical cell densities than WT females (Fig. 2B, p=0.0315; M=227.9, F=378.9). PGE2-exposed

males also had lower cell densities than PGE2-exposed females (Fig. 2B, p=0.0448; M=238.8, F=417.8).

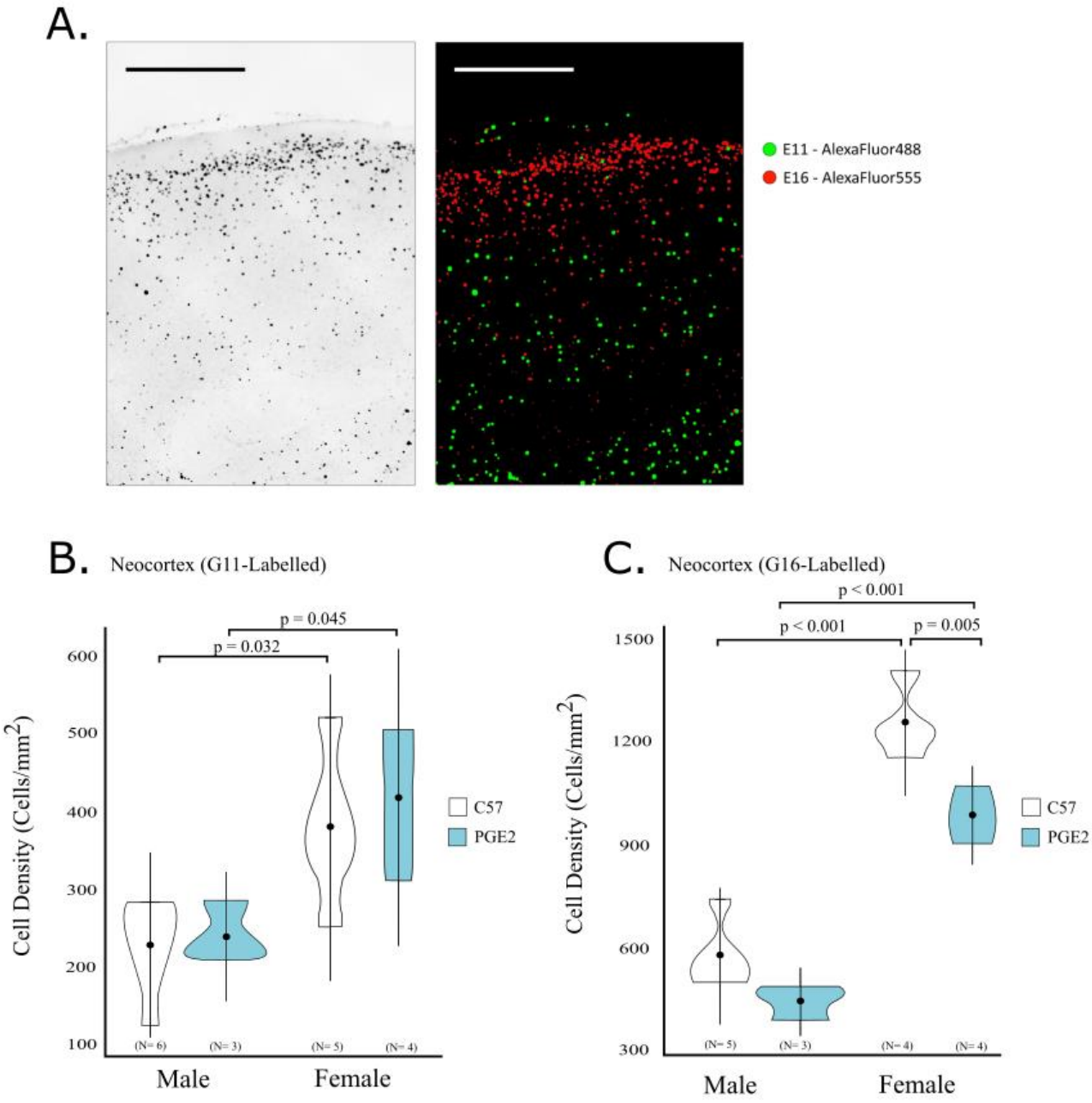

Figure A-2: G11 and G16 Cohort-labelled cell densities in the Neocortex. (A) Cell density of E11 and E16 labelled cell populations were counted in the neocortex (B) PGE2 exposure did not significantly affect cell density of G11-labelled cells in the neocortex. Within control and PGE2-exposed groups, females had a greater G11 cell density than males. (B) Decreased cell density of G16-labelled cells in the neocortex of PGE2-exposed females. Neocortical G16 cell densities were greater in females than

males. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SD, with significant differences shown above.

Two-way ANOVA analysis on neocortical G16 cell density values were also completed (Fig. 2C). Though we observed no significant interaction between condition and sex ($F(3,12) = 2.329$, $p = 0.1529$) we observed a significant effect of condition ($F(3,12) = 7.838$, $p = 0.0161$) and sex ($F(3,12) = 192.21$, $p < 0.0001$). There were also no significant differences in G16 neocortical cell densities between PGE2-exposed males compared to WT males (Fig. 1B, p=0.2238; WT=575.3, PGE2=442.5). In contrast, PGE2-exposed females had lower cell densities than WT females (Fig. 2C, p=0.0048; WT=1254.6, PGE2=984.3). Sex-dependent differences were observed in the WT and PGE2-exposed groups. WT males had a lower G16 neocortical cell density than WT females (Fig. 2C, $p < 0.0001$; M=575.3, F=1254.6). Similarly, PGE2-exposed males also had a lower cell density than PGE2-exposed females (Fig. 2C, $p< 0.0001$; M=442.5, F=984.3).

To summarize, PGE2-exposure did not change the neocortical cell densities for G11 and G16 cell cohorts in males. However, PGE2-exposed females had lower neocortical cell densities than WT controls for the G16 cell cohort. Sex differences were observed in neocortical cell densities, males had lower cell densities for G11, and G16-originating cells compared to females.

A.3.3 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 (Sidham 1973). The migration distances in the neocortex of P8 brain samples were measured from the subplate to the cells labelled at G11 (CldU-labelled) and G16 (IdU-labelled) (Fig.3A). Migration percentages were calculated for each labelled cell as described in the methods.

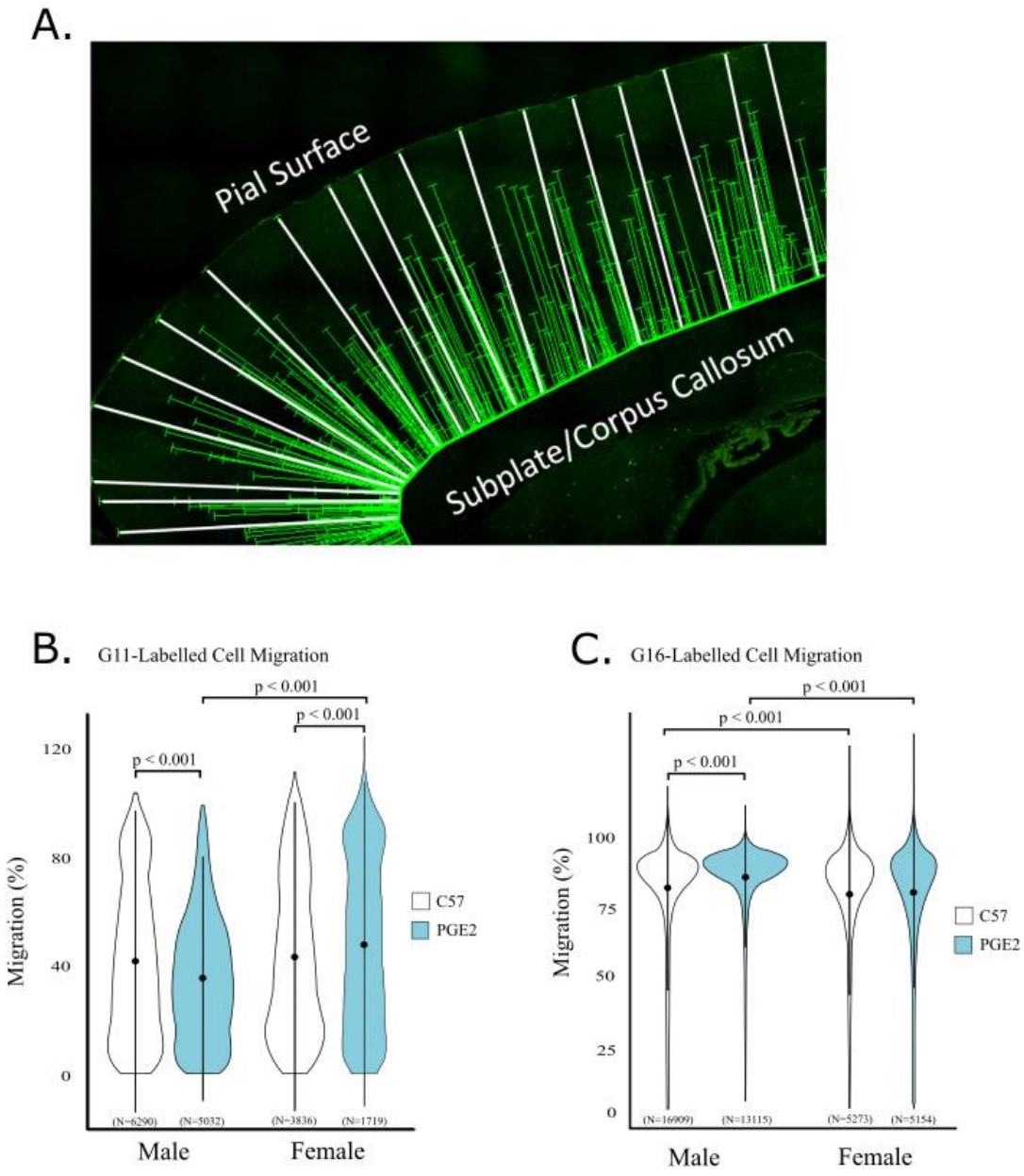

Figure A-3: G11 and G16 Cohort-labelled cell migration in the Neocortex. (A) Migration percentages were calculated by taking the migration distance of each cell and dividing it by the total distance between the subplate and cortical plate. E16 cells only shown here (B) Decreased neocortical cell migration of G11-labelled cells in PGE2-exposed males and increased migration in PGE2 exposed females. In the control and PGE2-exposed group, G11-labelled cell migration was greater in females than males. (C) Increased neocortical cell migration of G16-labelled cells in PGE2-exposed males compared to sex-matched controls. G16-labelled cells migrated further in males compared to females within control and PGE2-exposed groups. Means represent at least 3 independent animals for each experimental group. Data are presented as mean ±SD, with significant differences shown above.

Two-way ANOVA analysis was conducted on the migration percentages for G11-born cells of WT and PGE2-exposed males and females (Fig. 3B). We examined the effects of condition, sex and the interaction between condition and sex. We found a significant interaction between condition and sex ($F(3,6286)=58.322$, $p<0.0001$) and a significant effect of sex ($F(1,6286) = 94.130$, $p < 0.001$). However, we observed no significant effect of the condition main effect ($F(1,6286) =0.017$, $p = 0.895$). Given the significant interaction we further investigated pairwise comparisons. The migration percentage of G11 cells for PGE2-exposed males was significantly decreased from WT males (Fig. 3B, $p < 0.0001$; WT=36.9, PGE2=35.4). In contrast, the migration percentage of G11 cells for PGE2-exposed females was statistically greater than WT females (Fig. 3B, $p<0.0001$; WT=43.5, PGE2=48.2). Sex differences were not seen in WT animals (Fig. 3B, $p = 0.2716$; M=36.9±0.58, F=43.5±0.62) but were observed in PGE2-exposed animals. G11 cells of PGE2-exposed males migrated less than G11 cells of PGE2-exposed females (Fig. 3B, $p<0.0001$; M=35.4, F=48.2).

Two-way ANOVA analysis was also conducted on the migration percentages for G16-born cells of males and female (Fig. 3C). We again examined condition, sex and the interaction between condition and sex. We found a significant interaction between condition and sex ($F(3,16909)=39.92$, $p<0.0001$) as well as for the condition ($F(1,16909) = 50.67$, $p < 0.0001$), and sex ($F(1,16909) = 165.37$, $p < 0.0001$) main effects. Given the significance of our interaction and our main effects we further investigated pairwise comparisons. The migration percentage of G16 cells for PGE2-exposed males was significantly greater from WT males (Fig. 3C, $p<0.0001$; WT=81.4, PGE2=85.8). However, the migration percentage of G16 cells for PGE2-exposed females was not statistically different than WT females (Fig. 3C, $p=0.0573$; WT=79.3, PGE2=81.2). Sex differences were seen in both WT and PGE2-exposed animals. G16-labelled cells of WT males travelled a greater distance than those of WT females (Fig. 3C, $p<0.0001$; M=81.4, F=79.3). Similarly, G16 cells of PGE2-exposed males also migrated further than G16 cells of PGE2-exposed females (Fig. 3C, $p<0.0001$; M=85.8, F=81.2).

In summary, males exposed to PGE2 had decreased migration of G11 labelled cells and increased migration of G16-labelled cells. PGE2-exposed females had increased migration of only the G11-labelled cells. Sex differences were seen in G11-labelled and G16-labelled cells. While there was no significant difference in migration of G11-labelled cells in WT male and female mice, G11-labelled cells in PGE2-exposed females migrated further than in PGE2-exposed males. G11-labelled cells in females had greater migration percentages than G11-labelled cells in males for WT and PGE2-exposed groups. In contrast, G16-labelled cells in males had greater migration percentages than G16-labelled cells in females for both WT and PGE2-exposed groups.

A.3.4 Three-Chamber Sociability Test of Social Behaviour

Given the sex-dependent differences in density of cells originating from G11 and G16 in the neocortex and the olfactory bulb as well as the abnormal migration of these cells in the neocortex we also examined associated social, anxious, and repetitive behaviours linked to these brain regions (Takumi, Tamada, Hatanaka, Nakai, & Bolton, 2020; Varghese et al., 2017) First, the three-chamber sociability test was used to characterize changes in social behaviour (Fig. 4A, methods). The time spent in the novel object, centre, or novel mouse chamber was recorded and reported in seconds.

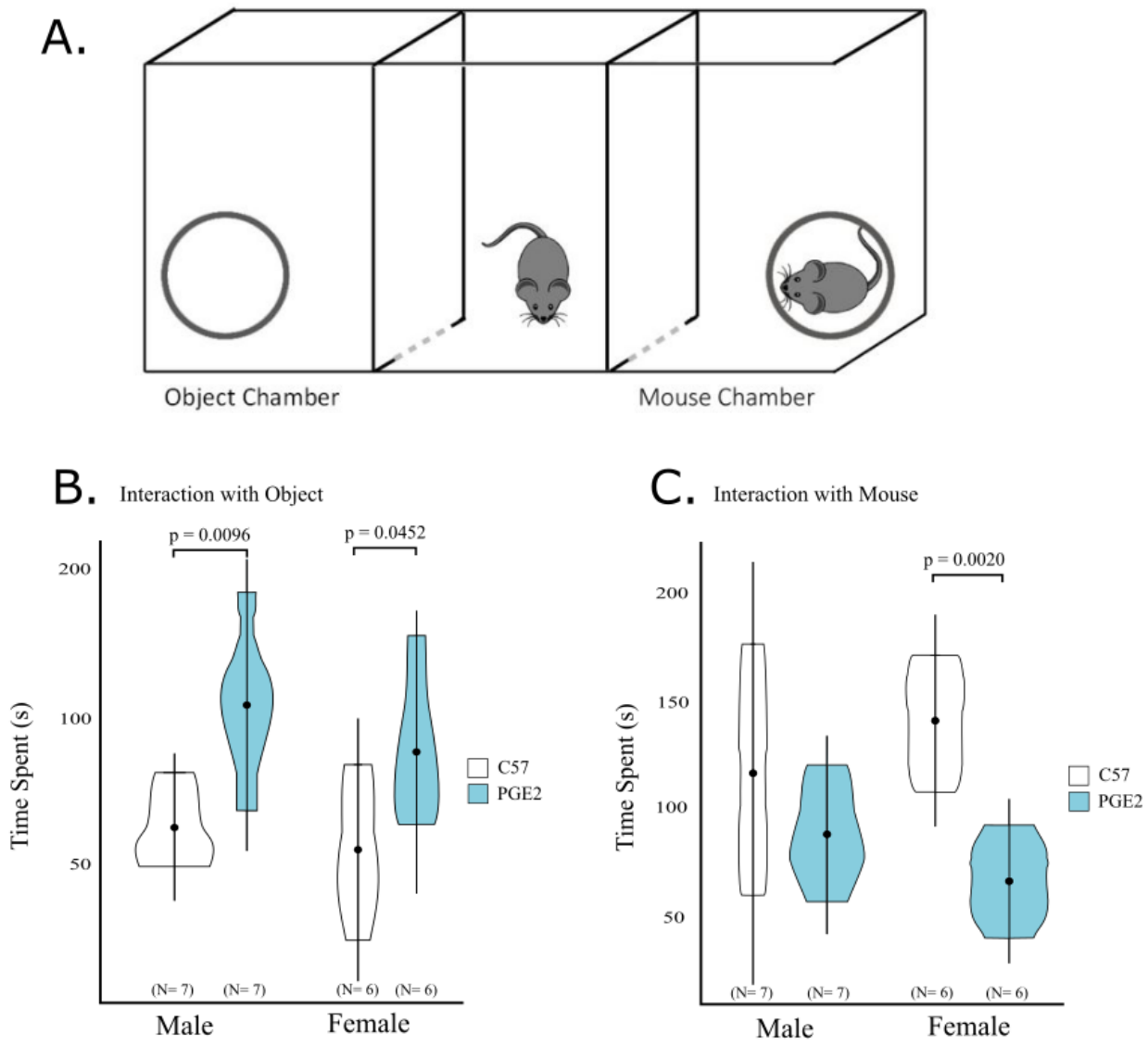

Figure A-4: Sociability behaviour was determined using the three-chamber test. (A) Video recordings were analyzed, and the time spent in the chambers or interacting with the novel object or novel mouse was measured. (B). PGE2-exposed males and females spent significantly more time interacting with the novel object than sex-matched controls. (C) PGE2-exposed females spent less time interacting with the novel mouse compared to WT females. N represents the number of animals tested in each experimental group, originating from at least 3 different litters. Data are presented as mean ±SD, with significant differences shown above.

The time spent interacting with the novel object or the novel mouse was 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. 4B). We examined condition, sex and the

interaction between condition and sex. While there was no significant effect of the interaction between condition and sex F(3,22)=0.243, p=0.3267), or the sex main effect (F(3,22) = 1.899, p =0.1821), the condition main effect was significant (F(3,22) = 20.133, p=0.0002). Given the significant condition effect we performed further pair-wise comparisons to examine specific condition comparisons. Pair-wise comparisons showed that PGE2-exposed males spent more time interacting with the novel object compared their respective control (p=0.0096; WT=60.1, PGE2=115.7). PGE2-exposed females also spent more time interacting with the novel object (p = 0.0453, WT = 53.7, PGE2 = 81.7).

Two-way ANOVA analysis on the time spent sniffing and touching the novel mouse was also conducted (Fig. 2C), again comparing condition, sex and the interaction between condition and sex. While there was no significant effect of the interaction (F(3,22) = 3.338, p = 0.0814) or the sex main effect (F(3,22) = 0.8043), we saw a significant effect of condition (F(3,22) = 16.726, p =0.0005), on the time spent interacting with the novel mouse. PGE2-exposed females spent less time interacting with the novel mouse than respective WT controls (p=0.0028; WT=140.1, PGE2=81.7), While there was no significant difference between PGE2-exposed males and WT Males, the trend follows the females (p=0.3991; WT=115.7, PGE2=87.2).

In summary, the three-chamber sociability test revealed that PGE2-exposed animals displayed abnormal social behaviour. When compared to WT controls, PGE2-exposed mice spent overall significantly more time interacting with (sniffing or touching) the novel object, while PGE2 females also spent significantly less time sniffing or touching the novel mouse.

A.3.5 Marble Burying Test of Anxiety-linked and Repetitive Behaviours

To assess repetitive and anxiety-like behaviour, the marble burying test was conducted (Fig. 5A). The number of marbles that were completely buried after the 30-minute trial was counted. The total time spent digging and grooming were also measured. Two-way ANOVA analysis was conducted

to compare the effect of condition, sex and the interaction between condition and sex on marble burying counts (Fig. 5B). While there was no significant effect of the interaction of condition and sex on marbles buried ($F(3,26) = 0.374$, $p = 0.546$), or on the sex ($F(3,26) = 2.628$, $p = 0.117$) main effect, we saw a significant effect of the condition main effect ($F(3,26) = 37.369$, $p < 0.001$). Given the significance of the condition main effect, further pair-wise comparisons were performed to examine specific condition differences. Significant difference in marbles buried between PGE2-exposed males and females and WT matched controls were observed. PGE2 males buried more marbles than WT males ($p<0.001$, WT = 1.63, PGE2 = 7.83), and PGE2-exposed females buried more marbles compared to matched WT controls ($p=0.003$; WT=0.60, PGE2=5.67).

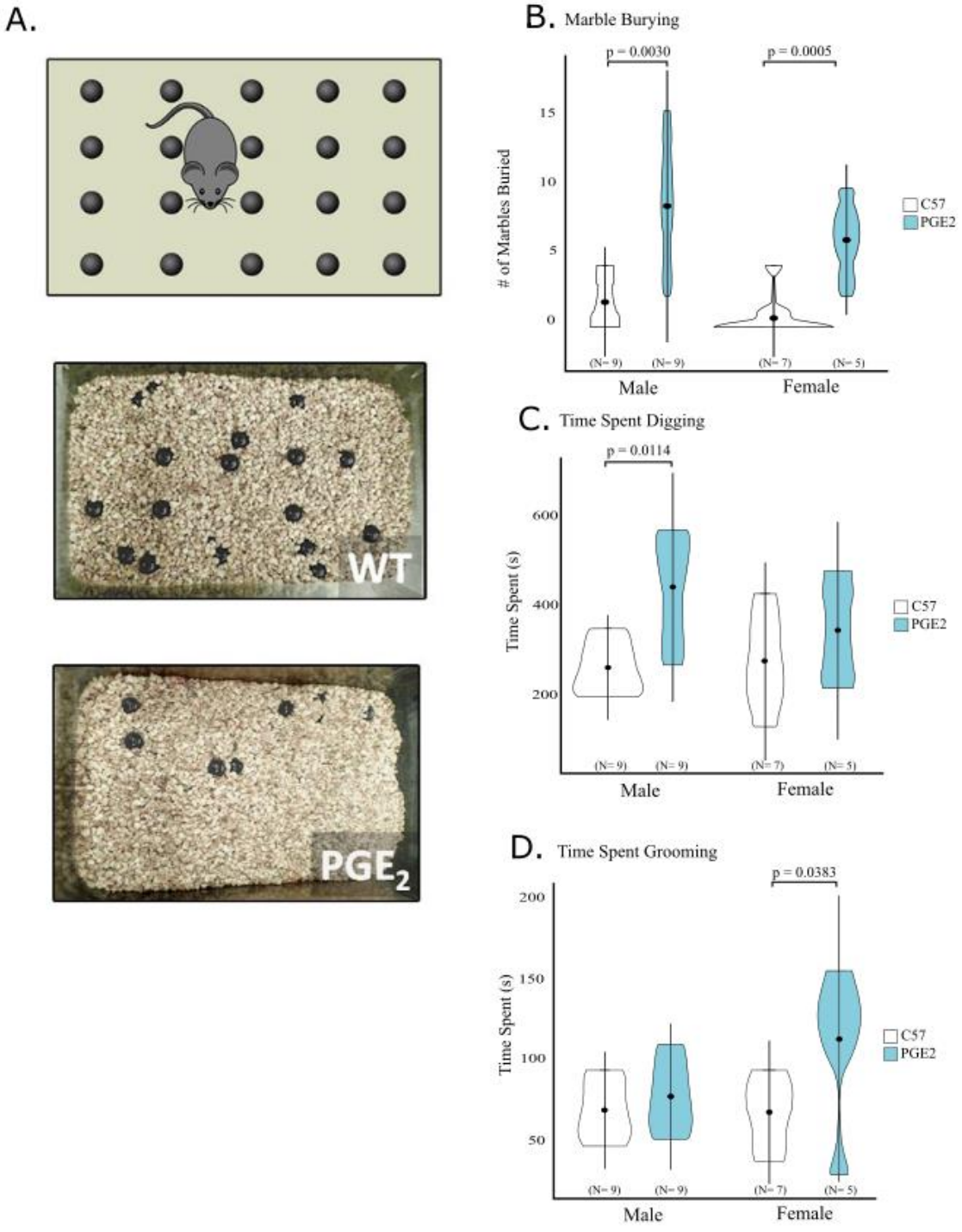

Figure A-5: Repetitive and anxiety-linked behaviour in the marble burying test. (A) Video recordings were taken, and total marbles buried were counted. (B) PGE2-exposed males and females buried more marbles than sex-matched WT controls. (C) PGE2-exposed males spent more time digging than controls. (D) PGE2-exposed females spent more time grooming than controls. N represents the number of animals tested in each experimental group, originating from at least 3 different litters. Data are presented as mean ±SD with significant differences for each shown above.

Two-way ANOVA analysis was also completed on digging behaviour again comparing the effects of condition, sex and the interaction between condition and sex (Fig. 5C). We saw no significant effect of the interaction between condition and sex ($F(3,24) = 1.930$, $p = 0.1775$), or the sex main effect ($F(3,24) = 0.919$, $p = 0.3474$), but saw a significant effect of condition ($F(3,24) = 10.676$, $p = 0.0033$). Given the significant condition effect we performed further pair-wise analysis between conditions. Statistically increased time of digging behaviour was seen PGE2-exposed males compared to WT controls ($p=0.0114$; WT=206.1, PGE2=390.3). No significant differences in digging time were seen between WT and PGE2-exposed females ($p=0.6708$; WT=220.8, PGE2=291.0), though a similar trend to males was observed.

Two-way ANOVA analysis was also completed on grooming behaviour again comparing the effects of condition, sex and the interaction between condition and sex (Fig. 5C). While we saw no significant effect of the interaction between condition and sex ($F(3,24) = 3.131$, $p = 0.0895$), or the sex main effect ($F(3,24) = 2.374$, $p = 0.1365$), but saw a significant effect of condition ($F(3,24) = 6.032$, $p = 0.0217$). Given the significant condition effect we performed further pair-wise analysis to examine specific differences in conditions. Statistically increased time of digging behaviour was seen PGE2-exposed females compared to WT controls ($p=0.0383$; WT=67.3, PGE2=112.7). No significant differences in digging time were seen between WT and PGE2-exposed males ($p=0.9275$; WT=68.7, PGE2=77.1).

To summarize, the marble burying test revealed that PGE2-exposed mice buried more marbles than WT controls. While PGE2-exposed males spent more time digging than WT controls, PGE2-exposed females spent more time grooming than WT controls.

A.3.6 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. 6A). Movement behaviour was assessed by measuring the total pathlength (cm) that was travelled (Fig. 6B), while anxiety-like behaviour was determined by quantifying the time spent in the centre of the apparatus (Fig. 6C).

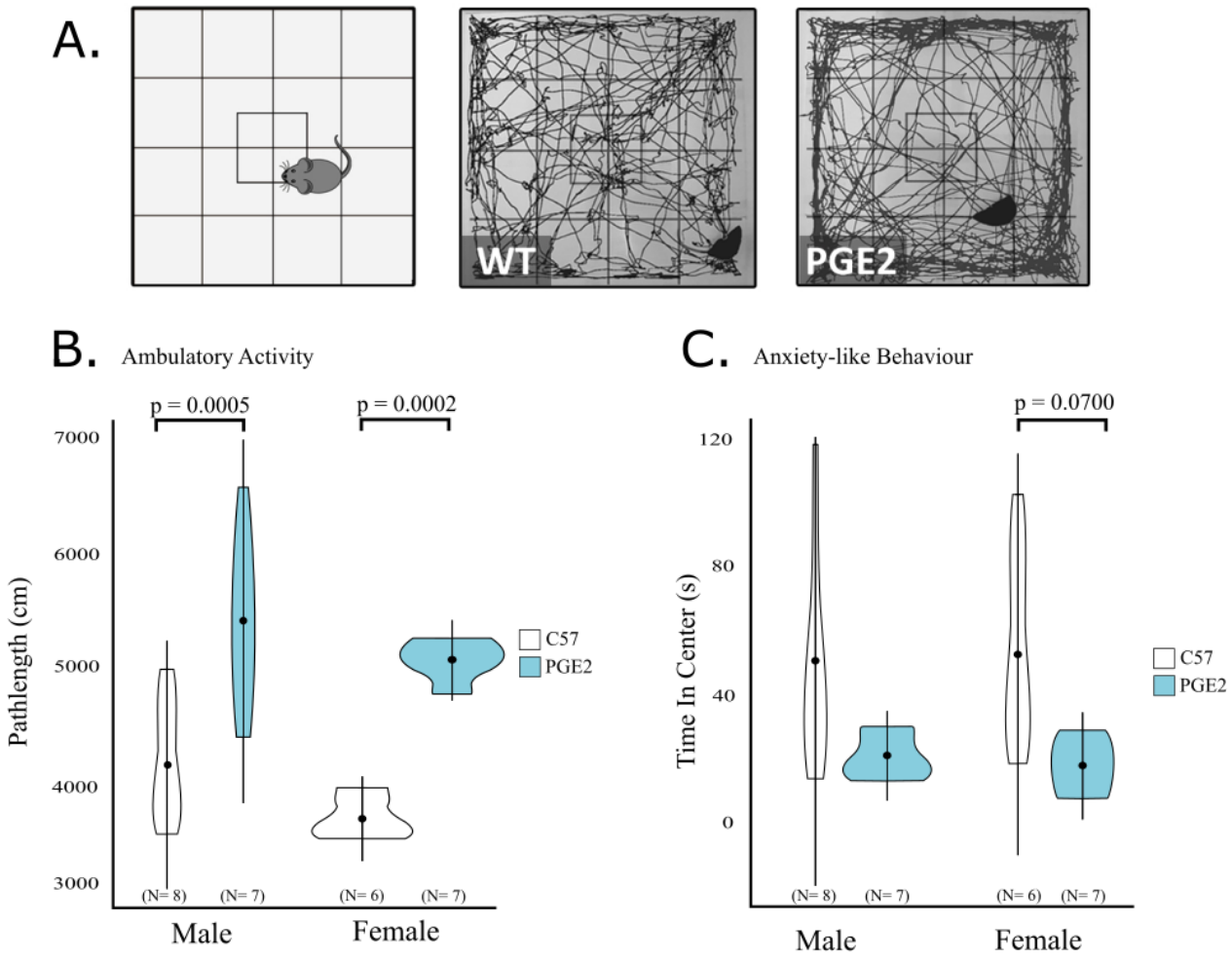

Figure A-6: Ambulatory activity and anxiety-linked behaviour in the open field test. (A) An automated tracking program determined the pathlength travelled. (B) PGE2-exposed male and female mice travelled further than sex-matched controls. (C) PGE2-exposed females spent less time in the center than WT-female controls. N represents the number of animals tested in each experimental group, originating from at least 3 different litters. Data are presented as mean ±SD with significant differences shown above.

The effect of condition, sex, and the interaction between condition and sex on pathlength values were analyzed using two-way ANOVA analysis (Fig. 6B). While we found no significant effects of the interaction between condition and sex ($F(3,23) = 0.109$, $p = 0.7439$), there were significant effects of the condition ($F(3,27) = 0.249$, $p = 0.622$)and sex ($F(3,27) = 0.622$) main effects. Given the significance of the condition and sex main effects we performed further pairwise analysis to examine specific comparisons. PGE2-exposed males and females travelled a greater distance than WT-males ($p=0.0005$; WT=4178.8, PGE2=5414.2) and females ($p=0.0002$; WT=3720.2, PGE2=5079.4) respectively. We saw no significant differences between WT males and females ($p=0.0383$; WT=4178.8, PGE2=3720.2) or PGE2-exposed males and females ($p=5414.2$; WT=67.3, PGE2=5079.4).

Two-way ANOVA analysis was conducted on the amount of time spent in the centre of the apparatus (s) to evaluate anxiety-like behaviour, again comparing condition, sex and the interaction between condition and sex (Fig. 3C). We again found no significant effect of the interaction between condition and sex ($F(3,24)=0.077$, $p=0.7833$), or the main effect of sex ($F(3,24) = 0.000$, $p = 0.9849$), but saw a significant effect of the condition main effect ($F(3, 24) = 11.370$, $p = 0.0025$) main effects. Given the significance of the condition main effect we performed further pairwise analysis on condition comparisons. PGE2-exposed females spent significantly less time in the center of the apparatus than WT-females ($p=0.0719$; WT=52.7, PGE2=17.9). While we saw no significant difference between PGE2-exposed and WT males ($p=0.1612$; WT=50.4, PGE2=20.9), the trend mirrored what was seen in females.

Overall, PGE2-exposed mice (both males and females) had increased pathlength, and PGE2-exposed females spent less time in the center of the open field test. These findings suggest there are increases in anxious behaviours in our PGE2-exposed mice.

A.3.7 Expression of developmental genes in PGE2-exposed mouse offspring

We previously showed that in PGE2-exposed NE-4C cells there was increased proliferation and decreases Wnt-dependent migration of neuronal stem cells(C. T. Wong et al., 2014). In this study, we examined sex specific differences in the expression of migration and proliferation genes *Spn, Actb,* and *Ccnd1* in PGE2-exposed offspring. Full brain samples from P8, the time analogous to infanthood in humans (Pressler & Auvin, 2013; B. D. Semple et al., 2013), were collected for analysis. Gene expression profiles were determined using quantitative real-time RT-PCR analysis in comparison to WT males (RQ=1) as a reference (methods). The expression of *Spn* was decreased in PGE2-exposed males ($t(4)=4.540$, $p=0.045$; RQ=0.487) and increased in females ($t(4)=-4.371$, $p=0.049$, RQ=1.525). The expression of *Actb* were not statistically different in PGE2-exposed males ($t(4)=1.721$, $p=0.227$; RQ=0.753) but increased in females ($t(4)=-5.389$, $p=0.033$, RQ=1.309). Lastly, the expression of *Ccnd1* was not statistically different in PGE2-exposed males ($t(4)=2.875$, $p=0.103$; RQ=0.725) or females ($t(4)=-2.179$, $p=0.161$; RQ=1.198). Overall, we found sex-dependent dysregulation of ASD gene expression in PGE2 exposed offspring.

Table A-2: Expression of Autism-linked genes in PGE2-exposed mice compared to WT controls

Gene expression analysis on autism-linked genes were completed on postnatal day 8 samples as shown as RQ units for male and female PGE2 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

PGE2-exposed compared to WT controls: Autism-Linked Genes					
Males			**Females**		
Gene	**RQ Mean Values**	***p*-values**	**Gene**	**RQ Mean Values**	***p*-values**
Spn	**0.487**	**0.045***	***Spn***	**1.526**	**0.049***
Actb	0.753	0.227	***Actb***	**1.309**	**0.033***
Ccnd1	0.725	0.103	***Ccnd1***	**1.198**	**0.161**
Wnt2	**0.8859**	**0.024***	***Wnt2***	**1.1072**	**0.33298**
Tcf4	**0.5781**	**0.009****	***Tcf4***	**1.3067**	**0.17462**
Glo1	0.8618	0.157	***Glo1***	**0.6259**	**0.00079*****
Grm5	0.8575	0.209	***Grm5***	**1.2689**	**0.17402**

Our previous *in vitro* studies in NE4C cells and *in vivo* studies in PGE2-exposed and COX2-KI mouse models collectively found differential expression of the genes: *Wnt2*, *Tcf4*, *Glo1*, and *Grm5* (R. Rai-Bhogal, E. Ahmad, et al., 2018b; R. Rai-Bhogal, C. Wong, et al., 2018a; C. T. Wong et al., 2014; C. T. Wong et al., 2019; C. T. Wong et al., 2016), which have all been previously associated with ASD(Abdallah et al., 2012; Junaid et al., 2004; Marui et al., 2010; Skafidas et al., 2014) . Here, we found that PGE2-exposed males had statistically decreased expression of *Wnt2* (t(4)=6.282, p=0.0244; RQ=0.8859) and *Tcf4* (t(4)=4.803, p=0.0086; RQ=0.5781) but no statistical differences were found in PGE2-exposed females for *Wnt2* (t(4)=-1.266, p=0.3330; RQ=1.1072) and *Tcf4* (t(4)=-2.067, p=0.17462; RQ=1.3067) (Table 2). The expression of *Glo1* was not statistically different in PGE2-

exposed males (t(4)=1.739, p=0.1570; RQ=0.8618) but it was decreased in females (t(4)=35.563, p=0.0008, RQ=0.6259). *Grm5* expression was not statistically different in either PGE2-exposed males (t(4)=1.831, p=0.20863; RQ=0.8575±0.078) or females (t(4)=I2.072, p=0.1740; RQ=1.2689)

In summary, PGE2 exposure decreased the expression of *Spn* in males but increased it in females. There was also a female specific increase in the expression of *Actb*. Lastly, we observed a male specific decrease in the expression of ASD-related genes *Wnt2,* and *Tcf4* and a female specific decrease in *Glo1* expression.

A.4 Discussion

Our study demonstrates that a single maternal injection of PGE2 during pregnancy can lead to cellular and molecular changes in the developing brain of mice offspring resulting in postnatal behavioural abnormalities. We report that in PGE2-exposed offspring cells originating at G11 or G16 displayed aberrant sex-dependent changes in density in ASD-associated brain regions including the olfactory bulb and the neocortex as well as abnormal neocortical migration (Table 3). In addition to cellular effects, we observed that PGE2-exposed male and female offspring exhibited postnatal behavioural alternations linked to ASD, including abnormal sociability, increased repetitive behaviours, and anxiety (Table 4), with distinct sex-specific differences. Finally, there were also changes in expression of cellular migration and proliferation genes (*Spn*, *Actb*) and ASD-related genes (*Wnt2*, *Tcf4*, *Glo1*, *Grm5*) in the offspring of PGE2-injected mice. Our findings add new and convincing evidence that maternal exposure to PGE2 may impact brain development and ultimately contribute to ASD-related symptoms in offspring. We propose that prenatally PGE2-exposed mice may serve as a novel experimental model system for studying a subset of ASDs.

A.4.1 PGE2-exposure affects migration and proliferation in the olfactory bulb and neocortex

ASDs are a group of neurodevelopmental disorders that is characterized by behavioral difficulties involving social interaction, communication, and repetitive or restricted behaviors. Over the years research has established that this atypical behavior reported in individuals with ASD results from various molecular and morphological changes in corresponding brain regions that occurred during critical times points in development (Bandim et al., 2003; M. T. Miller et al., 2004; Qasem, Al-Ayadhi, Bjørklund, Chirumbolo, & El-Ansary, 2018; Strömland et al., 2002). Although often overlooked, olfaction can affect one's social life, behaviours, emotions, memory, and thought (Boesveldt, Yee, McClintock, & Lundström, 2017; Kadohisa, 2013; Sarafoleanu, Mella, Georgescu, & Perederco, 2009). The olfactory bulb is a large structure in the mouse and is important for processing many social signals (D. Y. Lin, Zhang, Block, & Katz, 2005). In our study, we observed that PGE2-exposed males had greater density of G11 and G16 cell in the olfactory bulb compared to matched controls. In contrast, PGE2 exposure did not affect olfactory bulb cell densities in females. Perhaps abnormal cell densities in the olfactory bulb could be correlated to atypical olfactory processing present in cases of ASDs (Tonacci et al., 2017; Wicker, Monfardini, & Royet, 2016). Sex differences were specific to PGE2-exposed mice, with greater G11 and G16 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).

The neocortex, the largest part of the cerebral cortex, coordinates and processes sensory and motor information and is important in higher brain functions including cognition and language. In both the mouse and the human, processes of cell proliferation, specification, and migration lead to an 'inside-out' development of six neocortical layers, where post-mitotic neurons that arise earliest migrate to the deepest cortical layers and later-formed neurons move past them to progressively more

superficial layers (Sidman & Rakic, 1973). In this study, we report that PGE2 exposure in females were correlated with lower neocortical cell densities at G16. PGE2 exposure also resulted in decreased neocortical migration in males and increased migration in females at G11, while producing an increase in migration in males at G16 compared to matched controls. Sex differences were also observed: neocortical cell densities were greater in females compared to males in both control and PGE2-exposed groups. Interestingly, in PGE2-exposed mice, cells originating from G11 travelled a greater distance in females compared to males, while cells originating from G16 in both PGE2, and control groups travelled a greater distance in males compared to females. Abnormal cell proliferation and migration 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 & Marín, 2010). Several anatomical irregularities in the cortex have been reported in ASD such as increased neuron numbers, decreased interneuron numbers, disorganization of neurons and layering, disrupted minicolumn cell organization, and abnormal neuronal migration (Buxhoeveden et al., 2006; Casanova et al., 2002b; Courchesne et al., 2019; Hashemi et al., 2017; Stoner et al., 2014; Wegiel et al., 2010). Moreover, sex differences have also been described in the general human population, although findings are varied. For example, the cell density in cortical layer II and IV is higher in females than males (Witelson, Glezer, & Kigar, 1995), while other studies have found greater cortical neuronal numbers in males than females (de Courten-Myers, 1999; Rabinowicz et al., 2002). Similar to research in humans, our results also demonstrate abnormal migration and cell numbers in the cortex of PGE2-exposed mice as well as sex-dependent differences.

A.4.2 Gene expression in PGE2-exposed mice

In addition to atypical cellular densities and cortical migration, PGE_2-exposed mice also exhibited altered gene expression of spinophilin (*Spn*) and β-actin (*Actb*) in whole brain samples at P8. *Spn* expression was decreased in PGE_2-exposed males and increased in females compared to sex-matched

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. Spinophilin and β-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 (Feng et al., 2000; Sarrouilhe, di Tommaso, Metaye, & Ladeveze, 2006). Similarly, β-actin regulates cell growth and motility, wiring of neuronal circuitry through growth cone development, and synaptogenesis (Bassell et al., 1998; Cheever, Li, & Ervasti, 2012; Tondeleir, Noelanders, Bakkali, & Ampe, 2014). β-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, Swinnen, & Wenderoth, 2016; Mottron et al., 2015). We have previously observed an increase in total spinophilin and an increase in unbound ser94 phosphorylated spinophilin in neuronal stem cells treated with PGE2 during differentiation (A. Kissoondoyal & D. A. Crawford, 2021). We have also observed gene expression of actin and proteins that interact with actin in whole genome microarray analysis of COX-2 deficient COX-$2^{-/-}$ mice(R. Rai-Bhogal, E. Ahmad, et al., 2018b).

A.4.3 ASD-related behaviours in PGE2-exposed

Our molecular data described above was complemented by the autism-related behavioural tests. In the three-chamber sociability test, PGE2-exposed male and female mice spent more time in the novel object chamber than the novel mouse chamber, suggesting object preference over social preference. 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; F. Volkmar et al., 2014). Similar to decreased social interaction observed in the three-chamber test, clinical studies have reported that individuals with

ASD are less likely to engage in social interactions (Hiller, Young, & Weber, 2016; M. C. Lai et al., 2015). Although we saw a male specific increase in olfactory bulb E11 and E16 cell density in PGE2-exposed mice, we observed that both male and female PGE2-exposed mice interacted more with a novel object than WT controls. This could be due to the sex-specific role the olfactory bulb plays in smell recognition (B. C. Ryan, Young, Moy, & Crawley, 2008) and social interaction (Brunert & Rothermel, 2021; A. Li, Rao, Zhou, & Restrepo, 2020; Lizbinski & Dacks, 2017). Findings in other ASD mouse models have shown that olfaction is often impaired, resulting in decreased odor-evoked responses (Burket, Young, Green, Benson, & Deutsch, 2016; Cheaha, Bumrungsri, Chatpun, & Kumarnsit, 2015; M. Dean, Harwood, & Kasari, 2017; Geramita, Wen, Rannals, & Urban, 2020; Huang et al., 2019; M. C. Lai et al., 2011).

In the marble burying test, PGE2-exposed males and females mice buried a greater number of marbles than sex matched controls indicating increased anxious/repetitive behaviours. While PGE2 males spent more time digging during the test than sex matched controls, PGE2 females spent more time grooming than sex-matched controls, an indication of repetitive(Jahan et al., 2020; Kalueff et al., 2016) and anxious behaviour(Gandhi & Lee, 2021; Y.-E. Yoo et al., 2019). This is in line with many clinical studies showing that anxiety disorders are a common comorbidity of ASDs (Bartolotti, Sweeney, & Mosconi, 2020; van Steensel, Bögels, & Perrin, 2011; Wijnhoven, Creemers, Vermulst, & Granic, 2018). The open field test revealed that both male and female PGE2-exposed mice also displayed increased ambulatory activity, indicative of hyperactivity (Seibenhener & Wooten, 2015). This seems to parallel studies on attention deficit hyperactivity disorder (ADHD) and ASD, which have reported that ADHD-linked symptoms are often exhibited by individuals with ASD (Leitner, 2014; Sikora, Vora, Coury, & Rosenberg, 2012). Additionally, PGE2-exposed female mice also spent a decreased amount of time in the centre during the open field test suggestive of greater anxiety (Seibenhener & Wooten, 2015). The increases in anxious behaviour observed in the PGE2-exposed

females may be related to the effects we observed in neocortical cell density in which we saw a decrease in cell density specific to the PGE2-exposed female. Reductions in neocortical connectivity resulting from factors such as cell density, and reduced arborization are often correlated to increases in anxious behaviour ASD mouse models including VPA exposed mice (Kataoka et al., 2013; Varghese et al., 2017)

The abnormal behaviours observed in PGE2-exposed mice resemble those from our previous study in COX-2 deficient mice (C. T. Wong et al., 2019). The PGE2-exposed and COX-2 deficient experimental models both exhibited social defects, repetitive behaviours, hyperactivity, and anxiety-like behaviour. Social abnormalities and anxiety-like behaviours were more distinct in PGE2-exposed mice of this current study. Collectively, the abnormal autism-related behaviour observed in both mouse models provides additional evidence that elevated or reduced PGE2 levels lead to ASD-like pathologies, in line with the clinical studies(Tamiji & Crawford, 2010b; Wong & D. A. Crawford, 2014). Other studies have reported that postnatal changes in PGE2 levels also contribute to social irregularities in male rodents (S. L. Dean, Knutson, et al., 2012; Hoffman et al., 2016). The findings from our study and others are comparable to evidence from clinical studies, where increased PGE2 levels—for example, from exposure to maternal inflammation (Madore et al., 2016) or pesticides (De Felice, Greco, Calamandrei, & Minghetti, 2016; Holzman, 2014)—or diminished PGE2 levels—from acetaminophen use (Masarwa et al., 2018; Parker et al., 2017)—have been associated with increased risk for ASD. Similarly, clinical studies have reported that exposure to the drug misoprostol (structurally similar to PGE2) at the critical prenatal period in order to terminate the pregnancy resulted in Moebius syndrome and ASD (Bandim et al., 2003). Altogether, results from these studies strengthen the evidence that the COX-2/PGE2 signalling pathway is an autism candidate pathway.

In addition to investigating behaviours of PGE2-exposed mice, we also examined the expression of known autism-associated genes including *Wnt2*, *Tcf4*, *Glo1*, and *Grm5*. The expression

of three of these key developmental genes, *Wnt2*, *Tcf4*, and *Glo1*, was affected at P8 in PGE2-exposed mice, with different effects in males and females. We found decreased expression of *Wnt2* and *Tcf4* in PGE2-exposed males and decreased *Glo1* expression in PGE2-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 & Vleminckx, 2017) and has been associated with ASD (Kalkman, 2012). *Tcf4* (transcription factor 4) is a key protein initiated through canonical Wnt signalling (Cadigan & Waterman, 2012) and has also been linked to ASD (Kwan, Unda, & Singh, 2016). 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). Decreased *Wnt2* and *Tcf4* levels measured in PGE2-exposed males may contribute to abnormal sociability and repetitive behaviours that were also prominent in this group. Moreover, these results provide further evidence for an interaction between the PGE2 and Wnt signalling pathways in the brain, which has been previously shown in our lab in cell and animal model systems, including PGE2-exposed and COX-2 deficient mice (R. Rai-Bhogal, C. Wong, et al., 2018b; C. T. Wong et al., 2014; C. T. Wong et al., 2016).

A.4.4 Conclusions

This study complements our previous *in vitro* data showing that PGE2 affects neuronal proliferation, migration, and differentiation(Davidson et al., 2016; A. Kissoondoyal & D. A. Crawford, 2021; Tamiji & Crawford, 2010c; C. T. Wong et al., 2014; C. T. Wong et al., 2016). Here, we provide the first *in vivo* evidence that PGE2 can also alter expression of essential genes, cell density of two cohorts of migrating cells in the developing brain and results in manifestation of autism behaviour postnatally. In conclusion, our results demonstrate that a single dose of maternal PGE2 exposure can 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 olfactory bulb, and neocortex.

Moreover, prenatally PGE2-exposed mice also exhibited abnormal sociability, repetitive behaviour, anxiety, and hyperactivity, as well as aberrant expression of developmental and ASD-linked genes in a sex-dependent manner. Our study highlights that the maternal environment or prenatal exposure to risk factors that affect PGE2 levels during critical stages of development may contribute to ASD-related pathology. Finally, we emphasize that it is imperative that molecular and behavioural research be conducted in both males and females especially related to research on neurodevelopmental disorders, which is currently underrepresented in the literature.

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Appendix B: Publication List and Conference Presentations

PEER-REVIEWED ARTICLES

Articles Submitted or in Preparation:

Kissoondoyal A, Ho K, Hussain S, Crawford DA. (*in preparation 2021*) Maternal exposure to Prostaglandin E2 affects dendritic morphology in cerebellum and related motor coordination in offspring.

Kissoondoyal A, Rai-Bhogal R, Crawford DA. (*in preparation 2021*) Abnormal dendritic morphology in the cerebellum of Cyclooxygenase-2- Knockin mice. *Submitted to European Journal of Neuroscience*

Kissoondoyal A, Wong C, Bestard-Lorigados I, Crawford DA. (*in preparation 2021*) Maternal exposure to prostaglandin E2 alters cell density and migration in the brain of C57bl/6 mouse offspring and results in autism-like behaviours. *Submitted to Genes, Brain and Behaviour*

Peer-Reviewed Research Articles:

Kissoondoyal A, Crawford DA. (2021) Prostaglandin E2 Increases Neurite Length and the Formation of Axonal Loops, and Regulates Cone Turning in Differentiating NE4C Cells Via PKA. *Cell Mol Neurobiol.*

Rai-Bhogal R, Wong C, **Kissoondoyal A**, Davidson J, Li H, Crawford DA. (2018) Maternal exposure to prostaglandin E2 modifies expression of Wnt genes in mouse brain - An autism connection. *Biochem Biophys Rep.*

PUBLISHED ABSTRACTS AND CONFERENCE PRESENTATIONS

Kissoondoyal A, Ho K, Hussain S, Crawford DA. (2021) Maternal exposure to Prostaglandin E2 affects dendritic morphology in cerebellum and related motor coordination in offspring. *Canadian Association of Neuroscience (2021)*

Kissoondoyal A, Rai-Bhogal R, Crawford DA. (2020) A Quantification of Dendritic and dendritic spine morphology in Cyclooxygenase - 2 – Knockin Mice. *Neuromatch Conference*

Iyer S, **Kissoondoyal A** and Crawford DA. The Role of Lipids in Neuronal Plasticity - Link to Autism Spectrum Disorders. 8th Annual Multidisciplinary Research Fair. York University. 2020

Kissoondoyal A., Crawford D.A. Prostaglandin E2 regulates phosphorylation of spinophilin and growth cone morphology via PKA. Poster 117.06 / C8, Society for Neuroscience, Washington DC, USA. 11/2017

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Manuscript 1 Citation:
Kissoondoyal A, Crawford DA. (2021) Prostaglandin E2 Increases Neurite Length and the Formation of Axonal Loops, and Regulates Cone Turning in Differentiating NE4C Cells Via PKA. *Cell Mol Neurobiol.*

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