DIFFERENTIAL GENE EXPRESSION IN THE PRENATAL BRAIN OF CYCLOOXYGENASE-1
AND 2 KNOCKOUT MALE MICE - A MODEL SYSTEM OF AUTISM SPECTRUM DISORDERS

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ABSTRACT

Prostaglandin E_2 (PGE$_2$) is an endogenous lipid-derived signalling molecule, synthesized in part by the rate-limiting enzymes Cyclooxygenase-1 and 2 (COX-1 and 2). Along with inducing inflammation, PGE$_2$ is important for brain development, neuronal transmission, plasticity and modulation of an important developmental pathway known as the Wnt pathway. Due to its role in brain development, any genetic or environmental factors interfering with PGE$_2$ levels may result in abnormal brain development and Autism Spectrum Disorders (ASDs). To explore the role of PGE$_2$ in the developing brain we examine changes in the expression of genes and their associated pathways using RNA microarray analysis of mice brains lacking PGE$_2$ producing enzymes COX-1 and 2 at embryonic days 16 and 19. We also aim to determine whether there is an interaction between PGE$_2$ and Wnt pathways in the brain of COX$^{-/-}$ mice by western blot analysis of β-catenin, a key signal transducer in the Wnt pathway. We also aim to quantify the expression of selected developmental and ASD candidate genes in our cellular models exposed to a higher level of PGE$_2$. Overall, we found a greater number of differentially expressed genes, of which more were down-regulation than up-regulation, in the E16 COX$^{-/-}$ mice than E19 mice. In addition, several important developmental and ASD candidate genes along with their corresponding neuronal pathways, including synaptic transmission and Wnt signalling pathways, were affected, particularly in the E16 COX-2$^{-/-}$ mice. We also found greater Protein Kinase A (PKA) induced β-catenin activation in the COX-2$^{-/-}$ E16 mice. PGE$_2$ exposed neuroectodermal (NE-4C) stem cells and neuronal cells indicated changes in the expression of genes that were also differentially expressed in COX$^{-/-}$ microarray results. This research provides an in-depth look at the role of COX/PGE$_2$ during prenatal brain development and the implications for the COX$^{-/-}$ mouse to serve a potential ASD model system.

ACKNOWLEDGMENTS

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ABBREVIATIONS

AA, Arachidonic acid
ACC, Animal Care Committee
ACTβ, β-actin
AGE, Advanced glycation end-products
ANOVA, Analysis of Variance
APC, Adenomatous polyposis coli
ASD, Autism Spectrum Disorders
ATCC, American Tissue Culture Collection
AutDB, Autism Database
β-ME, β-mercaptoethanol
BLAT, BLAST-like alignment tool
Bp, Basepairs
CAM, Cell Adhesion Molecule
CAMK4, Calcium/calmodulin dependent protein kinase IV
CaN, Calcineurin
CCND, Cyclin D
CDH, Cadherin
cDNA, Complementary DNA
DEPC, Diethyl pyrocarbonate
CK1α, Casein kinase 1, alpha
CK1ε, Casein kinase 1, epsilon
CNTN2, Contactin 2
CNTNAP, Contactin associated protein
CNV, Copy number variation
COX-1, Cyclooxygenase-1
COX-2, Cyclooxygenase-2
CREB, C-AMP response element-binding protein
CT, Threshold Cycle
CTNNB1, β-catenin gene
DAAM1, Disheveled-associated activator of morphogenesis 1
DHA, Docosahexaenoic acid
DNA, Deoxynucleic acid
DNase, Deoxyribonuclease
dNTP, Deoxynucleotide triphosphates
dsDNA, Double-stranded DNA
Dvl, Dishevelled
E, Embryonic day
ECL, Enhanced chemiluminescence
EDTA, Ethylenediaminetetraacetic acid
EP, E-Prostanoid
ESC, Embryonic stem cells
FA, Fatty acid
FC, Fold Change
FDR, False discovery rate
FMR1, Fragile X metal retardation 1

FZD, Frizzled
GABA, gamma-aminobutyric acid
GAPDH, Glyceraldehyde 3-phosphate dehydrogenase
gDNA, genomic DNA
GLO1, Glyoxylase 1
GO:BP, Go ontology: Biological Processes
GPCR, G-protein-coupled receptors
GRM5, Glutamate receptor, metabotropic 5
GSK-3B, Glycogen synthase kinase 3β
GWAS, Genome-Wide Association Studies
HPRT, Hypoxanthine phosphoribosyl transferase
HSC, Hematopoietic stem cells
KEGG, Kyoto encyclopedia of genes and genomes
KIF1B, kinesin family member 1 beta
LRP, Lipoprotein receptor-related protein
LTP, Long-term potentiation
MECP2, Methyl-CpG binding protein 2
MEEBO, Mouse exonic evidence based oligonucleotide
MEM, Minimum essential medium
MG, Methylglyoxal
mGluR5, metabotropic glutamate receptor 5
MMP9, matrix metalloproteinase 9
MRI, Magnetic resonance imaging
mRNA, messenger ribonucleic acid
mTOR, mechanistic target of rapamycin
MYT1L, Myelin transcription factor 1 like
N, Total number
NBM, Neurobasal Media
NCBI, National center for biotechnology information
NE-4C, Neuroectodermal stem cells
NGS, Normal Goat Serum
NLGN, Neuroligin
NRXN, Neurexin
NSAIDs, Non-steroidal anti-inflammatory drugs
NT, Neurotransmitter
OGS, Official gene symbol
PBS, Phosphate buffered saline
PCDH, protocadherin
PCP, Planer cell polarity
PCR, Polymerase chain reaction
PGE, Prostaglandin type e
PGE2, Prostaglandin E2
PGES, Prostaglandin E synthase
PGH2, Prostaglandin H2
PGK1, Phosphoglycerate kinase 1
PI-3K, Phosphoinositide 3-kinase

PKA, Protein kinase A
PKB, Protein kinase B
PLA2, Phospholipase A2
PLC, Phospholipase C
PPP3CA, Protein phosphatase 3 catalytic subunit alpha
PTGS, Prostaglandin-Endoperoxide Synthase gene
PTM, Post translational modification
PUFAs, Polyunsaturated fatty acids
qRT-PCR, Quantitative real-time polymerase chain reaction
QC, Quality control
RNA, Ribonucleic acid
RNases, Ribonucleases
RQ, Relative quantity
S, Serine
SCN1A, Sodium voltage-gated channel alpha subunit 1
SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEMA5A, Semaphorin 5A
SFRP, Secreted frizzled-related protein
SHANK, SH3 and multiple ankyrin repeat domains protein
SNP, Single nucleotide polymorphism
SOX11, SRY-related HMG box 11
SRY, Sex determining region of Y chromosome
T, Threonine
TAE, Tris-acetate-EDTA
TBS-T, Tris-Buffered Saline and Tween 20
TCF/LEF, T-cell factor/lymphoid enhancer-binding factor
TF, Transcription factor
TSC, Tuberous sclerosis complex
TSC1/2, Tuberous sclerosis 1/2
VPA, Valproic acid
µM, Micromolar
WB, Western blot
Wnt, Wingless-type MMTV integration site family
WT, Wild-type

1.0. INTRODUCTION

1.1. Autism Spectrum Disorders

Autism spectrum disorders (ASDs) are a highly complex set of neurodevelopmental disorders that manifest around the age of three and remain throughout an individual's lifetime (Tamiji and Crawford, 2010b). ASDs are considered a spectrum of disorders consisting of sub-categories in accordance with severity and types of symptoms. The most commonly cited behavioural characteristics for ASD include deficits in qualitative social interaction, impairments in language development and repetitive or restricted behaviour (Watts, 2008, Cao et al., 2012). Epidemiological data from 2010 suggests ASD prevalence is at an alarming one in 68 children of age 8 which is a 29% increase from previous estimates (Wingate et al., 2014). In addition, the 4-5 times greater prevalence of ASD in boys than girls is still not well understood or explained (Fombonne, 2003, Wingate et al., 2014). Advancing ASD research to understand etiology, potential risks and modes of treatment has become important as these disorders impact an increasing number of individuals and families. Clinical observations of physiological and neurological characteristics of the brain of individuals with ASDs have been an important foray for understanding ASD etiology.

Neuroanatomical studies have found larger brain size, or macrocephaly, early in postnatal life of children and adults with ASD (Courchesne and Pierce, 2005). More specifically, while being born with normal head circumference, larger brain volume followed by slower brain growth has been a predictor of later diagnosis of ASD in boys (Courchesne et al., 2001). Interestingly, the growth of the head is proportional to the severity of clinical symptoms in children with ASD (Courchesne et al., 2004). In addition, volumetric abnormalities have been observed in the amygdala, hippocampus, cerebral cortex, cerebellum and white matter (Courchesne et al., 2001, Sparks et al., 2002). One study has found that the size of the amygdala

in children becomes normal in adolescents, whereas the hippocampus remains enlarged throughout the life of individuals with ASD (Schumann et al., 2004). Furthermore, another study suggests that the frontal and temporal lobe may be affected more by the overgrowth than other regions of the brain (Carper et al., 2002). These changes have served as early warning signs before behavioural symptoms can confirm a diagnosis of ASD, allowing for early behavioural intervention. These structural changes can be helpful in predicting the behavioural symptoms corresponding to the abnormally sized brain regions (Ecker et al., 2013). However, it is important to examine neuronal function and the prenatal conditions in which these changes may arise.

Dysfunction of synaptic signalling has been a key interest in ASD pathophysiology and a revealing phenotype of these disorders (Ecker et al., 2013). An imbalance between excitatory and inhibitory neural signals has become one of the most cited characteristics of ASD pathophysiology (Rubenstein and Merzenich, 2003, Ecker et al., 2013). Excitation and inhibition of neurons is governed by the action of neurotransmitters (NTs) γ-aminobutyric acid (GABA) and glutamate, respectively, and their corresponding receptors (Lee et al., 2016). In addition, brain imaging techniques have also revealed imbalances in the activity of serotonin, another NT, which governs mood and other complex behaviour (Ecker et al., 2013). In addition to the imbalance of signals, the synaptic function through which these signals are received has also been found to be altered (Ecker et al., 2013). Overall, these findings result in abnormal neural systems/circuits which makes up the connectome, a term for the neuronal pathways connecting the entire brain (Ecker et al., 2013). This has prompted the need for the characterization of the connectome in individuals with ASD using diffusion tensor imaging which provides an image of white matter tracts (Mevel et al., 2015). The study of structural and physiological phenotypes

have led to the identification of similarities between ASD and potential animal models for ASD (Lee et al., 2016). The continued effort to understand ASDs has indicated a complex interplay of several genetic and environmental processes resulting in the heterogeneous characteristics of ASDs (Zhang et al., 2009, Betancur, 2011, Ratajczak, 2011).

1.2. ASD etiologies

Several mechanisms underlying the etiology of ASDs have been described; one of the most studied is genetic mutations (Lichtenstein et al., 2010). Early epidemiological family studies indicated a high concordance rate in monozygotic twins, demonstrating an underlying genetic cause of Autism (Folstein and Rosen-Sheidley, 2001, Guo et al., 2011). These studies, however, have been criticized for being limited in scope and sample size (Mevel et al., 2015). Several genetic mutations including single nucleotide polymorphisms (SNPs), and rare inherited or *de novo* Copy-Number Variations (CNVs), which are large insertions or deletions, have been associated with ASD (Guo et al., 2011). Individuals with known SNPs or CNVs often display syndromic ASD which results in co-morbidities such as intellectual disability, epilepsy and schizophrenia, while genetically idiopathic ASDs are less severe and are non-syndromic (Basu et al., 2009, Betancur, 2011, Guo et al., 2011). Interestingly, SNPs and CNVs have been estimated to account for only 10-30% of all ASD cases, while the remaining are idiopathic or non-syndromic (Guo et al., 2011, Kelleher et al., 2012, Szczaluba, 2014). Despite the limited ability for DNA sequence analysis to explain all ASD cases, these studies have identified a staggering number of ASD risk or associated genes.

Genome-Wide Association Studies (GWAS), which compare polymorphisms and other mutations between two groups, have identified several cell adhesion molecules (CAMs) as ASD

susceptible genes. CAMs are cell surface proteins that bind to CAMs of neighboring cells or the extracellular matrix. The CAM proteins implicated in ASD primarily belong to the superfamily of cadherins (CDHs) and a subfamily known as protocadherins (PCDHs) (Guo et al., 2011, Redies et al., 2012, Talkowski et al., 2014). In particular, studies have identified genetic variants in a region containing the *CDH8*, *9* and *10* genes in ASDs (Wang et al., 2009, Pagnamenta et al., 2011). These CDHs have been associated with several neuropsychiatric disorders and are important for neural tube development, neuronal migration, synaptic formation and synaptic modulation (Redies et al., 2012).

An additional set of synaptic CAMs and scaffolding proteins have also been implicated in ASD pathophysiology (Bourgeron, 2009, Ecker et al., 2013, Gong and Wang, 2015). These proteins belong to three families; Neurexins (NRXNs), Neuroligins (NLGNs) and SH3 and multiple ankyrin repeat domain proteins (SHANKs), making up the NRXN-NLGN-SHANK pathway. More specifically, the ASD associated genes primarily include *NRXN1*, *2* and *3* along with another NRXN known as *contactin associated protein-like 2* (*CNTNAP2*) (Autism Genome Project et al., 2007, Gauthier et al., 2011, Vaags et al., 2012); *NLGN3* and *4* (Jamain et al., 2003); and *SHANK1*, *2*, and *3* (Moessner et al., 2007, Berkel et al., 2010, Sato et al., 2012). Together, these groups of proteins make up the protein complexes that ligate pre- and postsynaptic membranes (Bourgeron, 2009, Gong and Wang, 2015). NRXNs and NLGNs serve as adhesion molecules and localize in the pre- and postsynaptic membrane, respectively, to form the synaptic connection (Scheiffele et al., 2000, Autism Genome Project et al., 2007). Interestingly, the specific subtypes of NLGNs and NRXNs involved in the formation of the synapse can determine whether it will be excitatory or inhibitory (Dalva et al., 2007). Furthermore, mutations of SHANK3, a key scaffolding protein in the postsynaptic glutamatergic

signalling complex, have also been association with ASD (Durand et al., 2007). Furthermore, *SHANK3* null mice not only display repetitive behaviour and deficits in social interaction, but also reduced glutamatergic (excitatory) synaptic transmission and long-term potentiation which are key processes for learning and memory (Peca et al., 2011, Yang et al., 2012). The formation and proper distribution of excitatory vs inhibitory synapses is a key event during brain development and strongly associated with ASD pathophysiology (Autism Genome Project et al., 2007).

Several other genes that have been associated with ASD can involve specific co-morbidities, such as *Tuberous Sclerosis 1* and *2* (*TSC1* and *2*; tuberous sclerosis), *Fragile X Mental Retardation 1* (*FMR1*; intellectual disability), *Methyl CpG binding Protein 2* (*MECP2*; Rett's syndrome), and other genes resulting in epilepsy, neurofibromatosis and macrocephaly (Bourgeron, 2009, Ey et al., 2011). In addition, *SEMA5A* which encodes for Semaphorin 5A, a protein involved in axon guidance, has also been implicated in a GWAS (Weiss et al., 2009). While conservative estimates have listed 103 genes, up to 845 ASD susceptible genes have been identified along with 44 genomic loci associated with non-idiopathic ASDs (Basu et al., 2009, Betancur, 2011). For idiopathic ASDs, the role of the environment during early development has become particularly important due to the interaction between genes and the environment (Guo et al., 2011). Recent evidence suggests an etiological role of altered epigenetics as a result of maternal immune response and environmental exposure to pollutants in ASDs (Keil and Lein, 2016, Nardone and Elliott, 2016). Thus, it is important to understand the interaction between environmental risk factors and gene expression which can have an impact on brain development and ASD pathogenesis.

1.3. Environmental insults during brain development

During pre-and postnatal neural development, many widespread changes involving cellular organization, neuronal wiring and patterning (specialization and segmentation of brain regions) occur. These events rely on precisely regulated cellular and extracellular signalling molecules including morphogens which temporally and spatially regulate gene expression required for neuronal differentiation, migration, synaptogenesis and maturation (Inestrosa and Varela-Nallar, 2015, Gonzalez et al., 2016). Several environmental risk factors can interfere with these events resulting in abnormal brain development which can result in ASD pathogenesis. In particular, brain inflammatory immune response triggered by maternal or prenatal infections has been implicated in ASDs (Hwang and Chen, 2010, Ohkawara et al., 2015, Nardone and Elliott, 2016). Other critical environmental factors include exposure to teratogens and oxidative stress (Chauhan and Chauhan, 2006, Tamiji and Crawford, 2010b, Dufour-Rainfray et al., 2011, Reynolds et al., 2012). Teratogens are chemicals that result in developmental anomalies in newborns depending on timing and dosage of prenatal exposure (Dufour-Rainfray et al., 2011). Due to the sequential nature of early brain development, time periods when the brain is particularly vulnerable to a specific type of insult are known as critical periods which can vary depending on the nature of the insult or teratogen (Courchesne and Pierce, 2005, Chaudhury et al., 2016). Teratogens can disrupt events taking place in varying rates and locations of the developing brain, some of these include cell division, migration and neurotransmission (Dufour-Rainfray et al., 2011). With exposure during a critical period of brain development, several teratogens have been identified to cause autism-like characteristics, including valproic acid (VPA) (a mood stabilizer and antiepileptic drug), alcohol and thalidomide (Dufour-Rainfray et al., 2011, Middleton et al., 2012).

A particular teratogen known as misoprostol, a prostaglandin type E (PGE) analogue, has been found to result in abnormal brain development (Wong and Chan, 2005, Bos-Thompson et al., 2008, Miller et al., 2009). Normally, misoprostol is used to treat gastric ulcers as it decreases stomach acid secretion (Wong and Chan, 2005). However, prenatal exposure to misoprostol arises from attempts at using this drug to induce labour or terminate pregnancy (Miller et al., 2009). Inappropriate administration of misoprostol, results in the child having Mobius syndrome and ASD like characteristics (Miller et al., 2009). Misoprostol exerts its action on cells thorough E-prostanoid (EP) receptors which normally bind to prostaglandin E_2 (PGE_2) which is the major endogenous lipid molecule in the brain (Bandim et al., 2003). The effects of misoprostol is an important finding as it suggests that lipid signalling pathways play an essential role in brain development, function and behaviour (Tamiji and Crawford, 2010b, Wong and Crawford, 2014). These results point towards a developmental role of lipids such as PGE_2 and their connection with developmental brain disorders such as ASDs (Gharami et al., 2015, Guillermo et al., 2015, Schneider et al., 2016).

1.4. The role of lipids in brain development

Polyunsaturated fatty acids (PUFAs) are a key component of lipids found in the brain and include docosahexaenoic acid (DHA) and arachidonic acid (AA) fatty acids (FAs), these are derived from dietary omega-6 and -3 FAs and are the most abundant PUFAs in the brain (Tamiji and Crawford, 2010b, Brigandi et al., 2015). Along with playing an important role in cell membrane composition and fluidity, PUFAs and their derivatives interact with transmembrane signalling proteins such as G-Protein Coupled Receptors (GPCR) to modulate developmental events including synaptogenesis, neurogenesis, neurotransmission, neurotrophic factor

expression, signal transduction and gene expression (Schuchardt et al., 2010, Bradbury, 2011, Brigandi et al., 2015, Morgese and Trabace, 2016). More specifically, DHA has been found to play an essential role in the proliferation of neuronal cells, cell migration, differentiation and synaptogenesis during brain development (Gharami et al., 2015). Furthermore, DHA is highly concentrated in synapses for the maintenance of synaptic membrane potentials (Richardson, 2004, Bradbury, 2011). One particular study has indicated that maternal supplementation of DHA and AA can enhance spatial learning and neuroprotection of brain lesions in adult rodent offspring (Godor-Kacsandi et al., 2013). Inappropriate dietary intake and imbalances of these lipids not only have an important role in brain development and behaviour, but also have been linked to neuropsychiatric disorders, including attention deficit disorder, Alzheimer's disease, Schizophrenia and depression (Muller et al., 2002, Wenk, 2005, Young and Conquer, 2005, Colter et al., 2008).

Mounting evidence has linked abnormal lipid intake and metabolism to developmental neuropsychiatric disorders, especially ASDs (Young and Conquer, 2005, Schuchardt et al., 2010, Tamiji and Crawford, 2010b). Several clinical studies have consistently found an imbalance or deficiency of PUFAs in blood samples of individuals with ASD compared to healthy groups (Vancassel et al., 2001, Bell et al., 2004, Sliwinski et al., 2006, Meguid et al., 2008, Wiest et al., 2009, Bell et al., 2010, Mostafa et al., 2010, Brigandi et al., 2015). Interestingly, dietary supplementation of PUFAs has been shown to improve behaviour, especially eye contact, concentration and motor skills, as well as the balance of blood PUFAs in individuals with ASD (Meguid et al., 2008). Additionally, supplementation with omega-3 fatty acids has been shown to reduce hyperactivity associated with autism compared to placebo in a randomized double-blind study (Amminger et al., 2007). Interestingly, female mice that were fed an increased amount of

omega-6 PUFAs during gestation and lactation resulted in adult offspring exhibiting ASD like

behaviour (Jones et al., 2013). Omega-6 PUFAs are used to synthesis AA which serves as a

precursor for lipid derived signalling molecules, particularly PGE_2 which has a diverse role in

brain development and has been linked to ASD (Breyer et al., 2001). Taken together, these

results not only indicate a key role of lipid molecules in ASD pathogenesis, but also the need to

understand the role of downstream synthetic pathways and products of these molecules, such as

PGE_2. In addition to levels of PUFAs, other genetic and environmental factors have been shown

to interfere with PGE_2 levels resulting in abnormal brain development and ASD (Wu et al., 2007,

Andreasson, 2010, Tamiji and Crawford, 2010b).

1.5. The COX/PGE$_2$ pathway

PGE_2 is primarily derived from AA which is released from the plasma membrane by

phospholipase A_2 (PLA_2) (Nomura et al., 2011). Cyclooxygenase-1 and 2 (COX-1 and 2)

facilitate the rate limiting step of converting AA into Prostaglandin H_2 (PGH_2) which is then

converted to various prostanoids by prostaglandin E synthases (PGESs), including PGE_2 which

is the main prostanoid in the brain (Figure 1) (Coleman et al., 1994, Tamiji and Crawford, 2010b,

Furuyashiki and Narumiya, 2011). The downstream pathway of PGE_2 is facilitated by 4 isoforms

of GPCRs known as EP1-4 (Coleman et al., 1994, Furuyashiki and Narumiya, 2011).

Interestingly, functional EP receptors can be found both in the cell membrane and the nuclear or

perinuclear membranes, thus PGE_2 not only results in paracrine but also intracellular and

autocrine signalling (Bhattacharya et al., 1999). Protein kinase A (PKA) and

phosphotidylinositide 3-kinase (PI-3K) are key facilitators of EP receptor signal transduction

(Andreasson, 2010). PGE_2 is the main molecule involved in the COX induced immune response

as its pathway plays an important role in triggering inflammation and fever (Lazarus, 2006, Straccia et al., 2013). During an immune response, PGE_2 levels rise due to an increased production and activity of COX-2 (Straccia et al., 2013). The role of triggering inflammation has direct implications for neurodevelopmental disorders associated with inflammation, caused by maternal and pre or postnatal infections, especially ASDs (Pepicelli et al., 2005, Smith, 2006, Benvenuto et al., 2009, Patterson, 2011). In non-neuronal cells, the PGE_2 pathway has been involved in the modulation of cellular growth and motility (Raisz et al., 1993, Charo et al., 2013). In neuronal cells, this pathway has been shown to modulate a wide range of processes including synaptic maturation, transmission and plasticity (Chen and Bazan, 2005, Sang and Chen, 2006, Furuyashiki and Narumiya, 2011). PGE_2 signaling also contributes to perinatal dendritic spine formation and learning and memory by regulating membrane excitability and plasticity in the hippocampus (Chen et al., 2002, Burks et al., 2007). Studies focusing on the inductive behaviour and function of COX enzymes have yielded further evidence for the role of PGE_2 in the brain.

The COX isoforms have distinct functions depending on differential cellular localization, regulatory mechanisms and expression levels throughout the body (Aid and Bosetti, 2011). While inducible by cytokines and growth factors in most tissues, COX-2 is constitutively expressed in the brain, especially during development, and COX-1 is constitutively expressed throughout the body (Vane, 1998, Maslinska et al., 1999, Amateau and McCarthy, 2004, Kirkby et al., 2012). In the adult brain COX-1 is primarily expressed in microglia and vascular cells (Hoozemans et al., 2001, Garcia-Bueno et al., 2009). COX-2, interestingly, is localized in post-synaptic dendrites and excitatory terminals of neurons in the cortex, hippocampus and amygdala suggesting an important role in learning and memory through the modulation of neuronal transmission and plasticity (Wang et al., 2005, Hewett et al., 2006, Aid and Bosetti, 2011).

Interestingly, several over-the-counter anti-inflammatory drugs, known as Non-Steroidal Anti-inflammatory Drugs (NSAIDs), function by inhibiting COX enzymes and have been used to study the function of COX enzymes in the brain (Silberstein and Stirpe, 2014). COX-2 selective inhibitors have been shown to significantly reduce postsynaptic membrane excitability and induction of long-term potentiation (LTP) in the hippocampus (Chen et al., 2002). Furthermore, this reduction in neuron excitability was reversed by PGE_2 and not by other prostaglandins (Chen et al., 2002). Overall, expression of COX-2 in the cerebral cortex, hippocampus, amygdala, hypothalamus and spinal cord has been shown to play a role in fundamental brain processes such as synaptic remodelling, synaptic plasticity, memory consolidation, functional hyperemia and nerve transmission for fever and pain (Yamagata et al., 1993, Appleby et al., 1994, Breder et al., 1995, Breder and Saper, 1996, Vane et al., 1998, Minghetti, 2004). The inflammatory, neurological and neurodevelopmental role of PGE_2 and the environmental insults that can alter the levels of PGE_2 point towards a link between the PGE_2 pathway and ASD pathogenesis.
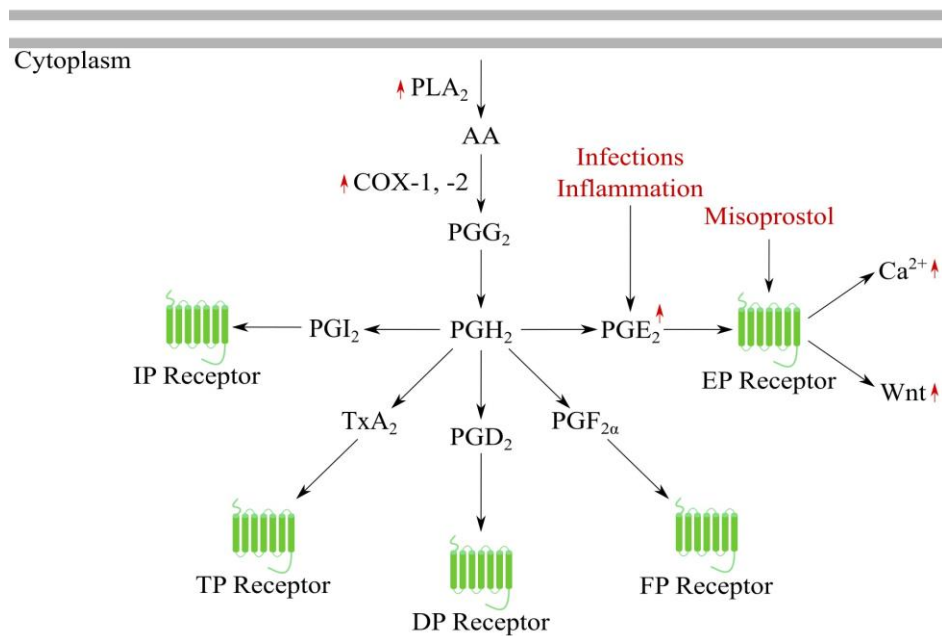
Figure 1: An overview of the prostanoid synthesis pathway and environmental interactions.
The red arrows indicated the up or down regulatory responses during environmental changes such as immune response. As shown, misoprostol and inflammation can up-regulate or activate the PGE$_2$ pathway which can also modulate the Wnt pathway and intracellular calcium homeostasis. This image has been modified from our previous work in Tamiji and Crawford (2010b).

1.6. The PGE$_2$ pathway in ASDs

Abnormalities in the PGE$_2$ signaling pathway due to genetic or environmental causes have been linked to ASDs (Tamiji and Crawford, 2010b, Wong et al., 2014, Wong et al., 2015). In addition to environmental factors outlined above, our recent review discusses additional ASD environmental risk factors, including pollutants and toxins which can also alter PGE$_2$ levels (Wong et al., 2015). Genetic mutations of enzymes involved in the synthesis of PGE$_2$ have been implicated in several neurological disorders such as schizophrenia in which PLA$_2$ mutations and abnormal expression has been found (Pae et al., 2004, Smesny et al., 2005). In individuals with Autism and Asperger's syndrome, significantly increased PLA$_2$ activity has been reported in blood cells (Bell et al., 2004). This increase in activity alters the functional capabilities of the cell membrane by reducing the amount of PUFAs incorporated into the membrane (Bell et al., 2004, Bell et al., 2010). Furthermore, several studies have implicated abnormal function and expression levels of COX-2 in patients with ASDs. More specifically, COX-2 immunoreactivity in the cortex has been found to be altered in individuals with Rett syndrome, a condition that exhibits ASD like behaviour (Kaufmann et al., 1997). The most striking evidence linking COX-2 and ASD has been provided by Yoo and colleagues who found polymorphisms in the COX-2 gene (*PTGS2*) in a large cohort of children with ASD but not in healthy parents (Yoo et al., 2008). Additional studies with a focus on the PGE$_2$ pathway and its exposure have also indicated a significant role in neuronal development and ASD pathogenesis.

The COX/PGE$_2$ pathway is associated with several etiologies of ASD including genetic mutation, maternal diet, pollutant exposure, inflammation, neuronal transmission and plasticity. However, the molecular mechanisms by which PGE$_2$ alters brain development are unclear. Our lab aims to understand the role of this pathway in neuronal development using cell culture and

mouse models. We have shown that PGE$_2$ and, its analogue, misoprostol can alter intracellular and growth cone calcium homeostasis via PKA, resulting in neurite retraction in differentiating neuroblastoma cells (Tamiji and Crawford, 2010b, a, Davidson et al., 2016)**.** More recently, we have demonstrated that higher levels PGE$_2$ can not only accelerate proliferation and neuronal differentiation but also differentially regulate important genes in neuroectodermal (NE-4C) stem cells (Wong et al., 2016). Some of the developmental genes we found to be differentially regulated are known target genes of the Wingless-type MMTV integration site family (Wnt) pathway, a critical developmental pathway (Wong et al., 2016). This not only provided supporting evidence implicating Wnt signalling in ASD, but also for the role of PGE$_2$ in brain development and ASD pathogenesis shown in our lab (Garber, 2007, Wang et al., 2010a, Cao et al., 2012, Wong et al., 2014).

1.7. The Wnt pathway

The Wnt pathway plays an overarching role in embryogenesis and is highly conserved throughout the animal kingdom as it is a key developmental morphogen (van Amerongen and Nusse, 2009, Solis et al., 2013). Wnt ligands are a large family of excreted glycoproteins that bind to Frizzled (FZD) receptors along with co-receptors such as LRP5/6 and tyrosine kinase receptors Ryk and Ror (Okerlund and Cheyette, 2011, Kuhl and Kuhl, 2013). Depending on the Wnt ligand and the receptor activated, Wnt signalling can activate two types of pathways; the canonical Wnt/β-catenin pathway (Figure 2) or the non-canonical pathway, which is independent of β-catenin and involves the release of Ca^{2+} stores (Okerlund and Cheyette, 2011). The canonical Wnt pathway utilizes β-catenin when activated as the central transducer protein, however, in the absence of a Wnt signal, β-catenin is continuously degraded by the destruction

complex (Okerlund and Cheyette, 2011). The destruction complex consists of four proteins that interact with β-catenin: Adenomatous polyposis coli (APC), Casein Kinase 1α (CK1α), Axin and glycoprotein synthase kinase-3 (GSK-3β) (Liu et al., 2002). The degradation of β-catenin is halted by the Wnt receptor activated protein Disheveled (Dvl) which causes the dissociation of the destruction complex (Okerlund and Cheyette, 2011). Upon disassociation, the discontinued degradation of β-catenin results in its accumulation and translocation into the nucleus where it binds heterodimers of T-Cell-specific/Lymphoid Enhancer-binding Factor (TCF/LEF) transcription factors (TFs) to induce transcription (Okerlund and Cheyette, 2011). Interestingly, two known post-translational modifications (PTMs) have been shown to indicate non-degradable or active β-catenin, this includes phosphorylation at serine (S)552 and non-phosphorylated at S33, S37 and threonine(T)41 (S33/S37/T41) (Wong et al., 2014). The downstream activation of gene expression results in a large variety of regulatory changes involving cell fate specification, polarity, differentiation, proliferation, migration, neuronal axon guidance and synapse formation (Logan and Nusse, 2004, Okerlund and Cheyette, 2011).

The main role of the Wnt pathways is in early development, however, activation of this pathway in adults is often associated with cancer or adult stem cells (Clevers and Nusse, 2012, Mah et al., 2016). During early brain development, the expression of Wnt ligands is tightly regulated as specific ligand concentration gradients are crucial for appropriate spatial and temporal cell differentiation to occur (Kiecker and Niehrs, 2001, Solis et al., 2013). It has been demonstrated that treatment of a Wnt ligand and Wnt inhibitor on human embryonic stem cells (ESCs) results in the generation of endodermal/cardiac cells and neuroectodermal cells, respectively (Blauwkamp et al., 2012). Wnt ligands also regulate neuronal cell specialization, into dopamine neurons for example, and cell morphogenesis resulting in functional axons and

dendritic networks (Bodmer et al., 2009, Cajanek et al., 2009, Rosso and Inestrosa, 2013). Mounting evidence for a direct link between the canonical Wnt signalling pathway and ASD exists (Wassink et al., 2001, Zhang et al., 2009, Marui et al., 2010, Okerlund and Cheyette, 2011, Kalkman, 2012, Martin et al., 2013, Zhang et al., 2014).

Several Wnt related genes have been implicated in ASD, especially the Wnt2 ligand gene (*WNT2*). With an earlier report by Wassink et al. (2001) and the identification of 9 SNPs in the *WNT2* gene among 170 ASD patients compared to 214 controls, the *WNT2* gene is considered a strong ASD candidate gene (Marui et al., 2010). Furthermore, mutations of the β-catenin gene (*CTNNB1*) have been identified in individuals with ASD (O'Roak et al., 2012). Several mouse knockout or knock-in studies of Wnt associated genes have indicated impaired behavioural traits similar to ASDs (Lijam et al., 1997, Kalkman, 2012, Dong et al., 2016). In particular, mouse studies have shown that altered functional levels of β-catenin, APC, Dvl1 and GSK-3 in the brain result in a significant increase in repetitive behaviour and decrease in social interaction (Lijam et al., 1997, Mines et al., 2010, Mohn et al., 2014, Dong et al., 2016). Interestingly, an ASD mouse model created from VPA exposure, a known teratogen, has been shown to activate the Wnt pathway by inhibiting the activity of GSK-3β (Hall et al., 2002, Bug et al., 2005, De Ferrari and Moon, 2006). Similarly, we have also shown a link between PGE$_2$ and the Wnt pathway (Wong et al., 2014, Wong et al., 2016).

1.8. Interaction between PGE$_2$ and Wnt pathways

In non-neuronal cells, a considerable amount of evidence indicates the existence of an interaction between PGE$_2$ signalling and the Wnt pathway (Oshima et al., 2006, Goessling et al., 2009, Liu et al., 2010). This interaction is primarily studied in cancer, particularly gastric

tumourigenesis, and stem cells (Oshima et al., 2006, Oshima et al., 2009, Yoshida et al., 2013).

Goessling and colleagues (2009) have used Wnt reporter activity to demonstrate *in vivo* Wnt

responsiveness to PGE$_2$ exposure in hematopoietic stem cells (HSCs) of zebrafish. This was

done in order to demonstrate the role of PGE$_2$/Wnt in organ regeneration but warrants careful

study due to risks of inducing tumourigenesis (Goessling et al., 2009). To date, there has not

been any evidence for this interaction, not only in neuronal cells but also in the context of early

development. In our lab we have shown for the first time that the PGE$_2$ can alter Wnt pathways

in our *in vitro* (Wong et al., 2014, Wong et al., 2016) and *in vivo* (Rai-Bhogal, 2016) models of

the nervous system by altering β-catenin activity and Wnt target gene expression.

Our research has shown that PGE$_2$ can modify the activity of and cellular responses

governed by the canonical Wnt pathway in NE-4C stem cells via PKA and PI-3K (Figure 2)

(Wong et al., 2014, Wong et al., 2016). More specifically, PGE$_2$ treatment of cells activated by a

Wnt agonist (WntA) alters migration and proliferation of NE-4C cells (Wong et al., 2014). We

also found increased levels of active β-catenin in the PGE$_2$ treated WntA cells compared to

WntA only treated cells. Furthermore, these changes in NE-4C cells were accompanied by

altered expression of Wnt target genes that are important for cell proliferation, migration and

PGE$_2$ synthesis, including *β-catenin* (*CTNNB1*), *PTGS2*, cyclin D1 (*CCND1*) and *matrix*

metalloproteinase 9 (*MMP9*) (Wong et al., 2014). Similarly, our *in vivo* study of embryonic

exposure to PGE$_2$, via maternal subcutaneous injection, also indicated differential expression of

Wnt-target genes (Rai-Bhogal, 2016). The brains of offspring maternally exposed to PGE$_2$

during prenatal development also indicated an elevated level of active β-catenin (Rai-Bhogal,

2016). Considering the neurodevelopmental processes that are regulated by PGE$_2$ and Wnt

signalling, the interactive link between them particularly important for the study of ASD.
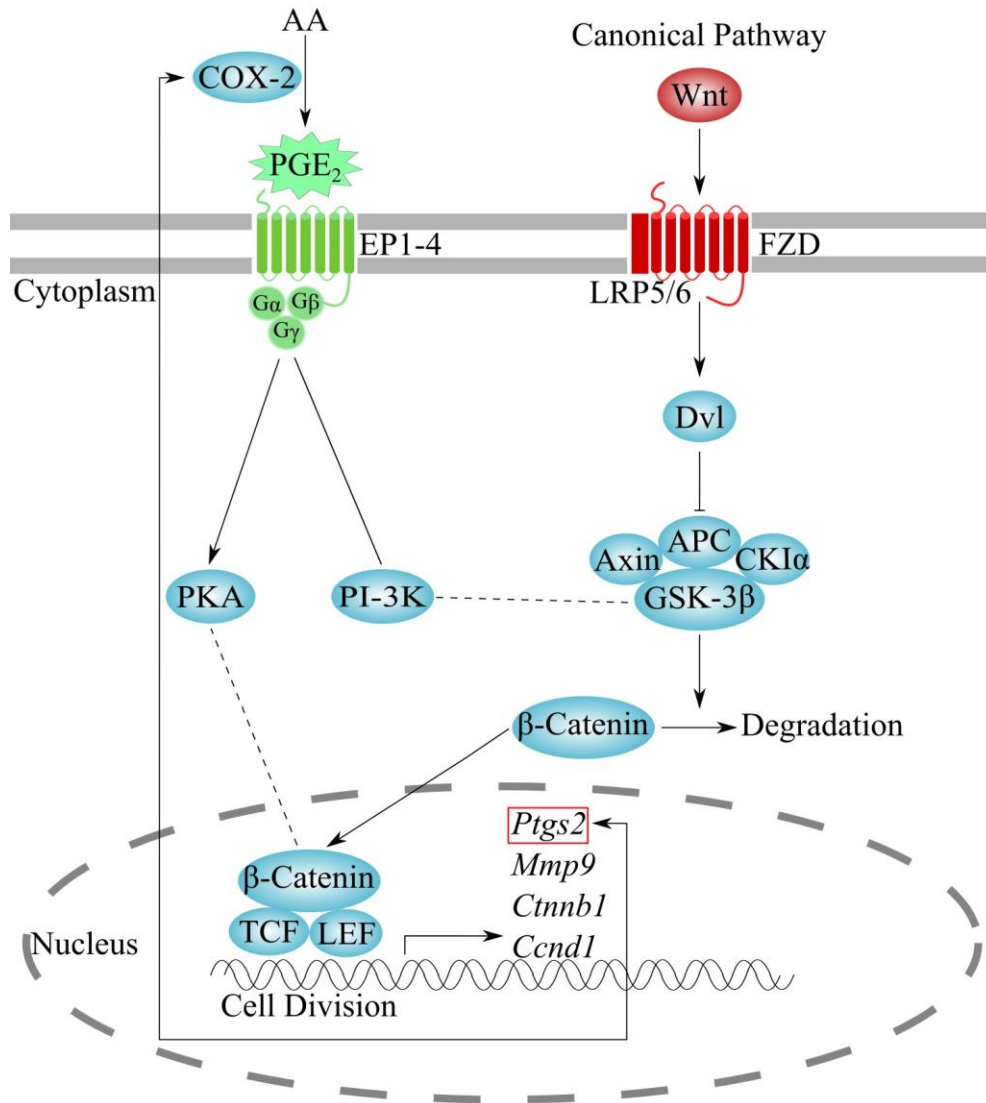
Figure 2: A simplified overview of the Wnt/β-catenin (canonical) pathway. The destruction complex consists of axin, GSK-3β, CK1α and APC. If a Wnt signal is present, β-catenin accumulates and activates TFs TCF and LEF in the nucleus. This figure has been modified from our previous work in Wong et al. (2014).

1.9. Objectives

Recent literature provides evidence for the diverse role of the COX-PGE$_2$ pathway during healthy brain development and association of its abnormal signalling with ASD pathogenesis. Stemming from the current *in vitro* research emerging in our lab, the main objective of this thesis is to provide an in-depth global analysis of gene expression in the prenatal brain lacking COX-1 and COX-2 enzymes producing PGE$_2$. The goals of this research are the following:

1. To provide novel insight on the transcriptome of the homozygous COX-1$^{-/-}$ and 2$^{-/-}$ male mice, during both embryonic day (E) 16 and 19. This study will investigate a global overview of differentially regulated genes and their associated functional signalling pathways in a model lacking PGE$_2$ producing enzymes.

2. To examine whether the cross-talk between the PGE$_2$ and Wnt signalling pathway occurs in the prenatal brain of the COX$^{-/-}$ models.

3. To characterize the expression level of selected differentially regulated genes in the existing *in vitro* cell models of undifferentiated and neuronally differentiated neuroectodermal NE-4C cells treated with PGE$_2$.

These results will provide the first *in vivo* evidence for the involvement of the COX/PGE$_2$ pathway in brain development and how it may contribute to the pathology of ASD.

1.10. Hypothesis

We propose the following hypotheses for the studies presently conducted:

Study 1: Microarray analysis in COX$^{-/-}$ mice models at E16 and E19

1. Defect in the COX/PGE$_2$ pathway results in abnormal expression of developmental genes linked to autism and important neurodevelopmental pathways.

2. The comparisons in gene expression between E16 and E19 within each knockout will identify abnormalities in stage specific gene expression.

3. A comparison of COX-1$^{-/-}$ to COX-2$^{-/-}$ prenatal brains will reveal distinct differentially expressed genes and pathways within both stages of development.

4. Due to defective COX/PGE$_2$ activity in the developing brain of our mouse models, we expect to find differential levels of active β-catenin and thus altered activity of the canonical Wnt pathway.

Study 2: Gene expression of selected genes in PGE$_2$ treated cell cultures

1. Gene expression of selected developmental genes in our microarray study will also be affected in undifferentiated NE-4C stem cells and neuronally differentiated NE-4C cells exposed to PGE$_2$.

1.11. Experimental Model Systems

For *in vivo* prenatal brain gene expression analysis, homozygous male mice lacking PGE$_2$ producing enzymes COX-1$^{-/-}$ or COX-2$^{-/-}$ (Taconic Biosciences) were used. These mice contain disruptions in the genomic DNA (gDNA) sequence of *Ptgs1* or *Ptgs2* genes encoding COX-1 or COX-2 enzymes, respectively (Langenbach et al., 1995, Yu et al., 2006). These mice models were compared to wild-type (WT) mice as controls. Due to the important role of critical periods in the manifestation and severity of neurodevelopmental disorders, we chose to observe gene

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expression during E16 and 19. E16 spans the occurrence of neurogenesis in most brain regions, with especially elevated neurogenesis in the cerebral cortex, particularly in layers II/III, whereas E19 is the stage prior to birth with a slower rate of neurogenesis (Rodier, 1980, Rice and Barone, 2000, Clancy et al., 2001). Furthermore, E16 has been identified as a critical period in which there is vulnerability for environmentally triggered hyperactivity and abnormal learning and memory in mice (Rodier, 1980).

Several phenotypes of COX knockout mice or mice treated with COX inhibitory drugs have been characterized in the past. The reviews by Loftin et al. (2002), Morita (2002), and Langenbach et al. (1999a) outline many phenotypes across several organ systems where various prostanoids play important functional and developmental roles. While the function of COX-1 has been associated with gastric ulcerations, disruption of the COX-1 gene has only resulted in a significantly reduced stomach acid pH (Langenbach et al., 1995, Langenbach et al., 1999a). Interestingly, however, the inhibition of both COX enzymes using either two COX specific NSAIDs or a non-specific NSAID does result in gastric ulcerations in rats (Wallace et al., 2000). COX enzymes are also important for the female reproductive system; COX-1 deficiency results in a prolonged gestation period due to delayed parturition (Gross et al., 1998), while COX-2$^{-/-}$ female mice indicate more severe defects in ovulation, fertilization, implantation and decidualization (Dinchuk et al., 1995, Morham et al., 1995, Lim et al., 1997). Similarly, COX-1 has not been shown to be critical for kidney development and function, while COX-2 is crucial for proper renal morphology and development, especially in postnatal life (Morham et al., 1995, Komhoff et al., 2000, Norwood et al., 2000). In addition to reduced intestinal tumorigenesis, COX-2 deficiency also results in protection against brain injury induced by ischemia (Oshima et al., 1996, Iadecola et al., 2001). Lastly, COX-2$^{-/-}$ mice also shown cardiac fibrosis, peritonitis,

and reduced ability for the liver to regenerate after hepatectomy (Langenbach et al., 1999b, Cheng et al., 2001, Rudnick et al., 2001). Overall, these studies have revealed several phenotypes and further indicate the more vital role of COX-2 over COX-1. Relevant for this study, however, are behavioural phenotypes which are not yet known and to be discovered.

For *in vitro* experiments, we used NE-4C stem cells as the experimental model. These cells were obtained from American Tissue Culture Collection (ATCC). NE-4C cells are derived from primary brain cell cultures of E9 forebrain and midbrain vesicles of transgenic mouse embryos. These cells are known to mimic processes occurring in the developing brain including proliferation, migration, aggregation and differentiate into neuronal cells (Schlett and Madarasz, 1997). These attributes make undifferentiated NE-4C stem cells a suitable model for understanding prenatal brain development. In addition to undifferentiated NE-4C stem cells, we also examined gene expression in neuronally differentiated NE-4C cells using differentiating media. Interestingly, we have previously found that the formation of neurospheres (3D aggregates of precursor neuronal cells), occurs two days earlier at Day 6 due to PGE_2 treatment, while neurospheres formed at Day 8 in untreated differentiating cells, thus PGE_2 accelerates differentiation (Wong et al., 2016). This was further demonstrated by an RNA marker known as *Gfap*, indicating the absence of astrocytes; and *Mapt*, indicating late neuronal cells, at Day 6 and 8 for treated and untreated cells, respectively (Wong et al., 2016). Thus, to examine mature neurons for our study we examine Day 8 neuronally differentiated cells.

2.0. MATERIALS AND METHODS

2.1. Animals

Homozygous male mice deficient in COX-1 or COX-2 (COX-1$^{-/-}$ or COX-2$^{-/-}$), and female heterozygous (COX-1$^{+/-}$ or COX-2$^{+/-}$) mice were obtained from Taconic Biosciences (B6;129P2-*Ptgs1^{tm1Unc}* and B6;129P2-*Ptgs2^{tm1Unc}*). These mice contained disruptions in *Ptgs1* or *Ptgs2* genes, encoding for COX-1 or COX-2, respectively (Langenbach et al., 1995). Mice were acclimatized for one week at the York University animal facility with a 12 h light/dark cycle and *ad libitum* food and water. We used heterozygous females as homozygous females have substantially reduced fertility (Wang et al., 2010b). Wild-type (WT) mice (C57BL/6) were bred to obtain brain samples for control. For the microarray, we obtained three homozygous male offspring from each COX-1$^{-/-}$, COX-2$^{-/-}$ and WT genotypes at days E16 and E19. COX-1$^{-/-}$ E16, COX-1$^{-/-}$ E19, COX-2$^{-/-}$ E16, COX-2$^{-/-}$ E19, WT E16 and WT E19 samples were collected. The animal protocol was approved by the Research Ethics Board of York University, Toronto.

2.2. Genotyping

gDNA was isolated from tail samples boiled in alkaline lysis reagent (25mM NaOH) and subsequently neutralized with Tris-HCL. The resulting gDNA was used to determine sex of the embryos using PCR with *sex determining region Y (SRY)* primers (Table 1). Knockout zygosity was determined using primer designs obtained from Taconic for both COX$^{-/-}$ mice (Table 1). The PCR reaction was carried out using dNTP (2500 µM of each), 5U/µL OneTaq DNA Polymerase and 5x OneTaq Reaction Buffer (BioLabs). 30 cycles of denature, anneal and extension; and a final extension step (Eppendorf Mastercycler) was used. 100 base pair (bp) ladder (Biolabs) and PCR products were resolved using a 1.2% agarose set in 1x Tris-acetate-EDTA (TAE) buffer with 2.5 µL of Ethidium Bromide for double-stranded DNA (dsDNA) labelling.

Table 1: Primers designed for genotyping using PCR. Forward (F) and reverse (R) primers are written in the 5' to 3' direction. To determine zygosity of the mutant mice, an additional primer was needed for amplification of the mutant version of the gene.

Gene	Sequence (5'→3')	Amplicon Length (bp)
SRY	F - TCCCAGCATGCAAAATACAGAGATC	300 bp
	R - TTGGAGTACAGGTGTGCAGCTCTAC	
COX-1 Genotyping (Taconic)	F(-/-) - CAGCCTCTGTTCCACATACAC	(-/-): 700 bp,
	R - CTGACTTTCTGAGTTGCCAAC	(+/-): 700 and
	F – GAGATGGCTGCTGAGTTGG	600 bp
COX-2 Genotyping (Taconic)	F(-/-) - ACGCGTCACCTTAATATGCG	(-/-): 950 bp,
	R - TCCCTTCACTAAATGCCCTC	(+/-): 950 and
	F - ACACACTCTATCACTGGCACC	800 bp

2.3. Microarray

Total RNA isolation from brain samples was carried out using the TRIzol method (Fisher Scientific). Sample collection and preparation was done by Ravneet Bhogal (Ph.D Candidate). The RNA for each sample was sent to Princess Margaret Genomics Centre (Toronto, Canada; www.pmgenomics.ca) for completion of microarray, quality control (QC) and statistical data analysis. Mouse WG-6 V2 BeadChip (Illumina) microarrays contained 45,281 target probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI) *RefSeq (Build 38 Release 22)* (November 7, 2009), Mouse Exonic Evidence Based Oligonucleotide (MEEBO) and RIKEN FANTOM2 database. QC was done using R (v3.0.2) with the Bioconductor framework and LUMI packages installed; all data pertaining to each sample passed QC for further analysis. Subsequent analysis involved normalization steps using GeneSpring v12.6.1. Only probes that were above the 20th percentile of the distribution of the detected signal were included. Overall, the analysis indicated that the data was reliable, especially since housekeeping genes were found to be regulated as expected between comparisons. One way ANOVA with post-hoc test (Tukey HSD) was done to compare the data and the *P*-values were subsequently corrected using the Benjamini-Hochberg False Discovery Rate (FDR). The end results for the COX$^{-/-}$ experimental samples are indicated as a fold change (FC) with WT equaling 1.

2.4. Bioinformatics

Prior to bioinformatics, lists of genes that were statistically significant with FDR corrected *P*-value < 0.05 and absolute FC > 1.5 were compiled. In addition, we converted the gene names from Illumina gene IDs to Official Gene Symbol (OGS) using DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/). These cut-offs resulted in lists of

significantly differentially expressed genes for COX-1$^{-/-}$ E16, COX-1$^{-/-}$ E19, COX-2$^{-/-}$ E16 and

COX-2$^{-/-}$ E19 (the list of all genes and FC can be found in Supplementary Table S1). To

determine genes in common between knockouts within corresponding developmental stages,

Venn diagrams were created using Venny 2.0 tool (http://bioinfogp.cnb.csic.es/tools/venny/). To

obtain pathways in which the genes belonged, we carried out functional annotation using Gene

Ontology: Biological Process (GO:BP) and Kyoto Encyclopedia of Genes and Genomes

(KEGG) categories using DAVID Bioinformatics with the mouse BeadChip background list

corresponding to the microarray chip used in this study (MouseWG-6_V2_0R2_11278593_A)

(Huang da et al., 2009). Enrichment of genes belonging to a pathway was determined using

Benjamini FDR corrected EASE score (one-tail Fisher Exact Probability Value) P-value < 0.1

cut-off in DAVID. Only non-redundant annotations containing a gene count > 3 are reported.

The annotations obtained from DAVID were divided using Venn diagram analysis to find

pathways in common between COX-1$^{-/-}$ and 2$^{-/-}$ during E16, and COX-1$^{-/-}$ and 2$^{-/-}$ for E19. Genes

associated with ASD in our lists were identified using Autism Database (AutDB) (Basu et al.,

2009) and cross referencing with additional genome wide studies of ASD patients. Much like

that EASE score generated for the biological pathway analysis, we carried out hypergeometric

tests using GeneProf (https://www.geneprof.org/GeneProf/tools/hypergeometric.jsp) for the ASD

candidate genes found. A probability value cut-off of 0.005 was set, meaning that there would

need to be a <0.5% chance of finding the same number of genes found with random sampling for

the finding to be interesting. For this test, the total number of genes in the microarray (44622)

and the total number of genes in AutDB (668) were used. Lastly, a KEGG pathway diagram was

used to illustrate all three Wnt signalling pathways, indicating Wnt genes found in our COX-2$^{-/-}$

E16 mice and AutDB search.

2.5. Cell cultures

Mouse NE-4C stem cells (ATCC) were kept in Minimum Essential Medium (MEM) from Eagle with 10% fetal bovine serum, 2 mM glutamine, 1 x penicillin-streptomycin mixture (Invitrogen). Cells were incubated in 95% humidity, 37°C and 5% CO_2 (Varga et al., 2009). Plating was done on 0.01% poly-L-lysine (Sigma) coated 100 mm culture plates (BD Falcon) and sub-cultured at 1:5. After 24 hours the cells were treated with either control or experimental conditions and left to incubate for 24 hours. 1 µM PGE_2 was added into the media for the experimental treatment, while media alone served as the control treatment. For the examination of undifferentiated cells, RNA/protein extraction was carried out after 24 hours of incubation in experimental or control treatments.

For the induction of differentiation, the cells were dissociated from stock using 0.05% trypsin in ethylenediaminetetraacetic acid (EDTA), pelleted and re-suspended in MEM with conditions described above. 0.01% poly-L-lysine (Sigma) coated 35 mm tissue culture dishes (Sarstedt) were used for plating. Incubation was done in the conditions described above. Induction of differentiation of NE-4C cells was done on day 0 using Neurobasal media (NBM; supplemented with L-glutamate, 1x penicillin-streptomycin and 1x B-27; Invitrogen). Supplemental differentiating media was replaced every 2 days during the progression of differentiation. The treatments included only the media (control) and 1 µM PGE_2 in media (experimental) from day 0 to 8. At maximum confluency, cells were lysed for RNA and protein extraction. Differentiation and sample preparation was done by Christine Wong (Ph.D Candidate).

2.6. RNA and protein isolation

NucleoSpin®RNA/Protein-Kit (Macherey-Nagel) for RNA and protein extraction was used with the cell culture protocol provided by manufacturer (Kramer et al., 2010). Cell lysis buffer contained β-mercaptoethanol (β-ME) to inactivate Ribonucleases (RNases) present in the cell lysis. The lysis was filtered and homogenized by incorporation of 70% ethanol. RNA and protein were separated using a filter that is selective for RNA. Flow-through was set aside for protein purification. RNA was removed from the filter and the RNA was extracted through a series of filters and washes. In the process of RNA isolation, the samples were treated with deoxyribonuclease (DNase) enzyme and all samples were handled with RNase free lab-ware. Protein precipitator was added to the protein flow-through and upon washing with 50% ethanol and pellet drying, the protein was dissolved in 1% SDS with protease inhibitors (β-glycerophosphate and sodium orthovandate).

2.7. Reverse transcription and PCR

Concentration of RNA samples were determined using RNA spectrophotometer analysis as done in previous studies (Kramer et al., 2010). The appropriate volume required for 4 µg of RNA was calculated and used for reverse transcription, this ensured that each reaction would result in the same amount of complementary DNA (cDNA). This is important to ensure that the variation in gene expression is not due to changes in loading of total RNA. As a precaution, isolated 4 µg RNA samples were treated with DNase I (0.2U of DNase I/µg of RNA and 1x DNase I reaction buffer at 37°C for 10 minutes) and, subsequently, 5 mM of EDTA was added for 10 minutes in 75°C to deactivate DNase I. Upon incubation (65°C for 5 minutes) with 0.04 µg/µL Oligo(dT)$_{18}$ and 2.5 mM dNTP (Biolabs) for annealing, 1x Reverse Transcriptase

Reaction Buffer and 200U of M-MuLV Reverse Transcriptase (Biolabs) were added to initiate

the reaction. The reaction was carried out at 42°C for 60 minutes and 90°C for 10 minutes.

Diethyl pyrocarbonate (DEPC)-treated water, RNase free pipette tips and tubes were used to

avoid degradation of RNA.

The completion of the reserve transcriptase reaction was confirmed by determining the

presence of cDNA in the sample using PCR. The PCR reaction was carried out using dNTP

(2500 µM of each), 5U/µL OneTaq DNA Polymerase and 5x OneTaq Reaction Buffer (BioLabs).

GAPDH primers were used as this gene is a housekeeping gene and its presence can indicate the

presence of cDNA. Final concentrations of 0.2 µM of forward (5'-CGGCCGCATCTTCTTGTG-

3') and reverse (5'-ACACCGACCTTCACCATTTTG-3') GAPDH primers were used. The

reaction was carried out using an initial denature step; 30 cycles of denature, anneal and

extension; and a final extension step in the Eppendorf Mastercycler. 100 bp ladder (Biolabs) and

PCR products were resolved using a 1.2% agarose set in 1x TAE buffer with 2.5 µL of Ethidium

Bromide for double-stranded DNA (dsDNA) labelling.

2.8. Quantitative real-time PCR

To validate genes of interest in our $COX^{-/-}$ mice brains and quantify gene expression in

undifferentiated and differentiated NE-4C cell models, we carried out quantitative real-time PCR

(qRT-PCR) of cDNA obtained from RT reactions as described previously (Wong et al., 2014).

Sequences of primers used in the microarray were aligned using The UCSC Genome Browser

Mouse BLAT search to precisely determine the genes for which the primers should be designed.

Primers were designed using Primer Express Software v3.0. The primers chosen resulted in the

optimal amplicon length of 50-150 bp. The primers were also void of repeat sequences, to

prevent non-specific binding; and contained exon-exon junctions in order to amplify only mature mRNA and not precursor mRNA or gDNA (Table 2).

Two standard housekeeping control genes were used *Phosphoglycerate Kinase 1 (PGK1)* and *hypoxanthine phosphoribosyl transferase* (*HPRT*) (Wong et al., 2014). The expression of *PGK1* and *HPRT* have been verified to be stable during development (Weingarten et al., 2012). We validated the expression of three genes in the $COX^{-/-}$ mice brains: *Glyoxalase 1* (*GLO1*), *Myelin Transcription Factor 1 Like* (*MYT1L*) and *Dishevelled-associated activator of morphogenesis 1* (*DAAM1*). For the examination of gene expression in our undifferentiated and differentiated NE-4C cell models, we quantified the expression of *NRXN1*, *NRXN3*, *GLO1*, *Glutamate Receptor, Metabotropic 5* (*GRM5*), *DAAM1* and *Sry-related hmg box 11* (*SOX11*). qRT-PCR was conducted using a 7500 Fast Real Time PCR System (Applied Biosystems) as done previously (Wong et al., 2014). Equal loading of 3 µL cDNA was added for 20 µL SYBR green reactions per well.

Quantitative values were obtained from the threshold cycle (C_T) number. The raw C_T values from experimental ($COX^{-/-}$ mice) and control (WT) samples were normalized using the geometric mean of the housekeeping genes, *PGK1* and *HPRT,* to obtain the ΔC_T values. The ΔC_T values of the samples were compared with the control to obtain the relative quantity (RQ) value which represents the fold change expression of each target sample compared to control samples, this is known as the comparative C_T method (Weingarten et al., 2012, Wong et al., 2014). Three independent experiments were carried out for statistical analysis.

Table 2: Primers designed for qRT-PCR. Forward (F) and reverse (R) primers are written in the 5' to 3' direction.

Gene	Sequence (5'→3')	Amplicon Length (bp)
HPRT	F - TCCATTCCTATGACTGTAGATTTTAT	75
	R - AACTTTTATGTCCCCCGTTGACT	
PGK1	F - CAGTTGCTGCTGAACTCAAATCTC	65
	R - GCCCACACAATCCTTCAAGAA	
NRXN1	F - CCCGAATGTAGAAGGCAAAGA	78
	R - TGACACACGGAACCTGATATG	
NRXN3	F - TGTGAACCAAGTACAGATAAGAGTC	71
	R - TTGGGCGCATGTGCTTTGTA	
GLO1	F - CAAGACCCTGACGGCTACTG	97
	R - ATCCATTAATCCTCAAAGGCACA	
GRM5	F - CATGGAGCCTCCGGATATAATG	89
	R - GTATCCAAGAGGAGTGACAACC	
DAAM1	F - CTAAGCGAAGAGTTGCGGGA	115
	R - ACTCCAGCTCCGTCTCTACA	
SOX11	F - CTCCATCACTCGGCTTTCTTAT	98
	R - GATACACTGCGTCTAGAGTTGG	

2.9. Western blot analysis

Total protein was extracted from WT and COX$^{-/-}$ brain tissue samples using the TRIzol method (Fisher Scientific). 40 ug samples were treated with loading buffer and resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on to nitrocellulose membranes. We quantified two post-translationally modified forms of β-catenin to assay Wnt activity in our knockout models. These included non-phospho (Active) β-Catenin (S33/S37/T41) and phospho β-Catenin (S552), using a 1:1000 dilution of non-phospho (active) β-Catenin (S33/S37/T41) rabbit monoclonal (Cell Signaling #8814) and phospho β-Catenin (S552) (Cell Signaling #9566) antibodies, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (mouse 1:5000; Abcam #ab8245) was used as a stable housekeeping protein for loading control. With treatment of anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies the membranes were incubated in enhanced chemiluminescence (ECL) substrate (Bio-Rad) and visualized using Geliance 600 Imaging System (Perkin Elmer).

2.10. Statistics

Statistical Analysis for the qRT-PCR and western blot experiments for relative quantification of gene expression and β-catenin protein expression, respectively, was conducted with the Student's t-Test. All numerical data is presented as mean±SEM determined from the average of three independent experiments. A P-value < 0.05 was considered significant.

3.0. RESULTS

3.1. Study 1

3.1.1. Differential gene expression impact and Venn diagram analysis in COX-1$^{-/-}$ or COX-2$^{-/-}$ mice models

To obtain a broad overview of the impact on gene expression, we determined the total number of genes differentially expressed in all four COX knockout mice. We found a total of 229, 323, 134 and 148 differentially expressed genes meeting our cut-offs in COX-1$^{-/-}$ E16, COX-2$^{-/-}$ E16, COX-1$^{-/-}$ E19 and COX-2$^{-/-}$ E19 mouse models, respectively (Figure 3). Supplementary Table S1 contains the entire list of genes and their FC for each knockout model. During E16, both COX-1$^{-/-}$ and COX-2$^{-/-}$ knockouts show a greater number of affected genes compared to E19. Generally, we also found more genes down-regulated than up-regulated in all mice models, although this is more apparent in the E16 mice. The COX-1$^{-/-}$ E16 differentially expressed genes include 163 down and 66 up-regulated genes, and COX-2$^{-/-}$ E16 indicates 208 down and 115 up-regulated genes. Similarly, COX-1$^{-/-}$ E19 differentially expressed genes include 74 down and 60 up-regulated genes, while COX-2$^{-/-}$ E19 indicates 89 down and 59 up-regulated genes. Overall, the knockout of either one of the COX enzymes has a greater impact on gene expression at the E16 stage with greater down-regulation than up-regulation, compared to E19. Furthermore, among all knockout conditions, COX-2$^{-/-}$ mice indicated the greatest number of differentially regulated genes. Thus, COX-2$^{-/-}$ has a greater impact than COX-1$^{-/-}$ during either stage of development, especially E16.

Next, we aimed to determine the number of genes in common between and exclusive to COX-1$^{-/-}$ and COX-2$^{-/-}$ knockouts within either developmental stage. At E16, 157 affected genes were in common between COX-1$^{-/-}$ and COX-2$^{-/-}$ knockouts, while 72 and 166 genes were unique to COX-1$^{-/-}$ and COX-2$^{-/-}$ mouse models, respectively (Figure 4A). During E19, COX-1$^{-/-}$ and COX-2$^{-/-}$ indicated 69 genes in common, while 65 and 79 genes were unique to the

respective COX$^{-/-}$ models (Figure 4b). Overall, we observed a greater number of genes in common between the E16 than E19 COX$^{-/-}$ mice, indicating a more apparent functional overlap between the two enzymes during the earlier stage. Despite the overlap, the COX-2$^{-/-}$ mice also have a relatively high number of genes exclusively differentially expressed at E16. These results indicate a potentially greater impact on development in the COX-2 deficient mice than COX-1, with greater changes in gene expression in the earlier stage during which neurogenesis is rapidly occurring throughout the brain.
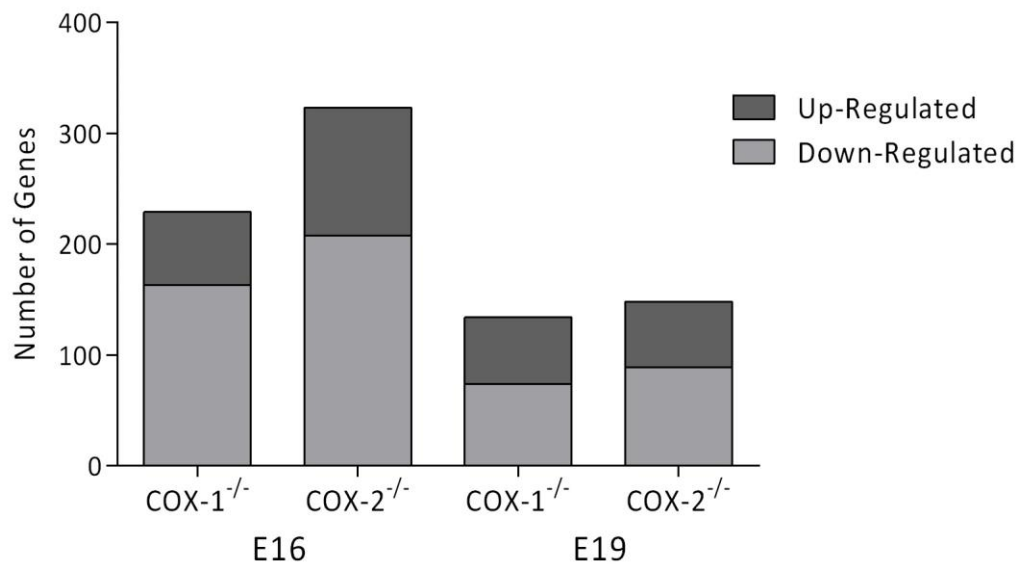
Figure 3: An overview of differentially expressed genes in COX-1$^{-/-}$ or COX-2$^{-/-}$ at E16 and E19. In the E16 stage we found 229 and 323 affected genes in the COX-1$^{-/-}$ and 2$^{-/-}$ mice, respectively. In the E19 stage, 134 and 148 genes were differentially expressed for COX-1$^{-/-}$ and 2$^{-/-}$ mice, respectively. At stage E16, COX-1$^{-/-}$ has 163 and 66; and COX-2$^{-/-}$ has 208 and 115 down- and up-regulated genes, respectively. At stage E19 however COX-1$^{-/-}$ has 74 and 60, while COX-2$^{-/-}$ has 89 and 59 down- and up-regulated genes, respectively.
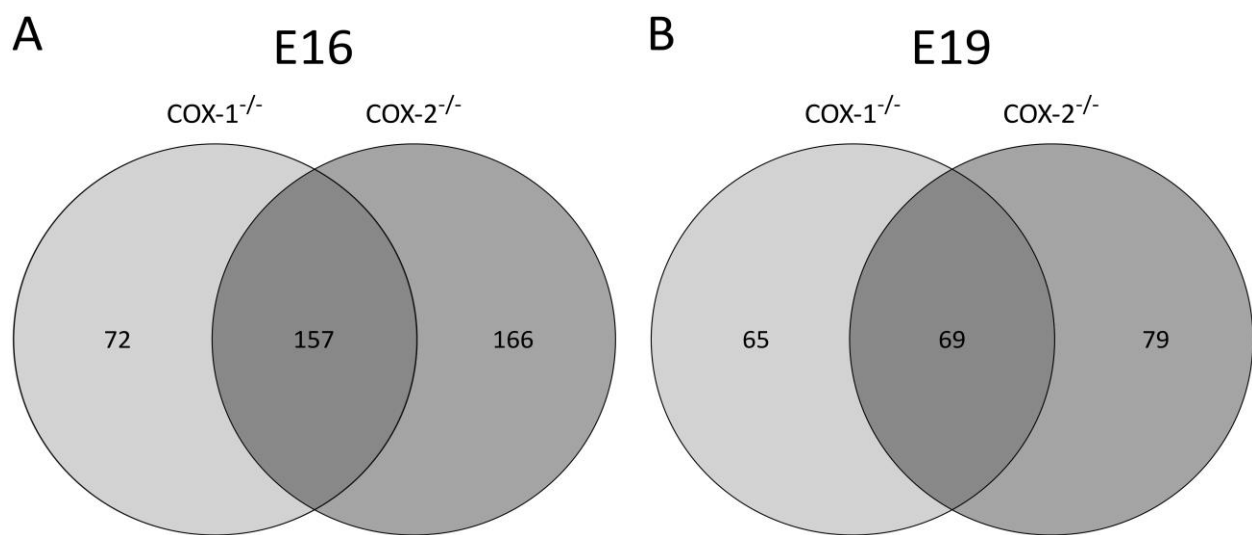
Figure 4: Comparison of the number of genes differentially expressed between the COX$^{-/-}$ animals of stage E16 and E19. (A) At E16, COX-1$^{-/-}$ indicated 72 genes, COX-2$^{-/-}$ indicated 166, and both have 157 genes in common. (B) At E19, COX-1$^{-/-}$ has 65 stage-specific genes, while COX-2$^{-/-}$ has 79 genes and both have 69 genes in common. Diagrams were created using Venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny/).

3.1.2. Functional pathway analysis

In order to gain insight into the biological and neurodevelopmental roles of the affected genes, we used GO:BP and KEGG databases to discover enriched functional pathways (see methods for detailed criteria). The generated pathway lists for both COX knockouts at either E16 or 19 were put through venn diagram analysis to determine pathways in common between the knockout models of the COX isozymes. At the E16 stage, 13 pathways exclusively enriched in the COX-1$^{-/-}$ knockout were found, 8 of which include at least one gene previously implicated in ASD (Table 3A, ASD genes in bold). The ASD genes containing functional pathways include: regulation of neurological system process (GO:0031644, $P = 0.002$), long-term potentiation (LTP) (KEGG: mmu04720, $P = 0.008$), adipocytokine signaling pathway (KEGG: mmu04920, $P = 0.041$), secretion (GO:0046903, $P = 0.047$), lipid biosynthetic process (GO:0008610, $P = 0.047$), blood vessel development (GO:0001568, $P = 0.071$), microtubule-based process (GO:0007017, $P = 0.093$) and vesicle-mediated transport (GO:0016192, $P = 0.095$). In the COX-2$^{-/-}$ knockout of the E16 stage, 14 pathways were exclusively affected, 10 of which included at least one gene previously implicated in ASD (Table 3B, ASD genes in bold). The functional pathways containing an ASD gene include: Wnt signaling (KEGG: mmu04310, $P = 0.023$), DNA metabolic process (GO:0006259, $P = 0.031$), chordate embryonic development (GO:0043009, $P = 0.035$), skeletal system development (GO:0001501, $P = 0.036$), intracellular transport (GO:0046907, $P = 0.043$), axon guidance (KEGG: mmu04360, $P = 0.044$), regulation of translation (GO:0006417, $P = 0.075$), long-term depression (KEGG: mmu04730, $P = 0.089$) and negative regulation of apoptosis (GO:0043066, $P = 0.094$). Lastly, 10 pathways were determined to be in common between both COX-1$^{-/-}$ and COX-2$^{-/-}$ mouse models of the E16 stage (Table 3C, ASD genes in bold). From the common pathways, the 7 consisting of at least one ASD risk gene include: transmission of nerve impulse (GO:0019226, $P = 0.00034; 0.011$),

regulation of synaptic transmission (GO:0050804, P = 0.001; 0.071), cell cycle processes (GO:0007049, P = 0.045; 0.069), heart development (GO:0007507, P = 0.050; 0.071), cell motion/migration (GO:0006928; 0016477, P = 0.063; 0.088) and transcription (GO:0006350, P = 0.080; 0.097). Overall, these pathways, especially synaptic transmission which was found in both E16 COX$^{-/-}$ mice, are crucial for brain development and behaviour. Interestingly, we found Wnt signalling exclusively enriched with 8 genes in the COX-2$^{-/-}$ mice during E16.

At the E19 stage, 8 pathways were enriched in COX-1$^{-/-}$ mice, from these, 3 include at least one ASD risk gene (Table 4A, ASD genes in bold). The ASD gene containing pathways include transmission of nerve impulse (GO:0019226, P = 0.014), secretion (GO:0046903, P = 0.051) and induction of apoptosis (GO:0006917, P = 0.086). In the COX-2$^{-/-}$ mice, 4 of the 6 functional pathways included at least one ASD risk gene (Table 4B, ASD genes in bold), these include sensory perception of sound (GO:0007605, P = 0.014), lipid transport (GO:0006869, P = 0.040), tissue morphogenesis (GO:0048729, P = 0.066) and intracellular protein transport (GO:0006886, P = 0.099). In addition, there were three pathways enriched with critical neurological functions and ASD candidate genes that were common between COX-1$^{-/-}$ and COX-2$^{-/-}$ models at E19 (Table 4C, ASD genes in bold). These affected pathways are involved in lipid localization (GO:0010876, P = 0.046; 0.009), neural tube development (GO:0035295, P = 0.087; 0.029), and morphogenesis of branching structure (GO:0001763, P = 0.046; 0.048).

Overall, a greater number of pathways were enriched in both COX knockouts of the earlier stage of development during which neurogenesis is active in most regions of the prenatal brain. This is consistent with the greater number of genes differentially expressed in the E16 knockouts and provides further evidence that this earlier developmental stage may be more critical for normal COX-PGE$_2$ activity. Furthermore, most of the enriched pathways included

ASD risk genes, indicating that evidence for the potential role of aberrant COX-PGE$_2$ pathway in the pathogenesis of ASDs. With our previous research, enrichment of the Wnt signaling pathway in the COX-2$^{-/-}$ E16 model is of particular importance (this finding is further outlined in section 3.1.4. of the Results).

Table 3: Enriched GO:BP and KEGG pathways affected in the COX-1⁻/⁻ and COX-2⁻/⁻ mice at E16. The chart shows affected biological pathways (A) exclusive to COX-1⁻/⁻ E16, (B) COX-2⁻/⁻ E16 and (C) common between COX-1⁻/⁻ and 2⁻/⁻. The database category for GO and KEGG, Benjamini FDR corrected EASE score *P*-value, gene count and gene lists are listed. Bold genes are ones that were found in our subsequent AutDB analysis. Both COX⁻/⁻ enzymes exhibit important developmental pathways including LTP, synaptic transmission, cell division, Wnt signalling pathway (red), cell migration and axon guidance.

(A) COX-1⁻/⁻ E16 only			
Database Category	**Biological Pathways**	**Corrected *P*-value**	**Gene Count**	**Differentially Expressed Genes**
GOTERM_BP_FAT	Regulation of neurological system process (GO:0031644)	0.002	7	*SYP*, ***GRM5***, *GRIA2, NCDN, CSPG5, PPP3CA, CHRNA3*
GOTERM_BP_FAT	Regulation of cell morphogenesis (GO:0022604)	0.005	6	*EPB4.1L2, PALM, MAPT, CHRNA3, MYH10, THY1*
KEGG_PATHWAY	Long-term potentiation (mmu04720)	0.008	5	***GRM5***, *GRIA2*, ***CAMK4***, *CAMK2B, PPP3CA*
GOTERM_BP_FAT	Female gamete generation (GO:0007292)	0.026	4	*REC8, ZMIZ1, HEXB, ATM*
KEGG_PATHWAY	Adipocytokine signaling pathway (mmu04920)	0.041	4	*IRS2*, ***PRKAG2***, *ACSL6, CAMKK2*
GOTERM_BP_FAT	Secretion (GO:0046903)	0.047	7	*SYT4, HPS1*, ***NRXN1***, *VPS33A, CHRNA3*, ***EXOC6B***, *MYH10*
GOTERM_BP_FAT	Lipid biosynthetic process (GO:0008610)	0.047	8	*AGPAT5, INSIG2, HEXB*, ***PRKAG2***, *MBOAT2, PBX1, ALG9, DHCR24*
KEGG_PATHWAY	Viral myocarditis (mmu05416)	0.070	4	*ACTB, DAG1, CXADR, MYH10*
GOTERM_BP_FAT	Blood vessel development (GO:0001568)	0.071	7	***MEF2C***, ***SEMA5A***, *RECK, HIF1A, IL18, ZMIZ1, THY1*
GOTERM_BP_FAT	Regulation of kinase activity (GO:0043549)	0.073	6	*PKIG, DGKH, PRDX2, ZEB2, CHRNA3, THY1*
GOTERM_BP_FAT	Response to oxidative stress (GO:0006979)	0.085	4	*HIF1A, GPX8, PRDX2, DHCR24*
GOTERM_BP_FAT	Microtubule-based process (GO:0007017)	0.093	6	*SPC25*, ***KIF1B***, *MTAP2, MAPT*, ***TUBB6***, *HOOK3*
GOTERM_BP_FAT	Vesicle-mediated transport (GO:0016192)	0.095	10	*SYP, GRIA2, SYT4, CAP1, NAPB, VPS33A, HOOK3*, ***EXOC6B***, *MYH10, TRAPPC2*
(B) COX-2⁻/⁻ E16 only				
GOTERM_BP_FAT	Sulfur metabolic process (GO:0006790)	0.015	6	*ADI1, AHCY, SEPP1, DCN, TPMT, MGST1*
KEGG_PATHWAY	Wnt signaling pathway (mmu04310)	0.023	8	*SFRP1, CCND2, CSNK1E, SFRP2,* **GSK3B**, *PPP3CA, DAAM1, ACTB*
GOTERM_BP_FAT	Somitogenesis (GO:0001756)	0.028	4	*SFRP1, SFRP2, FOXC2, ZEB2*
GOTERM_BP_FAT	Regulation of protein complex disassembly (GO:0043244)	0.031	4	*MTAP2, MAPT, TMOD3, CLASP1*
GOTERM_BP_FAT	DNA metabolic process (GO:0006259)	0.031	13	*RAD23B, UBE2A, MELA, LOC100046025, ESCO2, MCM6,* ***RPA3***, *GIN1, UBE2N, BCL11B, RRM1, MLL1, NFIB*
GOTERM_BP_FAT	Chordate embryonic development (GO:0043009)	0.035	13	*NRP, UBE2A, HIF1A, SFRP1, SFRP2, ZMIZ1, TGFBR2,* ***TSC2***, *FOXC2, ZEB2, KEAP1, DLK1, MYH10*
GOTERM_BP_FAT	Skeletal system development (GO:0001501)	0.036	10	***MEF2C***, ***RAI1***, *HIF1A, HEXB, TGFBR2,* ***SOX5***, *FOXC2, DLK1, SOX6, COL5A2*
GOTERM_BP_FAT	Intracellular transport (GO:0046907)	0.043	13	*AP1B1, NAPB, HOOK3,* ***KIF1B***, ***SRPR***, ***GSK3B***, *ARCN1,* ***TSC2***, *PPP3CA, KPNA2, SRP9, TRAPPC2, MYH10*

KEGG_PATHWAY	Axon guidance (mmu04360)	0.044	6	*NGEF, GNAI1, PLXNA2, CFL2, **GSK3B**, PPP3CA*
KEGG_PATHWAY	Tight junction (mmu04530)	0.046	6	*ACTB, EPB4.1L2, EPB4.1L1, GNAI1, JAM3, MYH10*
GOTERM_BP_FAT	Regulation of translation (GO:0006417)	0.075	5	*TNRC6C, **RBM8A**, PAIP1, TIA1, SRP9*
KEGG_PATHWAY	P53 signaling pathway (mmu04115)	0.077	4	*CCND2, **TSC2**, CCNG1, CCNG2*
KEGG_PATHWAY	Long-term depression (mmu04730)	0.089	4	***GRM5**, GRIA2, GNAI1, GUCY1A3*
GOTERM_BP_FAT	Negative regulation of apoptosis (GO:0043066)	0.094	8	*BCL11B, **GSK3B**, **FCER1G**, FOXC2, PRDX2, **GLO1**, DNAJC5, DHCR24*
(C) Common pathways for E16 COX-1^{-/-} (top) and COX-2^{-/-}(bottom)				
GOTERM_BP_FAT	Gas transport (GO:0015669)	0.000	4	*HBB-BH1, HBB-B1, HBA-X, HBB-Y*
		0.001	4	*HBB-BH1, HBB-B2, HBA-X, HBB-Y*
GOTERM_BP_FAT	Transmission of nerve impulse (GO:0019226)	0.000	11	*SYP, **SCN1A**, **KIF1B**, GRIA2, LOC100047888, **CAMK4**, SYT4, HEXB, **NRXN1**, PPP3CA, CHRNA3*
		0.011	10	*SYT1, **SCN1A**, **KIF1B**, GRIA2, LOC100047888, HEXB, **NRXN1**, SV2A, PPP3CA, USP14*
GOTERM_BP_FAT	Regulation of synaptic transmission (GO:0050804)	0.001	7	*SYP, **GRM5**, GRIA2, NCDN, CSPG5, PPP3CA, CHRNA3*
		0.071	5	***GRM5**, GRIA2, NCDN, CSPG5, PPP3CA*
GOTERM_BP_FAT	Cell cycle process (GO:0022402/0007049)	0.045	10	*SPC25, REC8, SIN3A, ANAPC5, CAMK2B, UBE2I, PPP3CA, CCNG2, STAG2, MYH10*
		0.069	16	*SF1, CETN2, UBE2I, CCNG1, CCNG2, ESCO2, MCM6, MLF1, SPC25, REC8, CCND2, **GSK3B**, CLASP1, PPP3CA, STAG2, MYH10*
GOTERM_BP_FAT	Cytoskeleton organization (GO:0007010)	0.015	10	*EPB4.1L2, PALM, SPC25, EPB4.1L1, MTAP2, MAPT, CAP1, HOOK3, MYH10, THY1*
		0.017	12	*EPB4.1L2, PALM, SPC25, EPB4.1L1, NAV1, MTAP2, LIMCH1, MAPT, DAAM1, **CDC42BPB**, HOOK3, MYH10*
GOTERM_BP_FAT	Hemopoietic or lymphoid organ development (GO:0048534)	0.016	9	*EGR1, HIF1A, BCL11B, ADAM17, PBX1, PRDX2, HBB-B1, VPS33A, HBA-X*
		0.076	9	*HIF1A, BCL11B, TGFBR2, PRDX2, SOX6, HBB-B2, HBA-X, MLL1, MLF1*
GOTERM_BP_FAT	Chromosome segregation (GO:0007059)	0.041	4	*SPC25, REC8, UBE2I, STAG2*
		0.088	4	*SPC25, REC8, UBE2I, STAG2*
GOTERM_BP_FAT	Heart development (GO:0007507)	0.050	7	***MEF2C**, HIF1A, ZMIZ1, CXADR, ATM, CHRNA3, MYH10*
		0.071	8	***MEF2C**, HIF1A, ZMIZ1, TGFBR2, **TSC2**, FOXC2, SOX6, MYH10*
GOTERM_BP_FAT	Cell motion/migration (GO:0006928/0016477)	0.063	9	***SEMA5A**, NRP, **KLF7**, HIF1A, CNTN2, ADAM17, ZEB2, CAP1, MYH10*
		0.088	8	*NRP, HIF1A, NAV1, PLXNA2, CNTN2, **FCER1G**, ZEB2, MYH10*
GOTERM_BP_FAT	Transcription (GO:0006350)	0.080	28	*ITGB3BP, **MEF2C**, **BACH1**, BACH2, ZFP68, ZEB2, KEAP1, **TFAM**, SIN3A, BCL11B, ACTL6B, GTF3C2, TWISTNB, EGR1, **KLF7**, TAF6, SOX11, SNAPC3, HMG20A, **MYT1L**, HIF1A, UBTF, ZMIZ1, PBX1, TRAPPC2, NFIB, PBX4*
		0.097	36	***BACH1**, **MEF2C**, POU6F1, **ZMYND11**, BACH2, **SOX5**, ZEB2, KEAP1, MED23, SOX6, ZBTB17, 1810035L17RIK, MLF1, BCL11B, ZFP131, PHTF1, GTF3C2, MLL1, **KLF7**, SOX11, SNAPC3, SF1, ZFP606, LOC100046343, SREBF2, MCM6, **MYT1L**, ZFP286, HIF1A, EBF3, ZMIZ1, FOXC2, ST18, TRAPPC2, NFIB, PBX4*

Table 4: Enriched GO:BP and KEGG pathways affected in the COX-1$^{-/-}$ and COX-2$^{-/-}$ at E19. Pathways (A) exclusive to COX-1$^{-/-}$ E19, (B) exclusive to COX-2$^{-/-}$ E19 and (C) common between COX-1$^{-/-}$ and 2$^{-/-}$ are shown. For each pathway, the database category, Benjamini FDR corrected EASE score *P*-value, gene count and gene list is presented. Bolded are genes are ASD candidate genes found in our subsequent AutDB analysis. Both COX$^{-/-}$ enzymes exhibit important developmental pathways including regulation of transcription, nerve transmission, lipid biosynthesis, vesicle secretion and forebrain development.

Database Category	Biological Pathways	Corrected *P*-value	Gene Count	Differentially Expressed Genes
(A) COX-1$^{-/-}$ E19 only				
GOTERM_BP_FAT	Regulation of transcription from RNA polymerase II promoter (GO:0006357)	0.005	11	*TXNIP, EGR1, FOS, CEBPB, SOX11, NKX6-2, PKIG, NKX2-1, ST18, KLF4, PITX2*
GOTERM_BP_FAT	Glucose metabolic process (GO:0006006)	0.010	5	*PPP1CA, UEVLD, PHKB, PKM2, PYGB*
GOTERM_BP_FAT	Transmission of nerve impulse (GO:0019226)	0.014	6	***NRXN2**, **CAMK4**, **NRXN3**, NKX6-2, HEXB, CHRNA3*
KEGG_PATHWAY	Focal adhesion (mmu04510)	0.029	5	*ACTB, PPP1CA, GRLF1, CAPN2, COL5A2*
GOTERM_BP_FAT	Lipid biosynthetic process (GO:0008610)	0.031	6	*AGPAT5, LASS4, INSIG2, HEXB, MBOAT2, ALG9*
GOTERM_BP_FAT	Secretion (GO:0046903)	0.051	5	***NRXN2**, **NRXN3**, STXBP2, VPS33A, CHRNA3*
GOTERM_BP_FAT	Forebrain development (GO:0030900)	0.084	4	*FEZF1, GRLF1, NKX2-1, PITX2*
GOTERM_BP_FAT	Induction of apoptosis (GO:0006917)	0.086	4	*SERINC3, CEBPB, **APOE**, PHLDA3*
(B) COX-2$^{-/-}$ E19 only				
GOTERM_BP_FAT	Translation (GO:0006412)	0.003	8	*RPL30, RPS3A, MRPL55, WARS2, LARS2, IARS2, RPS2, RPL29*
GOTERM_BP_FAT	Sensory perception of sound (GO:0007605)	0.014	4	*MYO6, **CDKN1B**, HEXB, GJB2*
GOTERM_BP_FAT	Sulfur metabolic process (GO:0006790)	0.021	4	*ADI1, MTHFD1, AHCY, MGST1*
GOTERM_BP_FAT	Lipid transport (GO:0006869)	0.040	4	***SORL1**, ATP10D, GLTPD1, ATP8A1*
GOTERM_BP_FAT	Tissue morphogenesis (GO:0048729)	0.066	5	*SFRP1, **TSC2**, GRLF1, PBX1, PITX2*
GOTERM_BP_FAT	Intracellular protein transport (GO:0006886)	0.099	5	*MYO6, **SRPR**, ARCN1, **TSC2**, IPO9*
(C) Common pathways for E19 COX-1$^{-/-}$ (top line) and COX-2$^{-/-}$ (bottom line)				
GOTERM_BP_FAT	Lipid localization (GO:0010876)	0.046	4	***APOE**, HEXB, SCP2, GLTPD1*
		0.009	5	*HEXB, SORL1, ATP10D, GLTPD1, ATP8A1*
GOTERM_BP_FAT	Tube development (GO:0035295)	0.087	5	***SEMA5A**, PPP1CA, GRLF1, NKX2-1, PITX2*
		0.029	6	***SEMA5A**, LHX1, **TSC2**, GRLF1, PBX1, PITX2*
GOTERM_BP_FAT	Morphogenesis of a branching structure (GO:0001763)	0.046	4	***SEMA5A**, PPP1CA, SFRP1, PITX2*
		0.048	4	***SEMA5A**, SFRP1, PBX1, PITX2*

3.1.3. ASD risk genes in COX$^{-/-}$ mice

Due to the important role of the COX-PGE$_2$ pathway in the brain and ASD pathogenesis, we used AutDB and additional human genome wide ASD studies to identify ASD risk or candidate genes in our knockout models (see methods) (Table 5). At the E16 stage, 23 out of 229 differentially expressed genes were identified as ASD risk genes (19 down- and 4 up-regulated) in the COX-1$^{-/-}$ model, while 37 out of 323 differentially expressed genes (29 down- and 8 up-regulated) were identified as ASD candidate genes for the COX-2$^{-/-}$ model. During E19, 7 out of 134 differentially expressed genes were ASD risk genes (5 down- and 2 up-regulated) in the COX-1$^{-/-}$ model and 10 out of 148 differentially expressed genes (8 down- and 2 up-regulated) were identified as ASD risk genes in the COX-2$^{-/-}$ model. The enrichment of these ASD genes was verified using a hypergeometric tests (see methods) resulting in values of 9.68e-13, 1.24e-21, 4.18e-3 and 8.47e-5 for COX-1$^{-/-}$ E16, COX-2$^{-/-}$ E16, COX-1$^{-/-}$ E19 and COX-2$^{-/-}$ E19, respectively. These probabilities indicate that there is a very low chance that the number of ASD genes found in all conditions can be due to random sampling and thus ASD genes are enriched in these knockouts. Overall, most of the ASD genes identified were down-regulated with a greater number enriched in the COX knockouts, especially COX-2$^{-/-}$ mice, of the E16 developmental stage. Some of the genes that we found to be noteworthy are *MYT1L*, in both COX-1$^{-/-}$ mice; *TSC2*, in both COX-2$^{-/-}$ mice; *MEF2C*, in both E16 knockouts; and *MDGA2*, in both E16 knockouts. Taken together, knockout of COX enzymes, especially COX-2, results in aberrant ASD risk gene expression of ASD risk genes indicating that this knockout model is useful for the study of ASD.

Table 5: ASD associated genes differentially expressed in (A) COX-1$^{-/-}$ E16, (B) COX-2$^{-/-}$ E16, (C) COX-1$^{-/-}$ E19 and (D) COX-2$^{-/-}$ E19. Along with official gene symbol, gene name, fold change (FC), chromosome location and number of gene association studies are listed from data found in the AutDB along with our own research findings of GWAS in humans.

(A) COX-1$^{-/-}$ E16					
Official Gene Symbol	Gene Name	Fold Change	Human Cytoband	Number of Studies listed in AutDB and additional autism studies	DAVID Biological Process
MYT1L	Myelin transcription factor 1-like	-5.48	2p25.3	8(AutDB)	Transcription (GO:0006350)
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	-3.97	7q36.1	Voineagu et al. (2011)	Fatty acid biosynthetic process (GO:0006633)
MEF2C	myocyte enhancer factor 2C	-2.85	5q14	14(AutDB), Hu et al. (2009)	Vasculature development (GO:0001944) Transcription (GO:0006350) Apoptosis (GO:0006915)
GRM5	Glutamate receptor, metabotropic 5	-2.81	11q14.2-q14.3	3(AutDB)	Behavior (GO:0007610) Learning or memory (GO:0007611) Regulation of synaptic plasticity (GO:0048167)
B3GALT6	similar to UDP-Gal:betaGal beta 1,3-galactosyltransferase, polypeptide 6; UDP-Gal:betaGal beta 1,3-galactosyltransferase, polypeptide 6	-2.70	1p36.33	Nishimura et al. (2007)	Polysaccharide biosynthetic process (GO:0000271) Glycosylation (GO:0070085)
BACH1	BTB and CNC homology 1	-2.24	21q22.11	Voineagu et al. (2011)	Transcription (GO:0006350)
KIF1B	kinesin family member 1B	-1.97	1p36.2	Hu et al. (2006)	Synaptic transmission (GO:0007268) Neuromuscular synaptic transmission (GO:0007274) Anterograde axon cargo transport (GO:0008089)
MDGA2	MAM domain containing glycosylphosphatidylinositol anchor 2	-1.81	14q21.3	5(AutDB), Voineagu et al. (2011)	Spinal cord development (GO:0021510) Neuron differentiation (GO:0030182)
PCDHA7	Protocadherin alpha 7	-1.79	5q31	3(AutDB)	Cell adhesion (GO:0007155)
CAMK4	Calcium/calmodulin-dependent protein kinase IV	-1.75	5q21.3	1(AutDB)	Synaptic transmission (GO:0007268) Phosphorylation (GO:0016310) Neurological system process (GO:0050877) Nuclear transport (GO:0051169)
CDH8	cadherin 8, type 2	-1.72	16q22.1	7(AutDB)	Cell adhesion (GO:0007155)
NRXN1	neurexin 1	-1.68	2p16.3	55(AutDB), Voineagu et al. (2011)	Regulation of neurotransmitter levels (GO:0001505) Cell adhesion (GO:0007155) Synaptic transmission (GO:0007268) Synaptogenesis (GO:0007416) Biological adhesion (GO:0022610)
KCND2	Potassium voltage-gated channel, Shal-related subfamily, member 2	-1.67	7q31	3(AutDB)	Ion transport (GO:0006811)
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	-1.66	5p15.2	11(AutDB), Voineagu et al. (2011)	Cell motion (GO:0006928) Axonogenesis (GO:0007409) Neuron differentiation (GO:0030182) Tube development (GO:0035295)
KLF7	Kruppel-like factor 7 (ubiquitous)	-1.66	2q32	Nishimura et al. (2007)	Transcription (GO:0006350) Cell motion (GO:0006928) Axonogenesis (GO:0007409) Dendrite development (GO:0016358)

					Neuron differentiation (GO:0030182)
SCN1a	sodium channel, voltage-gated, type I, alpha subunit	-1.61	2q24.3	19(AutDB)	Regulation of action potential (GO:0001508) Ion transport (GO:0006811) Locomotory behavior (GO:0007626)
AQP4	aquaporin 4	-1.60	18q11.2-q12.1	Gregg et al. (2008)	Water transport (GO:0006833)
EXOC6B	Exocyst complex component 6B	-1.52	2p13.3-p13.2	2(AutDB)	Exocytosis (GO:0006887)
A2BP1	ataxin 2 binding protein 1	-1.51	16p13.3	Garbett et al. (2008)	RNA processing (GO:0006396)
RBM8A	RNA binding motif protein 8A	1.50	1q12	2(AutDB)	mRNA processing (GO:0006397)
ARIH1	ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1 (Drosophila)	1.53	15q24	Voineagu et al. (2011)	Proteolysis (GO:0006508)
TFAM	transcription factor A, mitochondrial	1.54	10q21	Gregg et al. (2008)	Transcription (GO:0006350)
TUBB6	tubulin, beta 6	1.59	18p11.21	Chen et al. (2015)	Microtubule-based process (GO:0007017)
(B) COX-2$^{-/-}$ E16					
MEF2C	myocyte enhancer factor 2C	-2.96	5q14	14(AutDB), Hu et al. (2009)	Vasculature development (GO:0001944) Transcription (GO:0006350) Apoptosis (GO:0006915)
GRM5	Glutamate receptor, metabotropic 5	-2.83	11q14.2-q14.3	3(AutDB), (Voineagu et al., 2011)	Behavior (GO:0007610) Learning or memory (GO:0007611) Regulation of synaptic plasticity (GO:0048167)
BACH1	BTB and CNC homology 1	-2.59	21q22.11	Hu et al. (2009)	Transcription (GO:0006350)
KLF7	Kruppel-like factor 7 (ubiquitous)	-2.18	2q32	Nishimura et al. (2007)	Transcription (GO:0006350) Cell motion (GO:0006928) Axonogenesis (GO:0007409) Dendrite development (GO:0016358) Neuron differentiation (GO:0030182)
FCER1G	Fc receptor, IgE, high affinity I, gamma polypeptide	-2.10	1q23	Ghahramani Seno et al. (2011)	Regulation of cytokine production (GO:0001817) Chemotaxis (GO:0006935) Immune response (GO:0006955) Behavior (GO:0007610) Regulation of apoptosis (GO:0042981)
TSC2	tuberous sclerosis 2	-2.07	16p13.3	16(AutDB)	Neural tube formation (GO:0001841) Chemotaxis (GO:0006935) Cell projection organization (GO:0030030)
SRPR	signal recognition particle receptor ('docking protein')	-2.00	11q24.3	Nishimura et al. (2007)	Protein localization (GO:0008104)
NRXN1	neurexin 1	-1.98	2p16.3	55(AutDB), Voineagu et al. (2011)	Regulation of neurotransmitter levels (GO:0001505) Cell adhesion (GO:0007155) Synaptic transmission (GO:0007268) Synaptogenesis (GO:0007416) Biological adhesion (GO:0022610)
PCDHA7	Protocadherin alpha 7	-1.92	5q31	3(AutDB)	Cell adhesion (GO:0007155)
RAI1	retinoic acid induced 1	-1.88	17p11.2	8(AutDB), Voineagu et al. (2011)	Skeletal system development (GO:0001501) Regulation of growth (GO:0040008) Regulation of transcription (GO:0045449)
B3GALT6	similar to UDP-Gal:betaGal beta 1,3-galactosyltransferase, polypeptide 6; UDP-Gal:betaGal beta 1,3-galactosyltransferase, polypeptide 6	-1.88	1p36.33	Voineagu et al. (2011)	Polysaccharide biosynthetic process (GO:0000271) Glycosylation (GO:0070085)
MDGA2	MAM domain containing glycosylphosphatidylinositol anchor 2	-1.87	14q21.3	5(AutDB), Voineagu et al. (2011)	Spinal cord development (GO:0021510) Neuron differentiation (GO:0030182)
SOX5	SRY (sex determining region Y)-box 5	-1.81	12p12.1	3(AutDB)	Transcription (GO:0006350) Regulation of neurogenesis (GO:0050767)
TNIK	TRAF2 and NCK interacting kinase	-1.81	3q26.2-q26.31	Ghahramani Seno et al. (2011)	Phosphorylation (GO:0016310)

TRIO	TrioÃ‚Â Rho guanine nucleotide exchange factorÃ‚Â	-1.77	5p15.2	5(AutDB), Hu et al. (2009)	Phosphorylation (Rho/Ras) (GO:0016310)
SCN1a	sodium channel, voltage-gated, type I, alpha subunit	-1.74	2q24.3	19(AutDB)	Regulation of action potential (GO:0001508) Ion transport (GO:0006811) Locomotory behavior (GO:0007626)
GPD2	Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	-1.73	2q24.1	3(AutDB)	Glucose metabolic process (GO:0006006)
GSK3B	Glycogen synthase kinase 3 beta	-1.72	3q13.3	2(AutDB)	Re-entry into mitotic cell cycle (GO:0000320) Wnt receptor signaling pathway (GO:0016055)
KCND2	Potassium voltage-gated channel, Shal-related subfamily, member 2	-1.71	7q31	3(AutDB)	Ion transport (GO:0006811)
KIF1B	kinesin family member 1B	-1.70	1p36.2	Garbett et al. (2008)	Synaptic transmission (GO:0007268) Neuromuscular synaptic transmission (GO:0007274) Anterograde axon cargo transport (GO:0008089)
CDH8	cadherin 8, type 2	-1.69	16q22.1	7(AutDB)	Cell adhesion (GO:0007155)
A2BP1	ataxin 2 binding protein 1	-1.69	16p13.3	Voineagu et al. (2011)	RNA processing (GO:0006396)
SETBP1	SET binding protein 1	-1.64	18q21.1	4(AutDB)	
GRIP1	glutamate receptor interacting protein 1	-1.64	12q14.3	9(AutDB)	Protein localization (GO:0008104)
RNF182	ring finger protein 182	-1.63	6p23	Voineagu et al. (2011)	Proteolysis (GO:0006508)
SPAG9	sperm associated antigen 9	-1.57	17q21.33	Nishimura et al. (2007)	Activation of MAPK activity (GO:0000187) Phosphorylation (GO:0016310)
CDC42B PB	CDC42 binding protein kinase beta (DMPK-like)Ã‚Â	-1.54	14q32.3	2(AutDB)	Cytoskeleton organization (GO:0007010) Phosphorylation (GO:0016310)
MYT1L	Myelin transcription factor 1-like	-1.53	2p25.3	8(AutDB)	Transcription (GO:0006350)
ZMYND11	Zinc finger, MYND-type containing 11	-1.52	10p14	3(AutDB)	Transcription (GO:0006350)
RBM8A	RNA binding motif protein 8A	1.50	1q12	2(AutDB)	mRNA processing (GO:0006397)
PRPF40a	PRP40 pre-mRNA processing factor 40 homolog A (yeast)	1.54	2q23.3	Hu et al. (2009)	mRNA metabolic process (GO:0016071)
RSAD2	radical S-adenosyl methionine domain containing 2	1.55	2p25.2	Ghahramani Seno et al. (2011)	Immune response (GO:0006955)
TMED2	transmembrane emp24 domain trafficking protein 2; predicted gene 10698; predicted gene 7318	1.58	12q24.31	Gregg et al. (2008)	Vesicle-mediated transport (GO:0016192)
TM9SF2	Transmembrane 9 Superfamily Member 2	1.61	13q32.3	Hu et al. (2009)	
TUBB6	tubulin, beta 6	1.71	18p11.21	Garbett et al. (2008)	Microtubule-based process (GO:0007017)
RPA3	predicted gene 6195; replication protein A3	1.78	7p22	Hu et al. (2009)	DNA replication (GO:0006260)
GLO1	glyoxalase I	2.01	6p21.3 -p21.1	9(AutDB)	Anti-apoptosis (GO:0006916)

(C) COX-1⁻ᐟ⁻ E19

MYT1L	Myelin transcription factor 1-like	-4.05	2p25.3	8(AutDB)	Transcription (GO:0006350)
B3GALT6	similar to UDP-Gal:betaGal beta 1,3-galactosyltransferase, polypeptide 6; UDP-Gal:betaGal beta 1,3-galactosyltransferase, polypeptide 6	-2.85	1p36.33	Voineagu et al. (2011)	Polysaccharide biosynthetic process (GO:0000271) Glycosylation (GO:0070085)
APOE	apolipoprotein E	-1.88	19q13.2,19q13.32	Hu et al. (2006)	Vasculature development (GO:0001944) Steroid catabolic process (GO:0006706) Cellular ion homeostasis (GO:0006873) Cholesterol homeostasis (GO:0042632) Regulation of apoptosis (GO:0042981)
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short	-1.60	5p15.2	11(AutDB)	Cell motion (GO:0006928) Axonogenesis (GO:0007409) Neuron differentiation (GO:0030182) Tube development (GO:0035295)

	cytoplasmic domain, (semaphorin) 5A				
CAMK4	Calcium/calmodulin-dependent protein kinase IV	-1.56	5q21.3	1(AutDB)	Synaptic transmission (GO:0007268) Phosphorylation (GO:0016310) Neurological system process (GO:0050877) Nuclear transport (GO:0051169)
NRXN3	neurexin 3	1.76	14q31	9(AutDB)	Regulation of neurotransmitter levels (GO:0001505) Synaptic transmission (GO:0007268) Synaptogenesis (GO:0007416)
NRXN2	neurexin 2	1.79	11q13	3(AutDB)	Regulation of neurotransmitter levels (GO:0001505) Synaptic transmission (GO:0007268) Synaptogenesis (GO:0007416)
(D) COX2 $^{-/-}$ E19					
FCER1G	Fc receptor, IgE, high affinity I, gamma polypeptide	-3.12	1q23	Ghahramani Seno et al. (2011)	Regulation of cytokine production (GO:0001817) Chemotaxis (GO:0006935) Immune response (GO:0006955) Behavior (GO:0007610) Regulation of apoptosis (GO:0042981)
B3GALT6	similar to UDP-Gal:betaGal beta 1,3-galactosyltransferase, polypeptide 6; UDP-Gal:betaGal beta 1,3-galactosyltransferase, polypeptide 6	-2.19	1p36.3 3	Voineagu et al. (2011)	Polysaccharide biosynthetic process (GO:0000271) Glycosylation (GO:0070085)
TSC2	tuberous sclerosis 2	-1.83	16p13. 3	16(AutDB)	Neural tube formation (GO:0001841) Chemotaxis (GO:0006935) Cell projection organization (GO:0030030)
UAP1	UDP-N-acetylglucosamine pyrophosphorylase 1	-1.80	1q23.3	Hu et al. (2006)	
SRPR	signal recognition particle receptor ('docking protein')	-1.76	11q24. 3	Nishimura et al. (2007)	Protein localization (GO:0008104)
BDH1	3-hydroxybutyrate dehydrogenase, type 1	-1.57	3q29	Garbett et al. (2008)	Oxidation reduction (GO:0055114)
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	-1.55	12p13. 1-p12	1(AutDB)	Regulation of cyclin-dependent protein kinase activity (GO:0000079) Ion transport (GO:0006811) Cell cycle (GO:0007049) Neurological system process (GO:0050877) Cognition (GO:0050890)
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	-1.55	5p15.2	11(AutDB)	Cell motion (GO:0006928) Axonogenesis (GO:0007409) Neuron differentiation (GO:0030182) Tube development (GO:0035295)
SORL1	similar to sortilin-related receptor, LDLR class A repeats-containing; sortilin-related receptor, LDLR class A repeats-containing	1.55	11q23. 2-q24.2	Hu et al. (2009)	Lipid transport (GO:0006869)
GLO1	glyoxalase I	1.90	6p21.3 -p21.1	9(AutDB)	Anti-apoptosis (GO:0006916)

3.1.4. Cross-talk between COX-PGE$_2$ and Wnt signalling pathways

Previous *in vitro* research in our lab has shown the presence of a cross-talk between COX/PGE$_2$ and the canonical Wnt pathway (Wong et al., 2014, Wong et al., 2016). Our most recent study of mice exposed to PGE$_2$ during early prenatal development has also shown this interaction existing in the developing brain (Rai-Bhogal, 2016). In this study, we found 8 differentially expressed genes that belong to the Wnt pathways exclusively in the COX-2$^{-/-}$ mouse model at E16 (Table 3B). Interestingly, these differentially expressed genes belong to the canonical Wnt pathway, planer cell polarity (PCP) and Wnt/Ca^{2+} pathways (Figure 5; indicated in blue). The three up-regulated genes included *secreted frizzled-related protein 1* and *2* (*SFRP1* and *2*), and *Dishevelled Associated Activator of Morphogenesis 1* (*DAAM1*). While *casein kinase 1 epsilon* (*CK1ε*), *glycogen synthase kinase-3 beta* (*GSK3β*), *cyclin D2* (*CCND2*), *β-actin* (*ACTβ*), and *protein phosphatase 3 catalytic subunit alpha* (*PPP3CA*; CaN) were down-regulated. These genes encode for proteins that play an important role in Wnt signal transduction. CK1ε and GSK-3β are involved in the activation of β-catenin in the canonical Wnt pathway; CCND2, is encoded by a target gene of the canonical Wnt pathway; DAAM1 is a key component of the PCP pathway; and SFRP1 and 2 are extracellular modulators if Wnt signalling. PPP3CA is one of the three isozymes of calcineurin A which forms calcineurin (CaN) heterodimers that are subunits of the calmodulin binding catalytic subunit belonging to the Wnt/Ca^{2+} pathway. Interestingly, we also found down-regulation of the gene encoding for β-actin (*ACTβ*), belonging to the focal adhesion pathway (Figure 5). AutDB and human GWAS studies were used to determined ASD genes belonging to the Wnt pathways. These ASD genes include Wnt1, Wnt2, GSK-3β, β-catenin, APC, Prickle 1/2, an isoform of TCF, and an isoform of PLC (Figure 5; indicated in red). Overall, these findings indicate that defects in COX-2/PGE$_2$ signalling can affect the expression of many Wnt target genes critical for early brain development.
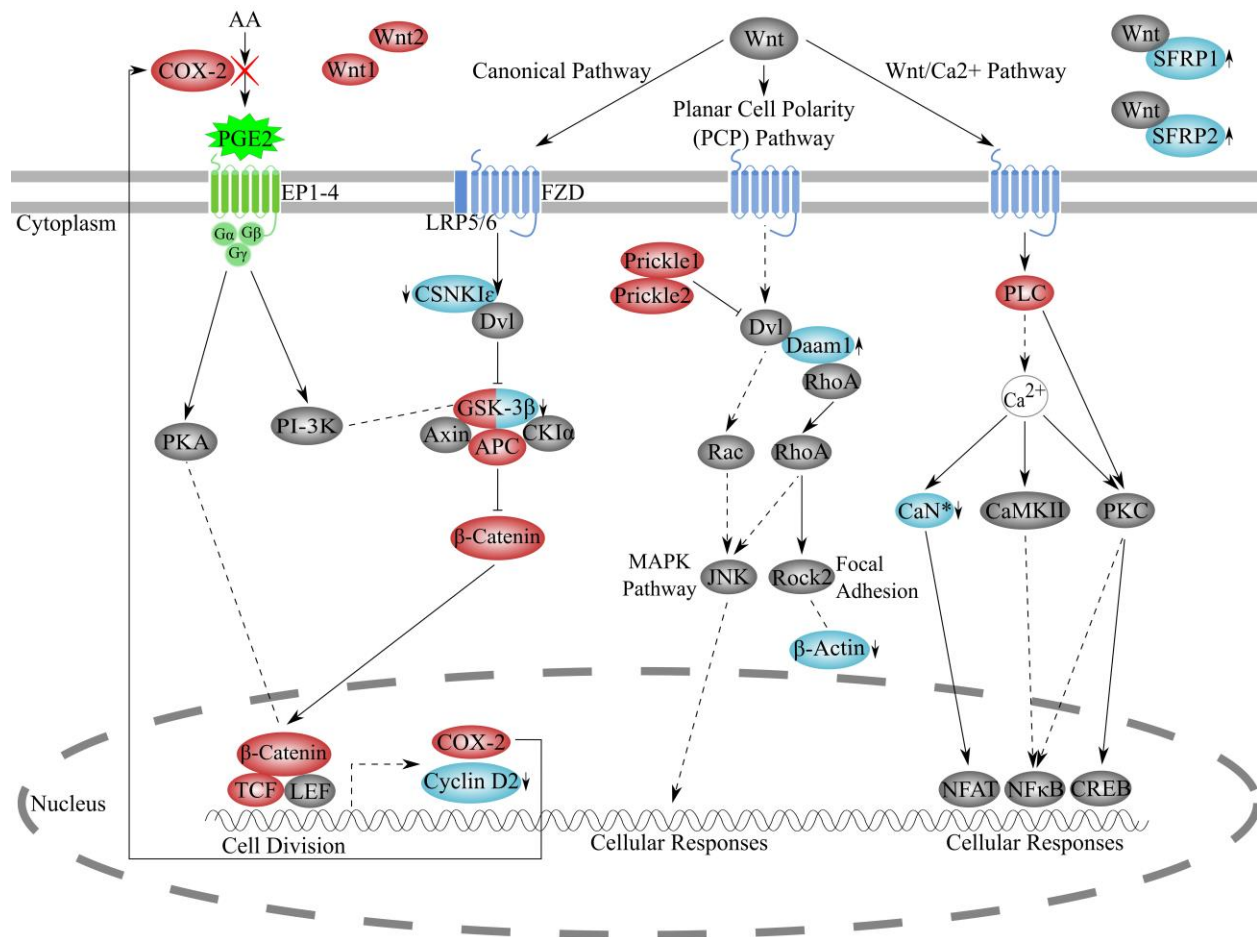
Figure 5: The interaction between Wnt and PGE$_2$ pathways with genes found in the COX-2$^{-/-}$ mice at E16. Affected genes found in COX-2$^{-/-}$ E16 are indicated in blue and genes associated with ASD (AutDB) are depicted in red. Here we show that the canonical, PCP and Wnt/Ca^{2+} pathway are affected due to COX-2 ablation. GSK-3β which is a crucial element of the canonical pathway has been affected in both the knockouts and implicated in ASD. Expression of some Wnt-target genes in the canonical pathway, including *Ptgs2* from Wong *et al*., 2014 is also shown. This figure was recreated from the KEGG Wnt Signalling Pathway (http://www.genome.jp/kegg-bin/show_pathway?hsa04310).

3.1.6. Validation of gene expression

To validate the microarray findings, we quantified expression of *MYT1L*, *GLO1* and *DAAM1* using qRT-PCR. We chose *MYT1L* and *GLO1* as they are both ASD risk genes and are highly differentially regulated in the microarray, while *DAAM1* is an important gene involved in Wnt pathways (Basu et al., 2009). qRT-PCR showed a similar pattern of down-regulated FC of the *MYT1L* gene as in the microarray analysis (Supplementary Table S1), in the COX-2$^{-/-}$ E16 (-1.40±0.04, *P* = 0.011) and COX-1$^{-/-}$ E19 (-1.66±0.01, *P* = 0.0004) knockouts. qRT-PCR also confirmed up-regulated expression of *GLO1* in both E16 and 19 stages of COX-2$^{-/-}$ with FCs of 2.28±0.05 (*P* = 0.0009) and 1.95±0.02 (*P* = 0.0001), respectively. Lastly, we also found an elevated expression pattern of *DAAM1* in the COX-1$^{-/-}$ E19 (1.23±0.21, *P* = 0.193) knockout, although not significantly different, and validated expression in the COX-2$^{-/-}$ E19 (1.67±0.19, *P* = 0.037) knockout. Overall, all knockout models, except COX-1$^{-/-}$ E16 which is to be further tested, were found to indicate qRT-PCR expression levels confirming the reliability of the microarray results.

3.1.5. Differential regulation of active forms of β-catenin in COX$^{-/-}$ mice

We have previously identified *in vitro* PGE$_2$ dependent activation of β-catenin, a major downstream regulator of the canonical Wnt pathway, in NE-4C stem cells (Wong et al., 2014). Along with the finding of differentially expressed Wnt genes, β-catenin was found to be activated in the brains of offspring maternally exposed to PGE$_2$ during prenatal development (Rai-Bhogal, 2016). These findings have clearly outlined a cross talk between the Wnt and PGE$_2$ pathways. To further investigate the cross-talk between COX-PGE$_2$ and the canonical Wnt pathway in the COX$^{-/-}$ mice, we determined the relative levels of two active forms of β-catenin using western blot analysis. β-catenin can be regulated with a complex set of post-translational modifications by several proteins. Phosphorylation of β-catenin at S552 by PKA results in

stabilization, activation, nuclear localization and Wnt pathway and target gene transcription (Taurin et al., 2006, Taurin et al., 2008). Furthermore, phosphorylation at Ser33, Ser37 and Thr41 (S33/S37/T41) by GSK-3β results in destabilization and degradation (Liu et al., 2002, Kimelman and Xu, 2006), thus we quantified both phospho (S552) and non-phospho (S33/S37/T41) forms of active β-catenin.

Among $COX^{-/-}$ mice of the E16 stage, we found a significant increase in FC of phospho β-catenin (S552) in the $COX-2^{-/-}$ mouse model. More specifically, while $COX-1^{-/-}$ did not show any change, we found phospho β-catenin (S552) to be up-regulated by a factor of 4.49 ± 0.13 ($P = 0.0015$) in the $COX-2^{-/-}$ mouse model compared to WT mice (Figure 6A). In contrast, non-phospho β-catenin levels were unchanged compared to WT for both $COX^{-/-}$ models at the E16 stage (Figure 6B). In the E19 stage, phospho β-catenin (S552) levels were unchanged in the $COX-1^{-/-}$ model, while a significant decrease in the $COX-2^{-/-}$ mouse model was observed with a FC of 0.52 ± 0.071 ($P = 0.022$) (Figure 7A). Non-phospho β-catenin levels in the E19 stage indicated a significant increase in the $COX-1^{-/-}$ mice with FC of 2.05 ± 0.14 ($P = 0.017$), while $COX-2^{-/-}$ indicated no change compared to WT (Figure 7B). These results indicate an altered level of PKA dependent phospho β-catenin (S552) in the brain of $COX-2^{-/-}$ mice. More specifically, in the $COX-2^{-/-}$ mice, phospho β-catenin levels were sharply elevated during E16 and decreased during E19, compared to the respective WT stages of development. These findings further add to our previous *in vitro* and *in vivo* studies involving PGE_2 exposure which have shown an interaction between $COX-PGE_2$ and the canonical Wnt pathway (Wong et al., 2014, Rai-Bhogal, 2016). In the COX knockout models, we have shown that the interaction is apparent in $COX-2^{-/-}$ mice at the E16 stage.
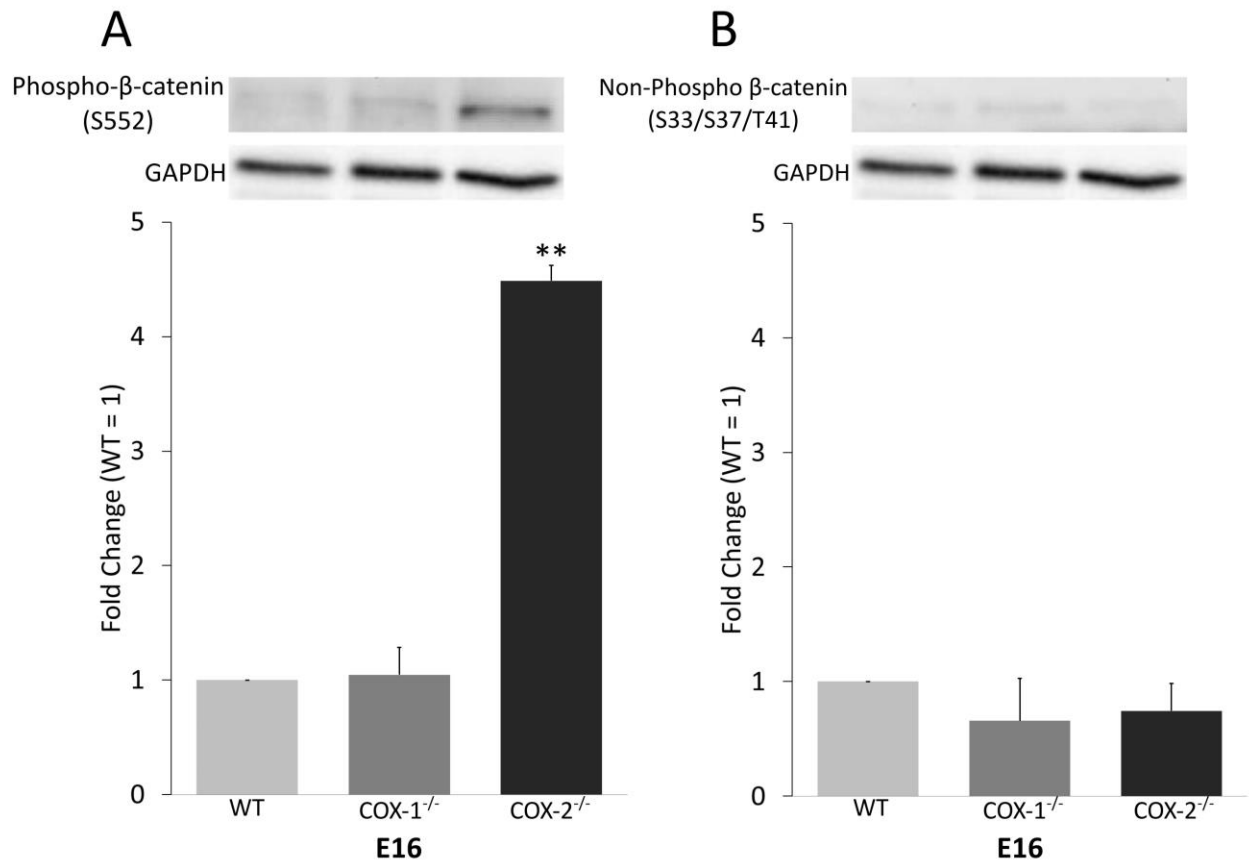
Figure 6: Levels of phospho β-catenin (S552) and non-phospho β-catenin (S33/S37/T41) in both COX-1$^{-/-}$ and 2$^{-/-}$ mice models, compared to WT, at the E16 stage. A marked increase in PKA dependent phospho β-catenin (S552) by the FC of 4.49±0.13 (P = 0.0015) in the COX-2$^{-/-}$ mice was observed (A). While there was no change in non-phospho β-catenin. Data shown as mean±SEM of 3 independent experiments (N = 3) with statistical significance represented by *P < 0.05 and **P < 0.01.
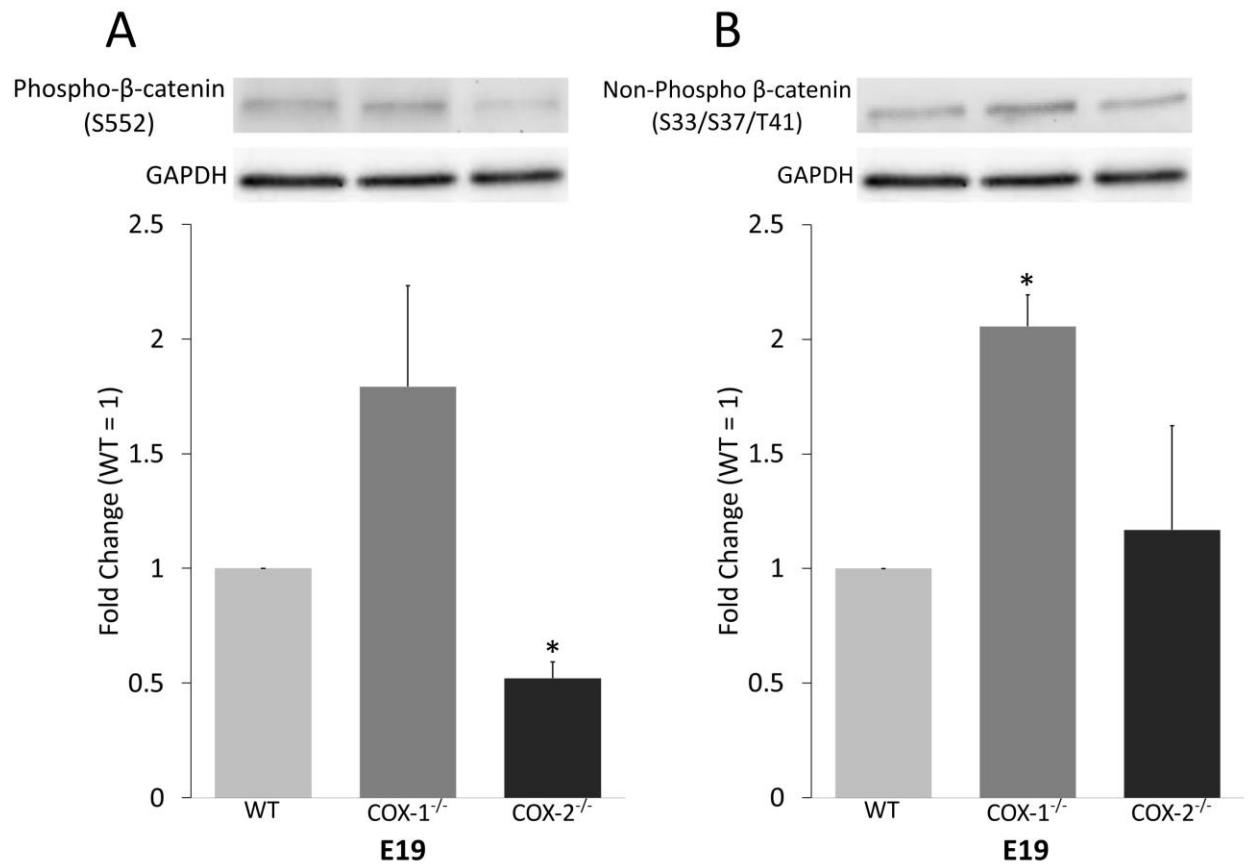
Figure 7: Levels of phospho β-catenin (S552) and non-phospho β-catenin (S33/S37/T41), in both COX-1-/- and 2-/- mice models, compared to WT, during E19. While there was no significant change for COX-1$^{-/-}$; a reduced level of phospho β-catenin (S552) by FC of 0.52±0.071 ($P = 0.022$) was observed for the COX-2$^{-/-}$ mice (A). Non-phospho β-catenin level was altered in the COX-1$^{-/-}$ mice with FC of 2.05±0.14 ($P = 0.017$), with no change observed for COX-2$^{-/-}$ (B). Data shown as mean±SEM of 3 independent experiments (N = 3) with statistical significance represented by *$P < 0.05$ and **$P < 0.01$.

3.2. Study 2

3.2.1. Gene expression in undifferentiated NE-4C stem cells

Although transcription of genes during development is tissue and cell specific, we aimed to examine the expression level of selected genes from our microarray study, in our neuroectodermal NE-4C stem cell models treated with PGE_2. We carried out qRT-PCR in our undifferentiated and differentiated NE-4C cell model treated with 1 µM PGE_2. The genes we selected have been outlined above and were chosen due to their association with ASD, and thus their importance for neuronal development, or their involvement in the Wnt pathway. The genes quantified for this study included: *NRXN1*, *NRXN3*, *GLO1*, *GRM5*, *DAAM1* and *SOX11*. In the NE-4C stem cell model treated with PGE_2 these genes were also differentially regulated. We found that *NRXN1*, *NRXN3*, *GLO1* and *GRM5* were all down-regulated compared to untreated cells with RQ values 0.63±0.10 ($P = 0.035$), 0.57±0.07 ($P = 0.013$), 0.70±0.08 ($P = 0.037$) and 0.38±0.17 ($P = 0.033$), respectively (Figure 8). *DAAM1* and *SOX11* expression was lower but not statistically different from the untreated NE-4C stem cells. The results presented have been normalized to the geometric mean of the housekeeping genes *HPRT* and *PGK1* which were not changed with PGE_2 treatment (Figure 8). Thus, the changes in expression of selected genes was due to the PGE_2 treatment. Although the regulation of gene expression may be tissue and cell specific, these results indicate that an increased level of PGE_2 in our *in vitro* model can also result in differential expression of genes that we found to be altered in the $COX^{-/-}$ microarray study. These results provide further evidence for COX/PGE_2 signalling modulating the expression of important neurodevelopmental genes in undifferentiated NE-4C stem cells.
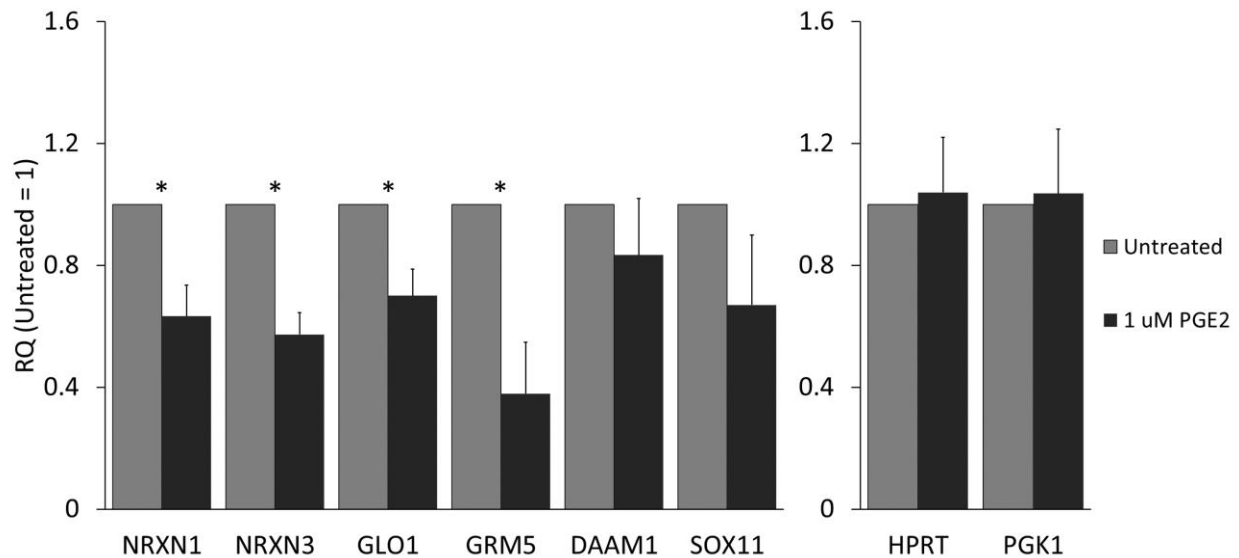
Figure 8: Differential gene expression in untreated and 1 μM PGE₂ treated NE-4C stem cells. A statistically significant down-regulation was observed for *NRXN1*, *NRXN3*, *GLO1* and *GRM5* with RQs of 0.63±0.10 (P = 0.035), 0.57±0.07 (P = 0.013), 0.70±0.08 (P = 0.037) and 0.38±0.17 (P = 0.033), respectively. *Daam1* and *Sox11* along with housekeeping controls, *HPRT* and *PGK1*, were not significantly different. Data shown as mean±SEM of 3 independent experiments (N = 3) with statistical significance represented by *P < 0.05 and **P < 0.01.

3.2.2. Gene expression in neuronally differentiated NE-4C stem cells

We also wanted to determine whether PGE_2 influences expression level of selected genes (as described in the previous section) in neuronally differentiated NE-4C stem cells. Differentiation of NE-4C stem cells has been carried out previously in our lab (Wong et al., 2016). The cells were treated with PGE_2 at the onset of differentiation (see methods) and on Day 8 the cells were confirmed to be fully differentiated into neurons in both treated and untreated conditions (Wong et al., 2016). In this study, as with the undifferentiated NE-4C cells, we studied *NRXN1*, *NRXN3*, *GLO1*, *GRM5*, *DAAM1* and *SOX11*. From these, we found statistically significant changes in expression level of *NRXN1* and *GLO1* in the neuronal cells treated with PGE_2 (Figure 9). In contrast to undifferentiated stem cells, *NRXN1* and *GLO1* were both up-regulated with an RQ of 1.27 ± 0.03 ($P = 0.0063$) and 1.23 ± 0.04 ($P = 0.016$), respectively. Along with the remaining genes, the housekeeping genes were stable between the treated and untreated neuronal cells (Figure 9). Since the housekeeping genes were not significantly different between treatments, the results were taken to be reliable. Taken together, these results further indicate that PGE_2 signalling can modulate gene expression of important developmental genes in neuronal cells, providing implications for the developing brain.
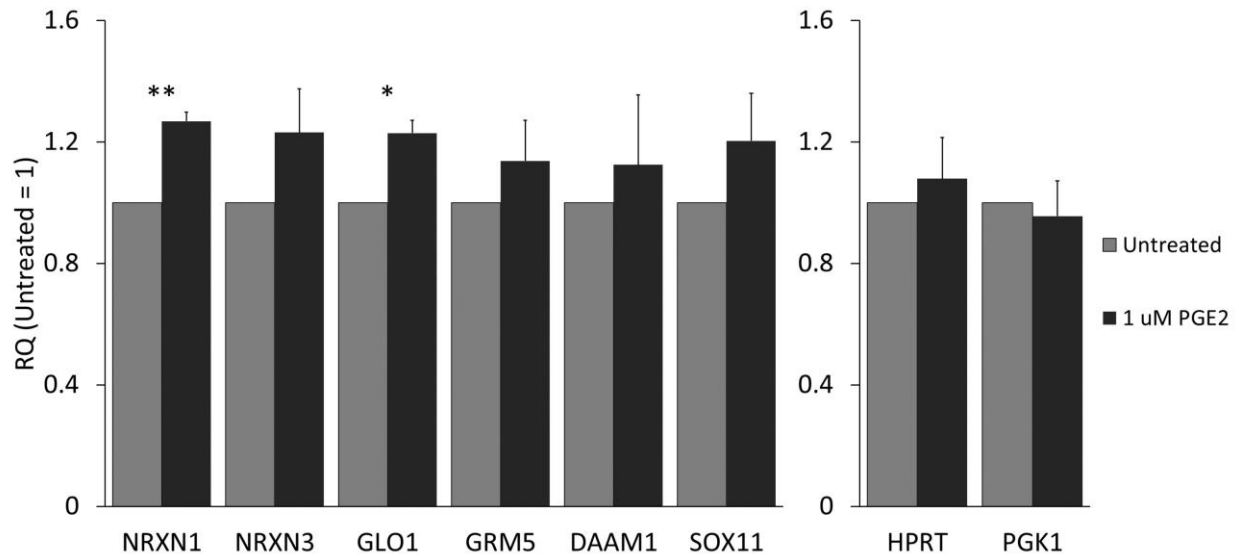
Figure 9: Gene expression in neuronally differentiated NE-4C stem cells. *NRXN1* indicated a significant 1.27±0.03 fold increase ($P = 0.0063$) compared to untreated neuronal cells. Similarly, *GLO1* expression was also higher with 1.23±0.04 ($P = 0.016$) increase in neuronal cells compared to untreated neuronal cells. *NRXN3, GRM5, DAAM1* and *SOX11* indicated no change between untreated and treated neuronal cells. Housekeeping genes indicated no change. Data shown as mean±SEM of 3 independent experiments ($N = 3$) with statistical significance represented by *$P < 0.05$ and **$P < 0.01$.

4.0. DISCUSSION

4.1. Study 1

Mounting evidence suggests that lipid signalling is an important component of brain development and pathogenesis of ASDs (Tamiji and Crawford, 2010b, Ning et al., 2015, Wong et al., 2015). Environmentally or genetically induced abnormalities in the activity of PGE_2, which is the most abundant prostanoid in the brain, have been implicated in ASD. Levels of essential FAs and lipid mediators have consistently been found to be increased or decreased in the blood plasma of children with ASD, which can potentially resulting in abnormal levels of PGE_2 (Tamiji and Crawford, 2010b, El-Ansary and Al-Ayadhi, 2012, Brigandi et al., 2015). Prenatal exposure to misoprostol, a PGE_2 analogue, has been found to result in symptoms of ASD in children (Bandim et al., 2003). Furthermore, polymorphisms in the *PTGS2* gene, which encodes for COX-2, have been enriched in children with ASDs (Yoo et al., 2008). While COX enzymes have distinct roles in the brain, both are responsible for the production of PGE_2 and both may play an important role in brain development. In addition to reduced levels of FAs in the plasma of children with ASD, common over the counter NSAIDs also act by inhibiting COX enzymes, thus it is important to understand cellular events that may arise from reduced COX activity (Silberstein and Stirpe, 2014). Here, we used $COX^{-/-}$ mice offspring as an experimental model of down-regulated PGE_2 and ASD to characterize the effects of abnormal PGE_2 signalling on gene expression during critical periods of prenatal brain development (Ayoub et al., 2004, Bosetti et al., 2004).

In this study, we examined global gene expression during prenatal days E16 and 19 in male mouse models lacking either COX-1 or COX-2 enzymes. Since abnormalities in the COX-PGE_2 signalling pathway have been associated with ASD, we hypothesized that many essential ASD associated developmental genes would be dysregulated in the brain of animals lacking

COX enzymes. We found that COX enzyme ablation at the earlier stage of E16 during which neurogenesis occurs has a greater impact on gene expression than the later stage (E19). This was demonstrated not only by the greater number of genes differentially expressed in the E16 mice compared to E19, but also by the greater portion of genes down-regulated in the E16 mice. Furthermore, COX-2 disruption resulted in a greater impact on gene expression compared to COX-1, especially for E16. Thus, this impact during E16 in the COX-2$^{-/-}$ mice also resulted in a greater number of biological pathways and ASD risk genes found. Interestingly, we have also shown the existence of a cross talk between the PGE$_2$ and Wnt pathways via activation of β-catenin exclusively in the COX-2$^{-/-}$ mouse model during E16. Taken together, these findings suggest that E16 spans a more critical period for disruption of normal COX-PGE$_2$ function than E19 and COX-2$^{-/-}$ plays a more important developmental role than COX-1$^{-/-}$. There are several reasons for why the distinction between the COX enzymes exists.

Although PGE$_2$ is the major prostanoid synthesized in the brain, due to marked functional and cellular differences between the two COX isozymes, the differences we have observed in our knockout mice are not surprising. Among many distinguishing characteristics of the COX enzymes, inducibility has been well described; with COX-1 being primarily constituent in microglia and vascular cells (Hoozemans et al., 2001, Amateau and McCarthy, 2004, Garcia-Bueno et al., 2009, Kirkby et al., 2012). Conversely, COX-2 is inducible except in neuronal cells, with subcellular localization in synapses of key brain regions, including cerebral cortex, hippocampus and amygdala, especially during early development (Vane et al., 1998, Maslinska et al., 1999, Wang et al., 2005, Hewett et al., 2006, Aid and Bosetti, 2011). In addition, Vazquez-Tello et al. (2004) have found enzymes important for downstream PGE$_2$ synthesis subcellularly colocalize with COX-1 in microsomal vesicles or COX-2 in both microsomal and

nuclear/perinuclear fractions of brain cells. Thus, it is possible that COX-1 may provide PGE_2

for local paracrine signalling, while COX-2 may produce PGE_2 for intracellular regulation of

gene expression through EP receptors present on intracellular membranes (Vazquez-Tello et al.,

2004, Zhu et al., 2006). These localization studies and their implications for the distinct roles of

the two COX enzymes may explain why we found a greater impact on gene expression in the

$COX-2^{-/-}$ mouse model compared to $COX-1^{-/-}$. Furthermore, the constitutive expression of COX-

$2^{-/-}$ in neuronal cells exclusively suggests that the changes we found in the $COX-2^{-/-}$ mice are due

to gene expression changes in developing neurons and not microglia. In addition, the differences

in localization and our results also suggest that the COX enzymes are not redundant in their

function and that knockout of only one COX enzyme and not both is still a relevant model to

study. However, despite these major differences, we did find some functional overlap between

the two COX enzymes.

The dysfunction of gene expression and pathways found in both $COX^{-/-}$ mouse models

indicate the importance of both COX enzymes during prenatal development. Our Venn diagram

analysis indicating many genes in common between the $COX^{-/-}$ mouse models during E16 and

E19. Specifically, the pathways involved in synaptic development were found to be effected in

both knockouts of the E16 developmental stage. Taken together, these results have shown that

both COX/PGE_2 signalling pathways are capable of regulating genes that carry out the same

biological function during brain development. This is especially true for the more critical day of

E16 with 10 pathways in common, while E19 indicated 3 pathways in common.

4.1.1. Syndromic ASD genes
Many of the genes and pathways found in this study are important for neuronal

development and implicated in ASD pathogenesis. Of interest were *MYT1L*, in both $COX-1^{-/-}$

mice; and *TSC2*, in both $COX-2^{-/-}$ mice (Basu et al., 2009). These genes were considerably

down-regulated in their respective model systems. MYT1L is a protein predominantly expressed in neurons and homologous to MYT1 which is an important TF for the regulation of myelin formation (Kim and Hudson, 1992). Mutations in this gene have been implicated in mental retardation, schizophrenia and ASD-like behaviour (Gruchy et al., 2007, Bonaglia et al., 2009, Meyer et al., 2012). Mutation of *TSC2* and *1* result in Tuberous Sclerosis Complex (TSC) which is a severe disorder involving intellectual disability, seizures, disorder in multiple organs, including benign tumours in the brain, and ASD like behaviour (Davis et al., 2015). The prevalence of ASD in TSC cases ranges from 26-50% (Jeste et al., 2008, Leclezio and de Vries, 2015). Interestingly, the TSC proteins are important components of the mechanistic target of rapamycin (mTOR) signalling pathway which regulates synaptic plasticity and memory as it responds to the signals coming from neuronal surface receptors (Lasarge and Danzer, 2014). Thus, ASDs in individuals with TSC involves neuronal specific and synaptic associated pathways found in our analysis of the $COX^{-/-}$ knockout mice transcriptome. Indeed, several studies, involving similar functional analyses of human or rodent ASD associated samples or gene lists, have indicated pathways found in our knockout models (Anney et al., 2011, Kumar et al., 2011, Sgado et al., 2013, An et al., 2014, Cristino et al., 2014, Ning et al., 2015). Here, we will discuss current research and implications of synaptic development and transmission along with Wnt pathways enriched in our analysis, which involved many other ASD associated genes of interest.

4.1.2. Synaptic development and transmission

Abnormal neuronal transmission found in both $COX^{-/-}$ mouse models at E16 has also been characterized in ASDs as abnormal synaptic transmission is considered a hallmark of ASD neuro-pathophysiology (Bourgeron, 2009, Ebert and Greenberg, 2013, Sala et al., 2015, Lin et al., 2016, Liu et al., 2016). While we found both COX enzymes to alter synaptic transmission during

the E16 stage they are both distinct in the way they can alter synaptic development and transmission (Chen et al., 2002, Yoo et al., 2008). COX-1 is constitutively expressed in microglial cells in the brain, these cells are highly involved in the early synaptic development and pruning, as well as synaptic function (Hoozemans et al., 2001, Roumier et al., 2004, Roumier et al., 2008, Paolicelli et al., 2011, Schafer et al., 2013). Furthermore, in adult mouse models, research indicating PGE_2 resulting from COX-1, not COX-2, with induced hippocampal inflammation is responsible for contextual memory impairment, a process which requires normal synaptic transmission (Matousek et al., 2010). In contrast, COX-2 is constitutively expressed in excitatory neuronal cells of the brain and localized in the post-synaptic buds of dendrites and developing dendritic spines (Kaufmann et al., 1996). With direct experimental techniques, COX-2 has consistently been found to play an important role in synaptic transmission and plasticity, predominantly through the production of PGE_2 (Chen et al., 2002, Chen and Bazan, 2005, Sang and Chen, 2006). Synaptic transmission is thought to be important for postnatal life when sensory stimulation and learning begins, however synaptic activity in the prenatal brain also occurs and is considered crucial for the formation of neuronal connections for proper development (Moody and Bosma, 2005, Moore et al., 2011). Taken together, both COX enzymes play crucial roles in synaptic transmission and, here, we have provided further evidence in the prenatal mouse brain lacking either COX enzyme. It is important to outline the pathways we found in our microarray analysis and their implications for neuronal development and ASD pathogenesis.

In the E16 stage of development, several important regulatory mechanisms were enriched, including long-term potentiation (KEGG:mmu04720), in the COX-1^{-/-} model; and long-term depression (KEGG:mmu04730) and axon guidance (KEGG:mmu04360) in the COX-2^{-/-} model.

Additionally, regulation of nerve impulse (GO:0019226) and synaptic transmission (GO:0050804) were enriched in both E16 COX$^{-/-}$ models. While we have shown cell culture neurite retraction induced by PGE$_2$ treatment in previous findings, this study indicates an *in vivo* link between COX-2 mediated PGE$_2$ and axon guidance (Tamiji and Crawford, 2010b). Several synaptic genes differentially expressed in the E16 knockouts have been implicated in ASDs, including *GRM5*, *calcium/calmodulin dependent protein kinase IV (CAMK4)*, *glycogen synthase kinase 3 beta (GSK3B)*, *sodium voltage-gated channel alpha subunit 1 (SCN1A)*, *kinesin family member 1B (KIF1B)*, and *neurexin 1 (NRXN1)*. In the pre-birth stage of E19, regulation of nerve impulse (GO:0019226) and secretion (GO:0046903) pathways were enriched in COX-1$^{-/-}$ mice with ASD-risk genes including *CAMK4, neurexin 2 (NRXN2)* and *neurexin 3 (NRXN3)* differentially expressed. The enrichment of pathways relating to synaptic development and function, and corresponding genes, indicates the significant role of COX enzymes during brain development. Next, we will outline some of the important genes outlined here.

GRM5, *encoding* for metabotropic glutamate receptor 5 (mGluR5), was considerably down-regulated in our E16 knockout mice and is of particular importance as it is not only crucial for brain development but also strongly associated with ASD pathophysiology (Reichelt et al., 2012, Zantomio et al., 2015). Interestingly, in a computer model that uses genomic SNP data to predict whether a child has ASD, *GRM5* was one of the three genes that were the most critical for the accurate prediction of a positive ASD diagnosis later in life (Skafidas et al., 2014). *GRM5* expression levels and mGluR5 positive neurons have been shown to be lower in the prefrontal cortex of individuals with ASDs (Chana et al., 2015). Furthermore, microglial density was significantly increased in *GRM5*$^{-/-}$ mice compared to controls (Chana et al., 2015). Functional variants of the *GRM5* gene have also been found to be enriched in 290 non-syndromic ASD

cases compared to 300 controls (Kelleher et al., 2012). It is important to note that while neurotransmission does not directly involve metabotropic receptors, such as mGluR5, they are integral for regulating and modulating neurotransmission (Wijetunge et al., 2008, She et al., 2009, Loerwald et al., 2015). A microarray study of post-mortem ASD cerebellum particularly found abnormal glutamate neurotransmission (Purcell et al., 2001). The normal development of functional neurons and the inhibitory/excitatory balance requires a crucial group of proteins, belonging to the NRXN-NLGN-SHANK pathway, which have consistently been implicated in ASD pathophysiology and have been implicated in our COX$^{-/-}$ study (Autism Genome Project et al., 2007, Dalva et al., 2007, Durand et al., 2007, Peca et al., 2011, Yang et al., 2012).

Several genes that have been differentially expressed in our COX$^{-/-}$ mice models belong to the NRXN-NLGN-SHANK pathway which is very important for synaptic development and function. *NRXN1*, which was highly down-regulated in our E16 mouse models, and other synaptic specific CAMs differentially expressed in our microarray have been implicated in ASD pathogenesis (Zoghbi, 2003, Garber, 2007). While *NRXN2* and *3* have some associations with ASD pathogenesis, significant and mounting evidence has strongly implicated *NRXN1* as an ASD susceptibility gene (Autism Genome Project et al., 2007, Gauthier et al., 2011, Reichelt et al., 2012, Vaags et al., 2012, Bena et al., 2013, Chen et al., 2014b). Deletions in the *NRXN1* gene have resulted in syndromic ASD cases displaying intellectual disability, severe language delay and seizures (Bena et al., 2013). Additional CAMs found in our knockouts include *CDH8*, *PCDHA7* and contactin 2 (*CNTN2*). *CNTN2* was differentially expressed in both COX knockout models of both stages of development, but it has not particularly been identified as an ASD candidate gene. However, CNTN2 interacts with CNTNAP2, which is a strong ASD candidate protein of the NRXN family (Arking et al., 2008, Bakkaloglu et al., 2008). Thus, abnormal

expression of the *CNTN2* gene may alter the function of CNTNAP2, resulting in abnormal neuronal development. Taken together, these differentially expressed genes code for proteins that are key for the NRXN-NLGN-SHANK pathway and proper synaptic development. Thus, these results provide further evidence supporting the function of COX-PGE$_2$ in the regulation of synaptic development and function, which is vital for early brain development and a hallmark of ASD pathogenesis (Chaudhury et al., 2016).

4.1.3. COX-PGE$_2$ and Wnt signalling

One of the more interesting findings in our microarray data is the enrichment the Wnt signalling pathway in the COX-2$^{-/-}$ knockout at the E16 stage. The interaction between COX/PGE$_2$ signalling and Wnt signalling has been characterized *in vitro* in non-neuronal cells (Yoshida et al., 2013, Li et al., 2014, Hiyama et al., 2015), and *in vivo* studies of zebrafish (Goessling et al., 2009, North et al., 2010). Interestingly, a PGE$_2$ dose dependent effect on Wnt signalling has also been described in osteoblast-lineage cells, with a high and low dose reducing and increasing Wnt transcription, respectively (Liu et al., 2010). Furthermore, our lab was the first to demonstrated the cross-talk between the PGE$_2$ and Wnt signalling pathways within the context of neuronal development in NE-4C neuronal stem cells and the developing mouse brain (Wong et al., 2014, Rai-Bhogal, 2016, Wong et al., 2016). This interaction has primarily been found to occur through the activity of PKA and PI-3K which are the main downstream kinases of PGE$_2$ signalling (Sheng et al., 2001, Fujino et al., 2002, Castellone et al., 2005). Using PKA and PI-3K inhibitors we have shown that greater attenuation of Wnt induced changes in cell behaviour result from PKA inhibition than PI-3K inhibition (Wong et al., 2014). Thus, PKA has a greater role in PGE$_2$ induced Wnt modulation than PI-3K in NE-4C cells (Wong et al., 2014). Interestingly, our previous *in vivo* study indicated an elevated level of phospho β-catenin (S552) in the E16 brain of mice prenatally exposed to PGE$_2$ (Rai-Bhogal, 2016). The evidence of PGE$_2$

modulating the Wnt pathway emphasizes the importance of maintaining normal lipid biochemistry during prenatal and early life.

The significance of finding the Wnt pathway enriched in our COX-2$^{-/-}$ mouse model during the E16 stage of prenatal development is highlighted by the importance of the Wnt pathway during development. The Wnt signalling pathway is responsible for global developmental patterning, particularly anterior-posterior patterning of the embryo (Arkell et al., 2013) and neural tube formaton (Ciani and Salinas, 2005, Ille and Sommer, 2005). The Wnt pathway also regulates cell fate determination, cell proliferation, neuronal migration, differentiation, axon development, dendritic spine development and synaptic development (Wassink et al., 2001, Rosso et al., 2005, Chen et al., 2006, Marui et al., 2010, Kalkman, 2012, Zhang et al., 2014). Interestingly, along with the Wnt signalling it self, neural migration, axon guidance and synaptic function have not only been effected in our COX knockout models, but also in individuals with ASD (Watts, 2008, Okerlund and Cheyette, 2011, Zhang et al., 2014). In this study, differentially expressed genes belonging to the Wnt pathway have provided further evidence for the interaction between COX-PGE$_2$ and Wnt signalling pathways.

Our previous findings mentioned above show that PGE$_2$ signalling can interfere specifically with the Wnt canonical pathway. The current study revealed for the first time that PGE$_2$ signalling can affect all three Wnt pathways. Altered function of Wnt signalling was revealed in the COX-2$^{-/-}$ model at the E16 developmental stage through the dysregulation of 8 genes that belong to either the canonical, PCP or Wnt/Ca^{2+} pathways (Figure 5). The genes *casein kinase 1 epsilon* (*CK1ε*), *glycogen synthase kinase-3 beta* (*GSK3β*) and *cyclin D2* (*CCND2*) belong to the canonical pathway; *dishevelled-associated activator of morphogenesis 1* (*DAAM1*) and *β-actin* (*ACTβ*) belong to the PCP pathway; *protein phosphatase 3 catalytic*

subunit alpha (*PPP3CA;* CaN) belongs to the noncanonical Wnt/Ca^{2+} pathway; and *secreted frizzled-related protein 1* (*SFRP1*), *secreted frizzled-related protein 2* (*SFRP2*) act as extracellular modulators of Wnt activity. GSK-3β, a key component of the destruction complex, was not only up-regulation in our COX-2$^{-/-}$ E16 mouse model but is also an ASD risk gene. Cyclin D2 is crucial for neuron proliferation and neurogenesis as shown by the ablation of adult neurogenesis in a *CCND2* knockout mouse model (Jedynak et al., 2014). Furthermore, macrocephaly, a well known characteristic of early ASD symptomology, and other severe abnormalities have been observed in children born with *de novo CCND2* mutations (Klein et al., 2013, Mirzaa et al., 2014). SFRP1 and 2 transcripts were found to be up-regulated, these proteins act as endogenous inhibitors of FZD receptors (Chung et al., 2009). While higher PGE$_2$ levels have been shown to modulate canonical Wnt activity as shown in our previous studies (Wong et al., 2014, Wong et al., 2016), here we have also shown that complete knockout of the PGE$_2$ producing enzyme COX-2 can result in abnormal regulation of genes that belong to all three Wnt pathways.

4.1.4. Altered β-catenin activation in COX$^{-/-}$ mice

To further assess whether the PGE$_2$ and Wnt pathways interact in our COX$^{-/-}$ mouse models, we determined relative levels of active β-catenin, the major downstream signalling molecule in the canonical Wnt pathway. β-catenin is normally subjected to PTMs that stabilizing or destabilizing its function. These PTMs can depend on the "on" or "off" state of canonical Wnt signalling or by kinases that can phosphorylate β-catenin. These kinases, acting independently of the destruction complex, include PKA, which phosphorylates S552 and S675; and protein kinase B (PKB, also known as AKT), which also phosphorylates S552 (Taurin et al., 2006, Fang et al., 2007). Phosphorylation of S675 has been shown to result in β-catenin binding to C-AMP response element-binding protein (CREB)-binding protein, which results in transcription (Taurin

et al., 2006). However, phosphorylation at S552 results in β-catenin nuclear translocation and TCF/LEF transcriptional activity as part of the canonical Wnt pathway (Fang et al., 2007, Taurin et al., 2008, Zhao et al., 2010). Phosphorylation at S552 by PKB also results in β-catenin induced transcriptional activity (Fang et al., 2007). PKB is primarily involved in growth factor receptor signalling, such as epidermal and insulin-like growth factors which regulate growth and glucose metabolism (Fang et al., 2007, Chen et al., 2014a). Interestingly, in addition to direct S552 phosphorylation and activation of β-catenin, insulin receptor signalling can activate PKB via PI-3K which inhibits GSK-3β, resulting in the stabilization of β-catenin and TCF/LEF transcriptional activity (Cross et al., 1995, Weston and Davis, 2001, Fang et al., 2007). This may also occur with PGE_2 signalling as PI-3K, along with PKA, is also activated downstream of the PGE_2 pathway. Thus, while PKA may directly activate β-catenin, PI-3K can indirectly ensure β-catenin stability by inhibiting the destruction complex via PKB inhibiting GSK-3β.

We have found that while both PKA and PI-3K play a role in Wnt modulation in our *in vitro* NE-4C stem cells, PKA plays a greater role in Wnt modulation (Wong et al., 2014). This may ensure that, if S552 phosphorylation does not protect β-catenin from degradation (this is likely the case), the inhibition of the destruction complex would prevent degradation. We have recently found altered levels of S552 phosphorylation in the brain of mice prenatally exposed to PGE_2 (Rai-Bhogal, 2016). In our current findings, a marked increase in phosphorylation of S552 suggests greater PKA activity in the E16 prenatal brain of male $COX-2^{-/-}$ mice. This increase was followed by a decrease in S552 β-catenin in the E19 $COX-2^{-/-}$ mouse model. The transient increase in β-catenin S552 phosphorylation at E16 followed by a seemingly over compensated decrease at E19 in the $COX-2^{-/-}$ may partly account for differential expression of Wnt pathway associated genes and Wnt target genes in the $COX-2^{-/-}$ mouse model. Similar to our previous

findings of PGE$_2$ altering Wnt activity in NE-4C cells (Wong et al., 2014, Wong et al., 2016), the *in vivo* COX-2$^{-/-}$ deficient mouse model indicates an increase of active β-catenin. These results collectively indicate that an increase or decrease of PGE$_2$ during development may alter canonical Wnt activity. Overall, these results provide further evidence for the developmental role of PGE$_2$ via modulation of the canonical Wnt pathway. The effect of PGE$_2$ signalling on the PCP or Wnt/Ca^{2+} pathways has not been documented and still needs to be investigated further.

In this study, we also found the relative quantity of stable non-phosphorylated β-catenin which arises when a Wnt ligand inhibits the destruction complex. In the absence of a Wnt signal, CK1 of the destruction complex primes β-catenin, by phosphorylating S45 (Amit et al., 2002, Liu et al., 2002, Yanagawa et al., 2002), this, in turn, allows GSK-3β to phosphorylate the residues S33/S37/T41 leading to ubiquitination and proteasomal degradation of β-catenin (Liu et al., 2002, Kimelman and Xu, 2006). Conversely, a canonical Wnt ligand can activate specific FZD receptors, leading to inhibition of the destruction complex. This allows stable β-catenin to remain non-phosphorylated at S33/S37/T41, this results in accumulation and translocation to the nucleus, allowing for the activation of gene transcription (Okerlund and Cheyette, 2011). Thus, the relative quantity of non-phospho β-catenin (S33/S37/T41) found in this study not only indicates the amount of stabilized β-catenin but also transcriptionally active β-catenin present, similar to phospho β-catenin (S552). This form of β-catenin was not altered in either COX$^{-/-}$ model during the E16 stage of development. However, at the later stage in the COX-1$^{-/-}$ model, there is an increase of non-phospho β-catenin (S33/S37/T41) level, suggesting that the destruction complex is inhibited at E19. The aforementioned inhibitory mechanisms that can act on the destruction complex involve Dvl, after Wnt ligand induced FZD activation; and PI-3K/PKB, from insulin and possibly PGE$_2$ signalling pathways. Evidently, neither of these are

very active in the earlier stage of development and only seem to be active in COX-1$^{-/-}$ E19 mouse models. Overall this data suggests that in the COX-2$^{-/-}$ mouse model, Wnt is modulated by mechanisms independent of the destruction complex and instead through kinases which can activate β-catenin.

4.1.5. Limitations and future studies

Growing evidence shows that COX-PGE$_2$ is a candidate pathway involved in the pathology of ASD. In this study, we identified many differentially expressed genes implicated in ASD in prenatal COX knockout mice brains; indicating that COX$^{-/-}$ mice may serve as model systems for studying ASDs. However, one of the limitations of this study was the use of COX knockout mice with a background of two mixed strains, one of those strains (C57BL/6) served as the WT mice in this study; this could have introduced genetic confounds between the experimental and control results. In addition, this study was limited to male offspring, thus, a similar microarray analysis in female offspring will be carried out to determine sex specific implications of COX enzymes in ASD pathogenesis. To further identify the link between COX enzyme knockout and ASD, we are carrying out ASD behavioural studies in male and female COX knockout model as well as mice treated with PGE$_2$ prenatally. Overall, these results provide several interesting aspects that may be explored further using a variety of experiments and model systems.

4.1.6. Conclusion

ASDs are a set of neurodevelopmental disorders which are becoming increasingly more prevalent. These disorders are enigmatic in the sense that much is known about contributing pathophysiological characteristics and yet categorical or preventable etiologies are not concretely understood. Due to this limited understanding of ASDs, functional annotation tools allow for the grouping of genes by function to make more applicable conclusions about ASDs. Furthermore,

most ASD risk genes have been identified using genomics in rare and syndromic ASD cases while other more common ASD cases remain idiopathic. This suggests it is important to use unconventional techniques such as analysis of the transcriptome, proteome and epigenetics. These techniques can help reveal functional changes that can result from the interaction between environmental factors, such as inflammation and dietary imbalances early in life, and differential gene expression. Thus, while genomic studies have revealed much and are very important, transcriptomic studies should also be carried out, particularly in idiopathic ASD cases. We have taken these factors in consideration by carrying out functional analysis and determining transcriptional changes in the prenatal brains of mice lacking COX enzymes. With this, we have not only shown that COX enzymes, especially COX-2, are important for brain development but also strongly implicated in ASD pathogenesis and etiology. While the central role of COX/PGE$_2$ in inflammatory response has consistently been associated with ASD, we have provided further evidence for its role in axon development, synaptic transmission and Wnt signalling pathways (COX-2 only) which are also implicated in ASDs. These results suggest that the COX-2$^{-/-}$ mouse model may be considered a potential mouse model for studying ASDs.

4.2. Study 2

4.2.1. Differential gene expression of key synaptic and developmental genes in differentiated and undifferentiated NE-4C stem cells

To further demonstrate whether abnormal PGE$_2$ signalling affects gene expression during neuronal development, we quantified gene expression of important genes from our microarray in NE-4C stem cells, an *in vitro* model for embryonic neuronal stem cells, treated with PGE$_2$. We found that *NRXN1*, *NRXN3*, *GLO1* and *GRM5*, all of which were also affected with PGE$_2$ treatment. Consistent evidence implicating NRXNs, particularly NRXN1 (Autism Genome

Project et al., 2007, Gauthier et al., 2011, Reichelt et al., 2012, Vaags et al., 2012, Bena et al., 2013, Chen et al., 2014b); and mGluR5 (Kelleher et al., 2012, Skafidas et al., 2014, Chana et al., 2015) in ASD pathogenesis already exist and the current study provides further evidence for the important role of PGE_2 signalling in the brain.

GLO1 is a ubiquitously expressed cytosolic zinc metalloenzyme responsible for the removal of glycotoxins, primarily methylglyoxal (MG) which is a product of glycolysis and other reactions in the cell (Thornalley, 2008, Roy et al., 2016). GLO1 is a rate limiting enzyme required for the removal of MG from the cytosol and ultimately leading to the formation of lactic acid and glutathione which are non-toxic and used for additional biochemical reactions (Junaid et al., 2004, Maher, 2012). Proteomic study of autopsied ASD brain samples indicated abnormal polarity and reduced function of GLO1 and with sequencing revealed SNPs in the *GLO1* gene significantly enriched in an ASD cohort (Junaid et al., 2004). Reduced function of GLO1 results in increased levels of MG which can react with other molecules in the cell to form advanced glycation end-products (AGEs) (Junaid et al., 2004, Maher, 2012). Both MG and AGEs contribute to oxidative stress, inflammation, mitochondrial dysfunction, age related neurological diseases and, more recently, ASDs (Thornalley, 2008, Maher, 2012). MG and AGEs can be found in common high heat treated foods such as deep-fried potatoes and fried meat (Tan et al., 2008, Birlouez-Aragon et al., 2010, Semba et al., 2010). Dietary MG and AGEs have been linked to oxidative stress and inflammation due to up-take from gut into the blood (Vlassara et al., 2002, Cai et al., 2008, Vlassara and Striker, 2011). Furthermore, these toxins can also be maternally transmitted to the fetus which may contribute to ASD pathogenesis (Mericq et al., 2010). Although more causal and direct studies are needed, these toxins appear to be a potential source

of stress during development arising from maternal transmission or reduced *GLO1* expression caused by mutations or abnormal PGE_2 signalling.

4.2.2. Differential gene expression in neuronally differentiated NE-4C stem cells

We have previously shown that PGE_2 accelerates the neuronal-lineage differentiation process in NE-4C stem cells (Wong et al., 2016). More specifically, PGE_2 results in the earlier formation of neuronal clusters or neurospheres along with earlier expression of neuronal markers. In addition, PGE_2 exposure leads to altered Wnt regulated genes previously implicated in neurodevelopmental disorders, and increased levels of active β-catenin (Wong et al., 2016). To further elucidate the way in which PGE_2 may alter gene expression, we quantified the transcripts of *NRXN1, NRXN3*, *GLO1*, *GRM5*, *DAAM1* and *SOX11* genes of interest from the microarray in neuronally differentiated cells. Both *NRXN1* and *GLO1* were significantly up-regulated, while the remaining genes were unaffected. The importance of these genes has been discussed above and their differential expression in neuronal cells further provide evidence that PGE_2 can regulate important genes in neuronal cells as well. Overall, these results add to the mounting evidence indicating that PGE_2 has a role in brain development and is associated with ASD pathogenesis.

4.2.3. Limitations and future studies

Although we have found significant changes in gene expression in both NE-4C stem cells and differentiated neuronal cells, these studies should be followed up with protein analysis to indicate translational changes in these genes. Furthermore, with several altered genes being important for synaptic neurobiology, cell based electrophysiological recordings can also be considered.

4.2.4. Conclusion

The *in vitro* studies carried out here have added evidence supporting what we have consistently shown in our lab; the important role of PGE_2 in early brain development. Previous cell behavioural studies with abnormal PGE_2 signalling, we have indicated altered division, migration, calcium homeostasis, extension growth and differentiation in *in vitro* cell models. Here we have provided additional candidate genes that may contribute to the previously observed cell behavioural changes caused by altered PGE_2 signalling. Overall, PGE_2 signalling pathway is important for normal neuronal stem cell and neuron cells, dysregulation of which may contribute to changes resulting in ASD pathogenesis.

5.0. CONCLUSION

The studies conducted here have provided a comprehensive outline of the role of COX enzymes and PGE_2 in prenatal mouse models of neuronal development. To our knowledge, we have conducted the first global gene expression analysis of male mouse brains lacking either COX-1 or COX-2 enzymes at two prenatal time periods. Additional evidence implicating PGE_2 signalling for altering expression of important genes in neuroectodermal stem cells and differentiated neurons was also provided. Examining expression during two different time periods was integral for demonstrating the importance of critical periods during prenatal brain development and we have shown that E16 falls in a more critical period for lipid signalling in the brain than E19. In addition, transcriptomic studies have been proven to be a valuable tool for identification of functional changes that may be associated with ASDs. The microarray analysis provided further evidence for COX/PGE_2 regulating synaptic neurobiology and Wnt signalling pathways (COX-2 only) which are vital for brain development and strongly implicated in ASDs. Taken together, we have provided sufficient evidence for the potential use of COX knockout models, especially COX-2$^{-/-}$, as viable ASD mouse models for further studies.

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7.0. APPENDIX

A.1. Supplementary Figure

Table S1: FC of all genes differentially expressed in COX-1$^{-/-}$ E16, COX-2$^{-/-}$ E16, COX-1$^{-/-}$ E19, and COX-2$^{-/-}$ E19. Only the genes with absolute FC > 1.5 and statistically significant (*P*-value < 0.05) genes were included in our bioinformatics analysis. We found a total of 229, 323, 134 and 148 differentially expressed genes in COX-1$^{-/-}$ E16, COX-2$^{-/-}$ E16, COX-1$^{-/-}$ E19 and COX-2$^{-/-}$ E19 mouse models, respectively.

COX-1$^{-/-}$ E16		COX-2$^{-/-}$ E16		COX-1$^{-/-}$ E19		COX-2$^{-/-}$ E19	
Official Gene Symbol	FC	Official Gene Symbol	FC	Official Gene Symbol	FC	Official Gene Symbol	FC
Hbb-y	4.34	Hbb-b2	10.88	Hbb-b2	11.15	Hbb-b2	12.83
SOX11	3.39	Hbb-y	5.15	C030014C12Rik	3.91	Paip1	3.74
slc35f1	2.09	Xlr4a	3.99	SOX11	3.17	PBX1	3.68
Lyplal1	2.03	SOX11	3.30	LOC100045240	2.89	Ankrd37	3.01
TMEM87A	2.02	Paip1	3.21	Ankrd37	2.75	SOX11	2.73
RBM45	2.01	0710001D07Rik	2.42	CAP1	2.66	Sfrp1	2.59
sdcbp	1.97	ABCC10	2.36	Paip1	2.58	GLTPD1	2.49
LOC100044314	1.92	emp1	2.34	GLTPD1	2.46	trim2	2.25
0710001D07Rik	1.89	LUM	2.32	Prkd3	2.45	Serpina3n	2.22
DCN	1.89	trim2	2.22	Egr1	2.42	DAAM1	2.12
Rnase4	1.88	LOC100044314	2.17	TMEM87A	2.35	C030014C12Rik	2.10
Hba-x	1.88	Lyplal1	2.09	LOC100044314	2.18	0710001D07Rik	2.08
Snapc3	1.86	COL3A1	2.05	trim2	2.13	LOC100044314	1.98
CAP1	1.86	slc35f1	2.01	FOS	2.13	ENSMUSG00000068790	1.93
Paip1	1.85	GLO1	2.01	Fezf1	2.11	4930455C21Rik	1.92
NFIB	1.84	gjb2	2.00	RBM45	1.97	GLO1	1.90
ITGB3BP	1.84	GLTPD1	1.96	Sfrp1	1.96	NEK3	1.89
LUM	1.82	DCN	1.96	Lyplal1	1.96	Lyplal1	1.87
DTD1	1.81	Hba-x	1.91	C530036F05Rik	1.88	Prkd3	1.83
IL18	1.79	sdcbp	1.88	GRLF1	1.84	WARS2	1.82
2810417H13Rik	1.76	Slc13a4	1.88	TXNIP	1.83	Ttc15	1.81
SCP2	1.76	Sfrp1	1.84	6330415B21Rik	1.82	GRLF1	1.80
ABCC10	1.74	IL22RA1	1.83	DAAM1	1.81	ADI1	1.78
ADI1	1.74	GNG10	1.79	mro	1.80	4833414E09Rik	1.77
LOC100045240	1.72	2810417H13Rik	1.79	2900011L18Rik	1.80	pitx2	1.74
vps33a	1.68	Hexb	1.79	NRXN2	1.79	LOC100046025	1.72
nosip	1.68	NUBP2	1.79	pitx2	1.78	LOC100045240	1.72
Rpa3	1.65	Rpa3	1.78	Ttc15	1.78	lhx1	1.69
Hbb-bh1	1.65	NDUFB10	1.78	Nrxn3	1.76	SSBP4	1.68

Hyexb	1.65	HEBP1	1.76	2310016E02Rik	1.76	LYPD1	1.68
CUEDC1	1.64	PFDN4	1.76	ADI1	1.73	cdkal1	1.67
emp1	1.63	MRPL47	1.75	GPM6B	1.71	Arcn1	1.67
ZBTB8OS	1.63	TMEM47	1.74	3830612M24	1.69	ahnak	1.65
itgb1bp1	1.62	Col6a3	1.74	0710001D07Rik	1.69	2310016E02Rik	1.65
SSBP4	1.62	Gpx8	1.73	ITGB3BP	1.69	UNC80	1.62
fign	1.62	Arcn1	1.73	FANK1	1.69	GNB4	1.61
LYPD1	1.61	Clcn4-2	1.71	Klf4	1.68	Psg23	1.61
Asnsd1	1.61	TUBB6	1.71	LYPD1	1.68	ATP10D	1.61
LOC280487	1.61	sfrp2	1.70	Nnat	1.67	Gins4	1.61
Clcn4-2	1.61	LOC100046025	1.69	9930031P18Rik	1.63	TMEM66	1.61
GTF3C2	1.60	TSPAN12	1.68	SCP2	1.62	pdia6	1.60
Col5a2	1.60	OGN	1.68	Hexb	1.61	C530036F05Rik	1.60
PFDN4	1.60	2310016E02Rik	1.67	SSBP4	1.61	MPP7	1.60
Ttc15	1.59	SEPP1	1.67	Hist1h1c	1.59	COL3A1	1.59
TUBB6	1.59	ADI1	1.66	Nkx6-2	1.59	ST18	1.59
AURKAIP1	1.58	SSBP4	1.66	nkx2-1	1.58	gjb2	1.58
Col6a3	1.57	CETN2	1.66	ST18	1.57	emp1	1.57
TRAPPC2	1.57	Col5a2	1.65	ZWINT	1.56	NUBP2	1.57
SH3BGRL	1.57	DAAM1	1.64	Uevld	1.54	myo6	1.56
Egr1	1.55	mgst1	1.64	Phkb	1.54	GMCL1	1.56
TSPAN12	1.55	ahnak	1.64	ZFYVE27	1.54	SORL1	1.55
Gpx8	1.55	2410076I21Rik	1.63	ZBTB33	1.54	FANK1	1.55
Prpf40a	1.54	Asnsd1	1.63	CEBPB	1.53	2900011L18Rik	1.55
TFAM	1.54	E130114P18Rik	1.62	ANGPT2	1.53	CHCHD8	1.55
2310016E02Rik	1.54	p2ry12	1.62	vps33a	1.53	Zfp286	1.54
E130114P18Rik	1.54	MLF1	1.62	NEK3	1.52	Hexb	1.54
HIF1A	1.54	ST18	1.61	Ppp1ca	1.51	esd	1.52
GNG10	1.54	COMMD1	1.61	Col5a2	1.51	Phkb	1.52
mrps10	1.53	Jam3	1.61	Arcn1	1.50	Col6a3	1.50
ARIH1	1.53	Tm9sf2	1.61	UNC80	1.50	EPM2AIP1	-10.02
GMPS	1.52	pdia6	1.61	Cox7a2l	-8.06	TMEM25	-7.27
STAG2	1.52	DTD1	1.60	Zfp68	-5.73	NDUFB10	-6.53
spc25	1.51	KPNA2	1.60	MCM6	-5.68	Cops8	-5.01
RECK	1.51	ppiC	1.60	SPARC	-5.35	UBE2I	-4.98
RBM8A	1.50	NFIB	1.60	ACTL6B	-5.29	Gpr137b-ps	-4.92
2810008M24Rik	1.50	EBF3	1.59	Cops8	-5.22	TM7SF3	-4.84
SPARC	-8.05	TRAPPC2	1.58	MYT1L	-4.05	Rn18s	-4.52
Zfp68	-6.10	Tmed2	1.58	Rn18s	-3.90	rpl29	-4.44
ACTL6B	-5.79	GMPS	1.58	Hbb-b1	-3.89	wdr82	-3.76
MYT1L	-5.48	DLK1	1.58	TM7SF3	-3.68	EMB	-3.74

Cops8	-5.34	FKBP7	1.58	ACTB	-3.12	SPARC	-3.32
CCNG2	-5.32	TPBG	1.57	Akr1c19	-2.91	Slc25a18	-3.19
TM7SF3	-4.27	spc25	1.57	B3galt6	-2.85	FCER1G	-3.12
Bach2	-4.20	Zfp606	1.57	arl5a	-2.78	MCM6	-3.05
MCM6	-4.15	ENSMUSG000000 68790	1.57	Gpr137b-ps	-2.64	FGFR1OP2	-2.69
cxadr	-4.14	Ttc15	1.57	PUSL1	-2.59	Mboat2	-2.66
PRKAG2	-3.97	fundc1	1.56	Mboat2	-2.55	Hbb-b1	-2.51
Cox7a2l	-3.84	fign	1.56	2900003A17Rik	-2.48	2900003A17Rik	-2.44
ACTB	-3.51	calml4	1.56	CAPN2	-2.36	mgst1	-2.44
ARV1	-3.31	SH3BGRL	1.56	twistnb	-2.19	CAPN2	-2.40
Akr1c19	-3.16	RSAD2	1.55	SFRS16	-2.18	C030014I23Rik	-2.37
Mtap2	-3.15	USP14	1.55	PKIG	-2.15	arrdc3	-2.32
Mboat2	-3.03	ESCO2	1.55	homer2	-2.13	SFRS16	-2.31
2900003A17Rik	-3.01	LOC280487	1.55	hpcal1	-2.09	SV2A	-2.27
twistnb	-2.99	Tspan6	1.55	CNTN2	-2.09	6330418B08Rik	-2.25
arl5a	-2.95	ccng1	1.55	DHRSX	-2.09	Nudt6	-2.25
MEF2C	-2.85	tpmT	1.55	FGFR1OP2	-2.04	TAGAP	-2.23
Grm5	-2.81	UBE2N	1.55	PKM2	-2.02	Defb11	-2.20
TAF6	-2.80	cdkal1	1.54	Cd200	-2.02	ATP8A1	-2.20
Hbb-b1	-2.78	HIST1H2BK	1.54	melA	-2.01	B3galt6	-2.19
Man2b1	-2.75	HIF1A	1.54	TAF6	-2.00	NT5C3	-2.14
Zmiz1	-2.75	tmod3	1.54	6330418B08Rik	-1.99	melA	-2.11
CNTN2	-2.74	KCNN1	1.54	ALG9	-1.97	Stk25	-2.10
B3galt6	-2.70	Tgfbr2	1.54	Stk25	-1.96	PUSL1	-2.10
melA	-2.63	HNRNPA2B1	1.54	Trappc5	-1.94	4833420G17Rik	-2.06
PCDH17	-2.63	Phkb	1.54	GIN1	-1.90	CNTN2	-2.04
Dpysl3	-2.58	RPGR	1.54	RpL30	-1.89	AI316807	-1.99
FGFR1OP2	-2.58	Zfp286	1.54	APOE	-1.88	GIN1	-1.98
ALG9	-2.57	Pcp4l1	1.54	AGPAT5	-1.87	ppp1r14c	-1.96
PKM2	-2.57	Prpf40a	1.54	Tia1	-1.87	PHTF1	-1.91
AGPAT5	-2.54	LYPD1	1.54	Znhit1	-1.86	rps3a	-1.91
1500035N22Rik	-2.53	THBD	1.53	MRPS27	-1.85	Cox7a2l	-1.90
ZDHHC21	-2.52	Rrm1	1.53	Rec8	-1.84	ahcY	-1.90
PCDH7	-2.43	3830406C13Rik	1.53	STXBP2	-1.83	D830030K20Rik	-1.88
Rn18s	-2.40	1810035L17Rik	1.53	TAGAP	-1.81	CCRN4L	-1.88
DENND1C	-2.36	UBE2A	1.52	lancl1	-1.81	Map4k5	-1.87
GRIA2	-2.36	STAG2	1.51	TMEM25	-1.78	Lars2	-1.86
hpcal1	-2.35	frmd3	1.51	Defb11	-1.78	TSC2	-1.83
PRDX2	-2.34	FOXC2	1.51	Mrpl55	-1.76	rps2	-1.82
BRD2	-2.28	GTF3C2	1.51	ahcY	-1.75	UAP1	-1.80
4833414E09Rik	-2.27	srp9	1.51	pts	-1.72	Tmed4	-1.80

Gene	Value	Gene	Value	Gene	Value	Gene	Value
Bach1	-2.24	Hbb-bh1	1.51	ZBTB45	-1.71	EME2	-1.78
THAP4	-2.24	2610036L11Rik	1.50	THAP4	-1.70	STXBP2	-1.78
NECAB1	-2.15	RBM8A	1.50	LASS4	-1.68	Ipo9	-1.77
Rec8	-2.09	3110052M02Rik	1.50	Fbxo46	-1.67	SRPR	-1.76
SLC4A4	-2.05	EPM2AIP1	-7.39	iars2	-1.62	Mrpl55	-1.76
DHRSX	-2.02	UBE2I	-5.38	Insig2	-1.62	THAP4	-1.74
RIOK1	-2.01	TM7SF3	-5.17	abcd3	-1.62	RpL30	-1.71
Tia1	-2.01	Bach2	-4.99	Gpr17	-1.61	AGPAT5	-1.68
MRPS27	-2.00	Cops8	-4.76	SEMA5A	-1.60	Fbxo46	-1.67
fam168a	-2.00	SPARC	-4.72	CHRNA3	-1.59	mthfd1	-1.66
KIF1B	-1.97	TMEM25	-4.69	ANAPC5	-1.59	Spsb3	-1.63
DHCR24	-1.95	wdr82	-4.47	KCNF1	-1.58	PEX11B	-1.63
SIN3A	-1.92	rpl29	-4.36	2900060N12Rik	-1.57	FLYWCH2	-1.61
PBX1	-1.91	Mtap2	-3.73	camk4	-1.56	klhl21	-1.61
homer2	-1.91	Gpr137b-ps	-3.44	9030624G23Rik	-1.56	1700029J07Rik	-1.60
6330418B08Rik	-1.91	GRIA2	-3.14	pygb	-1.56	Cirbp	-1.60
Stk25	-1.90	Rn18s	-3.12	ttc17	-1.55	ACTL6B	-1.59
Hook3	-1.90	Dpysl3	-3.12	SERINC3	-1.55	2900060N12Rik	-1.57
LOC100047888	-1.89	EMB	-3.07	9930105H17Rik	-1.54	BDH1	-1.57
AHDC1	-1.89	5330423I11Rik	-3.07	Gstm6	-1.53	Zfp160	-1.57
gtpbp2	-1.86	2900003A17Rik	-2.97	AI316807	-1.53	DMWD	-1.56
PKIG	-1.86	MEF2C	-2.96	TMEM158	-1.51	Znhit1	-1.56
PUSL1	-1.85	Mboat2	-2.95	Phlda3	-1.51	MED23	-1.55
Gcap14	-1.85	ODZ4	-2.90			CDKN1B	-1.55
Cd200	-1.85	Man2b1	-2.88			NLRX1	-1.55
mmp16	-1.85	PCDH7	-2.83			H2afj	-1.55
ODZ4	-1.84	CNTN2	-2.83			SEMA5A	-1.55
D430041D05Rik	-1.84	Grm5	-2.83			3110021A11Rik	-1.54
H2afj	-1.82	AGPAT5	-2.77			lancl1	-1.53
MDGA2	-1.81	FGFR1OP2	-2.76			iars2	-1.53
PCDHA7	-1.79	CCNG2	-2.71			fut10	-1.53
Epb4.1l2	-1.79	PRDX2	-2.68			Zfp39	-1.53
Gpr137b-ps	-1.78	Zmiz1	-2.68			DHRSX	-1.52
UBE2I	-1.78	SV2A	-2.65			Ccdc117	-1.51
igsf3	-1.78	PCDH17	-2.60			OLFML3	-1.51
AFAP1	-1.76	Bach1	-2.59			LOC674427	-1.51
Zeb2	-1.75	ARV1	-2.51			Trappc5	-1.51
RBBP9	-1.75	D4Ertd681e	-2.50				
SYT4	-1.75	BRD2	-2.47				
palm	-1.75	ZDHHC21	-2.46				
camk4	-1.75	melA	-2.43				

Fbxo41	-1.74	RIOK1	-2.38		
SERINC3	-1.73	DENND1C	-2.34		
CAMKV	-1.72	1200016E24Rik	-2.33		
9330164J24Rik	-1.72	LOC100047888	-2.32		
LOC100048372	-1.72	NECAB1	-2.32		
GLTPD1	-1.72	CSNK1E	-2.27		
CDH8	-1.72	BCL11B	-2.25		
Adam17	-1.71	Gcap14	-2.24		
Fbxl18	-1.71	4933407C03Rik	-2.19		
ATM	-1.69	POU6F1	-2.18		
C130045I22Rik	-1.69	KLF7	-2.18		
NRXN1	-1.68	6330418B08Rik	-2.17		
SLC4A3	-1.67	LOC100048372	-2.17		
irs2	-1.67	H2afj	-2.13		
KCND2	-1.67	Stk25	-2.10		
GLG1	-1.66	FCER1G	-2.10		
KLF7	-1.66	TSC2	-2.07		
SEMA5A	-1.66	Ppp1r1c	-2.06		
esd	-1.65	ACTB	-2.06		
gpsm1	-1.65	ATP8A1	-2.05		
Acsl6	-1.64	Fbxo41	-2.05		
DAG1	-1.64	Hook3	-2.04		
napB	-1.64	THAP4	-2.03		
CSNK1E	-1.63	9330164J24Rik	-2.02		
abcd3	-1.63	SRPR	-2.00		
Hps1	-1.63	TAGAP	-1.99		
SERPINE2	-1.63	NRXN1	-1.98		
SNX27	-1.63	TMEM66	-1.97		
ppp3ca	-1.62	ppp1r14c	-1.96		
Scn1a	-1.61	mmp16	-1.96		
2900060N12Rik	-1.60	igsf3	-1.96		
rpe	-1.60	C77370	-1.95		
H13	-1.60	PCDHA7	-1.92		
myh10	-1.60	2700089E24Rik	-1.92		
Insig2	-1.60	GLG1	-1.92		
AQP4	-1.60	CASKIN1	-1.91		
ttc17	-1.60	Gnai1	-1.91		
Epb4.1l1	-1.59	6330415B21Rik	-1.90		
pts	-1.59	D430007A19Rik	-1.90		
dnajc5	-1.58	hpcal1	-1.89		
nrp	-1.58	PKM2	-1.89		

6430548M08Rik	-1.58	gtpbp2	-1.89		
Adrbk2	-1.58	Epb4.1l2	-1.89		
BCL11B	-1.57	nrp	-1.89		
prpf40b	-1.57	B3galt6	-1.88		
camkk2	-1.57	RAI1	-1.88		
Syp	-1.57	MDGA2	-1.87		
Ptprd	-1.57	napB	-1.86		
UBTF	-1.56	Map4k5	-1.86		
CASKIN1	-1.56	SNX27	-1.84		
THY1	-1.56	Zeb2	-1.84		
Znhit1	-1.56	gpsm1	-1.83		
6430411K18Rik	-1.56	irs2	-1.83		
Ppp1r1c	-1.55	prpf40b	-1.82		
TAGAP	-1.55	Clasp1	-1.82		
SGSM1	-1.55	TNIK	-1.81		
keap1	-1.55	SOX5	-1.81		
DCTN4	-1.55	keap1	-1.81		
RAPGEFL1	-1.54	SLC4A3	-1.80		
ANAPC5	-1.54	Mll1	-1.79		
mapt	-1.53	TRIO	-1.77		
Zfp236	-1.53	ALG9	-1.77		
ZBTB45	-1.53	Adrbk2	-1.76		
Cops5	-1.52	DAG1	-1.76		
BAI2	-1.52	CAMKV	-1.76		
DGKH	-1.52	C030014I23Rik	-1.75		
Pbx4	-1.52	Tssc8	-1.75		
Camk2b	-1.52	Scn1a	-1.74		
GUCY1A3	-1.52	Gpd2	-1.73		
EXOC6B	-1.52	RBM28	-1.73		
CHRNA3	-1.52	rps2	-1.73		
KCNF1	-1.51	H13	-1.73		
A2bp1	-1.51	PHTF1	-1.73		
cspg5	-1.50	SLC4A4	-1.73		
Hmg20a	-1.50	GSK3B	-1.72		
Ncdn	-1.50	lsm12	-1.72		
GIN1	-1.50	ahcY	-1.72		
		SFRS16	-1.72		
		3110021A11Rik	-1.72		
		KCND2	-1.71		
		DHCR24	-1.71		
		palm	-1.71		

	4833414E09Rik	-1.70	
	KIF1B	-1.70	
	Rec8	-1.70	
	CDH8	-1.69	
	AFAP1	-1.69	
	dnajc5	-1.69	
	A2bp1	-1.69	
	MRPS27	-1.68	
	arrdc3	-1.68	
	MCM6	-1.67	
	D11Bwg0517e	-1.67	
	Nudt6	-1.67	
	NAV1	-1.67	
	6430548M08Rik	-1.67	
	E130307A14Rik	-1.66	
	LOC100046343	-1.66	
	C130045I22Rik	-1.66	
	AI316807	-1.65	
	ppp3ca	-1.65	
	usp48	-1.65	
	2900060N12Rik	-1.64	
	Ptprd	-1.64	
	ZCCHC6	-1.64	
	SETBP1	-1.64	
	mapt	-1.64	
	GRIP1	-1.64	
	SEZ6	-1.63	
	Epb4.1l1	-1.63	
	CLCN7	-1.63	
	AHDC1	-1.63	
	D830030K20Rik	-1.63	
	RNF182	-1.63	
	them4	-1.62	
	LDB2	-1.62	
	A130054J05Rik	-1.61	
	D430041D05Rik	-1.61	
	Zfp131	-1.61	
	Syt1	-1.61	
	myh10	-1.59	
	EME2	-1.59	
	LOC100045679	-1.59	

	SERPINE2	-1.58	
	Snapc3	-1.58	
	BAI2	-1.57	
	ANKMY2	-1.57	
	GIN1	-1.57	
	Fbxl18	-1.57	
	Tia1	-1.57	
	Spag9	-1.57	
	Pbx4	-1.57	
	AP1B1	-1.56	
	KLC4	-1.56	
	PUSL1	-1.56	
	Car11	-1.56	
	EXOSC10	-1.55	
	Spsb3	-1.55	
	LOC100046746	-1.55	
	Plxna2	-1.55	
	Sox6	-1.55	
	1700029J07Rik	-1.55	
	Zfp281	-1.55	
	LPHN1	-1.55	
	PIP5K1A	-1.54	
	ZBTB17	-1.54	
	LIMCH1	-1.54	
	vldlr	-1.54	
	homer2	-1.54	
	CHD5	-1.54	
	CDC42BPB	-1.54	
	GUCY1A3	-1.54	
	RAD23B	-1.53	
	GABARAPL1	-1.53	
	Defb11	-1.53	
	CCND2	-1.53	
	MYT1L	-1.53	
	DHRSX	-1.53	
	D930015E06Rik	-1.52	
	Ncdn	-1.52	
	6430590A07Rik	-1.52	
	zmynd11	-1.52	
	srebf2	-1.52	
	C030048H21Rik	-1.52	

	cspg5	-1.51	
	Sf1	-1.51	
	TNRC6C	-1.51	
	Ngef	-1.51	
	SPATA7	-1.51	
	Cfl2	-1.51	
	MED23	-1.51	