The Impact of Impaired Cyclooxygenase-2 Activity on Mouse Brain Development: A Focus on Sex Differences

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A Thesis submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science

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York University
Toronto, Ontario
August 2020

Abstract

There is a clear male bias in the prevalence of neurodevelopmental disorders (NDDs) such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD). Certain environmental factors have been shown to contribute to the etiology of these NDDs, including exposure to antipyretic drugs. Using cyclooxygenase-2 knockin (COX-2^{-}) and COX-2 knockout (COX-$2^{-/-}$) mice, genetic models which mimic exposure to antipyretic drugs, impaired COX-2 activity was found to induce sex-dependent changes in the expression of various neuroimmune markers in the brain during development. Further investigations also suggested that distinct subtypes of astrocytes may be dysregulated in male and female COX-2^{-} mice, with males exhibiting an increased prevalence of neurotoxic “A1” astrocytes, and females exhibiting an increased prevalence of neuroprotective “A2” astrocytes. A greater understanding of the sex-dependent effects of antipyretic drugs may ultimately facilitate the discovery of novel therapeutic targets for NDDs exhibiting a male bias, such as ASD and ADHD.

Acknowledgements

First, I would like to thank my supervisory and examining committee members: Dr. Dorota Crawford, Dr. Steven Connor, and Dr. Ali Abdul-Sater for their time, feedback, and contributions in strengthening my thesis. I would like to extend a special thank you to my supervisor Dr. Dorota Crawford for her continued guidance and support over the past two years. I entered her lab with absolutely zero research experience and would not be here today had she not taken a chance and given me this opportunity, one for which I am extremely grateful.

I would also like to extend my sincere appreciation and gratitude to the members of my lab team: Dr. Critistine Wong, Ashby Kissoondoyal, Isabel Bestard Lorigados, Sarah Wheeler, Sasha Udhesister, Shalini Lyer, and Kelly Ho. To Christine, my experience volunteering in the Crawford lab with you helped inspire me to pursue a career in science and your work laid the foundation that made my thesis project possible. To Ashby, your feedback and assistance in troubleshooting helped make me a better researcher. To Isabel, your training, feedback, and assistance with both troubleshooting and proofreading helped strengthen my technical skills and thesis. To Sarah, Sasha, Shalini, and Kelly, thank you for your support and friendship. To all, each of you helped cultivate a lab environment that made my experience doing research in the Crawford lab one that I will always remember fondly.

Lastly, I would like to extend my heartfelt thanks to my entire family. To my parents, none of this would have been possible without your unwavering support and encouragement throughout these years. Thank you for all the sacrifices you have made, and all that you have done for me.

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List of Abbreviations

ω-3 = Omega-3

ω-6 = Omega-6

ADHD = Attention-Deficit Hyperactive Disorder

ARA = Arachidonic acid

APAP = acetaminophen

Aqp4 = Aquaporin 4

ASD = Autism Spectrum Disorder

COX = Cyclooxygenase

COX-2^{-} = Cyclooxygenase-2 knockin

COX-$2^{-/-}$ = Cyclooxygenase-2 knockout

DNMT = DNA methyltransferase

E/I Imbalance = Imbalance between excitatory and inhibitory neuronal activity

E_2 = Estradiol

EP = E-prostanoid

ERF = Environmental risk factor

GAT-3 = GABA transporter 3

GD = Gestational day

Gfap = Glial fibrillary acidic protein

Glo1 = Glyoxalase 1

GLT-1 = Glutamate transporter 1

Grm5 = Metabotropic glutamate receptor 5

Gsk3β = Glycogen synthase kinase 3 beta

IL-1β = Interleukin-1beta

IL-6 = Interleukin-6

iNos = Inducible nitric oxide synthase

Itgam = Integrin alpha M

LPS = Lipopolysaccharide

mPOA = Medial preoptic area

MG = Methylglyoxal

NDD = Neurodevelopmental disorder

NO = Nitric oxide

Nox2 = NADPH oxidase 2

NSAID = Non-steroidal anti-inflammatory drug

PGE_2 = Prostaglandin E_2

PGs = Prostaglandins

PLA_2 = Phospholipase A_2

PND = Post-natal day

PUFA = Polyunsaturated fatty acid

RNS = Reactive nitrogen species

ROS = Reactive oxygen species

RRB = Restricted, repetitive and stereotyped patterns of behavior

S100a10 = S100 calcium binding protein A10

Serpina3n = Serpin family A member 3

Serping1 = Serpin family G member 1

VPA = Valproic acid

CHAPTER 1. INTRODUCTION

1.1 General Introduction

The prevalence rates of certain neurodevelopmental disorders (NDDs) have increased significantly over the last couple of decades [1–3]. For example, between 2009 to 2017, the prevalence of autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) in children across the United States rose from 1.1-2.5% and 8.5-9.5%, respectively [3]. Furthermore, the majority of NDDs, including ASD and ADHD, exhibit a male bias in prevalence rates and severity [4,5]. While the exact cause of these NDDs is not fully understood, a considerable body of research supports the notion that both genetic and environmental factors contribute to their etiology [6–9]. Given the relatively slow rate of change in genetics through evolution and rapid increase in environmental toxicants over the past several decades (e.g., traffic-related air pollution), it seems increasingly plausible that exposure to certain environmental risk factors (ERFs) have contributed to the rising prevalence rates of NDDs [8,10,11].

Two major ERFs implicated in nearly all NDDs include maternal immune activation during pregnancy (i.e., following infection or fever) [12–14] and prenatal exposure to antipyretics such as non-steroidal anti-inflammatory drugs (NSAIDs) or acetaminophen (APAP) [15–17]. Effectively, these factors antagonistically modulate the febrile response in the brain, which is believed to occur primarily through CNS initiation of cyclooxygenase-2 (COX-2) pathways and the subsequent production of prostaglandins such as prostaglandin E_2 (PGE_2) [18–21]. Interestingly, the COX-2/PGE_2 pathway is also found to play a significant role in the masculinization of the male brain during development [22,23]. Thus, the COX-2/PGE_2 pathway represents a point of convergence between ERFs and the male bias in NDDs. The research studies presented in this

thesis encompasses an overview of molecular techniques in COX-2 knockin (COX-2^{-}) and COX-2 knockout (COX-2$^{-/-}$) mice, genetic models which mimics exposure to antipyretic drugs, with the ultimate goal of providing insight into the sex-dependent effects of impaired COX-2 activity.

1.2 Background Literature

Although ASD and ADHD are distinct NDDs with technically no overlap in DSM-5 diagnostic criteria [4], a high degree of co-morbidity has been demonstrated between these disorders, with 30-50% of individuals with ASD also being diagnosed with ADHD [24–26] and 20-30% of individuals with ADHD also being diagnosed with ASD [25–27]. These disorders also display a certain degree of overlap between neuropsychological symptoms such as restricted, repetitive, and stereotyped patterns of behavior (RRB), inattention, and hyperactivity/impulsivity [28–31]. Notably, males consistently score higher on deficits in social communication, RRB, and hyperactivity/impulsivity across both disorders [28,30,31], and individuals with either ASD or ADHD typically show behavioral deficits in only one or two domains characteristic of the other disorder [29,30]. Thus, it has been suggested that a more useful approach may be to focus on symptom co-occurrence rather than complete co-morbidity between ASD and ADHD.

The high degree of phenotypical similarities between ASD and ADHD further suggests that certain pathophysiological mechanisms may be shared in at least some of these patients [25]. Evidence for this stems from the fact that both disorders arise during the developmental period, are more prevalent in males [4], and have ERFs that are common to both–including those relevant to the current thesis, such as prenatal exposure to antipyretics (e.g., NSAIDs and APAP) [17,32,33]. APAP is the medication recommended for pregnant women to relieve pain and reduce fever [16]. It

is used by approximately 60-70% of pregnant women in the US and Europe, whereas 10-20% of women typically use NSAIDs such as ibuprofen [34,35]. While the peripheral effects of NSAIDs and APAP differ significantly in their anti-inflammatory properties, in the brain, they both function to suppress the febrile response via CNS inhibition of the COX-2 pathway and subsequent prostaglandin production [36].

A number of studies have shown that antipyretic drugs can freely cross the placenta [37] and blood-brain barrier [38], and that prenatal exposure can be subsequently traced in an infant's urine following birth [39,40]. Recently, several studies have also established a clear dose-response relationship between APAP use during pregnancy and the risk of developing ADHD symptoms [16,17,33,41–43] or ASD symptoms [17,32,41]. Furthermore, exposure to antipyretic drugs has been associated with certain pathological mechanisms frequently implicated in both ASD and ADHD, including markers of oxidative stress [44–46], and impaired neuroinflammatory signaling in the brain [47–49]. Thus, it is likely that ERFs common to both ASD and ADHD (i.e., antipyretic drugs) that impact shared pathological mechanisms (i.e., neuroinflammatory signaling) may play a role in the underlying co-occurrence of behavioral symptoms and convergent pathology in these disorders.

1.3 Rationale

Previous studies in our lab have focused on how impaired COX-2/PGE_2 signaling impacts neurodevelopment in mice using both *in vitro* and *in vivo* models. Our *in vitro* experiments found that exogenous administration of PGE_2 to neuroectodermal stem cells derived from mice on gestational day (GD) 9 can impact several key neurodevelopmental processes such as neuronal migration, proliferation & differentiation [50,51]. Subsequent *in vivo* microarray studies

then investigated the role of this pathway using brain samples obtained from COX-2 knockout mice (COX-$2^{-/-}$) at GD 15 and GD 18. Male COX-$2^{-/-}$ mice exhibited changes in the expression of genes associated with biological pathways also found to be dysregulated in ASD, including synaptic transmission and the regulation of immune functions [52]. Interestingly, a follow-up study found that these same ASD-associated gene sets were not differentially expressed in age-matched female COX-$2^{-/-}$ mice (unpublished data), suggesting that the depletion of COX-2 may adversely impact prenatal brain development in a sex-dependent manner.

More recently, we used two mouse models to examine the effects of impaired COX-2/PGE_2 signaling on postnatal development. One of these models used COX-2 knockin mice (COX-2^{-}) mice to examine how lower PGE_2 levels effects development, while the other model involved a maternal injection of PGE_2 on GD 10 to examine how higher levels of PGE_2 effects development. Interestingly, ASD-related, microglial, and pro-inflammatory cytokine genes were found to be differentially expressed in the brain in a sex-dependent manner during early postnatal development (PND 8) in both models [53,54]. Subsequent behavioral analyses also found that both COX-2^{-} and PGE_2-injected mice exhibited deficits in behavioral domains common to ASD and ADHD, including increased hyperactivity, anxiety, and repetitive behavior. Conversely, deficits in social novelty were only observed in PGE_2-injected mice. Together, these findings suggest the existence of both convergent and divergent pathological mechanisms in these models, and that ERFs which impact COX-2/PGE_2 signaling (that is, those leading to increased or decreased PGE_2 levels) may contribute to an increased risk of behavioral deficits characteristic of ASD and ADHD. It should also be noted that males were found to exhibit more severe behavioral deficits in both models [53,54], supporting the notion that perturbations to the COX-2/PGE_2 pathway may have more significant consequences for proper brain development in males than females.

1.4 General Objectives & Hypothesis

The ***overall goal*** of my research is to investigate the effects of impaired COX-2 activity in the developing mouse brain, with a focus on identifying the underlying differences between males and females. More specifically, my thesis research aims to address two overarching objectives:

First, to determine the molecular mechanisms by which impaired COX-2 activity may affect brain development, thus providing further insight into how maternal exposure to antipyretic drugs may contribute to ASD- and ADHD-related pathologies. To do this, we will quantify the changes in the expression of genes and proteins in the brain of male and female 129S6 wildtype and COX-2^{-} mice at PND 25.

Second, to better understand the sex-dependent effects of impaired COX-2 activity on brain development and investigate whether these effects provide support for either the male vulnerability hypothesis or female protective effect in this model. To do this, we will investigate the developmental origins of these sex differences by quantifying the changes in the expression of epigenetic markers at GD 15 and GD 18, and genes found to be differentially expressed by sex at PND 8.

The ***overall hypothesis*** of my research is that impaired COX-2 activity will alter the expression of genes and proteins in the developing brain in a manner consistent with previous transcriptomic studies on sex-differences in ASD and rodent studies on brain masculinization (discussed in section 2.2). More specifically, with respect to my first objective, my hypothesis is that *impaired COX-2 activity will further upregulate gene sets typically expressed at higher levels in males* (i.e., those associated with the immune system and inflammation), *and*

downregulate gene sets typically expressed at higher levels in females (i.e., those associated with synaptic and neuronal function). With respect to my second objective, my hypothesis is that *males with impaired COX-2 activity will exhibit more profound alterations in the expression of genes and proteins than females, and that the sex-differences found will be better supported by the female protective effect* than the male vulnerability hypothesis in this model.

1.5 Research Aims

The following thesis is comprised of two sequential studies, with the results from the first study used to both address specific research aims and direct investigations in study two. Both studies were conducted on *in vivo* mouse models of impaired COX-2 activity. The specific research aims addressed by this thesis included an investigation of:

1) The effects of impaired COX-2 activity on the expression of ASD-risk genes and neuroinflammatory markers in the brain of male and female mice during early adolescence.
2) The sex-dependent and sex-independent effects of impaired COX-2 activity on the expression of genes related to biological pathway implicated in NDDs in the brain of mice during early adolescence.
3) How the sex-dependent effects of impaired COX-2 activity arise by measuring the changes in the expression of genes, previously found to be differentially expressed in the adolescent brains of COX-2^{-} males and females, during early postnatal development.
4) How the sex-dependent effects of impaired COX-2 activity arise by identifying the biological processes associated with enriched gene sets in the prenatal brains of male and female COX-2$^{-/-}$ mice.

1.6 Significance

Animal models are an essential tool for providing a better understanding of the biological mechanisms involved in brain development and pathology in NDDs. Previous work in our lab using COX-2$^-$ mice, a genetic model that mimics prenatal exposure to antipyretic drugs, found that impaired cyclooxygenase activity led to increased repetitive, hyperactive and impulsive behaviors during both adolescence (PND 28-42) and early adulthood (PND 56-77). Notably, COX-2$^-$ males were found to exhibit more profound deficits than COX-2$^-$ females across all behavioral domains [53]. A number of epidemiological studies have also suggested that the most frequent and severe behavioral symptoms observed in children following prenatal exposure to antipyretic drugs was increased hyperactivity/impulsivity, and that males were more susceptible to these behavioral phenotypes [33,42,55–57]. In summary, COX-2$^-$ mice have been found to exhibit a considerable overlap in both the sex bias and behavioral phenotypes observed following prenatal exposure to antipyretic drugs. Thus, a better understanding of the sex-dependent effects of impaired COX-2 activity in this model may provide valuable insights into the pathological mechanisms of antipyretic drugs on neurodevelopment, which may ultimately facilitate a better understanding of the male bias in NDDs such as ASD and ADHD.

1.7 Layout of Thesis

This thesis is organized into chapters beginning with a general introduction (Chapter 1). This is followed by a literature review of the role of lipids in the brain, the male bias in NDDs, and the molecular mechanisms governing the masculinization of the mammalian male brain (Chapter 2). A detailed overview of the methodology used for both studies is then presented

(Chapter 3). Chapters 4-6 then include the background, results and analysis for each study, with each chapter subdivided into the research aims discussed above. A graphical illustration of the timeline of these studies with respect to mouse development is provided in Figure 1 below.

The first study (Chapter 4) uses quantitative real-time polymerase chain reaction (qRT-PCR) experiments to examine the impact of impaired COX-2 activity on the expression of various genes in the adolescent brain of COX-2^{-} mice at PND 25 (Aim 1), including both the sex-dependent and sex-independent effects (Aim 2). The second study (Chapter 5) includes an investigation of the developmental mechanisms governing the sex-dependent effects of impaired COX-2 activity. To do this, we first re-examine the genes most differentially expressed by sex in the adolescent brain of COX-2^{-} mice during early postnatal development using qRT-PCR experiments from samples collected on PND 8 (Aim 3). We then use the results of previous microarray experiments in prenatal COX-2$^{-/-}$ mice to identify the biological processes associated with enriched gene sets in the brains of males and females on GD15 and GD18 (Aim 4).

The final chapter of this thesis (Chapter 6) then includes a summary of the most significant findings across both studies, a collective interpretation of these results, and a discussion of the implications of these findings and how they relate to current literature on the male bias in NDDs such as ASD and ADHD.

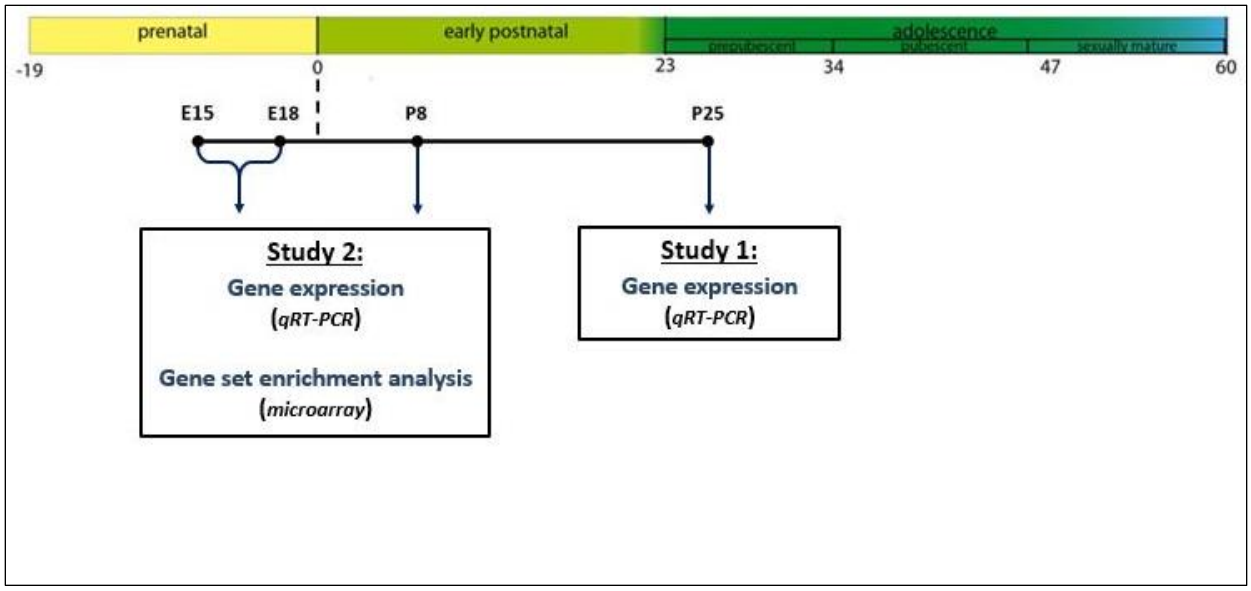

Figure 1. Mouse Developmental Timeline in Relation to Thesis Studies

The gestational period in mice typically lasts around 19 days with the observation of a vaginal plug being considered gestational or "embryonic" day 0.5 (E0.5). Following birth on postnatal day 0 (P0), mice undergo a period of early postnatal development lasting until around P23, which is followed by adolescence. Adolescence can be subdivided into prepubescent (P23-P34), pubescent (P34-P47), and sexually mature (P47-P60) stages. P60 then marks the end of adolescence and beginning of early adulthood in mice. Study 1 looks at the expression of various genes and proteins in the brain during early adolescence at P25. Study 2 looks at gene expression in the brain during early postnatal development at P8, and during late prenatal development at E15 and E18.

CHAPTER 2. LITERATURE REVIEW

2.1 Lipids in the Brain

2.1.1 Lipid Signaling during Brain Development

Lipids are the most abundant type of macromolecules in the brain [58]. They account for approximately 50-60% of the brain's dry mass [58–60], making the brain the most lipid-rich organ in the body next to adipose tissue. Lipid molecules also play an essential role in the structural formation of the brain [58,59]. For instance, the fatty acid profile of the brain is uniquely enriched with phospholipids and polyunsaturated fatty acids (PUFAs) that have been shown to play a critical role in a wide variety of developmental processes. In particular, phospholipids and PUFAs are essential structural components of cell membranes [58,59]. As such, they are both extremely abundant in and imperative to the formation of highly branched cells such as neurons and astrocytes. Additionally, the myelination of neuronal axons, a process where axons become ensheathed by fatty (lipid-rich) myelin, is a tightly regulated developmental process with the majority of it occurring during the perinatal period [61–64]. Interestingly, this developmental window for myelination is temporally correlated with an accumulation of PUFAs in the brain that happen to be enriched in myelinating cells (i.e., docosahexaenoic acid) [65–68]. Therefore, a sufficient supply of these PUFAs from the plasma may also be necessary for the structural formation of the myelin sheath by oligodendrocytes.

Lipids and their intermediates also serve as essential components of many functions in the brain. For instance, although the majority of brain lipids are found esterified to phospholipids in the cell membrane [69,70], they can also be released from the membrane and subsequently participate in various aspects of signal transduction. For example, PUFAs have been shown to

directly modulate the activity of various ion channels, receptors, and enzymes in the brain [71,72]. Furthermore, PUFAs liberated from the cell membrane can then be enzymatically converted to a variety of bioactive derivates [58–60]. These lipid signaling molecules have been shown to play a critical role in mediating various functions in the brain, such as neurogenesis [73], the promotion of neuronal survival [74], neuroplasticity and memory formation [75,76], the regulation of neuroinflammatory events [77–80], and cognitive development in children [81–84].

More recently, it has also been demonstrated that brain lipids are not merely static structures that, once incorporated into the cell membrane, will remain embedded for prolonged periods of time [59,85]. Rather, these macromolecules exhibit a highly dynamic pattern of kinematics that are undergoing continuous turnover. In fact, approximately 3-8% of PUFAs in the brain are replaced daily with fatty acids obtained from the plasma [85–88]. Notably, the brain also undergoes a period of rapid growth during development, starting around mid-gestation and continuing during the first few years of life [89,90]. Thus, any factors which adversely impact the supply and metabolism of lipids during development (i.e., dietary imbalances during pregnancy or pharmacological inhibition of lipid signaling pathways) may have detrimental effects on the proper development of the brain. Furthermore, considerable evidence now suggests that the occurrence of lipid imbalances or deprivation during perinatal development may result in lasting cognitive deficits that persist throughout adulthood [91–95].

2.1.2 Cyclooxygenase Enzymes & The COX-2/PGE_2 Pathway

The PGE_2 Signaling Pathway

One of the major PUFAs involved in lipid signaling in the brain is the pro-inflammatory omega-6 fatty acid derivative arachidonic acid (ARA) [96]. The release of ARA from the cell

membrane can initiate several intracellular signaling pathways, including the prostaglandin E_2 (PGE_2) pathway. As illustrated in Figure 1, the PGE_2 signaling pathway begins with the release of ARA from the cell membrane via the action of the cytosolic enzyme phospholipase A_2 (PLA_2). PLA_2 de-esterifies membrane phospholipids, thereby liberating bound PUFAs such as ARA. Previous research has shown that PLA_2 becomes enzymatically active in response to inflammatory, ischemic, and excitotoxic stimuli [97,98], as well as following the activation of phospholipase-bound receptors [99,100]. Once released, free ARA can then be metabolized by a variety of enzymes, including cyclooxygenase-1 and -2 (COX-1, -2). These enzymes catalyze the reaction of oxygen with ARA to create a variety of oxygenated PUFA derivatives, such as inflammatory prostaglandins (PGs), in a two-step process [101]. First, COX-1,2 enzymes temporarily convert ARA to the unstable prostaglandin precursor PGG_2 via the *cyclooxygenase reaction*. PGG_2 is then immediately converted by these same enzymes to the more stable precursor PGH_2 via the *peroxidase reaction*. Prostaglandin synthases subsequently convert PGH_2 to a variety of prostanoids, including PGE_2. Once synthesized, PGE_2 exerts its physiological effects through activation of one of four E-prostanoid (EP) receptors termed EP1-EP4 [102].

Cyclooxygenase Enzymes

Cyclooxygenases are considered the rate-limiting enzymes in the synthesis of PGs [103]. There are two major cyclooxygenase isoforms found in the body, COX-1 and COX-2, and they are differentially expressed throughout various tissues and cell types. The COX-1 enzyme is considered the constitutive isoform as it is ubiquitously expressed in the majority of tissues throughout the body in a relatively stable manner [104–106], and is suggested to primarily be involved in the synthesis of PGs required for homeostatic functions. COX-2, on the other hand,

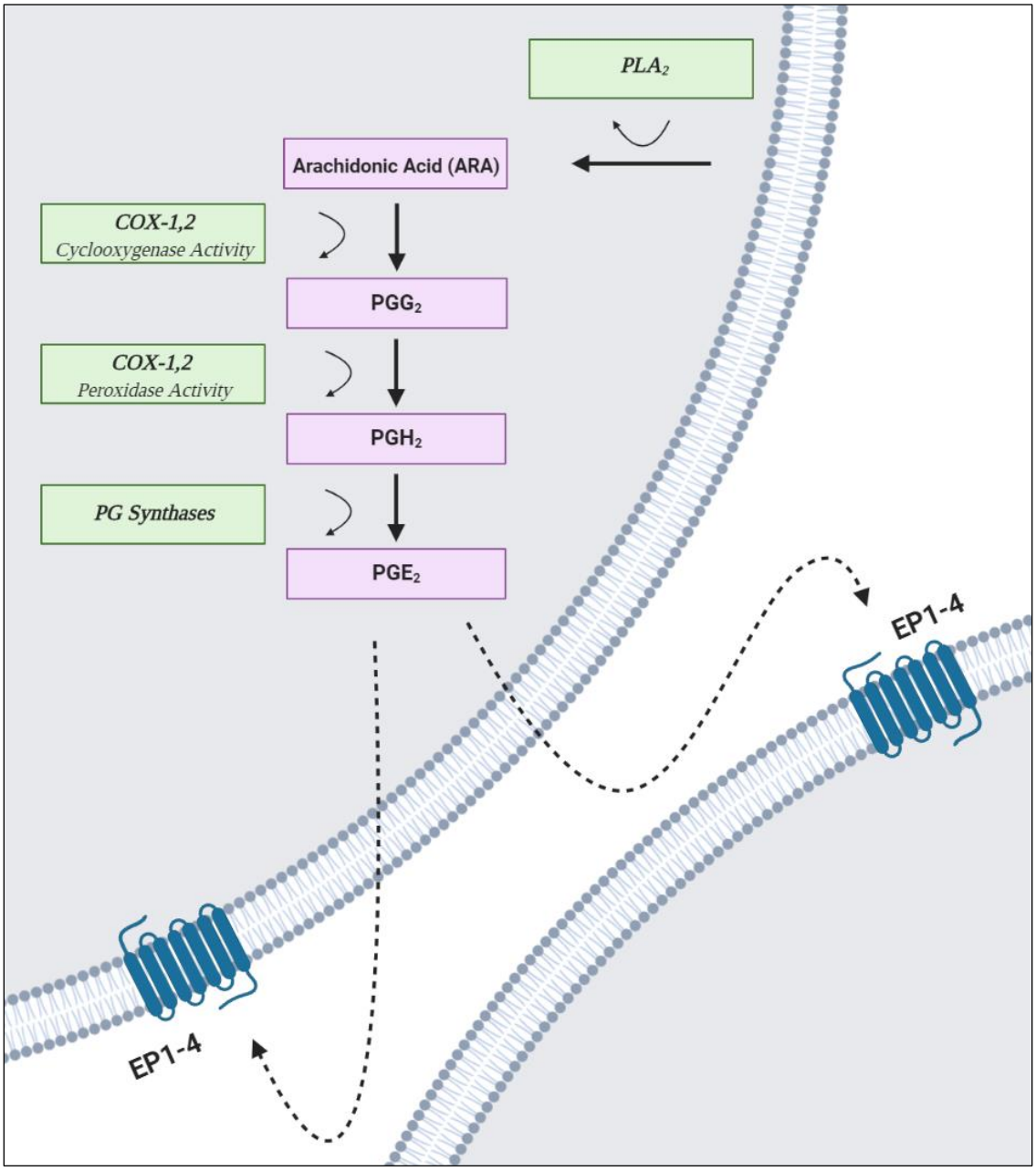

Figure 2. Overview of the PGE_2 Signaling Pathway

Arachidonic acid (ARA) is cleaved from membrane phospholipids by phospholipase A_2 (PLA_2) in response to various physiological and pathophysiological stimuli. Free ARA is then sequentially converted to the prostaglandin (PG) precursors PGG_2 and PGH_2 by cyclooxygenase enzymes (COX-1,2) in a two-step process. PGH_2 is then further metabolized by PG synthases into the highly potent autocrine and paracrine factor prostaglandin E_2 (PGE_2). PGE_2 subsequently exerts it's bioactivity by diffusing through the membrane and binding to one of four E prostanoid receptors (EP1-4) in the local environment.

is considered the inducible isoform as it is an immediate-early response gene that is strongly induced in response to inflammatory stimuli and oxidative stress [106–108]. The induction of COX-2 has also been shown to coincide with the upregulation of various neuroimmune signaling molecules in the brain, such as reactive oxygen species, as well as inflammatory cytokines and growth factors [107,109,110].

Furthermore, a variety of pathophysiological conditions, including inflammation [111], hypoxia [112], and ischemia [113], have also been associated with increased COX-2 expression in the brain. Under these conditions, COX-2 activity is typically induced in both neuronal and non-neuronal cells of the brain, including microglia and astrocytes [114,115]. Certain populations of neurons also express COX-2 at basal levels [116–118], although COX-2 expression in these neurons is often considered 'dynamic' rather than 'constitutive' [118] to reflect that COX-2 expression is further upregulated during inflammatory conditions [117] and is dependent on synaptic activity [116] in these cells.

EP Receptors & Role of PGE_2 in the Brain

EP receptors are high-affinity G-protein coupled receptors that exhibit differential expression patterns across varying tissues [119]. While all four EP receptor subtypes are expressed globally throughout the brain, numerous brain regions have also been found to exhibit unique patterns of expression that are distinct from one another [120,121]. EP receptors are also expressed in a variety of cell types in the brain, including neurons, microglia, and astrocytes [121–125]. Notably, the EP receptor subtypes are also linked to second-messenger systems capable of exhibiting functionally antagonistic effects [102,120,121]. These factors enable PGE_2 to have highly versatile actions in various regions and cell types throughout the brain, often with opposing effects.

PGE_2 is a highly potent autocrine and paracrine factor that primarily serves as a mediator of the inflammatory response in the brain by regulating neuroimmune signaling in the local environment [126]. Specifically, PGE_2 is able to rapidly diffuse across the cell membrane, bind to nearby EP receptors, and stimulate a number of signaling pathways that facilitate local immune responses in the brain (i.e., febrile and inflammatory responses) [127,128]. The activation of EP receptors by PGE_2 can also result in the modulation of several signaling cascades involved in brain development. For example, PGE_2 has been shown to play a role in the formation of dendritic spines [129,130], neuronal protection and survival [131], neurite retraction and apoptosis [132], synaptic plasticity [133], and learning and memory [134]. Increased expression of COX-2 and PG synthases [135,136], as well as EP receptor transcripts [137,138] during early- and mid-gestation, further supports the notion that PGE_2 may begin its involvement in shaping the brain at an early stage of development.

2.1.3 The COX-2/PGE_2 Pathway in Neurodevelopmental Disorders

Considerable evidence from both clinical and epidemiological studies suggests that various ERFs that impact the COX-2/PGE_2 pathway during development (illustrated in Figure 3) are associated with an increased risk of NDDs, including ASD and ADHD. For example, several studies have found that children with ASD and ADHD exhibit abnormal plasma concentration levels of pro-inflammatory omega-6 (ω-6) fatty acids and anti-inflammatory omega-3 fatty (ω-3) acids when compared to healthy controls. More specifically, children with ASD and ADHD have been found to exhibit either abnormally low plasma levels of ω-3 and ω-6 fatty acids [139–141] or a significantly lower ω-3/ ω-6 ratio [141–143] when compared to healthy age-matched children. Furthermore, supplementation of ω-3 and ω-6 fatty acids in children with ASD was found to

restore abnormal plasma levels and ratios of these fatty acids [142,144,145], and in some cases, even lead to improved performance in autism-related behaviors (i.e., language development [145], reciprocal communication [142,146], and stereotyped behaviors [144]). Similarly, the administration of PUFAs has been found to improve scores of inattention, hyperactivity, and impulsivity in children exhibiting elevated ADHD symptoms [147,148].

Several other classes of ERF's that can impact lipid signaling have also been associated with ASD and ADHD. These include maternal immune activation (MIA) resulting from maternal infections and fever during pregnancy [13,14,149–151], perinatal exposure to hazardous airborne pollutants (i.e., industrial and vehicular emissions, volatile organic compounds, and tobacco smoke) [152–158], and exposure to endocrine-disrupting chemicals (i.e., herbicides, pesticides, and synthetic plasticizers) during prenatal or early postnatal development [159–167]. For example, hazardous airborne pollutants have been shown to cause increased levels of inflammation and oxidative stress in the brain [168–170], both of which are believed to impact lipid signaling [96,171]. Specifically, oxidative stress can cause lipid peroxidation in cell membranes, thereby inducing the subsequent release of pro-inflammatory signaling molecules (i.e., PGE_2) upregulated during inflammatory events in the brain [172,173]. Several epidemiological studies have also indicated an association between prenatal exposure to antipyretic drugs and a subsequent clinical diagnosis of ASD or ADHD (see [15–17,46] for review). Notably, the results of recent studies investigating antipyretic drugs considered to be "low risk" (i.e., APAP) even suggest a dose-dependent relationship between the frequency of APAP use during pregnancy and an elevated risk of children developing NDDs, including ASD [17,32,41] and ADHD [16,17,41–43].

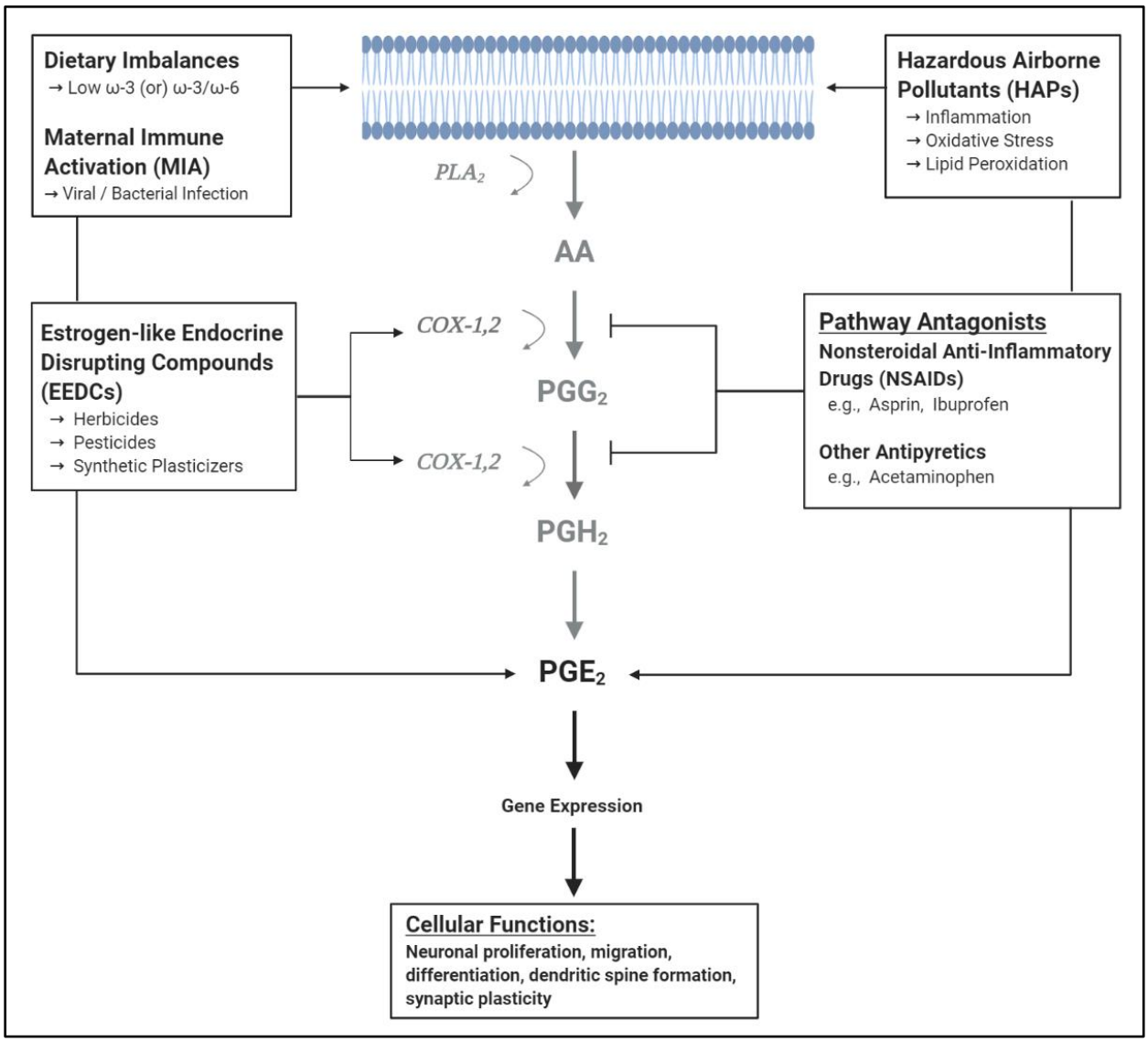

Figure 3. Environmental Risk Factors & the COX-2/PGE_2 Pathway
Environmental factors associated with an increased risk of neurodevelopmental disorders such ASD and ADHD that are linked to disruption of the COX-2/PGE_2 pathway in the brain (adapted from Wong et al., 2015).

2.2 Sex Differences in the Brain

2.2.1 Sex Differences in Neurodevelopmental Disorders

The majority of neurological and neuropsychiatric conditions exhibit differences in prevalence rates and severity among males and females, thereby providing some of the most

persuasive evidence for the existence of sex differences in the human brain [4]. With respect to adult-onset disorders, while many neurological conditions exhibit sex differences in prevalence rates, no clear sex bias can be found. That is, while males exhibit a higher incidence of certain neurodegenerative disorders (i.e., Parkinson's disease and amyotrophic lateral sclerosis [174–178]), others are found more commonly among females (i.e., Alzheimer's disease and multiple sclerosis [179–183]). Conversely, an explicit sex bias is found among childhood-onset disorders, with nearly all NDDs found to be more prevalent in boys than girls [4,5]. For example, the male-to-female ratio is approximately 4:1 for ASD [184,185], 2.5:1 in ADHD [186–188], and 2:1 in early-onset schizophrenia [189,190]. Additionally, evidence from etiological studies and animal models of these early-onset disorders suggest males exhibit more severe symptomology than females following exposure to a given etiological load [185,191]. As such, it would seem that being male is one of the most profound and significant risk factors for developing NDDs. Furthermore, while many extrinsic factors and experiences can contribute to an increased risk of developing adult-onset disorders, the male bias in NDDs is more likely to reflect inherent sex differences that occur naturally during brain development.

Current Theories on Sex Differences in NDDs: Insights from Studies on ASD

The following section will focus on research into the mechanisms underlying ASD in males and females, as it is among the most highly studied and sex-biased of the various NDDs [185,192]. Recent advances toward understanding the male bias in ASD have focused on addressing two critical questions. The first of which relates to whether higher rates of ASD can be attributed to an increased vulnerability in males or a reduced vulnerability in females [193]. More specifically, is normal male development associated with an upregulation of proteins and pathways which

render males more vulnerable to developing ASD, or is normal female development associated with inherently protective mechanisms that render females less susceptible to developing ASD?

The male vulnerability hypothesis suggests that if NDDs typically dysregulate genes expressed at higher levels in males, then they are more likely to adversely impact male brain development, thereby rendering males more vulnerable to genetic and environmental risk factors. Support for the male vulnerability hypothesis comes from transcriptomic studies showing that genes normally upregulated in male versus female brains were also those which were associated with biological pathways implicated in various neurological and neuropsychiatric conditions, including ASD [194–197].

Considerable support can also be found for the theory that inherently protective mechanisms arise during normal development in females [198]. For example, studies investigating familial patterns of ASD found that females with ASD are associated with significantly larger genetic changes or more explicit exposure to ERFs—indicating a larger etiological load may be required to induce adverse phenotypical behavioral characteristics in females than males [199,200]. Additionally, animal studies have found that inducing genetic mutations (or deletions) on ASD risk genes in rodents typically results in males exhibiting an increase in both the number and severity of ASD-related behavioral phenotypes [201,202]. Furthermore, in rats prenatally exposed to valproic acid (VPA), a common animal model used to study ERFs for ASD, males have been found to exhibit greater deficits in social behavior [203] and more profound alterations in glutamatergic synapse development [203,204]. VPA-exposed rats also exhibit a male-specific reduction in the expression of methyl-CpG-binding protein 2, a protein associated with silencing the transcription of other genes, suggesting females may be protected from epigenetic changes

following VPA exposure and that this may attenuate synapse dysregulation and behavioral abnormalities in females [204].

The second important question relates to whether the male bias in ASD can be attributed to sex differences in the expression of genes that are either directly or indirectly related to ASD. More specifically, are genes directly associated with ASD (or "ASD risk genes") expressed at different levels in males and females, or do ASD risk genes interact with sexually dimorphic biological pathways that play a role in normal male development?

Some previous transcriptomic analyses looking at gene expression patterns in individuals with ASD have provided indirect support for the latter hypothesis—that the genes upregulated in post-mortem autistic brains were those involved in molecular pathways and cellular processes which interact with ASD risk genes (i.e., those involved neuroinflammation and synaptic function), as opposed to ASD risk genes themselves [205,206]. However, one such study conducted by Werling and colleagues (2016) directly investigated the sex bias in ASD by comparing gene expression patterns in the post-mortem cortex of male and females with ASD (illustrated in Figure 4) [207]. In agreement with previous analyses, genes directly associated with ASD (ASD risk genes) were neither found to be expressed at higher levels in males, or at different levels between males and females. Conversely, genes that are indirectly associated with ASD—that is, those expressed at different levels in individuals with ASD, were also found to be differentially expressed by sex. More specifically, genes that were naturally upregulated in males compared to females were also expressed at higher levels in individuals with ASD compared to controls (Fig. 4a) [207].

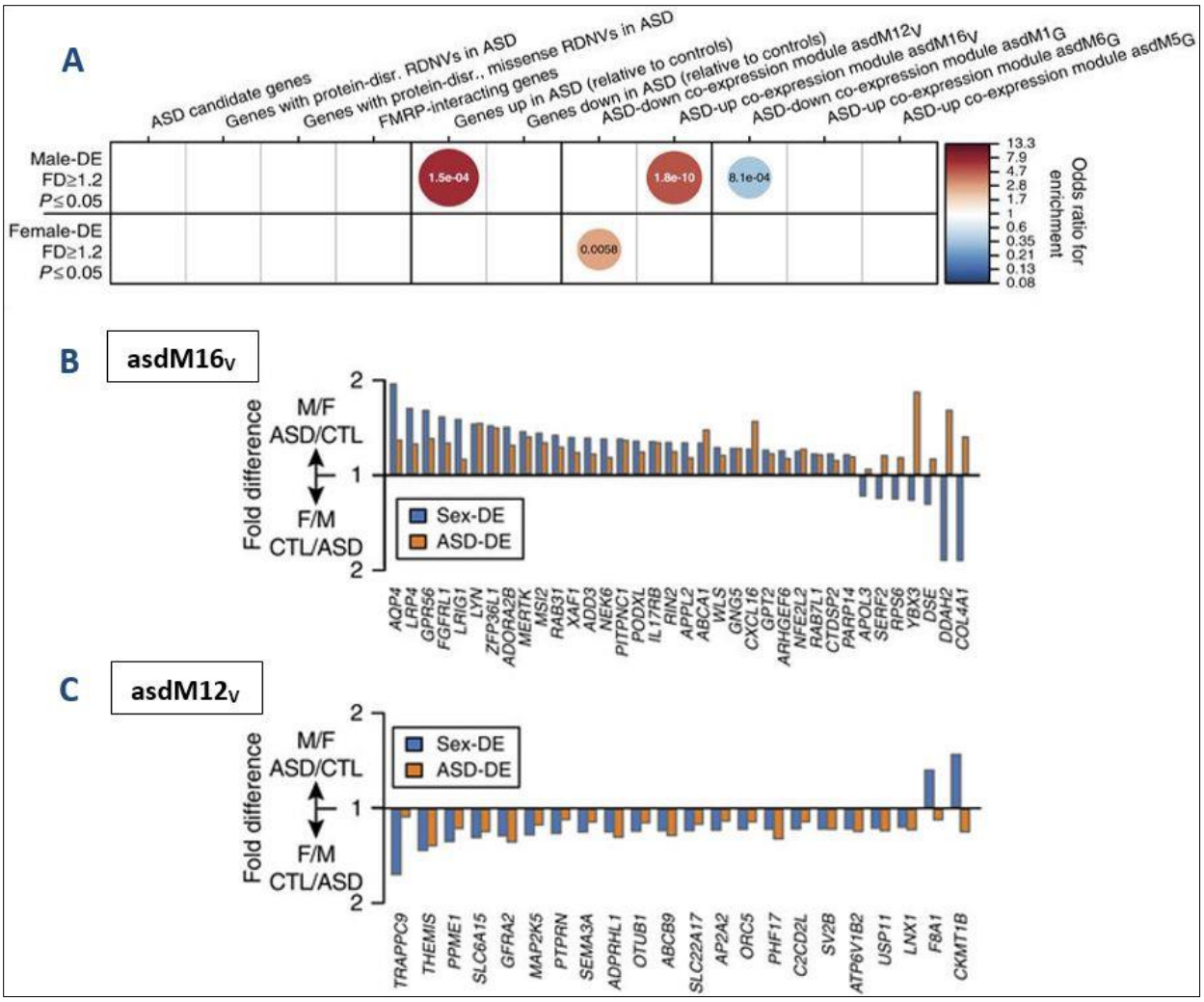

Figure 4. Sex-Dependent Gene Expression Pattern in ASD
Overview of transcriptomic analyses on gene expression patterns in the brains of males and females with and without ASD (adapted from Werling et al., (2016)). (**A**) Enrichment scores for both ASD-risk gene sets and ASD-associated gene sets in post-mortem cortical samples of individuals with ASD. (**B,C**) Fold-change of genes differentially expressed by sex and ASD status belonging to ASD co-expression modules. (**B**) Module asd16$_V$, which is enriched in inflammatory and immune system genes was upregulated males and individuals with ASD. (**C**) Module asd12$_V$, which is enriched in genes involved in neuronal and synaptic function, was upregulated in females and downregulated in individuals with ASD.

Notably, many of the genes in the Werling et al., (2016) study were disproportionately associated with neuroinflammation (i.e., activated microglia and reactive astrocytes) [207]. Furthermore, a re-analysis of the transcriptomic profiles of fetal cortices revealed that the male fetal cortex was also more indicative of inflammation compared to fetal females. As such, the

authors concluded that genes which interact with pathways involved in the regulation of normal male development were found to be elevated both in a sex-dependent manner (those higher in males vs. females were further upregulated in males vs. females with ASD), as well as among males with ASD compared to male controls [207,208]. In other words, rather than ASD risk genes being upregulated in males, it was the genes involved in normal development of the male brain that were being overexpressed.

In summary, support can be found for both the male vulnerability hypothesis and the female protective effect, although more evidence appears to support the latter [193,209]. However, it currently remains unknown whether the increased prevalence of ASD among males can be better explained by either theory alone or a combination of the two. Furthermore, it appears that ASD risk genes may interact with sex-specific biological pathways [207,208], thereby further upregulating gene sets typically expressed at higher levels in males, such as those associated with the immune system and inflammation (Fig. 4b), and downregulating gene sets typically expressed at higher levels in females, such as those associated with synaptic and neuronal function (Fig. 4c). Moreover, the finding that the neuroinflammatory gene sets enriched in ASD were also expressed higher in males than females during fetal development suggests that pathways involved in typical male brain development may be further upregulated in individuals with ASD [207,208].

2.2.2 Masculinization of the Male Brain

As discussed below, the masculinization of the male brain occurs at a specific timeline during development [210]. Therefore, any factors that might impact mechanisms governing brain masculinization are likely highlighted in brain regions that exhibit a sexually-dimorphic pattern

shortly thereafter—such as the medial preoptic area (mPOA). Development of the mPOA has been heavily studied in the context of sexual differentiation and can be used to illustrate possible neuroendocrine, neuroimmune, and neuroepigenetic factors underlying the emergence of sex differences in the morphology and neurochemistry of the brain. Furthermore, a better understanding of the process of brain masculinization during development may provide insight into the male bias in neurodevelopmental disorders such as ASD and ADHD.

Sexually Dimorphic Brain Regions – The Medial Preoptic Area (mPOA)

The mPOA has been long established as one of the most sexually dimorphic regions of the mammalian brain [211–215], and it has been shown to play a critical role in regulating male sexual behavior in rodents during adulthood [216,217]. Furthermore, the mPOA exhibits various sex-dependent neuroanatomical differences that arise shortly after the masculinization of the male brain. These include a two-fold increase in dendritic spine density among males [129,218], as well as a proportional increase in the number, morphological complexity, and activation of astrocytes [219] and microglia [220] compared to the mPOA of females. Notably, activated microglia are associated with increased production of pro-inflammatory cytokines (i.e., IL-6, IL-β, and TNFα). Additionally, these changes in mPOA glial and neuronal cells occur in parallel during development, reflecting the critical role of neuro-inflammatory signaling and communication in the process of brain masculinization [221].

Neuroendocrine System: A link between Sex Differences & the COX-2/PGE_2 Pathway

The primary driver of sexual differentiation in the male brain is an increase in both testosterone and estrogen following the onset of steroidogenesis in the fetal testes [222,223], typically late in the first trimester in primates [224], and around GD 16 in mice and GD 18 in rats [225].

Specifically, once in the brain, testosterone is aromatized to the estrogen steroid hormone estradiol (E_2), which has been well established as the dominant masculinizing hormone in the brain [226,227]. It should be noted that while the developing fetus is exposed to maternal E_2 during pregnancy, the presence of α-fetoprotein (a circulating binding globulin) binds E_2 with high affinity, thereby sequestering it in the peripheral circulation and preventing it from masculinizing the female brain [228]. Conversely, the aromatization of testosterone secreted by the fetal testes induces elevated E_2 levels in the male brain, thereby initiating masculinization [193]. Notably, if α-fetoprotein is ablated, maternal E_2 will penetrate and subsequently masculinize the female fetal brain [228]. Thus, while females are equally sensitive to E_2 as males, their brains are not typically exposed to significant amounts during development [225].

Elevated E_2 levels in the male brain during development has been shown to lead to an upregulation of both COX-1 and COX-2 enzymes, subsequently resulting in higher endogenous levels of the pro-inflammatory signaling molecule PGE_2 [23]. Additionally, similar increases in COX-1,2 mRNA and protein expression and levels of PGE_2 have also been observed in the mPOA of males during the critical period for sexual differentiation of the brain [22]. A series of studies also found that the administration of COX-2 inhibitors to newborn male rats blocked masculinization of the mPOA in the brain and impaired male copulatory behavior during adulthood [23]. Moreover, treating newborn females with a masculinizing dose of E_2 was found to increase the expression of COX enzymes and PGE_2 levels by PND 2. Similarly, a single dose of PGE_2 injected into the mPOA of newborn female rats was found to induce the stereotypical male synaptic profile in the mPOA, and masculine sexual behavior during adulthood [129]. Notably, the masculinizing effects of PGE_2 treatment in neonatal females were completely blocked by the co-

administration of COX inhibitors [229]. As such, these studies demonstrated that PGE_2 functions as the critical mediator of steroid-induced brain masculinization in rodents [193].

It should be noted that while the findings of these early rodent studies provided some insight into the neurochemical mechanisms governing masculinization of the male brain, they also raised new critical questions that remained unanswered. First, how could a single dose of PGE_2 have such a profound and long-lasting effect on females. Second, how could these changes in the synaptic profile of the mPOA occur so rapidly. The answers to these questions are provided, at least in part, by considering how sex differences in neuroendocrine and neuroimmune mechanisms interact during brain development.

Neuroimmune & Neuroinflammatory Signaling

Microglia are one of the brain's resident immune cells, and their communication with inflammatory signaling molecules has been found to play a critical role in regulating the sexual differentiation of the mPOA [220]. They migrate to the brain early in fetal development, beginning around the 4^{th}-5^{th} gestational week in humans [230] and GD 8-9.5 in mice and rats, respectively [231,232]. Several studies on rodents suggest that the mechanisms governing the masculinization of the male brain coincide with sex differences in the proliferation of microglia between males and females [233]. For example, one study found that on GD 17 (just prior to the fetal androgen surge following steroidogenesis), males and female rats had the same number of microglia in the brain [233]. When measured one week later, males were found to have significantly more microglia in several brain regions—including regions involved in brain masculinization (i.e., the mPOA) [215,223,227], as well as brain regions commonly implicated in ASD and ADHD (i.e., the hippocampus and amygdala) [234–236]. In another study, Lenz and colleagues (2013) found twice as

many microglia in an activated state in the mPOA of neonatal male rats than age-matched females. In contrast to quiescent microglia, which are characterized by a ramified morphology, activated microglia are characterized by an amoeboid morphology and serve as a major source of pro-inflammatory signaling molecules in the brain, including PGE_2 [233,237]. Interestingly, treating newborn females with a single dose of E_2 or PGE_2 increased the number of activated microglia and PGE_2 levels in the mPOA to what was found in males [220]. Additionally, administration of minocycline (a microglial inhibitor) to female rats during sexual differentiation was found to prevent exogenous E_2 from increasing PGE_2 levels and inducing masculinization of both dendritic morphology in the mPOA and sexual behavior during adulthood [220]. Conversely, temporary depletion of microglia from the neonatal brain of male rats led to complete loss of sexual behavior during adulthood [238]. Thus, microglia are thought to play an essential role in sex-dependent synaptic modifications by providing positive feedback of PGE_2, thereby facilitating the rapid rise in PGE_2 concentration in the developing mPOA in males following testicular androgen production [193].

In summary, a positive feedback loop between neurons and microglia seems to underlie much of the rapid changes in PGE_2 levels during sexual differentiation of the brain and male copulatory during adulthood [220,238] and thus helps to explain how a single dose of PGE_2 in neonatal female rats could have such a profound and long- lasting effect [129]. However, it should be noted that it does not account for how prostaglandins produce rapid changes in the formation of dendritic spines and synapses at the time of birth. For a more comprehensive understanding of how sex differences in prostaglandin levels can induce such rapid changes in the synaptic profile of the mPOA, and the role of neuroimmune and neuroinflammatory signaling during brain

masculinization, we must also consider the contribution of one of the brain's other glial cells involved in neuroimmune signaling—the astrocyte.

Astrocytes have also been shown to play a significant role in neuroimmune communication and synapse formation during development [239]. Additionally, the mPOA is characterized by the early maturation of astrocytes, and differences in astrocyte morphology between males and females are thought to play a significant role in establishing the sex differences in dendritic morphology of this region [219]. More specifically, astrocytes of the male mPOA exhibit a more highly branched and "stellate" (star-shaped) morphology than those of females. Furthermore, much like activated microglia, the high stellate morphology of male astrocytes is consistent with an increased "activational state", meaning they are more excitable, and "reactivity", meaning they release a greater number of inflammatory mediators [219,240].

Support for the involvement of astrocytes in brain masculinization comes from animal studies involving rats which found that treatment of neonatal females with either E_2, or its precursor (testosterone), masculinizes the morphology of astrocytes to the point of being indistinguishable from males [219]. This stellate morphology of astrocytes in the mPOA is accomplished in response to increased PGE_2 [218], which induces glutamate synthesis and release from neighboring neurons in the mPOA. In turn, this change in astrocyte morphology correlates with an increase in dendritic spine synapses on mPOA neurons, possibly via a positive feedback loop wherein glutamate released from presynaptic neurons promotes astrocytic release of glutamate onto post-synaptic neurons [239,241–243].

Thus, it seems a complex interplay between neuroinflammatory and neuroimmune signaling molecules interact to facilitate the masculinization of the mPOA. In summary, this is initiated following steroidogenesis where circulating testosterone crosses the blood-brain barrier

and diffuses into the excitatory neuronal cells of the mPOA [226–228]. Testosterone is then aromatized to E_2, which subsequently binds to estrogen receptors and leads to the increased expression of COX-2 and levels of the neuroinflammatory prostaglandin PGE_2 [22,23]. PGE_2, in turn, then undergoes a positive-feedback loop with both local microglia and astrocytes, ultimately resulting in the masculinization of the synaptic profile in the mPOA [218–220]. Notably, while the studies described so far facilitate an understanding of the mechanism of brain masculinization in the mPOA during healthy development, they do not provide a link between brain masculinization and the male bias in NDDs. For a better understanding of this link, we must also consider the impact of brain masculinization on epigenetic mechanisms in the mPOA.

Epigenetic Factors

The expression of DNA methyltransferase (DNMT) enzymes is significantly greater in the developing POA of female rats than males [244], suggesting that a set of genes in this region are epigenetically suppressed more strongly in females. To gain insight into the nature of those genes, newborn female rats were treated with DNMT inhibitors, which reduced methylation of these genes. Subsequent comparisons between the transcriptome of females administered DNMT inhibitors with age-matched controls revealed that the genes normally suppressed by epigenetic modifications in females were associated with immune regulation. Furthermore, administering DNMT inhibitors to neonatal females during the sensitive period for sexual differentiation was also found to induce masculinization of the mPOA— suggesting that the reduced methylation pattern among immune regulatory genes in the male mPOA may relate to the increased exposure to androgens in the male brain. This was confirmed by the discovery that administering exogenous E_2 to neonatal females during the critical period of sexual differentiation reduced

DNMT activity and DNA methylation to male levels in the mPOA [244].

An overview of the factors contributing to the masculinization of the rodent mPOA is illustrated in Figure 5. Notably, the human mPOA has is considered to organized in a manner that is functionally and biochemically analogous to these rodent models [215,245]. Therefore, collectively these studies provide strong support for the notion that the process of brain masculinization in humans is dependent on both inflammatory and endocrine-mediated factors. Additionally, epigenetic differences in the mPOA between males and females support the hypothesis that lower rates of ASD among females may result from inherently protective mechanisms arising during normal development [246].

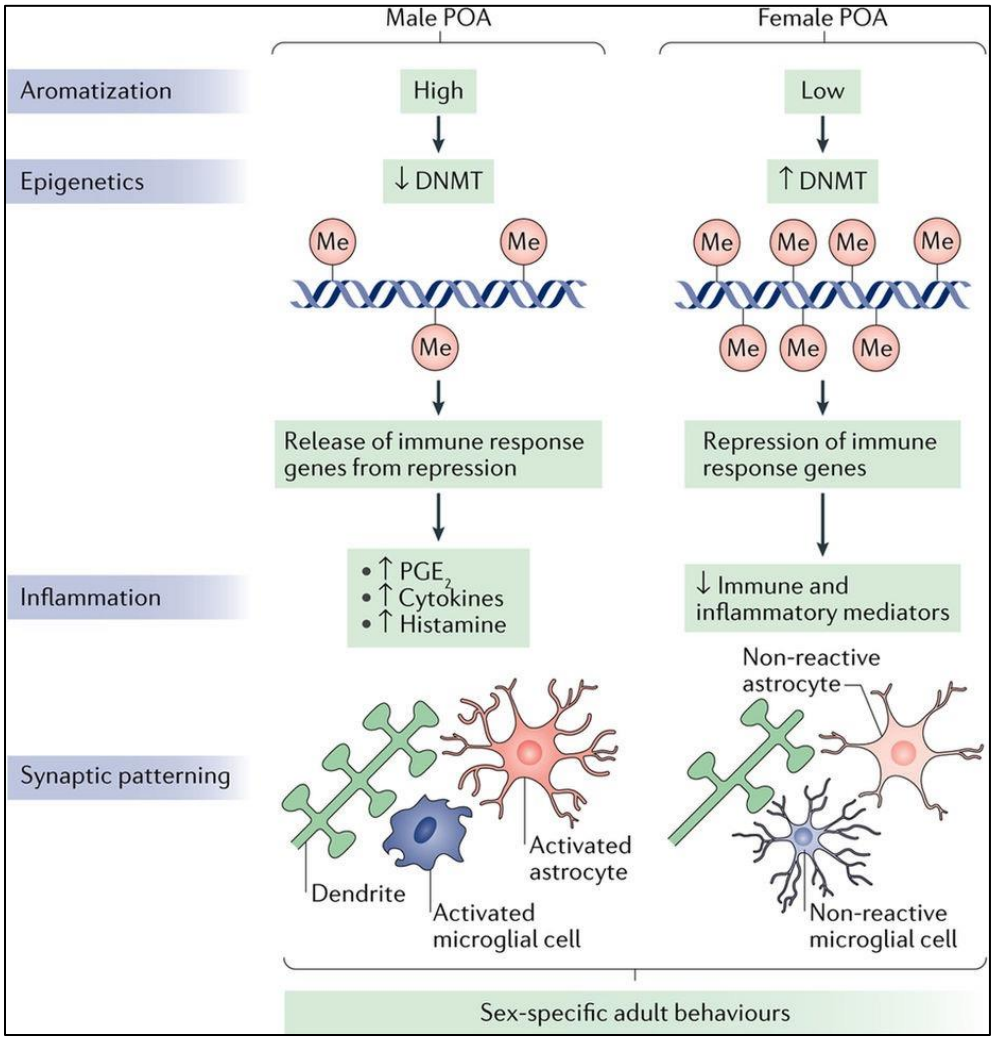

Figure 5. Sex Differences in the Developing Brain
Overview of factors contributing to masculinization of the preoptic area (POA) in the mammalian brain and the resulting sex differences in this region (from McCarthy et al., 2018).

CHAPTER 3: METHODS

3.1 Experimental Model

3.1.1 Cyclooxygenase-2 Knockin Mice (COX-2$^-$)

The following qRT-PCR experiments were conducted using COX-2$^-$ mice obtained from Jackson Laboratories (JAX, stock #008101). Briefly, these mice were developed as a genetic model of COX-2 specific inhibition via a targeted point mutation on the *Ptgs2* gene, resulting in an amino acid substitution [247]. In a manner analogous to the effects of selective COX-2 inhibitors, and other traditional NSAIDs, this mutation leads to complete inhibition of the cyclooxygenase activity of prostaglandin H synthase-2 (PTGHS2) but does not affect the associated peroxidase activity [248]. Thus, this model was chosen due to its ability to mimic the mechanism of action of COX-2 inhibition exhibited by these ERF's associated with NDDs such as ASD and ADHD [32,33,42,46]. Mice were backcrossed for at least 5 generations to 129S6/SvEvTac wildtype mice (Taconic Laboratory) which were used as control. Due to the infertility of homozygous COX-2$^-$ females, breeding in COX-2$^-$ mice was carried out by crossing homozygous COX-2$^-$ males with heterozygous COX-2$^-$ females to generate the homozygous COX-2$^-$ offspring that were used as the COX-2$^-$ experimental model in this research. All mice were bred and maintained in group housing under the same conditions at the York University Vivarium on a 12-hour light/dark cycle. All protocols and experiments were approved by the Research Ethics Board of York University and were conducted in accordance with the York University Animal Care Committee ethical guidelines.

3.1.2 Cyclooxygenase-2 Knockout Mice (COX-$2^{-/-}$)

The following microarray analyses were conducted using COX-$2^{-/-}$ mice and wildtype controls obtained from Taconic Laboratories (Taconic Biosciences, stock # 002181). Briefly, these mice were developed as a genetic model of a COX-2 specific constitutive knockout. This was accomplished by disrupting the *Ptgs2* gene via a combined insertion of a neomycin resistance gene and deletion of a portion of exon 8 [249]. The resulting COX-$2^{-/-}$ mice lack a functional *Ptgs2* gene and are characterized by a lack of a febrile response [250,251] and PGE_2 synthesis following exposure to LPS [249], as well as the absence of COX-2 mRNA induction or functional enzymatic activity. This model is functionally analogous to the COX-2^{-} mouse model with the major distinction being complete inhibition of both cyclooxygenase activity and peroxidase activity occurs in COX-$2^{-/-}$ mice [249]. Thus, with respect to effects of antipyretic drugs, the COX-$2^{-/-}$ mouse model more closely mimics the combined actions of NSAID and APAP exposure [248,252].

3.2 Genotype Analysis

Genotype analysis of all 129S6 and COX-2^{-} offspring was conducted using DNA collected from ear punch tissues samples. The collected tissue samples were then denatured and homogenized using an alkaline lysis buffer (25mM NaOH) at 95°C for 30 mins, followed by the addition of a neutralization buffer (Tris-HCl). To verify the sex of the animals used for experimentation, Polymerase Chain Reaction (PCR) analysis of the Sex-determining region Y gene (*Sry*) was conducted. For COX-2^{-} mice, PCR analysis of the gene encoding COX-2 (*Ptgs2*) was also used to identify the genotype of the offspring. All PCR reactions were conducted using

Taq DNA polymerase in accordance with the manufacturer's instructions (Bio Basic, #B0089). Standard PCR experiments were then performed in an Eppendorf 5331 Mastercycler according to the Jackson protocol for this strain (The Jackson Laboratory, Protocol #28516) using the primers listed in Table 1. DNA bands were then separated and visualized by gel electrophoresis on a 1.5% agarose gel containing SafeView Classic (Abm, #G108).

Table 1. PCR Primer Sequences

Name	Primer	Length	Sequence (5' to 3')
Gapdh	Forward	20	TTGTGATGGGTGTGAACCAC
	Reverse	20	GTCTTCTGGGTGGCAGTGAT
Ptgs2	Forward	20	ACCAGTCTCTCAATGAGTAC
	Reverse	22	AGAATGGTGCTCCAAGCTCTAC
Sry	Forward	28	TCCCAGCATGCAAAATACAGAGATCAGC
	Reverse	25	TTGGAGTACAGGTGTGCAGCTCTAC

3.2 Brain Extraction and RNA Isolation

On post-natal day 8 (PND 8) and PND 25, COX-2^{-} and 129S6 mice were first sedated using an isoflurane chamber and then immediately decapitated. Whole brain tissue was sequentially extracted, added to a tube containing 1 mL of Trizol (Invitrogen) and 1% protease inhibitor cocktail (Sigma, #P8340), and immediately placed on ice. RNA isolation was performed via a modified Trizol method (Invitrogen), where fresh brain tissue was homogenized shortly after extraction. Chloroform was then added to the supernatant to facilitate phase separation of RNA from DNA and protein. The RNA-containing aqueous layer was then precipitated using 100% isopropyl alcohol, washed with 75% ethanol, and resuspended in

RNAse-free water. Immediately following resuspension, the collected RNA samples underwent DNAse treatment (New England Biolabs, #M0303S) in accordance with the manufacturer's instructions.

3.3 Quantitative Real-Time PCR (qRT-PCR)

3.3.1 Primer Design & Validation

In accordance with guidelines listed in the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE guidelines) [253], the relevant aspects of primer design and validation steps taken have been included in the methods section. Primer sequences are listed in Table 2. Primer sequences for six of these genes (*Hprt*, *Glo1*, *Grm5*, *IL-6*, *IL-1β*, *Itgam*) were designed and validated previously by other students in my lab using Primer Express 3.0 software (Thermo Fisher Scientific). Primer sequences for these genes remained unchanged to allow for direct comparison between the current experiment (on samples from PND 25) and previous experiments (on samples from PND 8) from our lab.

Specific primers for the 9 remaining genes of interest (*Sdha*, *Aqp4*, *Gfap*, *Gsk3β*, *iNos*, *Nox2*, *S100a10*, *Serpina3n*, and *Serping1*) were designed using NCBI's Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) under the Primer3 default settings with the following exceptions. Primer melting temperatures were reduced to range 58- 62°C, and a max temperature difference of 2°C. Amplicons were designed to be between 80-150 bp in length as recommended by the manufacturer (Wisent, Cat # 801-001). To avoid the amplification of genomic DNA, forward and reverse primers either spanned an exon-exon junction, or included an intro of at least 1000 bp. To ensure specificity, both the primer and amplicon sequences were

checked using Primer-BLAST (above) and UCSC Blat (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start) platforms. Secondary structure of the forward and reverse primers was then checked using Beacon Designer (Premier Biosoft) under the default settings for SYBR Green assays. When predicted secondary structures could not be avoided, primers with predicted free energy more negative than -3.5 kcal/mol were rejected. At the 3' end of primers (last 5 base pairs), more stringent restrictions were used. Such primers with predicted free energy more negative than -2.0 kcal/mol, as well as those with greater than 2 bp matches were rejected. Predicted secondary structures in primer sequences were further checked using the Mfold software OligoAnalyzer (Integrated DNA Technologies) to ensure such structures should not hold together at the annealing temperature of 60°C. A similar Mfold software (UNAFold Tool, Integrated DNA Technologies) and procedure was used to check the amplicon for predicted secondary structures.

Once designed, oligonucleotides were subsequently ordered (Sigma Aldrich), and validated in the following manner. qRT-PCR primer efficiency was assessed in triplicates using a five-point 1:3 serial dilution of the cDNA and included a -RT (no cDNA) triplicate as a negative control. To be considered acceptable, primer pairs were restricted to those that fell within a range of 85-110% efficiency as determined by analysis of the standard curve. Both melt curve analysis and agarose gel visualization were also performed to assess primer specificity prior to primers being considered valid. To further optimize genes with low threshold cycle (Ct) values (between 30-32), a 4x4 primer concentration matrix with forward/reverse primer concentrations ranging from 100-600 nM was used determine the optimal primer concentration and combination. Gradient PCR (56-64°C) was also used to determine the optimal annealing temperature for these primers.

Table 2. Quantitative Real-Time PCR Primer Sequences

Name	Primer	Length	Sequence (5' to 3')
Aqp4	Forward	21	AGTCACCACGGTTCATGGAAA
	Reverse	21	CAGTTCGTTTGGAATCACAGC
Gfap	Forward	22	TTTGGAGAGAAAGGTTGAATCG
	Reverse	25	CTCGAACTTCCTCCTCATAGATCTT
Glo1	Forward	22	GGATTTGGTCACATTGGGATTG
	Reverse	20	CGTCATCAGGCTTCTTCACA
Grm5	Forward	22	CATGGAGCCTCCGGATATAATG
	Reverse	22	GTATCCAAGAGGAGTGACAACC
Gsk3β	Forward	21	GGTGTGGATCAGTTGGTGGAA
	Reverse	25	TCATTTCTCTAATTTGCTCCCTTGT
Hprt	Forward	29	TCCATTCCTATGACTGTAGATTTTATCAG
	Reverse	23	AACTTTTATGTCCCCCGTTGACT
IL-1β	Forward	24	CCACCTCAATGGACAGAATATCAA
	Reverse	22	GTCGTTGCTTGGTTCTCCTTGT
IL-6	Forward	24	TCGGAGGCTTAATTACACATGTTC
	Reverse	22	TGCCATTGCACAACTCTTTTCT
iNos	Forward	21	CAGCTGGGCTGTACAAACCTT
	Reverse	21	CATTGGAAGTGAAGCGTTTCG
Itgam	Forward	22	CATCCTGCGCCTCAATTATACA
	Reverse	24	GGGAAACATAGCTGTGAAGAACCT
Nox2	Forward	24	CAGGAACCTCACTTTCCATAAGAT
	Reverse	24	AACGTTGAAGAGATGTGCAATTGT
S100a10	Forward	19	GCAGGCGACAAAGACCACT
	Reverse	21	AGCCAGAGGGTCCTTTTGATT
Sdha	Forward	20	GCTCCTGCCTCTGTGGTTGA
	Reverse	19	AGCAACACCGATGAGCCTG
Serpina3n	Forward	24	TGAAACCCAGGATGATAGATGAGC
	Reverse	20	CCCTGATGCCCAGCTTTGAA
Serping1	Forward	20	GCCTCGTCCTTCTCAATGCT
	Reverse	20	CGCTACTCATCATGGGCACT

3.3.2 qRT-PCR & Analysis

DNAse-treated RNA samples were first reverse transcribed to cDNA with M-MuLV reverse transcriptase (New England Biolabs, Cat # M0253) in accordance with the manufacturer's instructions. By convention, the quantity of cDNA obtained from reverse transcription (RT) was assumed to reflect a 1:1 ratio of RNA converted to cDNA. Following RT, PCR for the gene *Gapdh* was conducted (using primer sequence in Table 1) to confirm the presence of cDNA in the RT samples. For the PCR reaction, 60 ng of cDNA was used with 1x Taq reaction buffer (Biobasic, Cat # 37A), $MgSO_4$ (2 mM), dNTP (200 µM), forward/reverse primers for *Gapdh* (1 µM each), and 2 units of Taq DNA Polymerase (Biobasic, Cat # B0089) diluted in ddH_2O.

For the following qRT-PCR reactions at PND 25, all 129S6 wildtype male samples were first pooled (5 individuals from 3 separate litters). Pooling of samples was also done with all 129S6 wildtype females (8 individuals from 3 separate litters). Conversely, both PND 25 COX-2^- males and females were measured individually (with each sex containing 3 individuals from 3 separate litters). On the other hand, for the qRT-PCR reactions at PND 8, samples were also pooled for all 129S6 wildtype males (11 individuals from 3 separate litters) and 129S6 wildtype females (6 individuals from 3 separate litters). Similarly, samples were also pooled for COX-2^- males (6 individuals from 3 separate litters) and COX-2^- females (8 individuals from 3 separate litters) at PND 8.

All qRT-PCR runs were carried out using a 7500 Fast RT-PCR system (Applied Biosystems) using SYBR green reagents (Wisent, Cat # 800-43) according to the manufacturer's instructions (Wisent). For each gene, three separate runs (3 technical replicates) were performed.

Two reference genes were used as endogenous controls—Hypoxanthine phosphoribosyl transferase (*Hprt*) and succinate dehydrogenase complex flavoprotein subunit A (*Sdha*). For each run, the relative gene expression was calculated using the comparative CT method (ΔΔCT), with the raw Ct values normalized using the difference between the mean Ct value of the gene of interest and the geometric mean of the endogenous controls to obtain a ΔCT value. Relative quantification (RQ) values were then computed. RQ values represented the fold change in gene expression of each sample (129S6 females, COX-2$^-$ males, and COX-2$^-$ females) compared to that of 129S6 males (RQ = 1), which by convention served as the reference sample for these experiments. These RQ values were calculated using the following formula:

$$RQ = 2^{-\Delta\Delta CT}$$

To facilitate a more meaningful interpretation of the qRT-PCR results, the relative fold-change (FC) values, representing the ratio of RQ values, for each comparison discussed in the text will be also be presented. Unless otherwise stated, when comparing males and females the FC values will be calculated as: RQ_{Female} / RQ_{Male}, whereas for comparisons between COX-2$^-$ and 129S6 mice the FC values will be calculated as: $RQ_{COX\text{-}2^-} / RQ_{129S6}$. Alternatively, negative FC values (-FC) represent the inverse of these ratios.

3.3.3 Statistical Analysis

The mean RQ value from each triplicate was used to calculate the mean value associated with each biological sample for a given qRT-PCR run or technical replicate. The numerical values illustrated in the bar graphs below represent the mean RQ values +/- standard error of the mean (SEM) obtained from three technical replicates for each biological sample. All statistical analyses were conducted using GraphPad Prism 8.0. Two-way (Genotype X Sex) ANOVAs were

performed first to assess differences in gene expression. Following significant interactions, *post hoc* comparisons (Bonferroni's pairwise comparison test) were performed to further distinguish differences between all groups of interest (that is, groups that only differed by one factor). In cases where interactions were not significant but main effects were, *post hoc* comparisons were also performed to assess differences between groups on factors that obtained significance (see [254,255] for a thorough rationale of this approach). For each comparison, the familywise error rate was controlled at $\alpha = .05$ and p-values were adjusted so that significance was assumed at $p < .05$.

3.3.4 Selection of Astrocyte Subtype Markers

The following criteria was used to identify and validate the selection of astrocyte subtype markers. First, data obtained from previous microarray experiments on astrocytes cultured from mice aged P30-P35 was used to identify PAN-reactive astrocyte markers (those upregulated in both LPS injected vs. saline injected, and middle cerebral artery occlusion (MCAO) vs. sham surgery mice), A1 astrocyte markers (those upregulated in LPS injected vs. saline injected mice only), A2 astrocyte markers (those upregulated in MCAO vs. sham surgery mice only), and non-reactive astrocyte markers (astrocyte markers not significantly upregulated in either LPS or MCAO mice models) [256]. Then, to be considered valid for qRT-PCR analysis in whole brain samples, the expression of each astrocyte subtype marker was cross-referenced against studies using whole brain RNA-expression profiles of each model (LPS vs. saline and MCAO vs. sham) to ensure similar findings between assays on cultured astrocyte ($FC \geq 5$) and whole brain assays ($FC \geq 2$) [256,257]. Lastly, each marker also had to be expressed at ≥ 5 fragments per kilobase of transcript per million mapped reads (FPKM) in previous analyses of whole brain samples obtained from C57BL6 wildtype mice at PND 7 and PND 32 [258].

3.4 Microarray Analyses

3.4.1 Microarray Experiments & Analyses

Data from two gene expression microarray experiments were used in our current analysis. The first experiment was conducted on COX-$2^{-/-}$ and 129S6 wildtype males at GD 15 and GD 18, and the second on COX-$2^{-/-}$ and 129S6 wildtype females at the same stages. All samples were previously collected (for each group, n = 3 individuals from a single litter) and prepared by another PhD student in our lab using the same methods described in section 3.2. Following brain collection and RNA isolation, the RNA samples were sent to the Princess Margaret Genomics Centre, Toronto, Canada (www.pmgenomics.ca) for completion of the microarray experiment and analysis. The male COX-$2^{-/-}$ microarray experiment was conducted using an Illumina microarray platform (Mouse WG-6 V2 BeadChip) containing 45,821 probe sets and then analyzed using GeneSpring (version 12.6.1). On the other hand, the female COX-$2^{-/-}$ microarray experiment was conducted using an Affymetrix microarray platform (Affymetrix Mouse Gene ST 2.0 arrays) containing 34,351 probe sets and then analyzed using GeneSpring (version 13.1.1).

The preliminary gene expression analysis of the microarray data was also performed by the Princess Margaret Genomics Center, Toronto, Canada (www.pmgenomics.ca). Briefly, the microarray data in each experiment was first $\log_{(2)}$ transformed, then normalized (using quantile normalization for Illumina data and RMA-16 normalization for Affymetrix data), and then filtered to improve the signal-to-noise ratio by removing probe sets falling in the lowest 20% of intensity in each group. For statistical analyses, a one-way ANOVA with Benjamini-Hochberg

false discovery rate (FDR) corrected p-values followed by Tukey HSD *post hoc* tests were performed to identify probe sets that varied significantly ($p < .05$) between groups of interest.

3.4.2 Functional Enrichment Analysis

For the current analysis, the previous microarray data was filtered to identify significantly upregulated (FC ≥ 1.5, $p < .05$) and downregulated (FC ≤ -1.5, $p < .05$) probes in COX-$2^{-/-}$ males and females at GD 15 and GD 18 using the statistical software R. All subsequent analyses were performed using the g:Profiler web server (http://biit.cs.ut.ee/gprofiler/). First, each set of differentially expressed probes were further refined to include only "non-ambiguous" and "annotated" genes. More specifically, only probes that could be mapped to a single protein-coding gene in the Ensemble database and had at least one annotation in the Gene Ontology (GO) database, hereafter referred to as "previously annotated, non-ambiguous genes", were included in the functional enrichment analysis. Since the adjusted p-values from the statistical analysis of the microarray data corresponded to each group of interest (COX-$2^{-/-}$ males at GD 15, COX-$2^{-/-}$ males at GD 18, COX-$2^{-/-}$ females at GD 15, and COX-$2^{-/-}$ females at GD 18), each gene set was submitted for functional enrichment analysis individually.

Differentially expressed genes from each group were first subdivided into upregulated and downregulated genes and organized into a ranked list (reflecting their differential expression values) that was then analyzed using the recommended settings for this type of cross-platform array data. In particular, the functional enrichment analysis was performed using g:Profiler (version e99_eg46_p14_f929183) under the gene set counts and sizes (g:SCS) multiple testing correction method (see [259] for details), with GO annotated genes as the background statistical domain and at a significance threshold of 0.05 [260]. Functional annotation data from three GO

subontologies—molecular function, biological process, and cellular compartment—were included in the analysis. Since large pathways are suggested to have limited interpretative value [261], functional categories larger than 2000 terms were omitted from the results.

CHAPTER 4: STUDY 1

4.1 Research Aim 1: The effect of impaired COX-2 activity on the expression of ASD-risk genes and neuroinflammatory markers in the adolescent mouse brain

The goal of Aim 1 is to examine the effects of impaired COX-2 activity on the expression of ASD-risk genes (Aim 1.1), inflammatory cytokines (Aim 1.2), and neuroglial markers (Aim 1.3) in the brain of male and female wildtype (129S6) and COX-2^{-} mice during early adolescence (PND 25). I hypothesize that impaired COX-2 activity will upregulate gene sets typically expressed at higher levels in males than females (inflammatory cytokines and neuroglial markers), and downregulate gene sets typically expressed at higher levels in females than males (ASD-risk genes). I also hypothesize that the overall effects will be more profound in COX-2^{-} males than COX-2^{-} females. This section will include the background, rationale, and results for Aim 1.1-1.3, followed by an analysis of the overall findings and conclusions for Aim 1. For each sub-aim, the results are organized as follows. qRT-PCR results are presented in figures (RQ and SEM values). The main body of the text will include all relevant statistical analyses (see section 3.3.2), including *post hoc* results (simple main effects) along with the relative fold-change (FC) values for each comparison where appropriate. For a summary of all statistical analyses, see the tables provided in Appendix A.

Aim 1.1: Expression of ASD-Related Genes in Male & Female COX-2- Mice at PND 25

Background & Rationale:

The objective of Aim 1.1 was to examine the effect of impaired COX-2 activity on the expression of ASD-related genes in the brain during early adolescence. As mentioned previously, ASD is believed to be due to an interaction of both genetic and environmental risk factors [6,7].

Since COX-2$^-$ mice are a genetic model of the ERF NSAIDs; it would be interesting to assess whether an interaction exists between this model and ASD-risk genes, as this may provide insight into whether prenatal NSAID exposure could alter the expression of ASD-risk genes, which would imply an interaction between these risk factors of ASD.

The term "ASD-risk" genes refers to genes that are implicated in autism susceptibility [262]. Previous experiments in our lab found that two ASD-risk genes, metabotropic glutamate receptor 5 (*Grm5*) and glyoxalase 1 (*Glo1*), were differentially expressed in the brain of COX-2$^-$ mice during early postnatal development [53]. The results from transcriptomic analyses of human post-mortem cortical samples obtained from males and females, both with and without ASD, suggested that genes associated with neuronal function (i.e., those including *Glo1*) and synaptic function (i.e., those including *Grm5*) were expressed higher in females than males [207]. Additionally, these gene sets were downregulated in individuals with ASD (Fig. 4b), with more substantial effects found in females than males with ASD [207,208]. Thus, I hypothesized that the expression of the ASD-risk genes *Grm5* and *Glo1* would be higher in the brain of females than males for 129S6 mice. I also hypothesized that impaired COX-2 activity would downregulate the expression of ASD-risk genes in the brain COX-2$^-$ mice, with more significant effects being found in COX-2$^-$ females than COX-2$^-$ males.

Results for Aim 1.1: Impaired COX-2 Activity Downregulates the Expression of ASD-Risk Genes

In this study, qRT-PCR was used to quantify the expression of the ASD-risk genes *Grm5* and *Glo1* in the brain of male and female COX-2$^-$ and 129S6 mice at PND 25 (RQ and SEM values are illustrated in Figure 6). A two-way ANOVA followed by Bonferroni *post-hoc* comparisons were then conducted to examine the effect of genotype (COX-2$^-$ vs. 129S6) and sex

(male vs. female) on the expression of *Grm5* and *Glo1*. A significant interaction between genotype and sex was found for both *Grm5* expression, $F(1,8) = 25.3$, $p = .001$, and *Glo1* expression, $F(1,8) = 33.6$, $p < .001$, in the brain.

As illustrated in Figure 6a, *Grm5* was found to be expressed significantly lower in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC = 0.69, $p < .001$. Conversely, no significant difference was found between 129S6 females compared to and 129S6 males, FC = 0.97, $p = .900$. These differences, with respect to the effect of sex in 129S6 and COX-2$^-$ mice, may reflect the fact that *Grm5* was significantly downregulated to a greater extent between COX-2$^-$ females and 129S6 females, FC =0.57, $p < .001$, than it was between COX-2$^-$ males and 129S6 males, FC = 0.80, $p < .001$. On the other hand, *Glo1* (Fig. 6b) was found to be expressed significantly higher in 129S6 females compared to 129S6 males, FC = 1.13, $p = .013$, and significantly lower in COX-2$^-$ females compared to COX-2$^-$ males, FC = 0.78, $p = .004$. Once again, these difference, with respect to the effect of sex in 129S6 and COX-2$^-$ mice, likely reflects the fact that *Glo1* was significantly downregulated to a greater extent between COX-2$^-$ females and 129S6 females, FC =0.47, $p < .001$, than it was between COX-2$^-$ males and 129S6 males, FC = 0.68, $p < .001$.

Overall, these findings partially agreed with our hypothesis that the expression of ASD-risk genes would be higher in the brain of 129S6 females than males, as this was the case for *Glo1* but not *Grm5*. Additionally, impaired COX-2 activity was found to downregulate the expression of ASD-risk genes in the brain COX-2$^-$ mice, with greater effects being found in COX-2$^-$ females than COX-2$^-$ males for both *Grm5* (FC = 0.57 vs. FC = 0.80) and *Glo1* (FC = 0.47 vs. FC = 0.68).

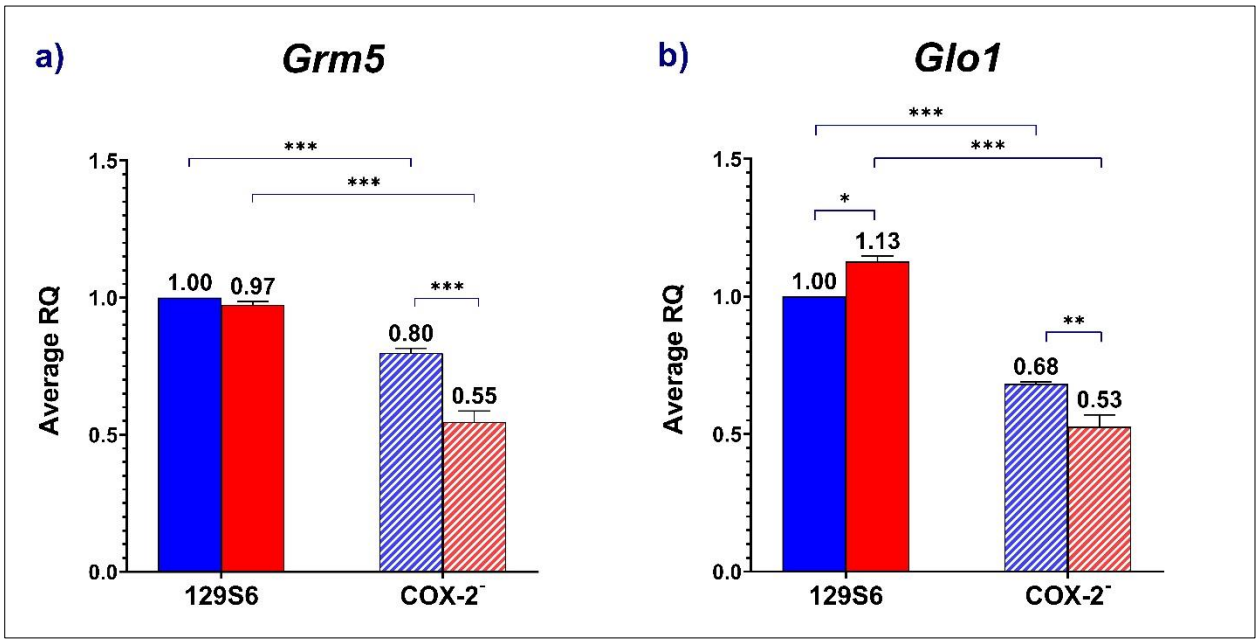

Figure 6. Expression of ASD-Risk Genes in the Brain at PND 25
Average RQ values for the expression of ASD-related genes *Grm5* (**Fig 6a.** left side) and *Glo1* (**Fig 6b.** right side) in the brain between COX-2$^-$ and 129S6 males (blue) and females (red) at PND 25. Error bars represent +/- SEM. Statistical significance was marked "*" for $p < .05$, "**" for $p < .01$, "***" for $p < .001$.

Aim 1.2: Expression of Pro-Inflammatory Cytokines in Male & Female COX-2- Mice at PND 25

Background & Rationale:

The objective of Aim 1.2 was to examine the effect of impaired COX-2 activity on the expression of pro-inflammatory cytokine genes in the brain during early adolescence. Pro-inflammatory cytokines are signaling molecules that are primarily released by immune cells in the brain, such as microglia and astrocytes [263,264]. They are generally expressed at low levels in the brain and are upregulated rapidly in response to certain stimuli (i.e., following exposure to pathogens or trauma) where they serve to activate signaling cascades involved in the initiation of immune responses [263]. Pro-inflammatory cytokines play a role in several functions during brain development, including the formation of synapses [265] and the differentiation of astrocytes [266]. They have also been extensively studied as mediators of altered brain function during inflammatory states following viral and bacterial infections [267,268].

Even low-doses of antipyretic drugs can trigger activation of the immune system in healthy adults [269]. Additionally, animal studies have found that both NSAID and APAP exposure in mice leads to elevated production of the same pro-inflammatory cytokines [270,271] that are also elevated in children with ASD [48,272] and ADHD [49]. Furthermore, male and female mice have recently been shown to exhibit a differential expression of cytokines the brain following immune challenge, with males exhibiting more delayed, longer-lasting, and more pronounced rises in the concentration of pro-inflammatory cytokines [273]. Thus, it would be interesting to see if impaired COX-2 activity leads to an altered expression of pro-inflammatory cytokines in the adolescent brain of male and female COX-2^{-} mice, as this may provide insight into whether prenatal NSAID exposure could alter the neuroinflammatory profile of individuals with NDDs.

Previous experiments in our lab found that two pro-inflammatory cytokines, interleukin 1 beta (*IL-1β*) and interleukin 6 (*IL-6*), were differentially expressed in the brain of COX-2^{-} mice during early postnatal development [53]. The results from transcriptomic analyses of human post-mortem cortical samples obtained from males and females, with and without ASD, suggested that gene sets associated with the inflammatory response were expressed higher in males than females during both fetal development and adulthood [207,208]. Additionally, these neuroinflammatory gene sets were upregulated in individuals with ASD (Fig. 4c), with more substantial effects found in males than females with ASD [207,208]. Thus, I hypothesized that the expression of the pro-inflammatory cytokine genes *IL-1β* and *IL-6* would be higher in the adolescent brain of males than females for 129S6 mice. I also hypothesized that impaired COX-2 activity would upregulate the expression of inflammatory genes in the brain COX-2^{-} mice, with more significant effects being found in COX-2^{-} males than COX-2^{-} females.

Results for Aim 1.2: Impaired COX-2 Activity Upregulates the Expression of Pro-Inflammatory Cytokines in a Sex-Dependent Manner

In this study, qRT-PCR was used to quantify the expression of the pro-inflammatory cytokine genes *IL-1β* and *IL-6* in the brain of male and female COX-2^{-} and 129S6 mice at PND 25 (RQ and SEM values are illustrated in Figure 7). A two-way ANOVA followed by Bonferroni *post-hoc* comparisons were then conducted to examine the effect of genotype (COX-2^{-} vs. 129S6) and sex (male vs. female) on the expression of *IL-1β* and *IL-6*. A significant interaction between genotype and sex was found for *IL-1β* expression, $F(1,8) = 11.2$, $p = .010$, but not *IL-6* expression, $F(1,8) = 2.92$, $p = .113$, in the brain.

As illustrated in Figure 7a, *IL-1β* was found to be expressed significantly higher in the brain of 129S6 females compared to and 129S6 males, FC = 1.99, $p = .006$. Conversely, no significant difference was found between COX-2$^-$ females and COX-2$^-$ males, FC = 0.94, $p > .999$. These differences, concerning the effect of sex in 129S6 and COX-2$^-$ mice, reflects the fact that *IL-1β* was significantly upregulated in COX-2$^-$ males compared to 129S6 males, FC = 2.20, $p = .002$, whereas no significant difference in *IL-1β* expression was found between COX-2$^-$ females and 129S6 females, FC =1.04, $p > .999$. On the other hand, *IL-6* expression (Fig. 7b) was not found to differ between males and female in either 129S6 or COX-2$^-$ mice, $F(1,8) = 1.41$, $p = .258$. However, a significant main effect of genotype on *IL-6* expression was found, $F(1,8) = 11.7$, $p = .005$, demonstrating impaired COX-2 activity lead to an overall increase in *IL-6* expression. Interestingly, further analyses revealed that *IL-6* was significantly upregulated in COX-2$^-$ females compared to 129S6 females, FC = 2.58, $p = .007$, whereas no significant difference in *IL-6* expression between COX-2$^-$ males and 129S6 males was found, FC =1.46, $p = .497$.

Overall, these findings contradicted our hypothesis that the expression of pro-inflammatory cytokine genes would be higher in the brain of 129S6 males than females, as the opposite was found for *IL-1β,* and no difference was found for *IL-6.* However, impaired COX-2 activity was found to upregulate the expression of *IL-1β* in males and *IL-6* in females, providing partial support for the hypothesis that pro-inflammatory cytokine genes would be upregulated in the brain of COX-2$^-$ mice. Additionally, these results suggest that the neuroinflammatory profile of COX-2$^-$ males and females may be altered in a sex-dependent manner.

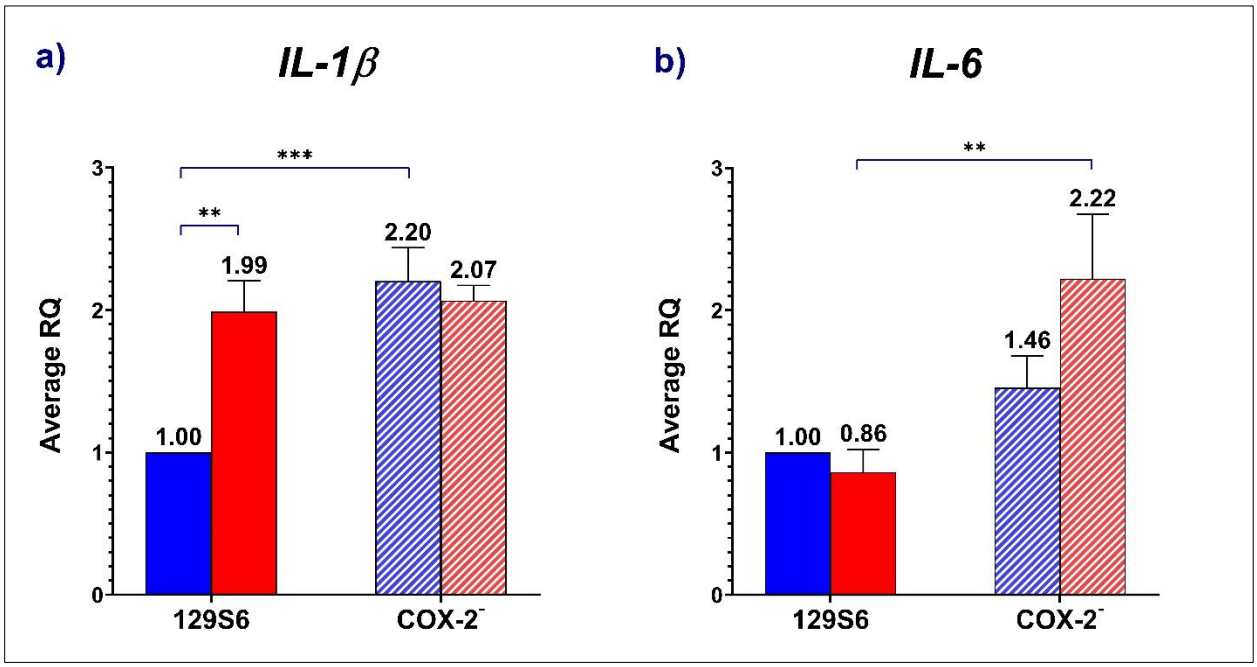

Figure 7. Expression of Pro-Inflammatory Cytokines in the Brain at PND 25

Average RQ values for the expression of pro-inflammatory cytokine genes *IL-1β* (**Fig 7a.** left side) and *IL-6* (**Fig 7b.** right side) in the brain between COX-2$^-$ and 129S6 males (blue) and females (red) at PND 25. Error bars represent +/- SEM. Statistical significance was marked "*" $p < .05$, "**" for $p < .01$, "***" for $p < .001$.

Aim 1.3: Expression of Neuroglial Markers in Male & Female COX-2- Mice at PND 25

Background & Rationale:

The objective of Aim 1.3 was to examine the effect of impaired COX-2 activity on the expression of neuroglial marker genes in the brain during early adolescence. The two main neuroglial cells that play a role in neuroinflammatory signaling in the brain are microglia and astrocytes [114,115]. As described previously (see section 2.2.2), both microglia and astrocytes are involved in the mechanisms governing brain masculinization. Additionally, the COX-2/PGE_2 pathway plays a major role in mediating neuroimmune signaling between these cells during both neuroinflammation and brain masculinization [218–220]. Thus, since COX-2/PGE_2 signaling is dysregulated in COX-2$^-$ mice, it would be interesting to assess whether an impaired COX-2 activity leads to an altered expression of microglia and astrocytes in the brain. Furthermore, given than these neuroglia mediate sexual differentiation of the brain, it would also be interesting to determine if impaired COX-2 activity alters the expression of microglia and astrocytes in a sex-dependent manner. These findings may provide insight into whether prenatal NSAID exposure could alter the expression profile of neuroglial genes in individuals with NDDs, and whether these effects are sex-dependent.

Previous experiments in our lab found that the microglial marker integrin alpha M (*Itgam*) was differentially expressed in the brain of COX-2$^-$ mice during early postnatal development [53]. *Itgam* is a common marker for microglia and other macrophages in the brain, where it is highly expressed both in resting microglia throughout development, and activated microglia during neuroinflammatory events [274]. A common marker of astrocytes in the brain is glial fibrillary acidic protein (*Gfap*) [275], an intermediate filament that forms a major component

of the cytoskeleton in astrocytes. *Gfap* is highly expressed in both recently differentiated and mature astrocytes during brain development and is well known to be upregulated in several pathological conditions (i.e., Alzheimer's disease and stroke) [257,275,276]. Evidence from transcriptomic analyses of human post-mortem cortical samples obtained from males and females, with and without ASD, suggested that gene sets associated with activated microglia (i.e., those including *Itgam*) and reactive astrocytes (i.e., those including *Gfap*) were expressed higher in males than females [207,208]. Additionally, these neuroglial gene sets were upregulated in individuals with ASD (Fig. 4c), with more profound effects found in males than females with ASD [207,208]. Thus, I hypothesized that the expression of the microglial marker *Itgam* and the astrocyte marker *Gfap* would be higher in the adolescent brain of males than females for 129S6 mice. I also hypothesized that impaired COX-2 activity would upregulate the expression of neuroglial genes in the brain of COX-2^{-} mice, with greater effects being found in COX-2^{-} males than COX-2^{-} females.

Results for Aim 1.3: Impaired COX-2 Activity Upregulates the Expression of Neuroglial Markers

In this study, qRT-PCR was used to quantify the gene expression of the microglial marker *Itgam* and the astrocyte marker *Gfap* in the brain of male and female COX-2^{-} and 129S6 mice at PND 25 (RQ and SEM values are illustrated in Figure 8). A two-way ANOVA followed by Bonferroni *post-hoc* comparisons were then conducted to examine the effect of genotype (COX-2^{-} vs. 129S6) and sex (male vs. female) on the expression of *Itgam* and *Gfap*. A significant interaction between genotype and sex was found for both *Itgam* expression, $F(1,8) = 7.84$, $p = .023$, and *Gfap* expression, $F(1,8) = 10.8$, $p = .011$, in the brain.

As illustrated in Figure 8a, the microglial marker *Itgam* was found to be expressed significantly higher in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC = 1.22, p = .005. Conversely, no significant difference was found between 129S6 females and 129S6 males, FC = 1.03, p > .999. These differences, concerning the effect of sex in 129S6 and COX-2$^-$ mice, reflects the fact that *Itgam* was significantly upregulated to a greater extent between COX-2$^-$ females and 129S6 females, FC = 2.03, p < .001, than it was between COX-2$^-$ males and 129S6 males, FC = 1.72, p < .001. On the other hand, the astrocyte marker *Gfap* (Fig. 8b) was found to be expressed significantly lower in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC = 0.71, p = .033. Conversely, no significant difference was found between 129S6 females and 129S6 males, FC = 5.00, p = .291. These differences, concerning the effect of sex in 129S6 and COX-2$^-$ mice, reflects the fact that *Gfap* was significantly upregulated to a much greater extent in COX-2$^-$ males compared to 129S6 males, FC = 26.30, p < .001, than it was between COX-2$^-$ females and 129S6 females, FC = 3.76, p = .001.

Overall, these findings contradicted our hypothesis that the expression neuroglial genes would be higher in the brain of 129S6 males than females, as no difference was found for *Itgam* and the opposite was found for *Gfap*. However, impaired COX-2 activity was found to upregulate the expression of both neuroglial markers in the brain COX-2$^-$ mice. Surprisingly, greater effects were found in COX-2$^-$ females than COX-2$^-$ males for the microglial marker *Itgam* (FC = 2.03 vs. FC = 1.72). Additionally, although in agreement with our hypothesis, impaired COX-2 activity had profoundly greater effects on the expression of the astrocyte marker *Gfap* in COX-2$^-$ males than COX-2$^-$ females (FC = 26.30 vs. FC = 3.76).

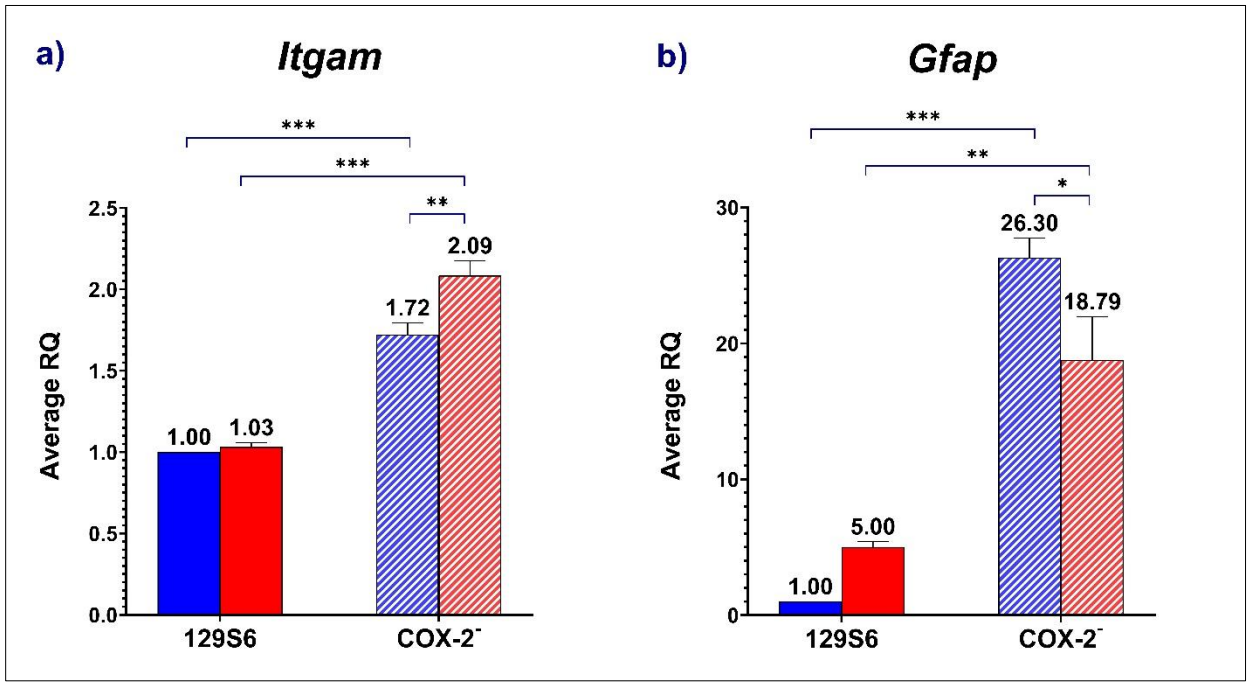

Figure 8. Expression of Neuroglial Markers in the Brain at PND 25

Average RQ values for the expression of the microglial marker *Itgam* (**Fig 8a.** left side) and the astrocyte marker *Gfap* (**Fig 8b.** right side) in the brain between COX-2^{-} and 129S6 males (blue) and females (red) at PND 25. Error bars represent +/- SEM. Statistical significance was marked "*" $p < .05$, "**" for $p < .01$, "***" for $p < .001$.

4.1.1 Analysis & Conclusions for Aim 1

Certain sets of genes have been shown to be differentially expressed by sex in the brain throughout development [207,208], suggesting that inherent differences in the activity of these biological pathways between males and females may account for some of the sex differences in prevalence and severity of NDDs. Interestingly, while our results did agree with some sex differences found in the literature—and for instance showed that the neuronal function gene *Glo1* was expressed higher in 129S6 females than males, and the pro-inflammatory cytokine *IL-1β* was expressed higher in 129S6 males than females—the remaining genes investigated either exhibited no sex differences or the opposite pattern of expression. It is important to note that these findings should be interpreted with caution, as our hypotheses were based on the results of transcriptomic studies involving large gene sets in humans [207,208], as opposed to only a couple of markers for each biological pathway in mice. However, these differences between our findings and the literature may also reflect the fact that several neurodevelopmental processes are suggested to occur earlier in the developing rodent brain of females than males, such as the maturation of microglia [277] and GABAergic neurons [278]. As such, inherent delays in the maturation of certain neurodevelopmental processes in males may have contributed to the unexpected trends in the expression pattern of genes in the brain of male and female 129S6 wildtype mice during early adolescence.

A guiding hypothesis for this study was that exposure to ERFs for ASD and ADHD may alter the expression of genes in the brain in a manner that parallels the differences found between males and females with these NDDs and healthy controls. In support of this, we found that impaired COX-2 activity led to changes in the expression of genes which mirrored those

observed in males and females with ASD compared to healthy age- and sex-matched controls (Fig. 4) [207,208]. More specifically, genes associated with synaptic and neuronal function were found to be downregulated, whereas genes associated with neuroinflammation and neuroglial were found to be upregulated in the adolescent brain of COX-2^{-} males and females compared to age- and sex-matched 129S6 wildtype mice (illustrated in Figure 9).

In agreement with our hypotheses, impaired COX-2 activity was also found to have a greater overall impact on the downregulation of genes associated with synaptic function (*Grm5*) and neuronal function (*Glo1*) in females than males (Fig. 9). Grm5 is a G-protein coupled receptor that is located peri-synaptically in glutamatergic and GABAergic neurons [279,280], as well as on microglia and astrocytes in several regions of the brain [281]. Grm5 signaling has also been shown to be important in the prevention of oxidative stress, [282] and the attenuation of neurotoxicity and microglial activation following exposure to the inflammatory mimetic lipopolysaccharide (LPS) [283,284]. Furthermore, *Grm5* has previously been found to be downregulated in ASD [279], and genetic deletions within *Grm5* have been linked to individuals with ADHD in a genome-wide association study [285]. Grm5$^{-/-}$ mice have also been shown to exhibit behavioral characteristics associated with these disorders, including cognitive impairments in learning and memory tasks [286], reduced pain perception [287], and increased hyperactivity [288].

On the other hand, Glo1 is a cytosolic enzyme that is ubiquitously expressed throughout the brain, where it participates in the metabolism of glucose [289]. It is the rate-limiting step in the glyoxalase pathway where it regulates detoxification of the metabolic byproduct methylglyoxal (MG). Increased MG concentration has been shown to lead to the formation of advanced glycation end-products, increase oxidative stress, and induce apoptosis [290]. Decreased Glo1

enzymatic activity has been implicated in individuals with ASD [291,292] and mouse models of both impaired Glo1 activity and maternal exposure to MG have been shown to exhibit impaired neurogenesis, cortical development, and behavioral abnormalities including increased scores on measured of RRB-related behavior [293]. Interestingly, the enzymatic activity of Glo1 has been found to be significantly greater in astrocytes than neurons in the brain [294]. Collectively, these findings suggest that downregulation of the ASD-risk genes *Grm5* and *Glo1* in the adolescent brain of COX-2$^-$ males and females may lead to increased oxidative stress, particularly in astrocytes, and increased microglial activation.

Also, in agreement with our hypotheses, impaired COX-2 activity more profoundly upregulated the expression of neuroinflammatory and neuroglial genes in males than females, as evidenced by the increased FC for both pro-inflammatory cytokines and the significantly larger upregulation of the astrocyte marker *Gfap* (Fig. 9). It is worth noting that the increased FC of inflammatory cytokine *IL-6* between COX-2$^-$ males and 129S6 males was not significant. However, IL-6 has been shown to be associated with both pro-inflammatory and anti-inflammatory mechanisms (discussed below). Thus, the overall conclusion that impaired COX-2 activity had a greater effect on the expression of neuroimmune genes in males is largely supported by the results.

The pro-inflammatory cytokine genes were upregulated in the adolescent brain of COX-2$^-$ mice, with *IL-1β* significantly upregulated in COX-2$^-$ males (Fig. 7a) and *IL-6* significantly upregulated in COX-2$^-$ females (Fig. 7b). Both IL-6 and IL-1β protein and mRNA levels have been found to be upregulated in the fetal brain of LPS and Poly I:C MIA mouse models of NDDs [295,296], and they have been shown to exhibit a differential expression pattern in the brain of male and female mice [273]. They have also been shown to mediate the febrile response in the brain

[297,298] and are found to be dysregulated following administration of antipyretic drugs [299]. While numerous cell types in the brain can produce IL-6 and IL-1β [300], their role in neuroinflammatory signaling is typically characterized first by an increased secretion of these signaling molecules from activated microglia and reactive astrocytes in response to an inflammatory event (i.e., the introduction of a pathogen or following trauma). The secretion of these cytokines by microglia and astrocytes occurs in a reciprocal manner and is subsequently followed by changes in the expression of cytokine and chemokine receptors in these same cells [300,301].

Activated microglia are believed to respond more rapidly than astrocytes and primarily secrete IL-1β [302]. This, in turn, leads to increased IL-6 secretion from astrocytes [302]. Interestingly, our results found an opposing trend—with *IL-1β* and the astrocyte marker *Gfap* more profoundly upregulated in COX-2$^-$ males, whereas *IL-6* and the microglial marker *Itgam* more profoundly upregulated in COX-2$^-$ females. This unexpected pairing between neuroinflammatory and neuroglial markers may reflect research suggesting that IL-6 can exhibit both pro- and anti-inflammatory properties depending on the receptor it binds to (see [303] for review). Briefly, the binding of IL-6 to membrane-bound receptors (IL-6R) on microglia [304] is believed to have a neuroprotective role in the brain, whereby IL-6 serves as an anti-inflammatory neurotrophic factor that promotes neuronal survival and regeneration [305,306], and has been associated with reduced glial activation [307,308]. However, IL-6Rs can be cleaved from the microglial membrane, leading to the liberation of a soluble IL-6R that can bind free IL-6 [303]. These complexes can bind to non-specific receptors (sIL-6Rs) expressed in a variety of cell types in the brain, including microglia [309], neurons [310], and astrocytes [311], and activate pro-inflammatory signaling cascades associated with neurotoxicity in the brain [312]. Therefore, the more pronounced upregulation of *IL-6* and *Itgam* in COX-2$^-$ females may reflect a neuroprotective compensatory response in these

mice. The anti-inflammatory aspect of such a mechanism may also partially explain the more profound effects of impaired COX-2 activity on the expression of *IL-1β* and *Gfap* expression in males than females (see Fig. 9).

In conclusion, impaired COX-2 activity seemed to have both sex-dependent and sex-independent effects on the expression of ASD-risk genes and neuroimmune markers in the adolescent mouse brain. Based on the results of Aim 1, the pathological mechanisms shared by both COX-2$^-$ males and females appear to relate to impaired glucose metabolism, increased oxidative stress, and microglial activation. Recent immunohistochemical analyses of microglial morphology in COX-2$^-$ mice in our lab suggested that there was no increase in activated microglia at this stage [313]. Thus, further exploration of the sex-independent effects of impaired COX-2 activity should focus on investigating markers associated with metabolic and oxidative stress. Conversely, the most notable sex-dependent effect of impaired COX-2 activity on gene expression observed in this study was the significantly larger upregulation of *Gfap* in COX-2$^-$ males. When combined with our interpretation of the pro- and anti-inflammatory mechanisms associated with increased levels of *IL-1β* and *IL-6*, respectively, these results suggest the possible existence of elevated astrocyte reactivity and neurotoxicity in COX-2$^-$ males, and that these effects may be mitigated by increased neuroprotective inflammatory signaling mechanisms in COX-2$^-$ females.

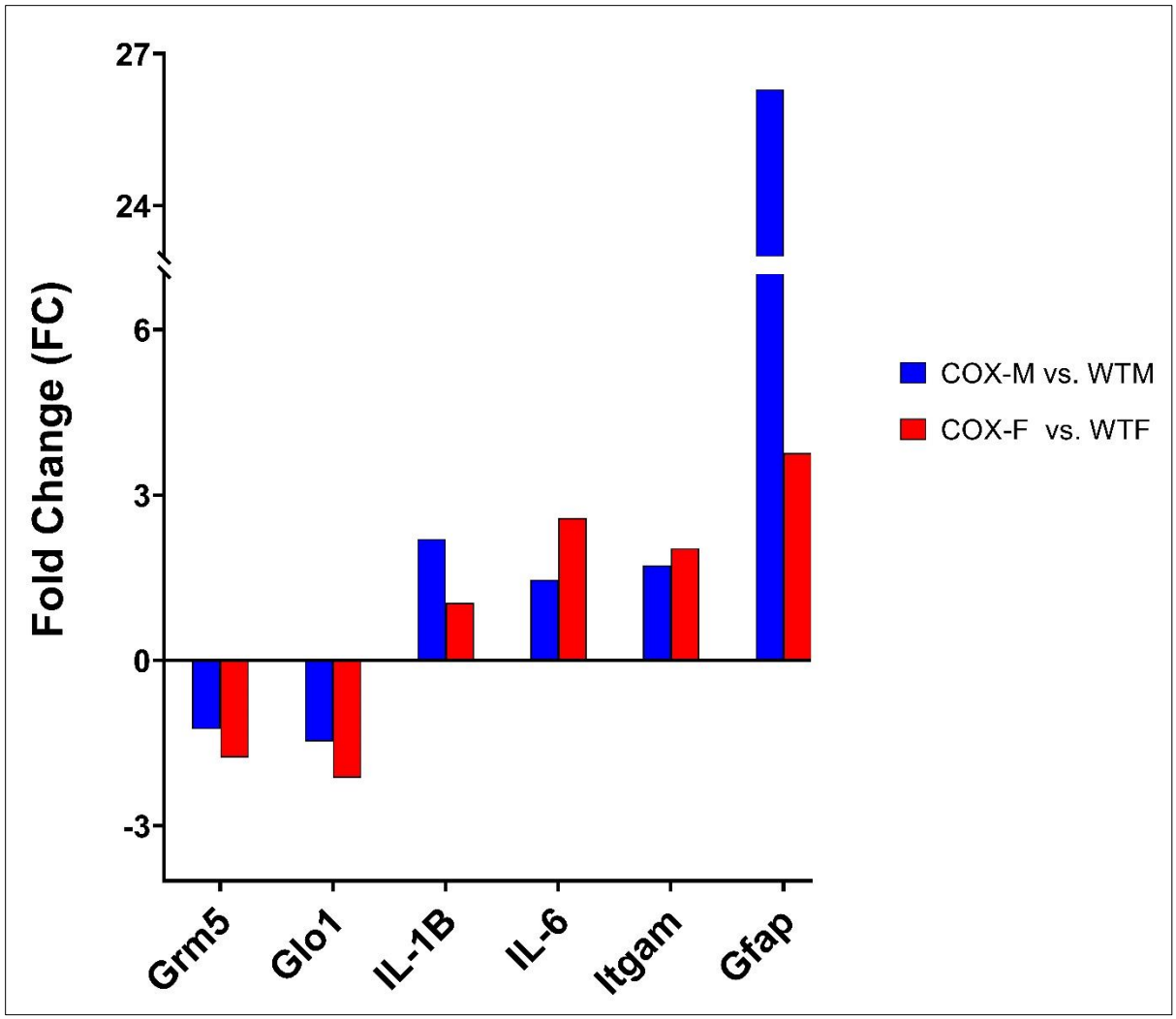

Figure 9. Summary of Results for Aim 1

Average positive and negative fold change values representing the differences in the expression of genes in the brain at PND 25 between COX-2$^-$ males ("COX-M") and 129S6 males ("WTM"), and between COX-2$^-$ females ("COX-F") and 129S6 females ("WTF").

4.2 Research Aim 2: The sex-dependent and sex-independent effects of impaired COX-2 activity on the expression of genes in the adolescent mouse brain.

The goal of Aim 2 is to identify the effects of impaired COX-2 activity on the expression of genes related to oxidative stress (Aim 2.1), and astrocyte reactivity (Aim 2.2) in the brain of male and female wildtype (129S6) and COX-2$^-$ mice during early adolescence (PND 25). I hypothesize that impaired COX-2 activity will increase the expression markers associated with metabolic and oxidative stress in COX-2$^-$ males and females in a sex-independent manner. I also hypothesize that impaired COX-2 activity will lead to an increase in astrocyte reactivity markers in COX-2$^-$ mice, and that the overall effects will be more profound in COX-2$^-$ males than COX-2$^-$ females. Lastly, I hypothesize that COX-2$^-$ males will exhibit an increased expression of astrocyte markers associated with neurotoxic signaling mechanisms, whereas COX-2$^-$ females will exhibit an increased expression of astrocyte markers associated with neuroprotective signaling mechanisms. An overview of the criteria used to select the markers of reactive astrocytes was described in the methods (see section 3.3.4). This section will be organized in the same manner as Aim 1, which was described previously (see section 4.1).

Aim 2.1: Expression of Oxidative Stress Genes in Male & Female COX-2$^-$ Mice at PND 25

Background & Rationale:

The objective of Aim 2.1 was to examine the effect of impaired COX-2 activity on the expression of gene markers of oxidative stress in the brain during early adolescence. One mechanism by which antipyretic drugs may interfere with normal brain development may relate to their tendency to induce oxidative stress [314]. Support for this comes from animal studies showing that high doses of antipyretic drugs such as APAP induces oxidative stress, promotes

the production of free radicals, and contributes to mitochondrial dysfunction in the brains of mice [47]. Oxidative stress is commonly marked by an imbalance between reduced glutathione (GSH) and its oxidized form GSSG, such that lower levels of GSH or a lower GSH/GSSG ratio indicate increased oxidative stress [315]. Some studies have shown that, even at therapeutic doses, exposure to antipyretic drugs can result in the downregulation of genes associated with mitochondrial oxidative phosphorylation [269,316]. These effects have been associated with decreases in GSH content in various brain regions, including the hypothalamus and cerebral cortex [317]. Both reduced GSH levels and a low GSH/GSSG ratio have also been implicated in individuals with ASD [45,318,319] and ADHD [44].

A hallmark of oxidative stress is an increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which is commonly associated with an increased expression of various enzymes [320]. These include the superoxide radical-forming enzyme NADPH oxidase 2 (*Nox2*), as well as the nitric oxide (NO) radical-forming enzyme, inducible NO synthase (*iNos*) [320,321]. Additionally, *Glo1*, which was found to be downregulated in the brain of COX-2^{-} mice, plays a major role in the detoxification of a metabolic byproduct of glucose metabolism [315,322]. Previous microarray analyses in our lab also found glycogen synthase kinase 3 beta (*Gsk3β*), to be dysregulated in COX-2$^{-/-}$ males [52]. *Gsk3β* is a major enzyme involved in the regulation of glucose and energy metabolism in the brain [323]. It is involved in the inactivation of glycogen synthase [324], and inhibition of *Gsk3β* is suggested to be required for the proper maintenance of oxidative phosphorylation in mitochondria within the brain [325]. Together, the downregulation of *Glo1* and dysregulation of *Gsk3β* may suggest that impaired COX-2 activity could result in impaired glucose metabolism and contribute to dysregulated mitochondrial enzymatic activity (i.e., *Nox2* and *iNos*). In turn, this may lead to increased ROS

and RNS production, ultimately resulting in increased oxidative stress in the brain. Thus, I hypothesized that impaired COX-2 activity would upregulate the expression of gene markers for oxidative stress and mitochondrial dysfunction in the brain COX-2$^-$ mice. Additionally, based on the results from Aim 1, I further hypothesized that these effects would be sex-independent.

Results for Aim 2.1: Impaired COX-2 Activity Alters the Expression of Oxidative Stress Markers

In this study, qRT-PCR was used to quantify the expression of genes linked to the production ROS and RNS, including *Nox2* and *iNos*, and a gene marker involved in the regulation of mitochondrial oxidative phosphorylation, *Gsk3β*, in the brain of male and female COX-2$^-$ and 129S6 mice at PND 25 (RQ and SEM values are illustrated in Figure 10). A two-way ANOVA followed by Bonferroni *post-hoc* comparisons were then conducted to examine the effect of genotype (COX-2$^-$ vs. 129S6) and sex (male vs. female) on the expression of *Nox2*, *iNos*, and *Gsk3β*. No significant interaction between genotype and sex was found for *Nox2* expression, $F(1,8) = 1.68$, $p = .231$, *iNos* expression, $F(1,8) = 0.083$, $p = .780$, or *Gsk3β* expression, $F(1,8) = 0.635$, $p = .449$, in the brain.

As illustrated in Figure 10, impaired COX-2 activity was found to lead to a significant downregulation of the ROS marker *Nox2* (Fig. 10a), $F(1,8) = 5.61$, $p = .045$, and the oxidative phosphorylation marker *Gsk3β* (Fig. 10c), $F(1,8) = 6.09$, $p = .039$, in the brain of COX-2$^-$ mice compared to 129S6 mice. Conversely, the RNS marker *iNos* was found to be significantly upregulated in the brain of COX-2$^-$ mice compared to 129S6 mice (Fig. 10b), $F(1,8) = 6.00$, $p = .040$. However, no significant effect of sex on the expression of *Nox2*, $F(1,8) = 0.31$, $p = .596$, *Gsk3β*, $F(1,8) = 3.17$, $p = .113$, or *iNos*, $F(1,8) = 0.191$, $p = .674$, was found. Furthermore, no significant differences between COX-2$^-$ males and 129S6 males was found for the expression of

Nox2, FC = 0.93, $p = .939$, *iNos*, FC = 1.32, $p = .330$, or *Gsk3β*, FC = 0.79, $p = .100$. Similarly, no significant differences between COX-2$^-$ females and 129S6 females was found for the expression of *Nox2*, FC = 0.77, $p = .064$, *iNos*, FC = 1.46, $p = .178$, or *Gsk3β*, FC = 0.87, $p = .542$.

In summary, the effects of impaired COX-2 activity on markers of oxidative stress were found to be sex-independent, as none of these markers were differentially expressed by sex in either COX-2 or 129S6 mice. Additionally, these findings partially agreed with our hypothesis that the expression of oxidative stress markers would be higher in the brain of COX-2$^-$ mice than 129S6 mice, as this was the case for the RNS marker *iNos*. Conversely, impaired COX-2 activity was found to downregulate the expression of the ROS marker, *Nox2*, and the marker of elevated mitochondrial oxidative phosphorylation, *Gsk3β*, in the brain of COX-2$^-$ mice compared to 129S6 mice. However, no significant differences in the expression of *Nox2*, *iNos*, or *Gsk3β* were found between COX-2$^-$ and 129S6 males, or between COX-2$^-$ and 129S6 females. Thus, the overall effect of impaired COX-2 activity on the expression of oxidative stress markers is limited.

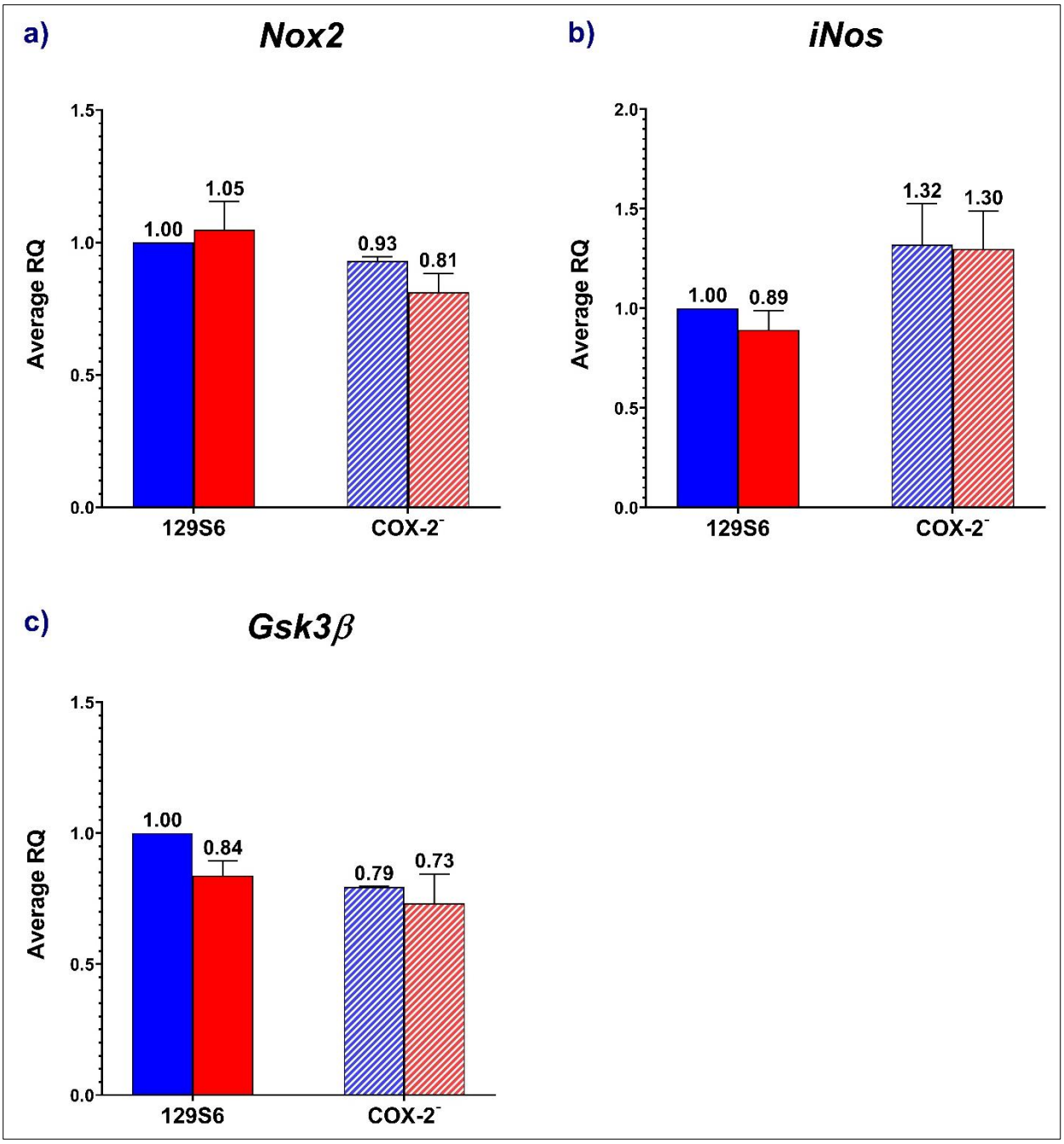

Figure 10. Expression of Metabolic & Oxidative Stress Genes in the Brain at PND 25

Average RQ values for the expression of *Nox2* (**Fig 10a.** top left), *iNos* (**Fig 10b.** top right), and *Gsk3β* (**Fig 10c.** bottom left) in the brain between COX-2$^-$ and 129S6 males (blue) and females (red) at PND 25. Error bars represent +/- SEM. Statistical significance was marked "*" for $p < .05$, "**" for $p < .01$, "***" for $p < .001$.

Aim 2.2: Expression of Reactive Astrocyte Markers in Male & Female COX-2^{-} Mice at PND 25

Background & Rationale:

The objective of Aim 2.2 was to examine the effect of impaired COX-2 activity on the expression of reactive astrocyte markers in the brain during early adolescence. While antipyretic drugs are traditionally associated with their ability to reduce glial activation under pro-inflammatory states, their administration has been shown to trigger immune and inflammatory responses in healthy individuals and in certain models of viral infection [269–271]. Furthermore, a recent study investigating the therapeutic potential of an APAP-derivative noted that administration of the antipyretic was able to reduce inflammatory responses in microglia but not in astrocytes, and it was unable to prevent these triggered astrocytes from, in turn, subsequently activating microglia [326]. Additionally, the chronic treatment of astrocytes with antipyretics have also been shown to upregulate the expression of pro-inflammatory cytokines in these cells [327]. Therefore, these studies suggest that while antipyretic drugs may not directly stimulate the classical pro-inflammatory response in microglia, they could still be capable of inducing these responses in astrocytes and may be unable to prevent astrocytes from subsequently activating microglia. Notably, these findings are consistent with the larger upregulation of the astrocyte marker *Gfap* than the microglial marker *Itgam* in the brain of COX-2^{-} mice.

The term "reactive astrocytes" typically refers to the observed hypertrophy of astrocytic processes and upregulation of intermediate filaments (Gfap, in particular) that is found following CNS injury [328], stroke [329], neuroinflammation [256], and certain neurodegenerative conditions (e.g., Alzheimer's disease & amyotrophic lateral sclerosis) [330,331]. A landmark study conducted by Zamanian et al., (2012) further discovered that inflammatory or ischemic insults induced two

distinct subtypes of reactive astrocytes. Notably, neuroinflammatory "A1" astrocytes were found to upregulate gene sets associated with inflammatory cytokines and neurodegeneration, and were therefore considered to exert primarily neurotoxic effects. Conversely, ischemic "A2" astrocytes were found to upregulate gene sets associated with the release of trophic factors and neuroregeneration, and were therefore considered to exert primarily neuroprotective effects [256]. Furthermore, the gene encoding COX-2 (*Ptgs2*) has been shown to be upregulated in reactive astrocytes under ischemic conditions but not in inflammatory conditions, suggesting a possible interaction between these different subtypes of reactive astrocytes and the COX-2/PGE_2 signaling pathway [256,257].

Interestingly, male- versus female-derived astrocytes have been shown to exhibit different levels of sensitivity to hypoxia [332], and male-derived astrocytes have been found to upregulate pro-inflammatory cytokines to a greater extent than female-derived astrocytes, despite having similar basal levels of these inflammatory mediators [333]. In agreement with these studies, and the male bias in NDDs, I hypothesized that impaired COX-2 activity would upregulate the expression of the neurotoxic "A1" reactive astrocyte marker serpin family G member 1 (*Serping1*) exclusively in COX-2^- males. Conversely, impaired COX-2 activity was hypothesized to upregulate the expression of the neuroprotective "A2" reactive astrocyte marker S100 calcium binding protein A10 (*S100a10*) exclusively in COX-2^- females.

To better understand the profile of astrocyte reactivity in this model, the gene expression of the "pan-reactive" astrocyte marker (that is, an astrocyte marker found to be upregulated under both neuroinflammatory and ischemic conditions), serpin family A member 3 (*Serpina3n*), was investigated [256]. Additionally, the expression of the "non-reactive" astrocyte marker (that is, an astrocyte marker that was not differentially expressed under either neuroinflammatory or

ischemic conditions), Aquaporin 4 (*Aqp4*), was also determined [256]. Based on our previous findings for *Gfap* expression, and the literature discussed above, I hypothesized that impaired COX-2 activity would upregulate the expression of the pan-reactive astrocyte marker (*Serpina3n*) in the brain COX-2^{-} mice, with greater effects being found in COX-2^{-} males than COX-2^{-} females. Additionally, the non-reactive astrocyte marker (*Aqp4*) was hypothesized to be upregulated in COX-2^{-} males only.

Results for Aim 2.2: Impaired COX-2 Activity Alters the Expression of Reactive Astrocyte Subtypes in a Sex-Dependent Manner

In this study, qRT-PCR was used to quantify the expression of gene markers for various subtype of reactive astrocytes in the brain of male and female COX-2^{-} and 129S6 mice at PND 25 (RQ and SEM values are illustrated in Figure 11). A two-way ANOVA followed by Bonferroni *post-hoc* comparisons were then conducted to examine the effect of genotype (COX-2^{-} vs. 129S6) and sex (male vs. female) on the expression of *Serpina3n*, *Aqp4*, *Serping1*, and *S100a10*. A significant interaction between genotype and sex was found for *Serpina3n* expression, $F(1,8) = 1448$, $p < .001$, *Aqp4* expression, $F(1,8) = 433$, $p < .001$, *Serping1* expression, $F(1,8) = 208$, $p < .001$, and *S100a10* expression, $F(1,8) = 205$, $p < .001$, in the brain.

As illustrated in Figure 11a, the pan-reactive astrocyte marker *Serpina3n* was found to be expressed significantly lower in the brain of COX-2^{-} females compared to COX-2^{-} males, FC = 0.81, $p < .001$, and significantly higher in 129S6 females compared to 129S6 males, FC = 2.47, $p < .001$. These differences, concerning the effect of sex in 129S6 and COX-2^{-} mice, reflects the fact that *Serpina3n* was significantly downregulated between COX-2^{-} females and 129S6 females, FC = 0.34, $p < .001$, whereas no significant difference in *Serpina3n* expression was found between COX-2^{-} males and 129S6 males, FC =1.03, $p = .856$. Similarly, the non-reactive

astrocyte marker *Aqp4* (Fig. 11b) was also found to be expressed significantly lower in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC =0.95, p = .048, and significantly higher in 129S6 females compared to 129S6 males, FC = 2.36, p < .001. However, these differences, concerning the effect of sex in 129S6 and COX-2$^-$ mice, likely reflect the fact that *Aqp4* was significantly upregulated between COX-2$^-$ males and 129S6 males, FC = 2.56, p < .001, whereas no significant difference in *Aqp4* expression was found between COX-2$^-$ females and 129S6 females, FC =1.03, p = .461.

As illustrated in Figure 11a, the pan-reactive astrocyte marker *Serpina3n* was found to be expressed significantly lower in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC = 0.81, p < .001, and significantly higher in 129S6 females compared to 129S6 males, FC = 2.47, p < .001. These differences, concerning the effect of sex in 129S6 and COX-2$^-$ mice, reflects the fact that *Serpina3n* was significantly downregulated between COX-2$^-$ females and 129S6 females, FC = 0.34, p < .001, whereas no significant difference in *Serpina3n* expression was found between COX-2$^-$ males and 129S6 males, FC =1.03, p = .856. Similarly, the non-reactive astrocyte marker *Aqp4* (Fig. 11b) was also found to be expressed significantly lower in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC =0.95, p = .048, and significantly higher in 129S6 females compared to 129S6 males, FC = 2.36, p < .001. However, these differences, concerning the effect of sex in 129S6 and COX-2$^-$ mice, reflects the fact that *Aqp4* was significantly upregulated between COX-2$^-$ males and 129S6 males, FC = 2.56, p < .001, whereas no significant difference in *Aqp4* expression was found between COX-2$^-$ females and 129S6 females, FC =1.03, p = .461.

The A1 reactive astrocyte marker *Serping1* (Fig. 11c) was found to be expressed significantly lower in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC = 0.79, p <

.001, and significantly higher in 129S6 females compared to 129S6 males, FC = 1.42, $p < .001$. These differences, concerning the effect of sex in 129S6 and COX-2$^-$ mice, reflects the fact that *Serping1* was significantly upregulated between COX-2$^-$ males and 129S6 males, FC = 1.79, $p < .001$, whereas no significant difference in *Serping1* expression was found between COX-2$^-$ females and 129S6 females, FC =1.00, $p > .999$. Conversely, the A2 reactive astrocyte marker *S100a10* (Fig. 11d) was found to be expressed significantly higher in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC =1.48, $p < .001$, and significantly lower in 129S6 females compared to 129S6 males, FC = 0.70, $p < .001$. These differences, concerning the effect of sex in 129S6 and COX-2$^-$ mice, reflects the fact that *S100a10* expression was significantly downregulated between COX-2$^-$ males and 129S6 males, FC = 0.73, $p < .001$, and significantly upregulated between COX-2$^-$ females and 129S6 females, FC =1.54, $p < .001$.

In conclusion, our results failed to support our hypothesis that impaired COX-2 activity would lead to an upregulation of pan-reactive astrocyte markers, although *Serpina3n* expression was found to be greater in COX-2$^-$ males compared to COX-2$^-$ females. On the other hand, the non-reactive astrocyte marker, *Aqp4*, and the neurotoxic A1 reactive astrocyte marker, *Serping1*, was found to be upregulated exclusively in COX-2$^-$ males as hypothesized. Additionally, the neuroprotective A2 reactive astrocyte marker *S100a10* was found to be upregulated in COX-2$^-$ females and downregulated in COX-2$^-$ males when compared to their same-sex wildtypes. Thus, these findings supported our hypothesis that the different subtypes of reactive astrocytes may interact with impaired COX-2 activity in a sex-dependent manner.

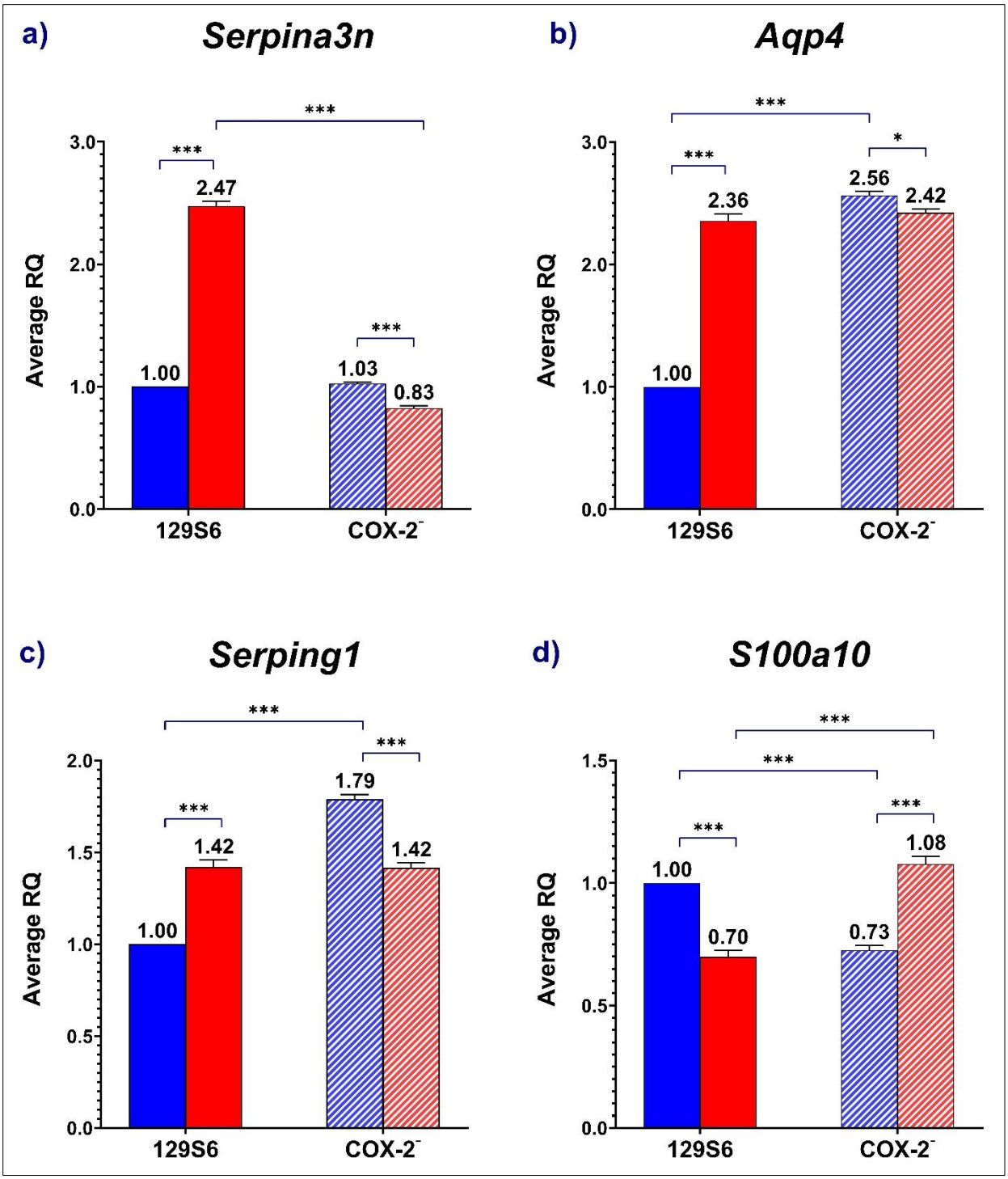

Figure 11. Expression of Reactive Astrocyte Subtype Markers in the Brain at PND 25
Average RQ values for the expression of *Serpina3n* (**Fig 11a.** top left), *Aqp4* (**Fig 11b.** top right), *Serping1* (**Fig 11c.** bottom left) and *S100a10* (**Fig 11d.** bottom right) in the brain between COX-2$^-$ and 129S6 males (blue) and females (red) at PND 25. Error bars represent +/- SEM. Statistical significance was marked "*" for $p < .05$, "**" for $p < .01$, "***" for $p < .001$.

4.2.1 Analysis & Conclusions for Aim 2

As discussed previously, studies on the effects of antipyretic drugs in the brain suggested that impaired COX-2 activity may be associated with increased oxidative stress [47] and neuroglial activation [269–271], which were hypothesized to have sex-independent and sex-dependent effects, respectively, based off the results from Aim 1. While the results of Aim 2.1 were in agreement with our hypothesis that the expression of oxidative stress markers would not be differentially expressed by sex, they failed to support the hypothesis that impaired COX-2 activity would results in elevated oxidative stress in the brain of COX-2$^-$ mice during early adolescence (summarized in Table 3). More specifically, previous literature suggests that increased expression and activity of Nox2 and Gsk3β is associated with increased production of ROS, including superoxide, in the brain [334,335]. Therefore, the downregulation of these enzymes suggests a lower production of superoxide in the brain of COX-2$^-$ mice. Additionally, increased RNS production and NO-derived nitrosative stress primarily results from the formation of peroxynitrite [336], which occurs via the reaction of NO with superoxide radicals. Notably, increases in both NO and peroxynitrite concentrations have been shown to result in increased superoxide production [336]. As such, while the upregulation of *iNos* expression suggests that impaired COX-2 activity may results in an increased production of the NO radical, when interpreted the reduced expression of *Nox2* and *Gsk3β* (and thus superoxide production), these results fail to suggest that impaired COX-2 activity leads to increased production of RNS in the brain of COX-2$^-$ mice.

Table 3. Summary of Results for Aim 2.1

	COX-2⁻ Mice vs. 129S6 Wildtype Mice
Nox2	↓
Gsk3β	↓
iNos	↑

The results of Aim 2.2 also agreed with our hypothesis that impaired COX-2 activity would affect the expression of reactive astrocyte markers in a sex-dependent manner (see Figure 12). However, contrary to our hypotheses, the expression of pan-reactive astrocyte marker *Serpina3n* was not found to differ significantly between COX-2⁻ and 129S6 males and was actually found to be significantly downregulated between COX-2⁻ and 129S6 females. Since *Serpina3n* was selected as a pan-reactive marker based on previous studies using inflammatory and ischemic mouse models [256,257], it is possible that *Serpina3n* expression may not serve as an accurate marker of astrocyte reactivity in models of NSAID exposure. Alternatively, a recent study found that the expression of certain pan-reactive and A1 astrocyte markers increased throughout development in mice, and that *Serpina3n* expression was actually downregulated in young versus mature astrocytes [258]. Thus, the downregulation of *Serpina3n* in COX-2⁻ females may reflect that impaired COX-2 activity may initially downregulate this pan-reactive astrocyte marker at earlier stages of development, and upregulation of this marker in COX-2⁻ mice may only occur later in development.

It should also be noted that the results from *Serpina3n* expression must be interpreted cautiously when attempting to draw any conclusions regarding astrocyte reactivity in COX-2- mice. Increased *Gfap* expression remains one of the most consistent indicators of astrocyte

reactivity as it is the major filament accounting for the hypertrophy of astrocytic processes [337]. Additionally, it is a highly-specific astrocyte marker whose expression is approximately 50-fold greater than *Serpina3n* in mouse cortical astrocytes during adolescence [338]. Thus, the significant upregulation of *Gfap* in both COX-2$^-$ males and females (see Fig. 8b) still suggests that impaired COX-2 activity likely leads to a profound increase in astrocyte reactivity in the adolescent brain. Furthermore, *Gfap* was found to be upregulated to a much larger degree between COX-2$^-$ and 129S6 males than between COX-2$^-$ and 129S6 females. Moreover, while only a slight non-significant increased fold-change in *Serpina3n* expression was observed between COX-2$^-$ and 129S6 males, *Serpina3n* was found to be significantly downregulated between COX-2$^-$ and 129S6 females (Fig. 12). Together, these results suggest that impaired COX-2 activity may lead to a greater increase in astrocyte reactivity in COX-2$^-$ males than females during early adolescence.

The Non-reactive astrocyte marker *Aqp4* was found to be upregulated between COX-2$^-$ and 129S6 males only, as no significant difference in *Aqp4* expression was found between COX-2$^-$ and 129S6 females (Fig. 11b). Aquaporin-4 is a water channel localized on the end feet of perivascular astrocytes, making it in direct contact with blood vessels [339]. It is believed to primarily be involved in maintaining water balance, osmotic pressure, and extracellular volume at synapses [340]. Notably, *Aqp4* is a highly specific astrocyte marker [338] whose expression was found to remain constant following LPS and MCAO treatment in these cells [256]. However, some studies have found that *Aqp4* expression may be increased in reactive astrocytes under certain conditions (i.e., edema), although this is suggested to largely reflect a loss of polarity in the distribution of *Aqp4* expression (that is, a bleeding of AQP4 immunoreactivity away from astrocytic end feet into other regions of the astrocyte) [257,341]. Therefore, the increased *Aqp4*

expression in COX-2$^-$ males suggests that impaired COX-2 activity throughout development may be associated with either a sex-dependent increase in the number of astrocytes, an elevated hypertrophy of astrocytic processes, or a loss in the polarity of Aquaporin-4 in the adolescent brain of male mice.

With regards to subtypes of reactive astrocytes, the A1 neurotoxic astrocyte marker *Serping1* was found to be upregulated only between COX-2$^-$ males and 129S6 males, as no difference in *Serping1* expression was found between COX-2$^-$ and 129S6 females (Fig. 11c). As mentioned previously, A1 astrocytes are considered to be neurotoxic due to their upregulation of pro-inflammatory mediators (i.e., complement cascades) that are associated with synaptic loss [342,343] and neurodegeneration, possibly via NF-κβ signaling mechanisms [257,344,345]. On the other hand, not only was the A2 neuroprotective astrocyte marker *S100a10* found to be significantly upregulated between COX-2$^-$ and 129S6 females, it was also found to be significantly downregulated between COX-2$^-$ and 129S6 males (Fig. 11d). As mentioned previously, A2 astrocytes are considered to be neuroprotective due to their upregulation of neurotrophic factors and cytokines (i.e., BDNF, VEGF, and IL-6) that are suggested to facilitate neuronal survival and the repair and regeneration of synapses [307,329], possibly via STAT3 mediated signaling mechanisms [256,346,347]. Therefore, these results suggest that impaired COX-2 activity during development may be associated with a sex-dependent increase in different subtypes of reactive astrocytes in the brain during early adolescence, with COX-2$^-$ males exhibiting an increase in neurotoxic A1 astrocytes and a decrease in neuroprotective A2 astrocytes, and COX-2$^-$ females exhibiting an increase in neuroprotective A2 astrocytes only (Fig. 12).

In conclusion, impaired COX-2 activity seemed to have both sex-dependent and sex-independent effects on the expression of oxidative stress markers and reactive astrocyte genes in

the adolescent mouse brain. Surprisingly, while oxidative stress genes were found to be differentially expressed in a sex-independent manner in COX-2$^-$ mice, the results failed to support previous literature on the effects of antipyretic drugs in the brain which suggested that impaired COX-2 activity would be associated with elevated oxidative stress [314]. It is possible that these results may relate to the increased expression of astrocytes, which are known to facilitate many homeostatic responses in the brain under normal physiological conditions and have been suggested to help protect neurons against oxidative stress [348]. Specifically, astrocytes serve as the primary source of extracellular GSH in the brain [349]. Therefore, the upregulation of astrocyte markers in response to impaired COX-2 activity may facilitate an increased production of antioxidants by these cells that serve to quench excess NO production and protect COX-2$^-$ mice against oxidative stress.

Alternatively, the various astrocyte markers were found to be differentially expressed in a sex-dependent manner, with impaired COX-2 activity leading to a more profound increase in reactive astrocyte markers in males than in females (Fig. 12). Furthermore, sex differences were observed in the expression of different subtypes of reactive astrocytes in COX-2$^-$ mice, and differences in the expression of A1 versus A2 markers in COX-2$^-$ males and females suggested that impaired COX-2 activity may impact astrocytes in a manner that exerts neurotoxic effects in males and neuroprotective effects in females. As such, these results suggest that prenatal exposure to antipyretic drugs may exert more adverse neurodevelopmental outcomes in males than females. However, it should be noted that the observed sex differences in the expression of reactive astrocyte markers are consistent with both the male vulnerability hypothesis and female protective effect, and thus no distinction can be made with regards to whether either of these hypotheses better accounts for the sex differences that were found.

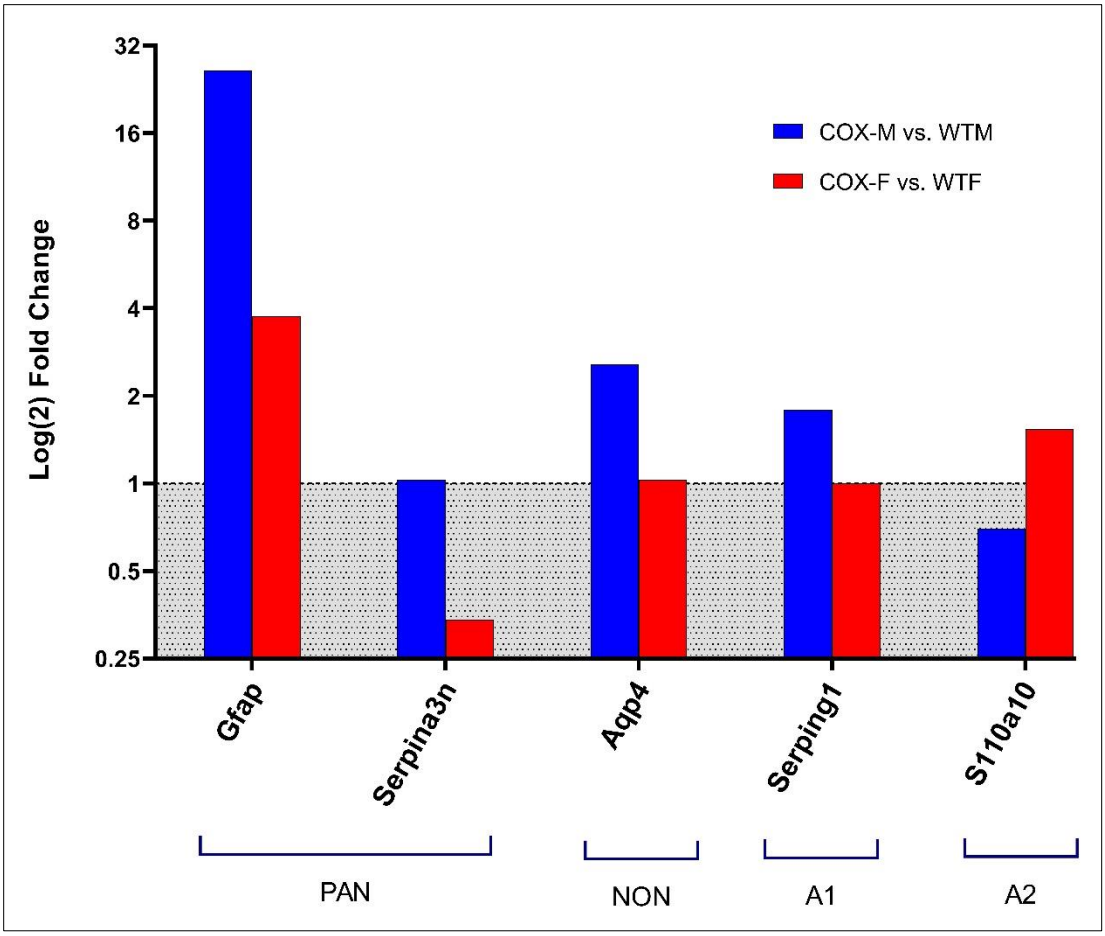

Figure 12. Summary of Results for Aim 2.2

Log(2) fold change values representing two-fold differences in the expression of reactive astrocyte genes in the brain at PND 25 between COX-2$^-$ males ("COX-M") and 129S6 males ("WTM"), and between COX-2$^-$ females ("COX-F") and 129S6 females ("WTF"). The *y*-axis is labeled using linear fold change values and *Gfap* expression from Aim 1.3 is included in the figure. "PAN" = Pan-reactive markers, "NON" = Non-reactive markers, "A1" = neurotoxic astrocyte markers, and "A2" = neuroprotective astrocyte markers.

CHAPTER 5: STUDY 2

5.1 Research Aim 3: The sex-dependent effects of impaired COX-2 activity on the expression of astrocyte markers in the mouse brain during early postnatal development

The goal of Aim 3 is to identify the effects of impaired COX-2 activity on the expression of astrocyte markers and genes related to astrocyte reactivity (Aim 3.1), as well as neurotoxic and neuroprotective astrocyte markers (Aim 3.2), in the brain of male and female wildtype (129S6) and COX-2$^-$ mice during early postnatal development (PND 8). I hypothesize that impaired COX-2 activity will lead to sex differences in both the onset and rate of progression regarding the expression of astrocyte reactivity markers in COX-2$^-$ mice throughout development. In agreement with this, only COX-2$^-$ males are expected to exhibit increased astrocyte reactivity during early postnatal development. I also hypothesize that these results will better support the female protective effect than the male vulnerability hypothesis. In support, COX-2$^-$ males and females are expected to exhibit no change in the expression of astrocyte markers associated with neurotoxic signaling mechanisms during early postnatal development when compared to their same-sex wildtype counterparts. Furthermore, the expression of astrocyte markers associated with neuroprotective signaling mechanisms are expected to be upregulated only in COX-2$^-$ females at this stage. This section will be organized in the same manner as Aim 1, which was described previously (see section 4.1).

Aim 3.1: Expression of Pan- & Non-Reactive Astrocyte Markers in Male & Female COX-2^{-} Mice at PND 8

Background & Rationale:

The objective of Aim 3.1 was to examine the effect of impaired COX-2 activity on the expression of pan-reactive and non-reactive astrocyte genes in the brain during early postnatal development on PND 8. In mice, the majority of astrogenesis occurs in two overlapping waves, the first of which is found to occur between GD 18 and PND 2 in the ventricular zone and sub-ventricular zones of the brain [350]. Once born, astrocytes quickly migrate out along radial glial processes or neuronal axons to their final destination during the first few days of postnatal development [351,352]. Following migration, the second wave of astrogenesis begins around PND 2 when resident astrocytes start to undergo local proliferation [353,354]. As radial glial cells begin to disappear shortly following birth, the local proliferation of differentiated astrocytes is believed to account for the majority of postnatal astrogenesis, which peaks around the end of the first postnatal week and continues until approximately PND 21 in the rodent brain. Shortly after invading the brain, developing astrocytes begin differentiating into mature astrocytes, a process that is primarily characterized by changes in morphology (i.e., a refinement and ramification of astrocytic processes) and electrophysiological properties [355–357]. The completion of astrocyte maturation during development roughly coincides with the end of the second wave of astrogenesis, with the majority of astrocytes found to exhibit mature morphological features between PND 21-28, depending on the brain region. Therefore, PND 8 roughly corresponds to an active growth stage of astrocyte development, overlapping with both the peak period of astrogenesis and the maturation of developing astrocytes in the murine brain.

Given that the local proliferation and maturation of astrocytes in the mouse brain begins during the first postnatal week [352,354], I hypothesized that no sex differences would be found in the expression of astrocyte markers between 129S6 males and females during early postnatal development (PND 8). Notably, with respect to the effects of impaired COX-2 activity at this stage, there were two overarching concepts that guided our hypotheses in this research aim. First, that impaired COX-2 activity would lead to an increased astrocyte reactivity in the brain of COX-2$^-$ mice in a manner that was sex-dependent, such that these effects would be greater in males than females. Second, the notion that astrocyte reactivity increases in a sex-dependent manner throughout development, such that the effects would manifest as a more profound change in the expression of astrocyte markers in COX-2$^-$ males between early postnatal development and adolescence.

During adolescence, impaired COX-2 activity was found to profoundly upregulate the expression of the pan-reactive astrocyte marker *Gfap* in the brain of COX-2$^-$ mice (Aim 1.3; Fig. 8b), with greater effects being found in COX-2$^-$ males than COX-2$^-$ females (Fig. 12). Thus, based off the notion that astrocyte reactivity increases in a sex-dependent manner throughout development, the following two hypotheses were made. First, I hypothesized that impaired COX-2 activity would upregulate *Gfap* expression only in COX-2$^-$ males during early postnatal development. Second, I hypothesized that *Gfap* expression would be significantly greater in COX-2$^-$ males compared to COX-2$^-$ females at this stage. Furthermore, in support of the notion that COX-2$^-$ males would exhibit an accelerated upregulation of reactive astrocyte genes, the following two additional hypotheses were also made. First, I hypothesized that the effects of impaired COX-2 activity on *Serpina3n* and *Aqp4* expression in both COX-2$^-$ males and females during early postnatal development would mimic what was previously found between COX-2$^-$

and 129S6 females during adolescence (Fig. 12). Thus, when compared to their same-sex wildtype counterparts, *Serpina3n* was expected to be downregulated and *Aqp4* was not expected be differentially expressed in either COX-2$^-$ males or females during early postnatal development. Second, I hypothesized that no differences in the expression of *Serpina3n* or *Aqp4* would be found between COX-2$^-$ males and females during early postnatal development.

Results for Aim 3.1: Impaired COX-2 Activity Alters the Expression of Reactive Astrocyte Markers in a Sex-Dependent Manner

In this study, qRT-PCR was used to quantify the expression of gene markers for various subtype of reactive astrocytes in the brain of male and female COX-2$^-$ and 129S6 mice at PND 8 (RQ and SEM values are illustrated in Figure 13). A two-way ANOVA followed by Bonferroni *post-hoc* comparisons were then conducted to examine the effect of genotype (COX-2$^-$ vs. 129S6) and sex (male vs. female) on the expression of the pan-reactive astrocyte markers *Gfap* and *Serpina3n*, as well as the non-reactive astrocyte marker *Aqp4*. A significant interaction between genotype and sex was found for *Gfap* expression, $F(1,8) = 26.7$, $p < .001$, and *Serpina3n* expression, $F(1,8) = 16.2$, $p < .004$, *Serping1* expression, $F(1,8) = 208$, $p < .001$, but not *Aqp4* expression, $F(1,8) = 1.80$, $p = .217$, in the brain.

As illustrated in Figure 13a, *Gfap* was found to be expressed significantly lower in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC = 0.66, $p < .001$. Conversely, no significant difference was found between 129S6 females and 129S6 males, FC = 1.06, $p > .999$. These differences, concerning the effect of sex in 129S6 and COX-2$^-$ mice, likely reflects the fact that *Gfap* was significantly upregulated in COX-2$^-$ males compared to 129S6 males, FC = 1.72, $p < .001$, whereas no significant difference in *Gfap* expression was found between COX-2$^-$

females and 129S6 females, FC =1.07, $p = .899$. On the other hand, *Serpina3n* expression (Fig. 13b) was found to be expressed significantly higher in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC = 1.61, $p < .001$, whereas no significant difference was found between 129S6 females and 129S6 males, FC = 1.06, $p > .999$. These differences, with respect to the effect of sex in 129S6 and COX-2$^-$ mice, may reflect the fact that *Serpina3n* was significantly downregulated to a greater extent between COX-2$^-$ males and 129S6 males, FC =0.28, $p < .001$, than it was between COX-2$^-$ females and 129S6 females, FC = 0.44, $p < .001$. Furthermore, the non-reactive astrocyte marker *Aqp4* was not found to be differentially expressed between males and females, $F(1,8) = 3.20$, $p = .111$, or between COX-2$^-$ and 129S6 mice, $F(1,8) = 1.54$, $p = .249$, during early postnatal development.

In summary, our results largely supported our hypotheses regarding the effect of sex on the expression of pan- and non-reactive astrocyte markers during early postnatal development. As expected, no differences in *Gfap*, *Serpina3n*, or *Aqp4* expression were found between 129S6 males and females, and *Gfap* was found to be expressed higher in COX-2$^-$ males than COX-2$^-$ females. However, *Serpina3n* was found to be expressed higher in COX-2$^-$ females than COX-2$^-$ males. Additionally, our findings were in complete agreement with our hypotheses regarding the effect of impaired COX-2 activity on the expression of pan- and non-reactive astrocyte markers during early postnatal development. Specifically, *Gfap* was upregulated only in COX-2$^-$ males, *Serpina3n* was downregulated in both COX-2$^-$ males and females, and *Aqp4* was not found to be differentially expressed in either COX-2$^-$ males or COX-2$^-$ females when compared to their same-sex wildtype counterparts.

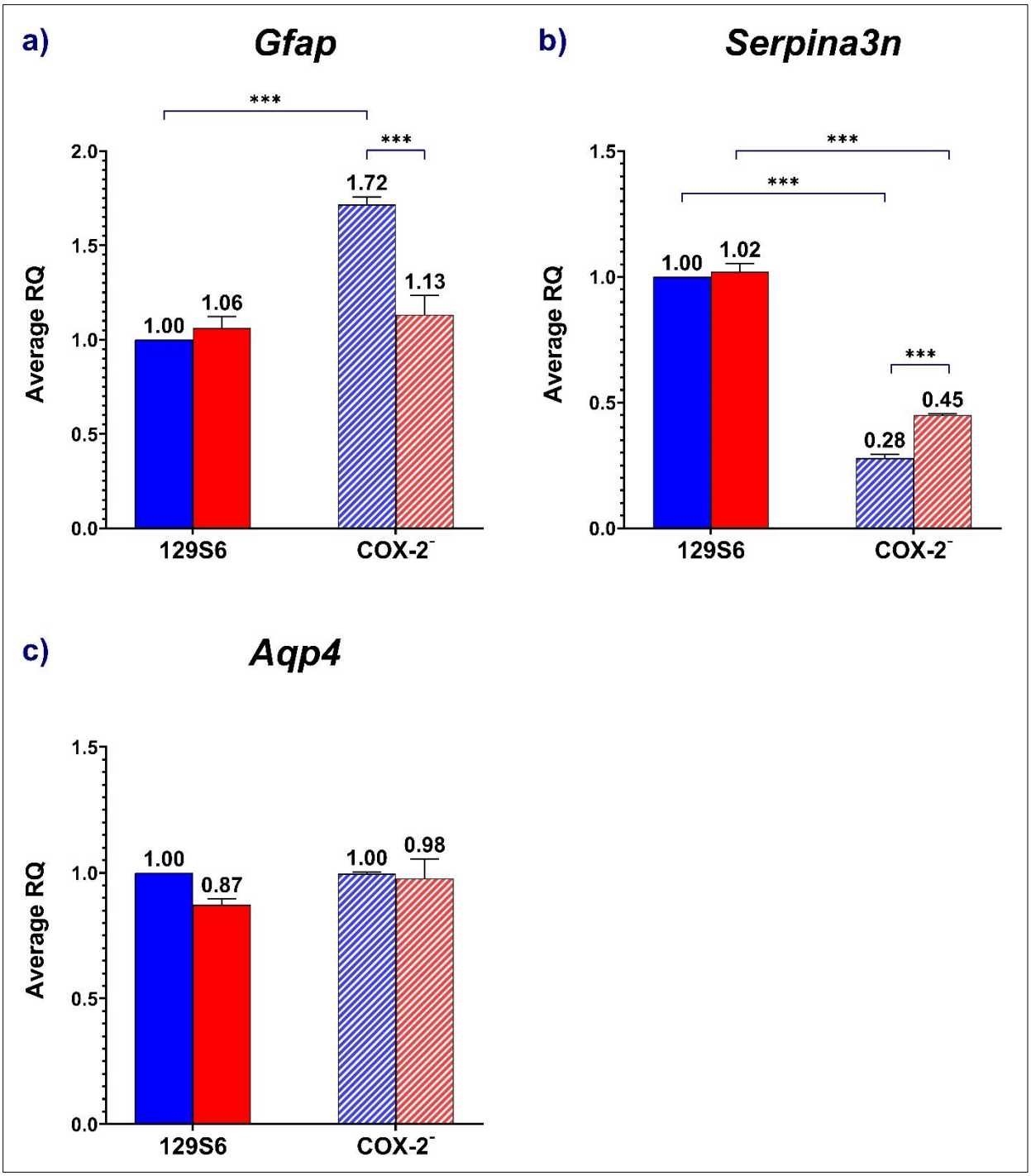

Figure 13. Expression of Pan- & Non-Reactive Astrocyte Markers in the Brain at PND 8
Average RQ values for the expression of *Gfap* (**Fig 13a.** top left), *Serpina3n* (**Fig 13b.** top right), and *Aqp4* (**Fig 13c.** bottom left) in the brain between COX-2$^-$ and 129S6 males (blue) and females (red) at PND 8. Error bars represent +/- SEM. Statistical significance was marked "*" for $p < .05$, "**" for $p < .01$, "***" for $p < .001$.

Aim 3.2: Expression of Neurotoxic & Neuroprotective Astrocyte Markers in Male & Female COX-2$^-$ Mice at PND 8

Background & Rationale:

The objective of Aim 3.2 was to examine the effect of impaired COX-2 activity on the expression of neurotoxic ("A1") and neuroprotective ("A2") reactive astrocyte genes in the brain during early postnatal development on PND 8. As discussed previously (see section 2.2.1), several studies have suggested that the increased prevalence of NDDs in males may reflect inherent sex differences in healthy development. While some research has implied that typical male development may render males more vulnerable to developing NDDs [194–197], the majority of research seems to indicate that typical female development may be associated with inherently protective mechanisms that render females less susceptible to developing NDDs [198–204]. Therefore, an investigation into the expression of A1 and A2 astrocyte genes during early postnatal development would not only facilitate an understanding of how sex differences emerged in this model, it may also provide insight into whether the sex differences in astrocyte reactivity between COX-2$^-$ males and females is supported by either the male vulnerability hypothesis or the female protective effect.

Given that astrocytes first begin to invade an mature in the mouse brain during the first postnatal week [352,354], I hypothesized that no sex differences would be found between 129S6 males and females during early postnatal development. With respect to the effects of impaired COX-2 activity, our findings were hypothesized to be consistent with previous literature suggesting the female protective effect would better account for the sex-differences in astrocyte reactivity [198–204]. In agreement with this hypothesis, the neurotoxic A1 astrocyte marker *Serping1* was not expected to be upregulated in either COX-2$^-$ males or COX-2$^-$ females when compared

to their same-sex wildtype counterparts, or differentially expressed between COX-2$^-$ males or COX-2$^-$ females, during early postnatal development. Additionally, the A2 neuroprotective astrocyte marker *S100a10* was hypothesized to be upregulated in COX-2$^-$ females but not COX-2$^-$ males when compared to their same-sex wildtype counterparts. *S100a10* was also expected to the expressed significantly higher in COX-2$^-$ females when compared to COX-2$^-$ males during early postnatal development.

Results for Aim 3.2: Impaired COX-2 Activity Alters the Expression of Reactive Astrocyte Markers in a Manner that Supports The Female Protective Effect

In this study, qRT-PCR was used to quantify the expression of gene markers for various subtype of reactive astrocytes in the brain of male and female COX-2$^-$ and 129S6 mice at PND 8 (RQ and SEM values are illustrated in Figure 14). A two-way ANOVA followed by Bonferroni *post-hoc* comparisons were then conducted to examine the effect of genotype (COX-2$^-$ vs. 129S6) and sex (male vs. female) on the expression of the neurotoxic A1 astrocyte markers *Serping1* and the neuroprotective A2 astrocyte marker *S100a10*. A significant interaction between genotype and sex was found for *S100a10* expression, $F(1,8) = 26.7$, $p < .001$, but not *Serping1* expression, $F(1,8) = 0.943$, $p = .360$, in the brain.

As illustrated in Figure 14a, *Serping1* was found to be differentially expressed by sex, $F(1,8) = 14.3$, $p = .005$, but not genotype, $F(1,8) = 1.79$, $p = .218$, at this stage. Furthermore, the expression of *Serping1* was found to be significantly greater in COX-2$^-$ females compared to COX-2$^-$ males, FC = 1.16, $p = .020$, whereas no significant difference in the expression of this neurotoxic astrocyte marker was found between 129S6 females and 129S6 males, FC = 1.09, $p = .164$. On the other hand, *S100a10* expression (Fig. 14b) was found to be expressed significantly

higher in the brain of COX-2$^-$ females compared to COX-2$^-$ males, FC = 1.72, $p < .001$. Conversely, no significant difference in the expression of this neuroprotective astrocyte marker was found between 129S6 females and 129S6 males, FC = 1.19, $p = .246$. These differences, with respect to the effect of sex in 129S6 and COX-2$^-$ mice, may reflect the fact that *S100a10* was significantly upregulated to a greater extent between COX-2$^-$ females and 129S6 females, FC = 2.05, $p < .001$, than it was between COX-2$^-$ males and 129S6 males, FC = 1.42, $p = .010$.

In summary, our findings seem to largely support our hypothesis regarding the effect of sex on the expression of A1 and A2 reactive astrocyte markers during early postnatal development, as no differences in *Serping1* or *S100a10* expression were found between 129S6 males and females, and the neuroprotective astrocyte marker *S100a10* was found to be expressed higher in COX-2$^-$ females than COX-2$^-$ males. However, contrary to our hypotheses, the neurotoxic astrocyte marker *Serping1* was found to be expressed higher in COX-2$^-$ females than COX-2$^-$ males at this stage. Additionally, our results also seem to largely support our hypothesis regarding the effect of impaired COX-2 activity on the expression of A1 and A2 reactive astrocyte markers during early postnatal development, as *Serping1* was not found to be upregulated in either expression in either COX-2$^-$ males or COX-2$^-$ females when compared to their same-sex wildtype counterparts. Furthermore, *S100a10* expression was found to upregulated in COX-2$^-$ females compared to 129S6 females as expected. Conversely, in disagreement with our hypotheses, the neuroprotective astrocyte marker *S100a10* was also found to be expressed higher in COX-2$^-$ males than 129S6 males at this stage.

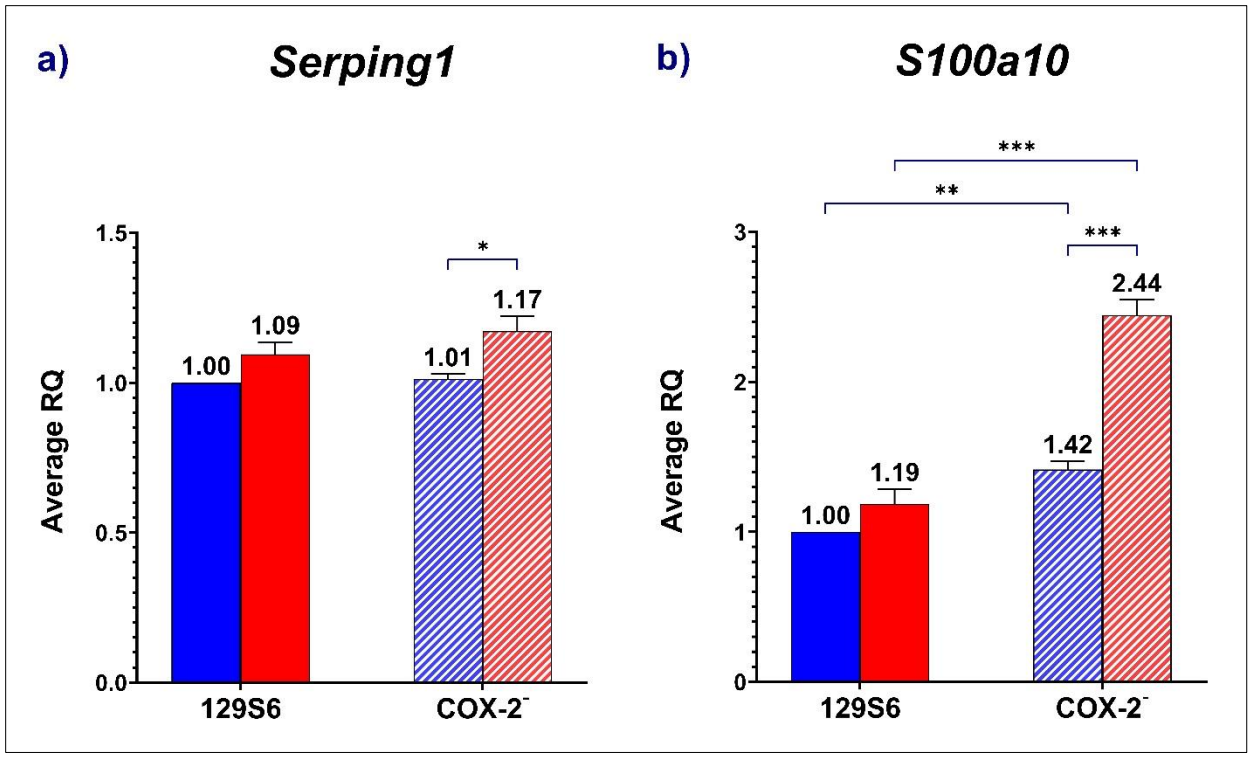

Figure 14. Expression of A1 & A2 Reactive Astrocyte Markers in the Brain at PND 8

Average RQ values for the expression of the A1 neurotoxic reactive astrocyte marker *Serping1* (**Fig 14a.** left side) and the A2 neuroprotective reactive astrocyte marker *S100a10* (**Fig 14b.** right side) in the brain between COX-2^{-} and 129S6 males (blue) and females (red) at PND 25. Error bars represent +/- SEM. Statistical significance was marked "*" $p < .05$, "**" for $p < .01$, "***" for $p < .001$.

5.1.1 Analysis & Conclusions for Aim 3

The effects of impaired COX-2 activity on the expression of all astrocyte markers between COX-2$^-$ males and 129S6 males and between COX-2$^-$ females and 129S6 females, during adolescence (study 1) and early postnatal development (study 2), has been summarized in Figure 15. It should be noted that since the results of study 1 were used to inform our hypotheses in study 2, no statistical analyses were performed to determine whether the expression of these genes differed between these developmental stages. Therefore, no direct conclusions can be drawn regarding whether the expression of any astrocyte reactivity marker changed (increased or decreased) throughout development. However, in this section we will use the expression of these astrocyte markers during both early postnatal and adolescent stages to infer trends across development, as this will facilitate our analyses and interpretation of the results in the current study.

Previous studies on brain masculinization have revealed the existence of sex differences in astrocyte maturation beginning as early as the first postnatal week in sexually dimorphic regions of the rodent brain [218,219]. However, given that these regions encompass a relatively small proportion of the total astrocyte population in the brain, our finding that none of the astrocyte markers were differentially expressed in the brain between 129S6 males and females during early postnatal development was expected (Fig. 13,14). As such, our results supported the assertion that PND 8 represents a good stage (or baseline) for investigating how the sex-dependent effects of impaired COX-2 activity on astrocyte reactivity arise during early postnatal development.

Our findings suggest that impaired COX-2 activity may initiate astrocyte reactivity earlier in males than females during brain development. In support of this, the main astrocyte reactivity marker, *Gfap*, was found to be upregulated in COX-2$^-$ males but not COX-2$^-$ females during early postnatal development, and was expressed significantly higher in the brain of COX-2$^-$ males than COX-2$^-$ females at this stage (Fig. 13a). Additionally, all pan-reactive and non-reactive astrocyte markers were found to be differentially expressed between COX-2$^-$ and 129S6 males during early postnatal development in a manner analogous to what was found in the adolescent brain between of COX-2$^-$ and 129S6 females (Fig. 15). Moreover, all pan-reactive and non-reactive astrocytes markers were found to be expressed higher in COX-2$^-$ males during adolescence than early postnatal development, when compared to 129S6 males at each stage, and this trend was not found between COX-2$^-$ and 129S6 females (Fig. 15). Collectively, these results suggest that impaired COX-2 activity may lead to a sex-dependent increase in astrocyte reactivity throughout development that is characterized by the appearance of reactive astrocytes in COX-2$^-$ males during early postnatal development, and the upregulation of pan-reactive and non-reactive astrocyte markers occurring at an accelerated rate in the male brain.

One possible consequence of this early and accelerated upregulation of reactive astrocyte genes in COX-2$^-$ males may relate to the suggested role of astrocytes in synapse formation during brain development. Specifically, previous studies have found that the peak period for astrogenesis during development immediately precedes that of synaptogenesis, which primarily occurs during the second and third postnatal weeks in the rodent brain [350,358,359]. Moreover, the formation of the first synapses typically occurs only after the first astrocytes are born. Together, these findings indicate that astrocytes may play a critical role in synaptogenesis. Further support for this comes from *ex vivo* studies showing that neurons cultured with astrocytes formed

considerably more synapses than neurons cultured alone [359–361]. Notably, these effects were also found to occur in cases where no direct contact was made between neuronal and glial cells, suggesting that signaling molecules secreted from astrocytes were responsible for promoting synapse formation between neurons. In support of this notion, a number of studies have identified several astrocytes-derived signaling molecules that facilitate formation and maturation of glutamatergic synapses in the developing brain [362–365]. Interestingly, several of these proteins (e.g., thrombospondin-1,2) are only expressed by astrocytes until PND 21 [362]. This suggests that the timeframe within which astrocytes can facilitate the formation of glutamatergic synapses may be both developmentally regulated and potentially limited to a particular window during development. Since reactive astrocytes have been associated with an altered production of various signaling molecules secreted by astrocytes [366], these findings suggest that impaired COX-2 activity may result in dysregulated neuroglial communication during synaptogenesis in a manner that could adversely impact the development of excitatory synapses in COX-2$^-$ males.

Our findings in this study also support the notion that the female protective effect would better account for the observed sex-differences in astrocyte reactivity between COX-2$^-$ males and females than the male vulnerability hypothesis. Specifically, one of the major tenets of the male vulnerability hypothesis is that genetic or environmental insults would have a more significant impact on genes expressed at higher levels in males, thereby rendering males more vulnerable [194–197]. However, none of the astrocyte markers were differentially expressed in the brain between wildtype 129S6 males and females during early postnatal development (Fig. 13,14). Additionally, during adolescence, 3 out of 5 astrocyte markers (*Serpina3n*, *Aqp4*, and *Serping1*; Fig. 11a-c) were expressed higher in 129S6 females compared to males, whereas only 1 out of 5 was expressed higher in 129S6 males compared to females (*S100a10*; Fig. 11d).

Moreover, *Gfap* expression was 5-fold greater in 129S6 females than males during adolescence, although this difference was not significant (Fig. 8b). These results collectively indicate that the vast majority of astrocyte markers were not upregulated in the brain of 129S6 males at either of these developmental stages. As such, our findings fail to support the notion that the observed sex differences in astrocyte reactivity between COX-2$^-$ males and females reflects a more significant impact of impaired COX-2 activity on genes typically expressed at higher levels in males than females. Therefore, the male vulnerability hypothesis likely does not account for the observed sex-differences in astrocyte reactivity in our model.

Additionally, an analysis of the expression of A1 and A2 astrocyte markers at both stages appears to support the female protective effect. Specifically, impaired COX-2 activity was not found to upregulate the A1 neurotoxic astrocyte marker *Serping1* in either COX-2$^-$ males or COX-2$^-$ females during early postnatal development (Fig. 14a). On the other hand, the A2 neuroprotective astrocyte marker *S100a10* was both upregulated in the brain of COX-2$^-$ females, and expressed significantly higher in the brain of COX-2$^-$ females compared to COX-2$^-$ males at this stage (Fig. 14b). These results suggest that the observed sex differences in the expression of neuroprotective astrocyte genes may precede those associated with neurotoxic astrocyte markers, indicating that neuroprotective mechanisms in females may be triggered earlier than neurotoxic mechanisms in males.

Notably, impaired COX-2 activity was found to upregulate the expression of *S100a10* in both COX-2$^-$ males and females during early postnatal development (Fig. 14b). Furthermore, while *S100a10* remained upregulated in COX-2$^-$ females at both developmental stages, the fact that in COX-2$^-$ males *S100a10* was initially found to be upregulated during early postnatal development and then downregulated during adolescence suggests that regulation of

neuroprotective astrocyte markers may be more stable in COX-2$^-$ females than the regulation of both A1 and A2 markers in COX-2$^-$ males (Fig. 15). Together, both the earlier onset and the increased stability of this A2 neuroprotective astrocyte marker in COX-2$^-$ females suggests that the female protective effect better accounts for the observed sex differences in the effect of impaired COX-2 activity on the expression of reactive astrocyte markers between COX-2$^-$ males and females.

As mentioned previously, considerable overlap exists between the timing of synaptic and astrocytic development, with the formation of synapses occurring immediately following astrocyte differentiation [350,358,359]. Similarly, the closing of the developmental period of peak synaptogenesis overlaps considerably with the maturation of astrocytes, with the major waves of synapse formation in the rodent brain finishing around PND 21 and astrocytes adopting a mature morphology by PND 28 [350,358,359]. Notably, astrocytes have been suggested to play large role in the refinement of excitatory synapses in the brain, as certain astrocyte secreted factors (e.g., glypicans) have been shown to induce post-synaptic modifications, including the trafficking of AMPA receptors, and facilitate the initiation of excitatory signaling in glutamatergic neurons [365]. Additionally, astrocytes play a number of roles in the maintenance of healthy synapses, including the prevention of glutamate excitotoxicity via the direct uptake of glutamate from the synapse (i.e., by the astrocytic glutamate transporters GLT-1) [367]. Astrocyte have also been shown to help protect against glutamate excitotoxicity by indirectly inhibiting presynaptic glutamate release [368]. Specifically, the release of GABA by inhibitory interneurons can lead to increased activity of the astrocytic GABAergic transporter GAT-3, a transporter that has been shown to promote the secretion of adenosine by astrocytes. In turn, the binding of adenosine to presynaptic receptors inhibits the release of glutamate [368]. These findings suggest that effect of impaired COX-2

activity on astrocytes may also impact the refinement and maintenance of glutamatergic synapses in COX-2^{-} mice.

Our findings indicate that different subtypes of reactive astrocytes may be upregulated in COX-2^{-} males and females throughout the developmental window of synaptogenesis. These effects of impaired COX-2 activity may have a more detrimental impact on brain development in males than females, as only COX-2^{-} males were found to exhibit an increased expression of A1 neurotoxic astrocytes and a reduced expression of A2 neuroprotective astrocytes, compared to 129S6 mice, by adolescence (Fig. 15). As such, the results of transcriptomic analyses of A1 vs. A2 astrocytes indicate the astrocytic profile of COX-2^{-} males may be associated with increased neurodegenerative inflammatory signaling (Nfκβ-mediated) and pro-inflammatory cytokine secretion, as well as reduced neuroprotective inflammatory signaling (STAT-3 mediated) and trophic factor release [256,258]. Additionally, insights from A1-associated (pro-inflammatory) conditions suggests that COX-2^{-} males may be more prone to glutamate excitotoxicity. Specifically, A1 reactive astrocytes have been found to be associated with an increased release of glutamate, as well as a reduced expression of receptors known to help mediate the effects of glutamate, including GLT-1 and GAT-3 [369–372]. These findings collectively suggest a mechanism whereby impaired COX-2 activity may promote increased neurotoxic pro-inflammatory signaling, enhanced glutamate release, and reduced glutamate uptake in COX-2^{-} males. Ultimately, this could result in elevated glutamate excitotoxicity and neuronal death during brain development, and potentially cause an imbalance between excitatory and inhibitory neuronal activity (E/I imbalance) in COX-2^{-} males.

In summary, the results from studies 1 and 2 suggest that impaired COX-2 activity may lead to an early and accelerated upregulation of reactive astrocyte genes in COX-2^{-} males, and

that COX-2$^-$ females may be protected against such effects (Fig. 15). Over time, impaired COX-2 activity may have a more detrimental impact on brain development in males than females, as COX-2$^-$ males appear to be associated with increased neurotoxic reactive astrocytes during adolescence, whereas COX-2$^-$ females appear to be associated with increased neuroprotective reactive astrocytes at this stage. As a result, COX-2$^-$ males may be subject to abnormal neuroglial communication during the formation of excitatory synapses, leading to increased glutamate excitotoxicity and ultimately contributing to an E/I imbalance in the brain. These sex differences in neurodevelopment may contribute to the increased hyperactivity and RRB-related behaviors exhibited by COX-2$^-$ males during adolescence and early adulthood [53]. In conclusion, these findings suggest that exposure to antipyretic drugs may adversely impact the development of astrocytes and synapses in the male brain during development, and may contribute to the male bias in NDDs by facilitating and E/I imbalance that has been implicated in the pathology of many of these disorders, including ASD and ADHD [373–376].

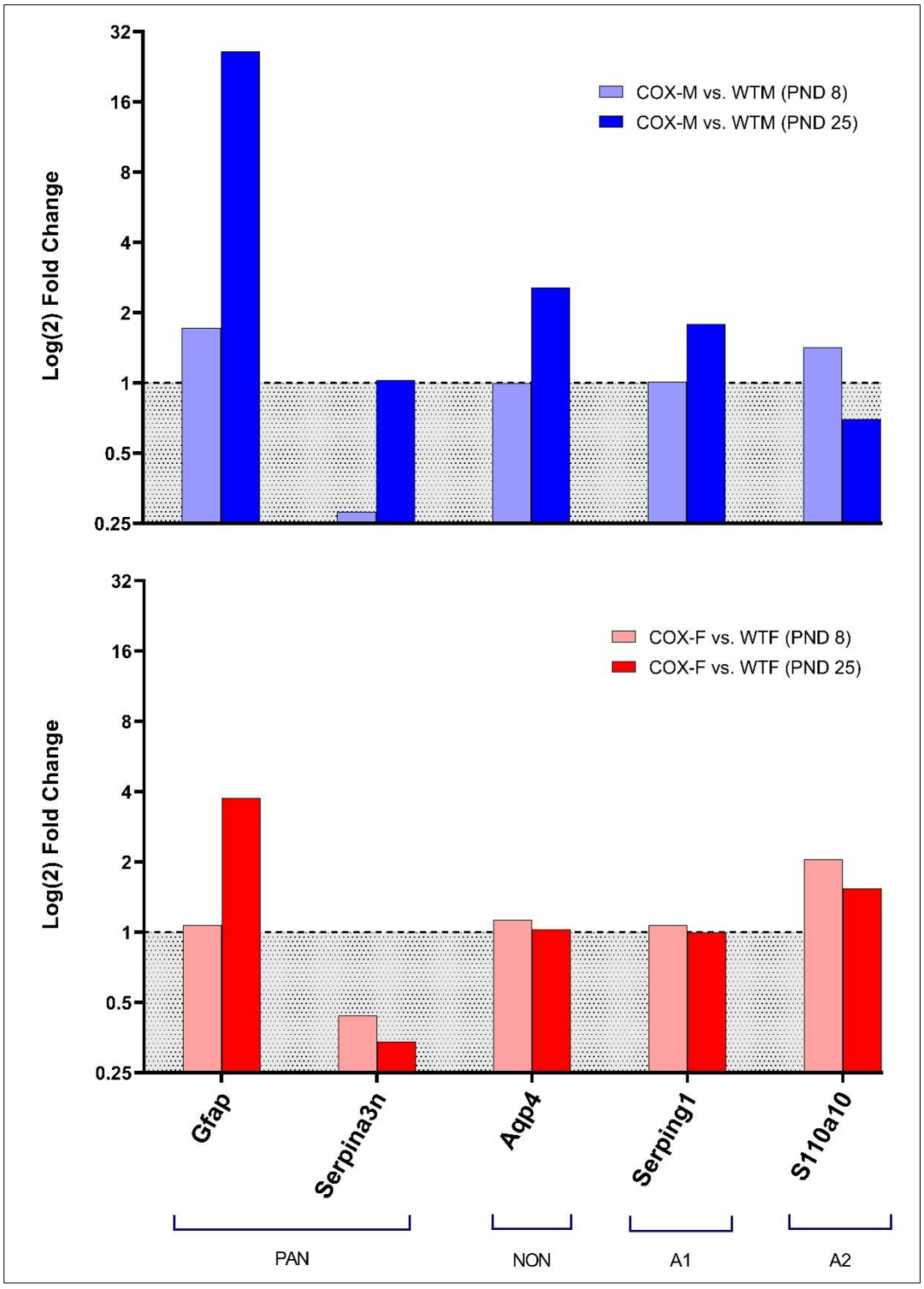

Figure 15. Summary of Results for Aim 3

Log(2) fold change values representing two-fold differences in the expression of astrocyte genes in the brain between COX-2$^-$ males ("COX-M") and 129S6 males ("WTM"), and between COX-2$^-$ females ("COX-F") and 129S6 females ("WTF") on postnatal day ("PND") 8 and PND 25. The *y*-axis is labeled using linear fold change values. "PAN" = pan-reactive, "NON" = non-reactive, "A1" = neurotoxic, and "A2" = neuroprotective.

5.2 Research Aim 4: The effects of impaired COX-2 activity on the enrichment of gene sets in the male and female mouse brain during prenatal development

The goal of Aim 4 is to identify the effects of impaired COX-2 activity on the enrichment of gene sets in the brain between COX-$2^{-/-}$ and wildtype 129S6 males (Aim 4.1), as well as between COX-$2^{-/-}$ and wildtype 129S6 females (Aim 4.2), during prenatal development (GD 15 and GD 18). Based on the results from Aims 1 to 3, I hypothesize that impaired COX-2 activity will alter the expression of gene sets associated with cytokine signaling and the neuroinflammatory response in COX-$2^{-/-}$ males. Additionally, gene sets associated with glutamatergic neurons and synapses are expected to be differentially expressed in COX-$2^{-/-}$ males. Furthermore, steroidogenesis in the male testes has been proposed to act as a trigger that facilitates epigenetic modifications that result in a lower threshold required to alter the expression of genes in the male brain [244,246] (see section 2.2.2). Since steroidogenesis occurs around GD 16 in mice, COX-$2^{-/-}$ males are expected to exhibit greater changes in transcriptional and regulatory gene sets at GD 18 than at GD 15. Based on the results from Aims 1 to 3, I also hypothesize that impaired COX-2 activity will alter the expression of gene sets associated with cytokine signaling and the neuroinflammatory response in COX-$2^{-/-}$ females. Since A2 astrocytes are not associated with E/I imbalances, gene sets associated with glutamatergic neurons are not expected to be differentially expressed in COX-$2^{-/-}$ females. In agreement with the female protective effect, I also hypothesize no differences in gene sets associated with transcriptional or regulatory functions in the brain of COX-$2^{-/-}$ females between GD 15 and GD 18.

Aim 4.1: Enriched Gene Sets in COX-2$^{-/-}$ Males on GD 15 & GD 18

Background & Rationale:

The objective of Aim 4.1 was to analyze the effect of impaired COX-2 activity on the expression of functional gene sets in the male brain during prenatal development (GD 15 and GD 18) and identify which biological processes, molecular functions, and cellular locations may be overrepresented in the brain of COX-2$^{-/-}$ males at this time. A previous omics analysis of human blood and urine samples found that exposure to antipyretic drugs can trigger a neuroimmune response, even at low doses [269]. Similar findings have been obtained from rodent studies, where both prenatal and postnatal exposure to antipyretics have led to an elevated production of inflammatory cytokines in the brain [270,271,377]. These findings are also in agreement with the results obtained from our lab, where COX-2^{-} mice have been found to exhibit an increased expression of inflammatory cytokines in the brain during early postnatal development [53], as well as in adolescence (see Aim 1.2). As such, I hypothesized that gene sets associated with inflammatory cytokines would be upregulated in the prenatal brain of COX-2$^{-/-}$ males.

As discussed previously, the developmental timeline between the formation and maturation of synapses and astrocytes is highly correlated. Typically, research investigating synaptic neuroglial signaling tends to focus on the role that astrocytes play in the formation, maturation, maintenance, and elimination of synapses. However, while the onset of astrogenesis in the mouse brain is suggested to occur around GD 18 – PND 0 [350], the production of neurons is predicted to begin around GD 11-16 and peak from GD 13-18 in the developing cortex, depending on the layer [378]. Given than the peak wave of neurogenesis occurs before that of astrogenesis, it is possible that deficits in neuronal development may serve to trigger reactive

astrocytes at a later date. Our findings in Aim 2 and 3 also suggested that impaired COX-2 activity may lead to an upregulation of neurotoxic astrocytes in the male brain during development. As discussed previously (see section 5.1.1), this may contribute to impaired neuroglial communication during the formation of excitatory synapses, and ultimately lead to increased glutamate excitotoxicity and an E/I imbalance in males. Thus, it would be interesting to investigate whether impaired COX-2 activity may adversely impact neuronal development, as this would suggest that the deficits in neuroglial signaling between reactive astrocytes and excitatory synapses may be reciprocal in nature. As such, I hypothesized that gene sets associated with glutamatergic neurons and synapses would be differentially expressed in COX-$2^{-/-}$ males during prenatal development.

Lastly, previous studies have suggested that steroidogenesis in the male testes may act as a trigger that facilitates certain epigenetic modifications (reduced DNA methylation, in particular) that result in a lower threshold required to alter the expression of genes in the male brain [244,246] (see section 2.2.2 for more details). Consistent with the notion that genetic and environmental insults interact in conferring their risk for NDDs, impaired COX-2 activity is hypothesized to have an additive effect (along with E_2 following steroidogenesis) on the modification of epigenetic markers in the male brain [244,246]. Since steroidogenesis is believed to occur around GD 16 in mice [225], COX-$2^{-/-}$ males are expected to exhibit a greater enrichment of gene sets associated with the regulation of transcription, molecular functions, and biological processes after this event (on GD 18) than immediately prior to it (GD 15).

Results for Aim 4.1: Impaired COX-2 Activity Alters the Enrichment of Biological Pathways in the Prenatal Male Brain

In this study, the results of previous gene expression microarray experiments using whole brain samples obtained from COX-$2^{-/-}$ and 129S6 wildtype males at GD 15 and GD 18 were analyzed for the enrichment of gene sets (see section 3.4 for methodology). Of the 483 probes found to be differentially expressed between COX-$2^{-/-}$ and 129S6 wildtype males at GD 15 (FC $\geq |1.5|$, $p < .05$), 279 were identified as being previously annotated, non-ambiguous genes that were used in our analysis—including 110 upregulated and 169 downregulated genes. The functional enrichment analysis was performed with g:Profiler using the gene set counts and sizes (g:SCS) multiple testing correction method. In total, 161 out of 279 of these profiled genes were mapped to 93 enriched GO terms at GD 15, $p < .05$. The top 10 differentially expressed (that is, most significantly upregulated or downregulated) GO categories in COX-$2^{-/-}$ males at this stage were included in our analysis and are illustrated in Figure 16. These findings suggest that upregulated genes in COX-$2^{-/-}$ males at GD 15 may be those associated with proteins involved in extracellular matrix structure and activity, and constituents of this class included ECM proteins, such as collagen (Col3a1,Col6a3,Col5a2). Proteins involved with the transport of oxygen also figured prominently in upregulated genes at this stage, which were particularly enriched with components of the hemoglobin complex (Hbb-y, Hba-x, Hbb-bh1). On the other hand, downregulated genes in COX-$2^{-/-}$ males on GD 15 may be those associated with proteins involved in synaptic organization and signaling and were particularly enriched in various cellular components of glutamate synapses (Fig. 16; Table 10 in Appendix A).

From the 207 probes found to be differentially expressed between COX-$2^{-/-}$ and 129S6 wildtype males at GD 18 (FC $\geq |1.5|$, $p < .05$), 115 were identified as being previously annotated,

non-ambiguous genes that were used in our analysis—including 50 upregulated and 65 downregulated genes. Following the functional enrichment analysis, 34 out of the 115 profiled genes were mapped to 26 enriched GO terms at GD 18, $p < .05$. For reference, the top 10 differentially expressed (that is, most significantly upregulated or downregulated) GO categories in COX-$2^{-/-}$ males at this stage were included in our analysis and are illustrated in Figure 17. These findings suggest that upregulated genes in COX-$2^{-/-}$ males at GD 18 may be those associated with proteins involved in DNA binding on regulatory regions and transcriptional factor activity, as well as proteins related to the positive regulation of biosynthetic processes and DNA transcription. Constituents of this class included various proteins localized on chromosomal regions and transcription factor complexes within the nucleus (Fig. 17; Table 11 in Appendix A). On the other hand, downregulated genes in COX-$2^{-/-}$ males on GD 18 were particularly enriched for proteins localized on histone acetyltransferase complexes (Fig. 17; Table 11 in Appendix A).

In summary, our findings failed to support our hypothesis that gene sets associated with neuroinflammatory signaling and cytokines would be differentially expressed between in COX-$2^{-/-}$ males and 129S6 males during prenatal development. On the other hand, in agreement with our hypotheses, genes downregulated in COX-$2^{-/-}$ males on GD 15 were found to be associated with proteins involved in glutamatergic neurons and synapses. Additionally, consistent with the hypothesis that impaired COX-2 activity may have an additive effect (along with E_2 following steroidogenesis) on the modification of epigenetic markers, COX-$2^{-/-}$ males were also found to exhibit a greater enrichment of gene sets associated with the regulation of transcription and biological processes on GD 18 than on GD 15.

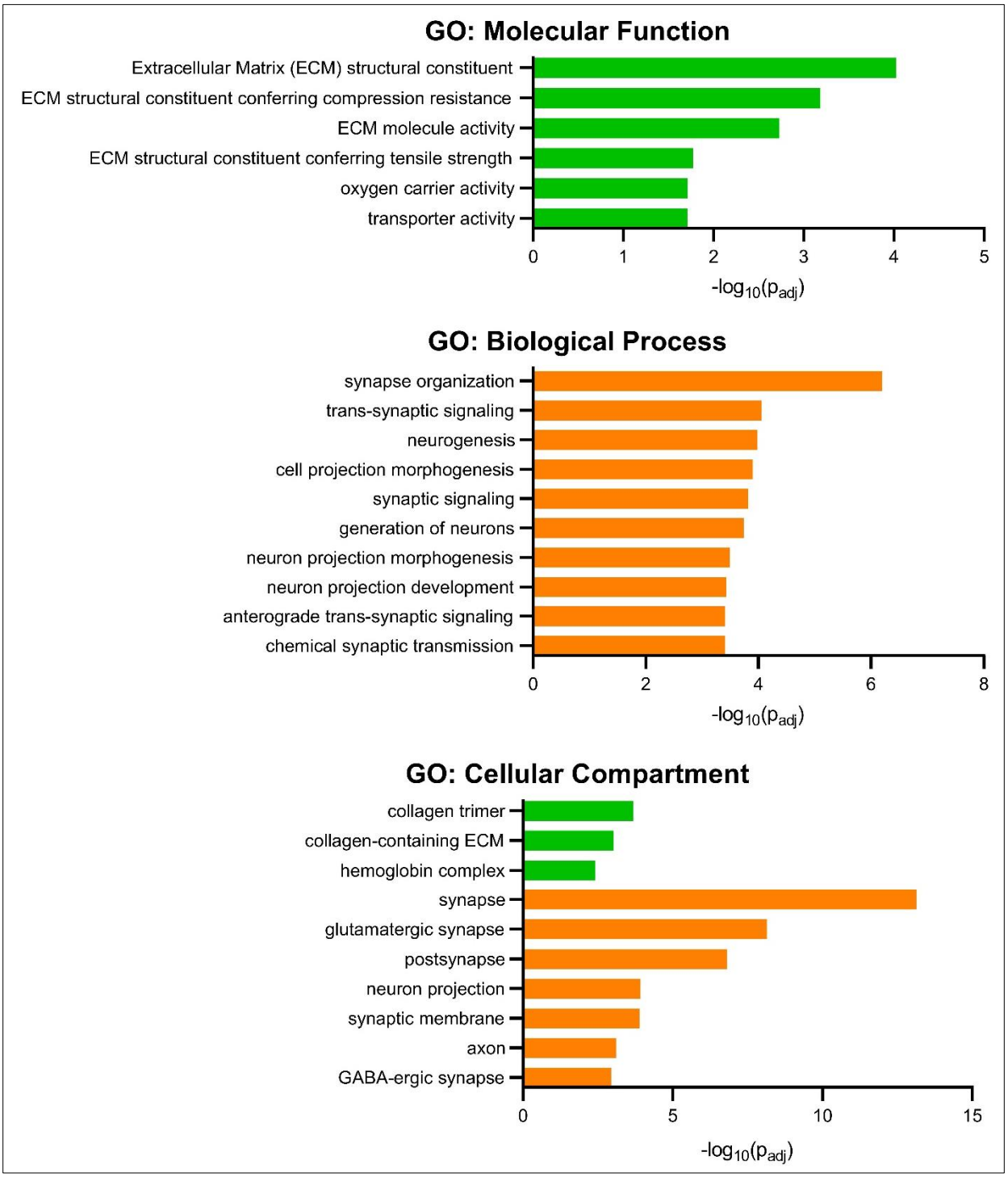

Figure 16. Gene Set Enrichment Analysis for COX-2$^{-/-}$ Males at GD 15

Gene Ontology (GO) enrichment analyses of molecular function, biological process, and cellular compartment for upregulated (*green*) and down-regulated (*orange*) genes between COX-2$^{-/-}$ males and 129S6 males at gestational day 15 (GD 15). The top 10 overall most significant results of each GO analysis are presented. Significance value was determined as the negative log(10) of the g:SCS adjusted p-values (p_{adj}) for each GO term.

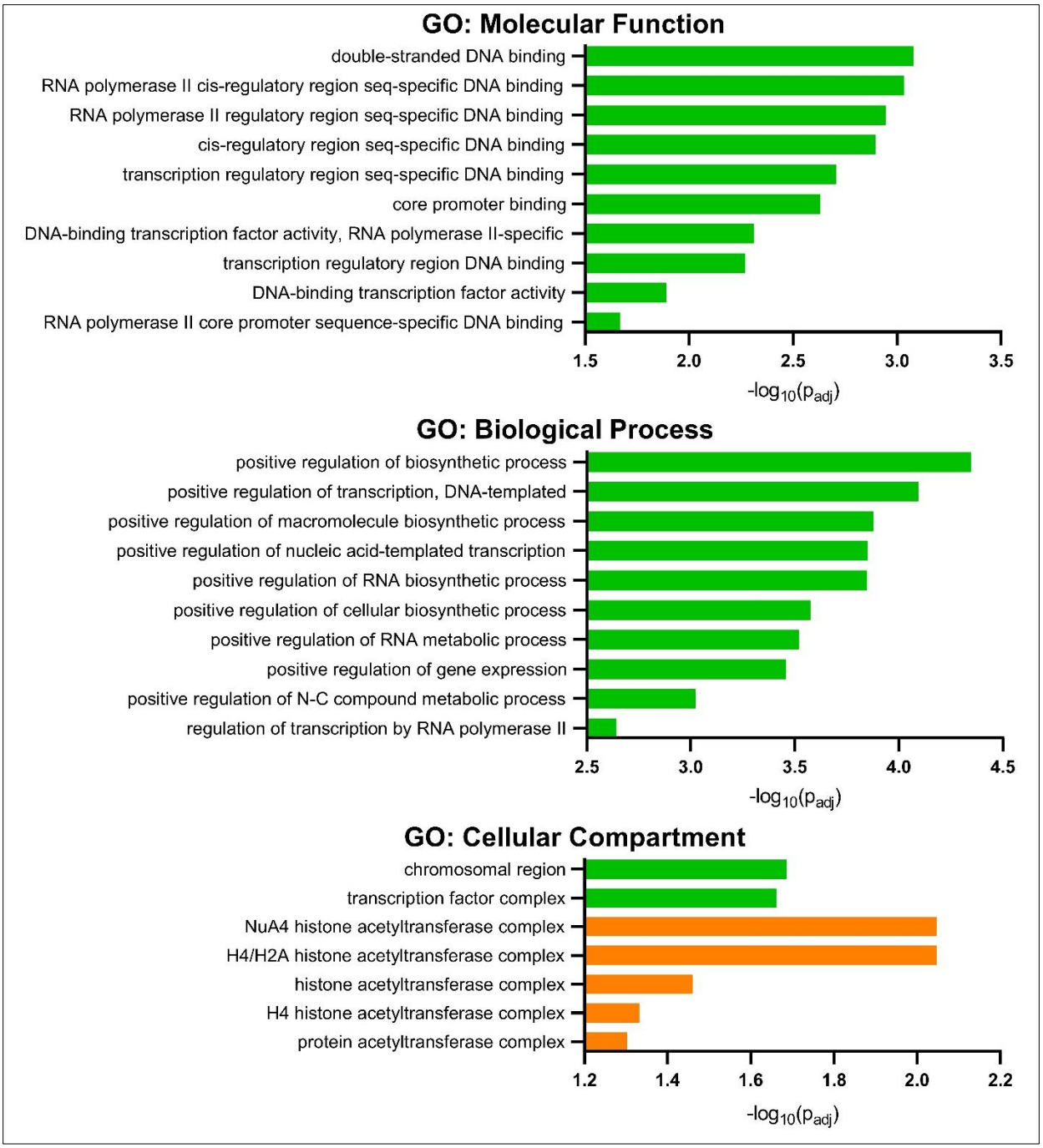

Figure 17. Gene Set Enrichment Analysis for COX-2$^{-/-}$ Males at GD 18

Gene Ontology (GO) enrichment analyses of molecular function, biological process, and cellular compartment for upregulated (*green*) and down-regulated (*orange*) genes between COX-2$^{-/-}$ males and 129S6 males at gestational day 18 (GD 18). The top 10 most significant gene sets results of each GO analysis are presented. Significance value was determined as the negative log(10) of the g:SCS adjusted p-values (p_{adj}) for each GO term. "N-C" = nucleobase-containing.

Aim 4.2: Enriched Gene Sets in COX-2$^{-/-}$ Females on GD 15 & GD 18

Background & Rationale:

The objective of Aim 4.2 was to analyze the effect of impaired COX-2 activity on the expression of functional gene sets in the female brain during prenatal development (GD 15 and GD 18) and identify which biological processes, molecular functions, and cellular locations may be overrepresented in the brain of COX-2$^{-/-}$ females at this time. As discussed previously, several studies in humans and rodents have found that exposure to antipyretic drugs can trigger a neuroimmune response, including an elevated production of inflammatory cytokines in the brain [269–271,377]. Their findings are also consistent with the results obtained from our lab, where an increased expression of neuroimmune genes and inflammatory cytokines was found in the brain of COX-2^{-} females during both early postnatal development [53], as well as in adolescence (see Aim 1.2). As such, I hypothesized that gene sets associated with inflammatory cytokines would be upregulated in the prenatal brain of COX-2$^{-/-}$ females.

Our findings in Aims 2 and 3 also suggested that impaired COX-2 activity may lead to an upregulation of A2 neuroprotective astrocytes in the female brain during development. Unlike the neurotoxic A1 astrocytes that are believed to be upregulated in COX-2^{-} males, the A2 astrocytes associated with COX-2^{-} females are not related to E/I imbalances. Thus, gene sets associated with glutamatergic neurons are not expected to be differentially expressed in COX-2$^{-/-}$ females. Additionally, as females are not exposed to the masculinizing effects of steroidogenesis occurring in mice around GD 16, I also hypothesized that gene sets associated with transcriptional or regulatory functions would not be differentially expressed in the brain of COX-2$^{-/-}$ females between GD 15 and GD 18. Our results in Aims 2 and 3 also appear to

support the female protective effect in this model, suggesting that typical female development may be associated with inherently protective mechanisms that render females mice less susceptible to developing NDD-related neurochemical and behavioral symptoms as a result of impaired COX-2 activity [198–204]. As such, I also hypothesized that certain compensatory mechanisms (i.e., negative regulation of apoptosis) might be upregulated to protect against the damaging effects of impaired COX-2 activity in the female brain.

Results for Aim 4.2: Impaired COX-2 Activity Alters the Enrichment of Biological Pathways in the Prenatal Female Brain

In this study, the results of previous gene expression microarray experiments using whole brain samples obtained from COX-$2^{-/-}$ and 129S6 wildtype females at GD 15 and GD 18 were analyzed for the enrichment of gene sets (see section 3.4 for methodology). Of the 263 probes found to be differentially expressed between COX-$2^{-/-}$ and 129S6 wildtype females at GD 15 ($FC \geq |1.5|$, $p < .05$), 200 were identified as being previously annotated, non-ambiguous genes that were used in our analysis—including 76 upregulated and 124 downregulated genes. The functional enrichment analysis was performed with g:Profiler using the gene set counts and sizes (g:SCS) multiple testing correction method. In total, 57 out of 200 of these profiled genes were mapped to 15 enriched GO terms at GD 15, $p < .05$. The top 10 differentially expressed (that is, most significantly upregulated or downregulated) GO categories in COX-$2^{-/-}$ females at this stage were included in our analysis and are illustrated in Figure 18. These findings suggest that upregulated genes in COX-$2^{-/-}$ females at GD 15 may be those associated with proteins involved in small nuclear RNA binding, and constituents of this class included members of a ribosome assembly intermediate, the small-subunit processome (Imp4,Nop58). Proteins involved with the

binding of signal peptides to the endoplasmic reticulum and oligopeptidase activity also appeared to figure prominently in upregulated genes at this stage. On the other hand, downregulated genes in COX-$2^{-/-}$ females on GD 15 may be those associated with proteins involved in ligase activity during the metabolic synthesis of acids (Acsl3, Asnsd1, Farsb, Dars). Proteins localized in GABAergic synapses and dendrites are also found to be associated with downregulated genes in a COX-$2^{-/-}$ females at this stage (Fig. 18; Table 12 in Appendix A).

From the 277 probes found to be differentially expressed between COX-$2^{-/-}$ and 129S6 wildtype females at GD 18 ($FC \geq |1.5|$, $p < .05$), 176 were identified as being previously annotated, non-ambiguous genes that were used in our analysis—including 128 upregulated and 48 downregulated genes. Following the functional enrichment analysis, 39 out of the 176 profiled genes were mapped to 34 enriched GO terms at GD 18, $p < .05$. The top 10 differentially expressed (that is, most significantly upregulated or downregulated) GO categories in COX-$2^{-/-}$ females at this stage were included in our analysis and are illustrated in Figure 19. These findings suggest that upregulated genes in COX-$2^{-/-}$ females at GD 18 may be those associated with proteins involved in protein translation, as well as peptide and amide biosynthesis and metabolism. Constituents of this class are largely comprised of various proteins localized on ribosomal subunits and complexes that are known to play a role in the binding of RNA and structural molecular activity in ribosomes (Fig. 19; Table 13 in Appendix A). On the other hand, downregulated genes in COX-$2^{-/-}$ females on GD 18 were not found to be significantly enriched in proteins that are associated with any particular biological processes.

In summary, our findings failed to support our hypothesis that gene sets associated with neuroinflammatory signaling and cytokines would be differentially expressed between in COX-$2^{-/-}$ females and 129S6 females during prenatal development. Similarly, our results failed to find

evidence of any well established compensatory or neuroprotective mechanisms (i.e., negative regulation of apoptosis) associated with upregulated genes in COX-$2^{-/-}$ females at either stage. On the other hand, in agreement with our hypotheses, genes sets in COX-$2^{-/-}$ females were not found to be associated with proteins involved in glutamatergic neurons and synapses, or exhibit a greater enrichment of gene sets associated with the regulation of transcription and biological processes on GD 18 than on GD 15.

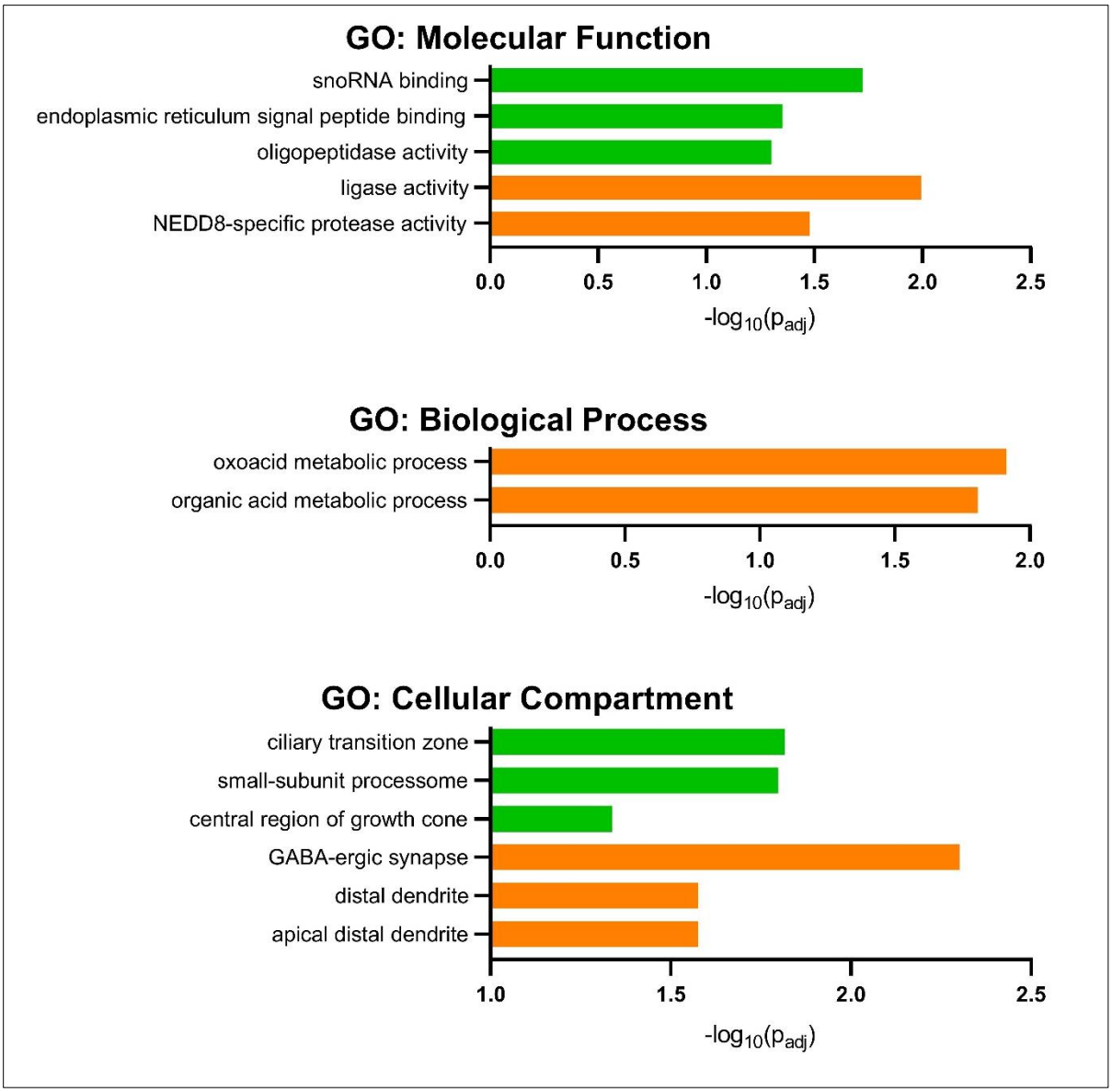

Figure 18. Gene Set Enrichment Analysis for COX-2$^{-/-}$ Females at GD 15

Gene Ontology (GO) enrichment analyses of molecular function, biological process, and cellular compartment for upregulated (*green*) and down-regulated (*orange*) genes between COX-2$^{-/-}$ females and 129S6 females at gestational day 15 (GD 15). The top 10 most significant gene sets results of each GO analysis are presented. Significance value was determined as the negative log(10) of the g:SCS adjusted p-values (p_{adj}) for each GO term.

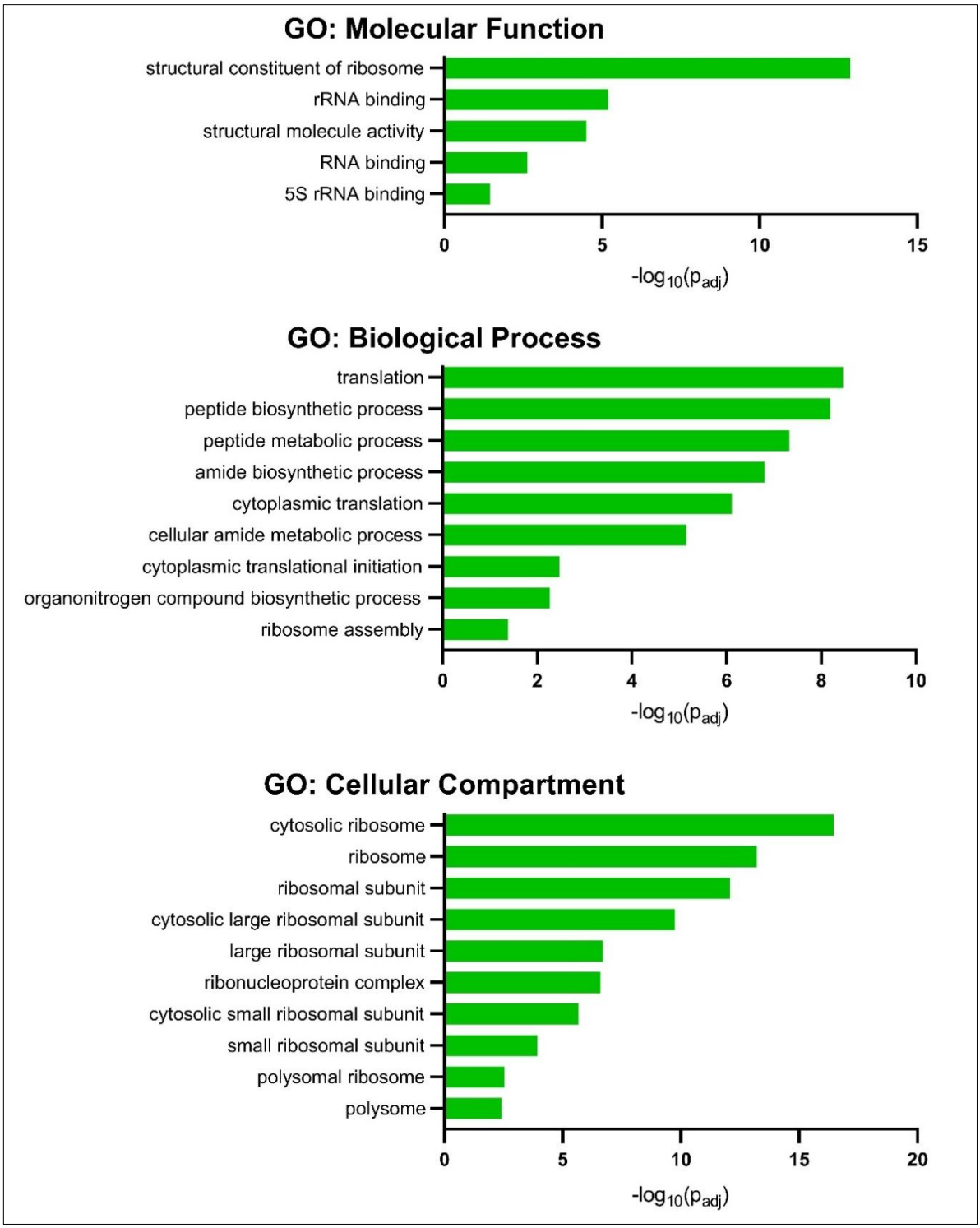

Figure 19. Gene Set Enrichment Analysis for COX-2$^{-/-}$ Females at GD 18

Gene Ontology (GO) enrichment analyses of molecular function, biological process, and cellular compartment for upregulated (*green*) and down-regulated (*orange*) genes between COX-2$^{-/-}$ females and 129S6 females at gestational day 18 (GD 18). The top 10 most significant gene sets results of each GO analysis are presented. Significance value was determined as the negative log(10) of the g:SCS adjusted p-values (p_{adj}) for each GO term.

5.2.1 Analysis & Conclusions for Aim 4

In Aim 4, we investigated the effects of impaired COX-2 activity on the enrichment of gene sets in the brain between COX-$2^{-/-}$ males and 129S6 males (Aim 4.1) and between COX-$2^{-/-}$ females and 129S6 females (Aim 4.2), during prenatal development. It should be noted that since our analyses were based on the results of two separate microarray experiments, no direct comparisons can be made between COX-$2^{-/-}$ males and females in this study. As such, our analysis of these results will focus on the impact of impaired COX-2 activity in the male prenatal brain, as well as the impact of impaired COX-2 activity in the female prenatal brain. However, differences between enriched gene sets in the brain of COX-$2^{-/-}$ males and females will be briefly discussed and used to infer potential sex differences, as this will facilitate our analyses and interpretation of the results in the current study.

As mentioned previously, studies on the effects of antipyretic drugs in the brain suggested that impaired COX-2 activity may be associated with an elevated production of inflammatory cytokines in the brain during both prenatal and postnatal development brain [269–271,377]. Our previous findings were in agreement with this literature as COX-2^{-} mice were found to exhibit an upregulation of inflammatory cytokines in the brain during early postnatal development [53], as well as in adolescence (see Aim 1.2). However, neither neuroinflammatory signaling nor cytokines were found to be enriched in differentially expressed gene sets in either COX-$2^{-/-}$ males or females in this study, suggesting that impaired COX-2 activity may not alter the expression of these signaling molecules during prenatal development.

One possible explanation for these differences between our prenatal and postnatal results is that they may reflect differences between the COX-2^{-} and COX-$2^{-/-}$ mouse models. While

cyclooxygenase activity is impaired in COX-2^{-} mice, this enzyme's subsequent peroxidase activity is spared [248]. On the other hand, both cyclooxygenase activity and peroxidase activity are disabled in COX-2$^{-/-}$ mice [249], suggesting COX-2 activity would be more profoundly impaired in COX-2$^{-/-}$ mice than in COX-2^{-} mice. However, previous literature has suggested that antipyretic drugs that primarily target cyclooxygenase activity (i.e., Ibuprofen) are more potent antagonists of inflammatory responses than those that primarily target peroxidase activity (i.e., APAP) [36], which would seem to partially conflict with this interpretation. Nonetheless, the combined effects of impaired cyclooxygenase and peroxidase activity in the COX-2$^{-/-}$ mouse model certainly offers a viable explanation for why neuroinflammatory signaling and cytokines appear to be elevated in the brain of COX-2^{-} mice and not COX-2$^{-/-}$ mice during development.

An alternative explanation for why the results of Aim 4 failed to support our previous findings in Aims 1 to 3, which had suggested that impaired COX-2 activity might cause increased neuroinflammatory and cytokine response in the brain, is that these findings may reflect developmental differences (that is, to differences in the effects of impaired COX-2 activity during prenatal versus postnatal development). One of the major cell types responsible for the production of cytokines in the brain is microglia. Microglia begin to colonize the brain early in development, around GD 10-11 [379], and are considered to be a major source of prenatal cytokine production [380]. On the other hand, early astrocytes don't begin invading the brain until around PND 0 [352,354]. Therefore, it is possible that impaired COX-2 activity was only found to results in an elevated neuroinflammatory and cytokine response postnatally because the upregulation of these signaling mechanisms is initially triggered in astrocytes. This interpretation would agree with the aforementioned results from our lab, as well as our previous findings,

which indicated that microglia in the brain of COX-2^{-} mice were not in an increased activational state on GD 18, PND 8, or PND 25 [54,313].

The results of Aim 4.1 indicated that gene sets upregulated in COX-2$^{-/-}$ males on GD 15 were primarily associated with proteins involved in extracellular matrix structure and activity (Fig. 16). Interestingly, these findings were consistent with the Zamanian et al., (2012) study, where a transcriptomic analysis of reactive astrocytes following both LPS and MCAO were found to be associated with proteins involved in extracellular matrix binding, modifications, and adhesion moreso than any other GO category [256]. This finding that extracellular matrix proteins was the largest class to be associated with upregulated genes in COX-2$^{-/-}$ males on GD 15, as well as both types of reactive astrocytes, suggests that the pathological mechanisms by which impaired COX-2 activity induces reactive gliosis in the male brain may be related to its impact on the extracellular matrix during development.

On the other hand, gene sets downregulated in COX-2$^{-/-}$ males on GD 15 appear to primarily be associated with proteins involved in the generation and organization of glutamatergic neurons and synapses, as well as synaptic signaling mechanisms (Fig. 16). This finding was interesting, as our results in Aims 1 to 3 suggested that impaired COX-2 activity may lead to an upregulation of neurotoxic astrocytes in the male brain during development. While some research has found that neurotoxic astrocytes can adversely impact the formation and healthy functioning of glutamatergic synapses [369–372], the majority of these studies have focused on the impact of neurotoxic astrocytes on glutamate synapses, and not the reverse. As discussed previously (see section 5.1.1), the timeline between the formation and maturation of synapses and astrocytes in the brain is highly correlated [350,358,359], suggesting neurons and astrocytes may communicate in coordinating these events during development. Additionally,

previous studies have demonstrated that astrocytic processes are highly responsive to neural activity [381]. Furthermore, glutamate receptors (i.e., Grm5) and transporters (i.e., GAT-1) appear to be expressed as early as the first postnatal week in astrocytes [382,383], shortly after astrocytes first begin to colonize and proliferate in the brain. Thus, astrocytes are likely primed to be responsive to neuronal activity even before having reached maturation. Furthermore, certain neuroglial signaling molecules (i.e., ephrins) are known to initiate bidirectional signaling between astrocytes and glutamatergic neurons, with astrocyte-derived ephrins shown to regulate the growth and morphology of dendritic spines [384], and neuron-derived ephrins shown to modulate the expression of glutamatergic transporters and morphology of astrocytic processes [385,386]. Therefore, given that the expression of genes associated with glutamatergic neurons and synapses are altered in COX-2$^{-/-}$ males before the onset of astrogenesis, it is possible that the impact of impaired COX-2 activity on glutamatergic neurons may serve to trigger the elevated expression of reactive astrocyte genes, as opposed to the reverse having occurred.

The results of Aim 4.1 also indicated that gene sets upregulated in COX-2$^{-/-}$ males on GD 18 were primarily associated with proteins involved in facilitating binding onto DNA regulatory regions, constituents of transcription factors, and proteins involved in the regulation of transcription factor activity (Fig. 17). As discussed previously, steroidogenesis in the male testes has been proposed to act as a trigger that results in certain epigenetic modifications, including reduced DNA methylation, that functions to lower the threshold required to alter the expression of genes in the male brain [244,246] (see section 2.2.2 for more details). Thus, these results agreed with our hypothesis that COX-2$^{-/-}$ males would exhibit a greater enrichment of gene sets associated with the regulation of transcription, molecular functions, and biological processes after steroidogenesis (on GD 18) than immediately prior to it (GD 15) [225]. As such, our results in

this study appear to support a multiple hit model with regards to the effects of impaired COX-2 activity in the male brain. More specifically, the release of E_2 following steroidogenesis may decrease the levels of DNA methylation in the male brain, lowering the threshold for additional insults (i.e., impaired COX-2 activity) to alter the expression of key signaling molecules during development. Ultimately, this may lead to a greater dysregulation of inherent developmental processes in the brain of males than females.

The results of Aim 4.2 indicated that gene sets upregulated in COX-$2^{-/-}$ females on both GD 15 and GD 18 were primarily associated with proteins localized on ribosomal subunits and complexes, that are involved in ribosomal RNA binding and molecular activity, as well as peptide signaling (Fig. 18 and 19). One possible explanation of these findings is that the upregulation of ribosomal proteins reflects an attempt being made by the brain of COX-$2^{-/-}$ females to compensate for impaired COX-2 activity by increasing its capacity to synthesize proteins. Support for this interpretation can be found in a previous study where chronic LPS treatment led to an impaired ability to synthesize cytosolic proteins [387]. Ultrastructural analysis of neurons in the brain revealed the presence of deep invaginations in the nuclear envelope that were found to be filled with numerous polyribosomes, which was interpreted by the authors as a possible mechanism that might compensate for the impaired translation of cytosolic proteins [387].

Notably, in addition to the association between ribosomal proteins in upregulated gene sets in COX-$2^{-/-}$ females at both prenatal stages, the most significant biological process related to upregulated genes in COX-$2^{-/-}$ females at GD 18 was translation, and the most significant cellular compartment upregulated at this stage was cytosolic ribosomes (Fig. 19). As such, our findings support the notion that the elevated expression of ribosome-associated genes in the brain of COX-$2^{-/-}$ females may reflect a compensatory mechanism that could protect against the

damaging effects of impaired COX-2 activity by increasing the brains capacity to synthesize proteins. This interpretation would also be in agreement with the female protect effect, offering a potential mechanism by which typical female development may be associated inherently protective responses that, in effect, render females mice less susceptible to developing NDD-related neurochemical and behavioral symptoms as a result of impaired COX-2 activity [198–204].

In conclusion, the results from studies 1-4 suggest that impaired COX-2 activity may impair the development of glutamatergic neurons and synapses in the male brain during prenatal development. The increased concentration of E_2 following steroidogenesis in the male testes may then act as a trigger that facilitates certain epigenetic modifications (i.e., reduced DNA methylation) that result in a lower threshold required to alter the expression of genes [244,246], in effect priming the male brain to be more susceptible to the adverse effects of impaired COX-2 activity. In turn, this may lead to an early and accelerated upregulation of reactive astrocyte genes in COX-2^- males during early postnatal development. Over time, impaired COX-2 activity appears to induce neurotoxic reactive astrocytes in males. As a result, COX-2^- males may be subject to abnormal neuroglial communication during the formation of excitatory synapses, leading to increased glutamate excitotoxicity and ultimately contributing to an E/I imbalance in the male brain. Alternatively, our results suggest that females may be largely protected against the adverse effects of impaired COX-2 activity. During prenatal development, elevated levels of ribosomes in the female brain may compensate for the damaging effects of impaired COX-2 activity by increasing the brains capacity to synthesize proteins. Postnatally, COX-2^- females appear to be associated with increased neuroprotective reactive astrocytes that may help in mitigating these harmful effects.

CHAPTER 6. GENERAL DISCUSSION

6.1 Research Aims Revisited: A Summary of the Main Findings

The goal of Aim 1 was to examine the effects of impaired COX-2 activity on the expression of ASD-risk genes and neuroinflammatory markers in the brain of male and female mice during early adolescence. The ASD-risk genes we investigated included markers for neuronal function (*Glo1*) and synaptic function (*Grm5*), both of which were found to be downregulated in COX-2$^-$ mice, and were more profoundly downregulated in COX-2$^-$ females than COX-2$^-$ males compared to their sex-matched wildtype counterparts (Fig. 9). Alternatively, the neuroinflammatory genes we investigated were found to be upregulated in COX-2$^-$ mice, with *IL-1β* and *Gfap* more profoundly upregulated in COX-2$^-$ males, and *IL-6* and *Itgam* more profoundly upregulated in COX-2$^-$ females, compared to their sex-matched wildtype counterparts (Fig. 9). Our results in Aim 1 were largely in agreement with previous transcriptomic analyses of cortical samples from males and females with ASD compared to healthy age- and sex-matched controls (illustrated in Fig. 4) [207,208]. The relatively high degree of overlap between our findings and previous analyses on individuals with ASD suggests that impaired COX-2 activity may produce changes to the developing brain of COX-2$^-$ males and females by interacting with sex-specific biological pathways in a manner that reflects the sex differences observed in the pathology of certain NDDs, such as ASD.

For Aim 2, the goal was to investigate the sex-dependent and sex-independent effects of impaired COX-2 activity on the expression of genes in the adolescent mouse brain. Previous literature on the effects of antipyretic drugs in the brain and our results from Aim 1 suggested that impaired COX-2 activity may lead to elevated oxidative stress in both COX-2$^-$ males and

females [314]. While the oxidative stress genes *iNos, Nox2* and *Gsk3β* were found to be differentially expressed between COX-2$^-$ and 129S6 wildtype mice in a sex-independent manner (Fig. 10), the results failed to support our hypothesis that impaired COX-2 activity would be associated with an upregulation of gene markers for oxidative stress [314]. More specifically, *iNos* expression was found to be upregulated in the brain of COX-2$^-$ mice (Fig. 10b), indicating that impaired COX-2 activity may results in an increased production of the NO radical. However, both *Nox2* and *Gsk3β* were found to be downregulated (Fig. 10a,c), suggesting the production of ROS, including superoxide (a precursor believed to be required for the production of NO-derived RNS) were decreased [334–336]. As such, these results failed to indicate that impaired COX-2 activity led to an increased production of ROS or RNS in the brain of COX-2$^-$ mice.

On the other hand, the most notable sex-dependent effect of impaired COX-2 activity on gene expression observed in Aim 1 was the significantly larger upregulation of *Gfap* in COX-2$^-$ males (Fig. 9). This suggested that the sex differences between COX-2$^-$ males and females may reflect elevated astrocyte reactivity occurring in COX-2$^-$ males. In Aim 2.2, the various astrocyte markers were found to be differentially expressed in COX-2$^-$ mice in a sex-dependent manner, with impaired COX-2 activity leading to a more profound increase in the expression of reactive astrocyte genes in males than in females (Fig. 12). Furthermore, sex differences were observed in the expression of different subtypes of reactive astrocytes in COX-2$^-$ mice, with the neurotoxic A1 astrocyte marker (*Serping1*) upregulated only between COX-2$^-$ males and 129S6 males, and the neuroprotective A2 astrocyte marker (*S100a10*) upregulated in COX-2$^-$ females and downregulated in COX-2$^-$ males, compared to their same-sex wildtype counterparts (Fig. 12). Therefore, these results suggested that impaired COX-2 activity may impact astrocytes in a manner that exerts neurotoxic effects in males and neuroprotective effects in females.

The goal of Aim 3 was to investigate how the sex-dependent effects of impaired COX-2 activity arise during early postnatal development (PND 8) and identify whether their developmental origins better support the male vulnerability hypothesis or the female protective effect. In this study, the major astrocyte reactivity marker (*Gfap*) was found to be upregulated only in COX-2- males during early postnatal development (Fig. 13a), suggesting that impaired COX-2 activity may initiate astrocyte reactivity earlier in males than females. Additionally, the expression pattern of reactive astrocyte markers between COX-2$^-$ and 129S6 males on PND 8 was noticeably similar to the pattern that was found between COX-2$^-$ and 129S6 females on PND 25 (trends visible in Fig. 15). Moreover, all pan-reactive and non-reactive astrocytes markers were found to be expressed higher in COX-2$^-$ males during adolescence than early postnatal development, when compared to 129S6 males at each stage (see Fig. 15). Together, these trends suggest that impaired COX-2 activity may also lead to astrocyte reactivity occurring at a faster rate in males than females.

Additionally, the male vulnerability hypothesis suggests that genetic or environmental insults would have a more significant impact on genes expressed at higher levels in males, thereby rendering males more vulnerable [194–197]. However, none of the astrocyte markers were differentially expressed in the brain between wildtype 129S6 males and females during early postnatal development (Fig. 13,14). Moreover, while the A2 astrocyte marker (*S100a10*) was found to be upregulated in the brain of COX-2- males and females during early postnatal development, the A1 astrocyte marker (*Serping1*) was not found to be differentially expressed in COX-2$^-$ mice at this stage (Fig. 14). Furthermore, while *S100a10* remained upregulated in COX-2$^-$ females at both developmental stages, *S100a10* was initially found to be upregulated during early postnatal development and then downregulated during adolescence in COX-2$^-$ males

suggesting that the regulation of neuroprotective astrocyte markers may be more stable in COX-2^{-} females than the regulation of both A1 and A2 markers in COX-2^{-} males (Fig. 15). Collectively, these findings indicate that the female protective effect may better account for the observed sex differences in the effect of impaired COX-2 activity on the expression of reactive astrocyte markers in the brain of COX-2^{-} mice.

For Aim 4, the goal was to investigate how the sex-dependent effects of impaired COX-2 activity arise during prenatal development (GD 15 and GD 18) by identifying the biological processes associated with enriched gene sets in the brain of male and female COX-$2^{-/-}$ mice. On GD 15, gene sets downregulated in COX-$2^{-/-}$ males on GD 15 appear to primarily be associated with proteins involved in the generation and organization of glutamatergic neurons and synapses, as well as synaptic signaling mechanisms (Fig. 16). Previous research has indicated that neurotoxic astrocytes may be associated with the abnormal development and signaling of glutamatergic synapses [369–372]. Since genes sets that are associated with proteins involved in the development of glutamatergic neurons and synapses were differentially expressed in COX-$2^{-/-}$ males prior to the onset of astrogenesis [350], it is possible that the impact of impaired COX-2 activity on glutamatergic neurons may serve to trigger the elevated expression of reactive astrocyte genes in the postnatal male brain.

On GD 18, gene sets upregulated in COX-$2^{-/-}$ males were primarily associated with proteins involved in facilitating binding onto DNA regulatory regions, constituents of transcription factors, and proteins involved in the regulation of transcription factor activity (Fig. 17). As discussed previously, steroidogenesis in the male testes has been proposed to act as a trigger that results in certain epigenetic modifications that functions to lower the threshold required to alter the expression of genes in the male brain [244,246]. Since steroidogenesis occurs

around GD 16 in mice [225], these findings suggest that the effects of impaired COX-2 activity may interact with the inherent mechanisms governing brain masculinization, thereby exerting a greater impact on developmental processes that occur after steroidogenesis than before it. On the other hand, gene sets upregulated in COX-$2^{-/-}$ females during prenatal development were primarily associated with ribosomal proteins that are involved in ribosomal RNA binding, translation, and molecular activity (Fig. 18 and 19). This upregulation of ribosomal proteins may reflect an attempt being made by the female brain to compensate for the effects of impaired COX-2 activity by increasing its capacity to synthesize proteins [387].

6.2 Limitations & Directions for Future Research

The studies conducted in this thesis have a few limitations. First, the identification of different astrocyte subtypes in COX-2^{-} males and females was inferred from gene expression experiments conducted on whole brain samples. While the selection of each astrocyte subtype marker was cross-referenced against studies using whole brain RNA-expression profiles in adolescent mice to ensure similar findings between assays on cultured astrocyte ($FC \geq 5$) and whole brain assays ($FC \geq 2$) in previous literature [256–258], our results should be interpreted with this caveat in mind. As such, future research should investigate the expression profile of astrocytes cultured from COX-2^{-} males and females directly by employing techniques such as immunopanning or fluorescence activated cell sorting (FACS).

A second limitation in this study was the reliance on gene expression-based techniques to investigate the sex-dependent and sex-independent effects of impaired COX-2 activity in the brain. While there are many instances where the relative abundance of a protein is well

correlated with the relative abundance of mRNA transcripts, several regulatory processes occur following transcription (i.e., post-transcriptional and translational regulatory mechanisms) that prevent relative gene expression levels alone from accurately predicting the relative level of proteins produced [388,389]. As such, further investigations into the sex-dependent effects of impaired COX-2 activity should use techniques such as immunofluorescence colocalization to accurately identify whether different subtypes of reactive astrocytes are upregulated in COX-2^{-} males and females. Similarly, further investigations into the sex-independent effects of impaired COX-2 activity should use techniques such as enzyme-linked immunosorbent assays (ELISA) to better determine the levels oxidative stress markers in the brain.

6.3 Concluding Remarks

The results of this thesis are noteworthy because they provide insight into how environmental factors that impair COX-2 activity, such as exposure to antipyretic drugs, may exert sex-dependent effects on the brain during prenatal and postnatal development. Previous studies have shown that certain sets of genes are differentially expressed by sex in the developing brain [207,208], suggesting that inherent differences in the activity of these biological pathways between males and females may account for some of the male bias in prevalence and severity of NDDs such as ASD and ADHD. While the exact cause of these NDDs is not fully understood, a considerable body of research supports the notion that certain environmental factors contribute to their etiology [6–9], including prenatal exposure to antipyretics drugs [15–17], and that males are more susceptible to these environmental insults [33,42,55–57]. Several epidemiological studies have also suggested that the most frequent and severe behavioral symptoms observed in

children following prenatal exposure to antipyretic drugs was increased hyperactivity/impulsivity, and that males were more susceptible to these behavioral phenotypes [33,42,55–57]. Previous research in our lab found that mice with impaired COX-2 activity exhibit a considerable overlap in the sex bias and behavioral deficits observed following prenatal exposure to antipyretic drugs, with COX-2$^-$ mice displaying increased repetitive, hyperactive and impulsive behaviors, and COX-2$^-$ males exhibiting more profound deficits than COX-2$^-$ females across all behavioral domains [53,54].

Exposure to antipyretic drugs has been associated with certain pathological mechanisms frequently implicated in both ASD and ADHD, including impaired neuroinflammatory signaling in the brain [47–49]. Thus, the results of the current thesis contributed to our previous research by helping to provide some insight into novel sexually dimorphic biological processes associated with impaired neuroinflammatory signaling, specifically demonstrating that impaired COX-2 activity may lead to increased neurotoxic astrocytes in males and increased neuroprotective astrocytes in females. These findings offer a potential mechanism whereby ERFs such as antipyretic drugs could confer a greater risk of developing neurobehavioral deficits in males that are characteristic of NDDs such as ASD and ADHD. By providing a better understanding of the sex-dependent effects of impaired COX-2 activity in this model, we hope to provide valuable insights into the pathological mechanisms of antipyretic drugs on neurodevelopment, with the ultimate aim of facilitating a better understanding of the male bias in these NDDs.

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APPENDICES

Appendix A: Statistical Results

Table 4. Summary of qRT-PCR Results at PND 25

Gene	RQ (± SEM)		
	129S6 Female	Male COX-2^{-}	Female COX-2^{-}
Grm5	0.975 (± 0.011)	0.798 (± 0.017)	0.546 (± 0.040)
Glo1	1.126 (± 0.020)	0.682 (± 0.008)	0.526 (± 0.044)
IL-1β	1.988 (± 0.216)	2.203 (± 0.234)	2.067 (± 0.106)
IL-6	0.862 (± 0.161)	1.455 (± 0.224)	2.223 (± 0.453)
Itgam	1.032 (± 0.026)	1.721 (± 0.073)	2.085 (± 0.090)
Gfap	5.003 (± 0.409)	26.302 (± 1.444)	18.786 (± 3.172)
Nox2	1.048 (± 0.106)	0.931 (± 0.015)	0.811 (± 0.072)
iNos	0.892 (± 0.097)	1.320 (± 0.205)	1.298 (± 0.191)
Gsk3β	0.838 (± 0.057)	0.794 (± 0.002)	0.732 (± 0.112)
Serpina3n	2.475 (± 0.038)	1.026 (± 0.013)	0.826 (± 0.018)
Aqp4	2.356 (± 0.057)	2.564 (± 0.032)	2.422 (± 0.031)
Serping1	1.419 (± 0.039)	1.788 (± 0.027)	1.415 (± 0.027)
S100a10	0.699 (± 0.026)	0.726 (± 0.019)	1.077 (± 0.033)

Table 5. Statistical Results for Research Aim 1

Gene	Statistical Analysis	Comparison	*F* value	*P* value
Grm5	Two-way ANOVA	**Interaction***	***F* (1, 8) = 25.3***	***p* = .001***
		Sex	*F* (1,8) = 37.8	p < .001
		Genotype	*F* (1,8) = 196	p < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* = .900
		Cox-M vs. Cox-F		*p* < .001
		WT-M vs. Cox-M		*p* < .001
		WT-F vs. Cox-F		*p* < .001
Glo1	Two-way ANOVA	**Interaction***	***F* (1, 8) = 33.6***	***p* < .001***
		Sex	*F* (1,8) = 0.377	*p* = .556
		Genotype	*F* (1,8) = 354	*p* < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* = .013
		Cox-M vs. Cox-F		*p* = .004
		WT-M vs. Cox-M		*p* < .001
		WT-F vs. Cox-F		*p* < .001
IL-1β	Two-way ANOVA	**Interaction***	***F* (1, 8) = 11.2***	***p* = .010***
		Sex	*F* (1,8) = 6.05	p = .035
		Genotype	*F* (1,8) = 14.6	p = .005
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* = .006
		Cox-M vs. Cox-F		*p* > .999
		WT-M vs. Cox-M		*p* = .002
		WT-F vs. Cox-F		*p* > .999
IL-6	Two-way ANOVA	Interaction	*F* (1,8) = 2.92	*p* = .113
		Sex	*F* (1,8) = 1.41	*p* = .258
		Genotype**	***F* (1,8) = 11.7****	***p* = .005****
	Bonferroni *post hoc*	WT-M vs. Cox-M		*p* = .497
		WT-F vs. Cox-F		*p* = .007
Itgam	Two-way ANOVA	**Interaction***	***F* (1, 8) = 7.84***	***p* = .023***
		Sex	*F* (1,8) = 11.2	*p* = .010
		Genotype	*F* (1,8) = 224	*p* < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* > .999
		Cox-M vs. Cox-F		*p* = .005
		WT-M vs. Cox-M		*p* < .001
		WT-F vs. Cox-F		*p* < .001
Gfap	Two-way ANOVA	**Interaction***	***F* (1, 8) = 10.8***	***p* = .011***
		Sex	*F* (1,8) = 1.00	*p* = .346
		Genotype	F (1,8) = 124	*p* < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* = .291
		Cox-M vs. Cox-F		*p* = .033
		WT-M vs. Cox-M		*p* < .001
		WT-F vs. Cox-F		*p* = .001

Note: WT-M = 129S6 males, WT-F = 129S6 females, COX-M = COX-2$^-$ males, Cox-F = COX-2$^-$ females

***** Interaction Significant. Thus, main effects not interpreted, and all multiple comparisons performed

****** Main effect for genotype significant. Thus, multiple comparisons across genotype performed.

Table 6. Statistical Results for Research Aim 2.1

Gene	Statistical Analysis	Comparison	*F* value	*P* value
Nox2	Two-way ANOVA	Interaction Sex **Genotype***	*F* (1, 8) = 1.68 *F* (1,8) = 0.305 ***F* (1,8) = 5.61***	*p* = .231 *p* = .596 ***p* = .045***
	Bonferroni *post hoc*	WT-M vs. Cox-M WT-F vs. Cox-F		*p* = .939 *p* = .064
iNos	Two-way ANOVA	Interaction Sex **Genotype***	*F* (1, 8) = 0.0834 *F* (1,8) = 0.191 ***F* (1,8) = 6.00***	*p* = .780 *p* = .764 ***p* = .040***
	Bonferroni *post hoc*	WT-M vs. Cox-M WT-F vs. Cox-F		*p* = .330 *p* = .178
Gsk3β	Two-way ANOVA	Interaction Sex **Genotype***	*F* (1, 8) = 0.635 *F* (1,8) = 3.17 ***F* (1,8) = 6.09***	*p* = .449 *p* = .113 ***p* = .039***
	Bonferroni *post hoc*	WT-M vs. Cox-M WT-F vs. Cox-F		*p* = .100 *p* = .542

Note: WT-M = 129S6 males, WT-F = 129S6 females, COX-M = COX-2$^-$ males, Cox-F = COX-2$^-$ females
***** Main effect for genotype significant. Thus, multiple comparisons across genotype performed.

Table 7. Statistical Results for Research Aim 2.2

Gene	Statistical Analysis	Comparison	*F* value	*P* value
Serpina3n	Two-way ANOVA	**Interaction***	***F* (1,8) = 1448***	***p* < .001***
		Sex	*F* (1,8) = 838	*p* < .001
		Genotype	*F* (1,8) = 1360	*p* < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* < .001
		Cox-M vs. Cox-F		*p* < .001
		WT-M vs. Cox-M		*p* = .856
		WT-F vs. Cox-F		*p* < .001
Aqp4	Two-way ANOVA	**Interaction***	***F* (1, 8) = 433***	***p* < .001***
		Sex	*F* (1,8) = 285	*p* < .001
		Genotype	*F* (1,8) = 513	*p* < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* < .001
		Cox-M vs. Cox-F		*p* = .048
		WT-M vs. Cox-M		*p* < .001
		WT-F vs. Cox-F		*p* = .461
Serping1	Two-way ANOVA	**Interaction***	***F* (1, 8) = 208***	***p* < .001***
		Sex	*F* (1,8) = 0.713	*p* = .423
		Genotype	*F* (1,8) = 204	*p* < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* < .001
		Cox-M vs. Cox-F		*p* < .001
		WT-M vs. Cox-M		*p* < .001
		WT-F vs. Cox-F		*p* > .999
S100a10	Two-way ANOVA	**Interaction***	***F* (1, 8) = 205***	***p* < .001***
		Sex	*F* (1,8) = 1.19	*p* = .308
		Genotype	*F* (1,8) = 5.23	*p* = .051
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* < .001
		Cox-M vs. Cox-F		*p* < .001
		WT-M vs. Cox-M		*p* < .001
		WT-F vs. Cox-F		*p* < .001

Note: WT-M = 129S6 males, WT-F = 129S6 females, COX-M = COX-2$^-$ males, Cox-F = COX-2$^-$ females
***** Interaction Significant. Thus, main effects not interpreted, and all multiple comparisons performed

Table 8. Summary of qRT-PCR Results at PND 8

Gene	RQ (± SEM)		
	129S6 Female	Male COX-2^{-}	Female COX-2^{-}
Gfap	1.062 (± 0.0.060)	1.717 (± 0.040)	1.133 (± 0.102)
Serpina3n	1.020 (± 0.034)	0.279 (± 0.016)	0.450 (± 0.006)
Aqp4	0.872 (± 0.024)	0.996 (± 0.008)	0.978 (± 0.078)
Serping1	1.095 (± 0.041)	1.012 (± 0.016)	1.172 (± 0.051)
S100a10	0.699 (± 0.026)	0.726 (± 0.019)	1.077 (± 0.033)

Table 9. Statistical Results for Research Aim 3

Gene	Statistical Analysis	Comparison	*F* value	*P* value
Gfap	Two-way ANOVA	**Interaction***	***F* (1, 8) = 26.7***	***p* < .001***
		Sex	*F* (1,8) = 17.4	p = .003
		Genotype	*F* (1,8) = 39.6	p < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* > .999
		Cox-M vs. Cox-F		*p* < .001
		WT-M vs. Cox-M		*p* < .001
		WT-F vs. Cox-F		*p* < .001
Serpina3n	Two-way ANOVA	**Interaction***	***F* (1, 8) = 16.2***	***p* = .004***
		Sex	*F* (1,8) = 25.7	*p* < .001
		Genotype	*F* (1,8) = 1179	*p* < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* = .961
		Cox-M vs. Cox-F		*p* < .001
		WT-M vs. Cox-M		*p* < .001
		WT-F vs. Cox-F		*p* < .001
Aqp4	Two-way ANOVA	Interaction	*F* (1, 8) = 1.80	*p* = .217
		Sex	*F* (1,8) = 3.20	p = .111
		Genotype	*F* (1,8) = 1.54	p = .249
Serping1	Two-way ANOVA	Interaction	*F* (1,8) = 0.943	*p* = .360
		Sex**	***F* (1,8) = 14.3****	***p* = .005****
		Genotype	*F* (1,8) = 1.79	*p* = .218
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* = .164
		Cox-M vs. Cox-F		*p* = .020
S110a10	Two-way ANOVA	**Interaction***	***F* (1, 8) = 30.2***	***p* < .001***
		Sex	*F* (1,8) = 63.0	*p* < .001
		Genotype	*F* (1,8) = 119	*p* < .001
	Bonferroni *post hoc*	WT-M vs. WT-F		*p* = .246
		Cox-M vs. Cox-F		*p* < .001
		WT-M vs. Cox-M		*p* = .010
		WT-F vs. Cox-F		*p* < .001

Note: WT-M = 129S6 males, WT-F = 129S6 females, COX-M = COX-2$^-$ males, Cox-F = COX-2$^-$ females
***** Interaction Significant. Thus, main effects not interpreted, and all multiple comparisons performed
****** Main effect for sex significant. Thus, multiple comparisons across sex performed.

Table 10. List of Top GO Enriched Gene Sets in COX-2$^{-/-}$ Males on GD 15:

Source	Term Name	Term ID	-Log$_{10}$(P$_{adj}$)	Intersection (Entrez IDs)
GO:MF	extracellular matrix structural constituent	GO:0005201	4.024	LUM, COL3A1, DCN, COL6A3, OGN, COL5A2
GO:MF	extracellular matrix structural constituent conferring compression resistance	GO:0030021	3.185	LUM, DCN, OGN
GO:MF	structural molecule activity	GO:0005198	2.728	LUM, COL3A1, DCN, MRPL47, COL6A3, TUBB6, OGN, COL5A2, AHNAK
GO:MF	extracellular matrix structural constituent conferring tensile strength	GO:0030020	1.770	COL3A1, COL6A3, COL5A2, COL4A5
GO:MF	oxygen carrier activity	GO:0005344	1.713	HBB-Y, HBA-X, HBB-BH1
GO:MF	transporter activity	GO:0005215	1.711	HBB-Y, ABCC10, SLC35F1, GJB2, BC002216, HBA-X, SLC13A4
GO:BP	synapse organization	GO:0050808	6.196	SPARC, MEF2C, CNTN2, GRM5, PCDH17, ACTB, NRXN1, MDGA2, SNX27, DAG1, CAMKV, GSK3B, PALM, CDH8, PTPRD, MAPT, SEZ6, MYH10
GO:BP	trans-synaptic signaling	GO:0099537	4.052	TMEM25, MEF2C, CNTN2, GRM5, SV2A, PCDH17, NRXN1, GNAI1, NAPB, DAG1, GSK3B, KIF1B, CDH8, PPP3CA, PTPRD, MAPT, SEZ6, SYT1, SERPINE2
GO:BP	neurogenesis	GO:0022008	3.982	DPYSL3, EMB, MEF2C, CNTN2, GRM5, BCL11B, KLF7, STK25, ACTB, HOOK3, NRXN1, MDGA2, ZEB2, TNIK, SOX5, TRIO, DAG1, GSK3B, NAV1, PPP3CA, PTPRD, MAPT, GRIP1, SEZ6, SYT1, MYH10, SERPINE2, SPAG9, PLXNA2, SOX6, VLDLR, CHD5, MYT1L, NCDN, CSPG5, NGEF
GO:BP	cell projection morphogenesis	GO:0048858	3.898	EMB, CNTN2, BCL11B, KLF7, STK25, ACTB, ZEB2, TNIK, TRIO, DAG1, GSK3B, PPP3CA, PTPRD, MAPT, GRIP1, SYT1, MYH10, PLXNA2, VLDLR, CSPG5, NGEF
GO:BP	synaptic signaling	GO:0099536	3.821	TMEM25, MEF2C, CNTN2, GRM5, SV2A, PCDH17, NRXN1, GNAI1, NAPB, DAG1, GSK3B, KIF1B, CDH8, PPP3CA, PTPRD, MAPT, SEZ6, SYT1, SERPINE2
GO:BP	generation of neurons	GO:0048699	3.740	DPYSL3, EMB, MEF2C, CNTN2, GRM5, BCL11B, KLF7, STK25, ACTB, HOOK3, NRXN1, MDGA2, ZEB2, TNIK, SOX5, TRIO, DAG1, GSK3B, NAV1, PPP3CA, PTPRD, MAPT, GRIP1, SEZ6, SYT1, MYH10, SERPINE2, SPAG9, PLXNA2, VLDLR, CHD5, MYT1L, NCDN, NGEF
GO:BP	neuron projection morphogenesis	GO:0048812	3.492	EMB, CNTN2, BCL11B, KLF7, STK25, ACTB, ZEB2, TNIK, TRIO, DAG1, GSK3B, PPP3CA, PTPRD, MAPT, GRIP1, SYT1, MYH10, PLXNA2, VLDLR
GO:BP	neuron projection development	GO:0031175	3.429	DPYSL3, EMB, MEF2C, CNTN2, BCL11B, KLF7, STK25, ACTB, NRXN1, ZEB2, TNIK, TRIO, DAG1, GSK3B, PPP3CA, PTPRD, MAPT, GRIP1, SEZ6, SYT1, MYH10, SERPINE2, PLXNA2, VLDLR, NCDN, NGEF
GO:BP	anterograde trans-synaptic signaling	GO:0098916	3.410	TMEM25, MEF2C, CNTN2, GRM5, SV2A, PCDH17, NRXN1, GNAI1, NAPB, GSK3B, KIF1B, CDH8, PPP3CA, PTPRD, MAPT, SEZ6, SYT1, SERPINE2
GO:BP	chemical synaptic transmission	GO:0007268	3.410	TMEM25, MEF2C, CNTN2, GRM5, SV2A, PCDH17, NRXN1, GNAI1, NAPB, GSK3B, KIF1B, CDH8, PPP3CA, PTPRD, MAPT, SEZ6, SYT1, SERPINE2
GO:CC	synapse	GO:0045202	13.139	UBE2I, SPARC, RPL29, GRIA2, DPYSL3, EMB, MEF2C, CNTN2, GRM5, SV2A, PCDH17, TSC2, ACTB, ATP8A1, NRXN1, GLG1, MDGA2, NAPB,

				SNX27, TNIK, ADRBK2, DAG1, CAMKV, SCN1A, RPS2, GSK3B, KCND2, PALM, KIF1B, CDH8, DNAJC5, PPP3CA, USP48, PTPRD, MAPT, GRIP1, SEZ6, SYT1, MYH10, SERPINE2
GO:CC	glutamate synapse	GO:0098978	8.145	SPARC, GRM5, SV2A, PCDH17, ACTB, NRXN1, MDGA2, NAPB, TNIK, DAG1, CAMKV, GSK3B, KCND2, CDH8, PPP3CA, PTPRD, GRIP1, SYT1, MYH10, HOMER2, CSPG5, NGEF
GO:CC	postsynapse	GO:0098794	6.821	GRIA2, MEF2C, CNTN2, GRM5, PCDH17, TSC2, ACTB, NAPB, SNX27, TNIK, ADRBK2, DAG1, CAMKV, GSK3B, KCND2, PALM, PPP3CA, USP48, MAPT, GRIP1, SEZ6, MYH10
GO:CC	neuron projection	GO:0043005	3.921	UBE2I, DPYSL3, EMB, CNTN2, GRM5, SV2A, CSNK1E, BCL11B, ACTB, NRXN1, ADRBK2, DAG1, SCN1A, GSK3B, AHCY, KCND2, PALM, KIF1B, CDH8, NAV1, PPP3CA, MAPT, GRIP1, SEZ6, SYT1, MYH10, HOMER2, GABARAPL1, NCDN, SREBF2, NGEF, SPATA7
GO:CC	synaptic membrane	GO:0097060	3.898	GRIA2, CNTN2, GRM5, PCDH17, NRXN1, DAG1, SCN1A, KCND2, PALM, CDH8, USP48, PTPRD, GRIP1, SYT1
GO:CC	axon	GO:0030424	3.106	DPYSL3, EMB, CNTN2, CSNK1E, ACTB, NRXN1, ADRBK2, DAG1, SCN1A, GSK3B, PALM, KIF1B, CDH8, NAV1, MAPT, SYT1, MYH10
GO:CC	GABA-ergic synapse	GO:0098982	2.945	SV2A, PCDH17, NRXN1, MDGA2, DAG1, KCND2
GO:CC	collagen trimer	GO:0005581	3.689	LUM, COL3A1, DCN, COL6A3, COL5A2
GO:CC	collagen-containing extracellular matrix	GO:0062023	3.024	LUM, COL3A1, DCN, SFRP1, COL6A3, OGN, COL5A2
GO:CC	hemoglobin complex	GO:0005833	2.409	HBB-Y, HBA-X, HBB-BH1

Table 11. List of Top GO Enriched Gene Sets in COX-2$^{-/-}$ Males on GD 18:

Source	Term Name	Term ID	-Log$_{10}$(P$_{adj}$)	Intersection (Entrez IDs)
GO:MF	double-stranded DNA binding	GO:0003690	3.079	SOX11,EGR1,FOS,FEZF1,PITX2,KLF4,HIST1H1C, NKX6-2,NKX2-1,ST18,CEBPB
GO:MF	RNA polymerase II cis-regulatory region sequence-specific DNA binding	GO:0000978	3.034	SOX11,FOS,FEZF1,PITX2,KLF4,NKX6-2,NKX2-1,ST18,CEBPB
GO:MF	RNA polymerase II regulatory region sequence-specific DNA binding	GO:0000977	2.945	SOX11,EGR1,FOS,FEZF1,PITX2,KLF4,NKX6-2,NKX2-1,ST18,CEBPB
GO:MF	cis-regulatory region sequence-specific DNA binding	GO:0000987	2.898	SOX11,FOS,FEZF1,PITX2,KLF4,NKX6-2,NKX2-1,ST18,CEBPB
GO:MF	transcription regulatory region sequence-specific DNA binding	GO:0000976	2.707	SOX11,EGR1,FOS,FEZF1,PITX2,KLF4,NKX6-2,NKX2-1,ST18,CEBPB
GO:MF	core promoter binding	GO:0001047	2.630	EGR1,FOS,NKX2-1,CEBPB
GO:MF	DNA-binding transcription factor activity, RNA polymerase II-specific	GO:0000981	2.312	SOX11,EGR1,FOS,FEZF1,PITX2,KLF4,NKX6-2,NKX2-1,CEBPB
GO:MF	transcription regulatory region DNA binding	GO:0044212	2.271	SOX11,EGR1,FOS,FEZF1,PITX2,KLF4,NKX6-2,NKX2-1,ST18,CEBPB
GO:MF	DNA-binding transcription factor activity	GO:0003700	1.890	SOX11,EGR1,FOS,FEZF1,PITX2,KLF4,NKX6-2,NKX2-1,ST18,CEBPB
GO:MF	RNA polymerase II core promoter sequence-specific DNA binding	GO:0000979	1.669	EGR1,FOS,CEBPB
GO:BP	positive regulation of biosynthetic process	GO:0009891	4.347	SOX11,PAIP1,EGR1,FOS,FEZF1,SFRP1,PITX2, FANK1,KLF4,SCP2,HEXB,SSBP4,HIST1H1C, NKX6-2,NKX2-1,ST18
GO:BP	positive regulation of transcription, DNA-templated	GO:0045893	4.094	SOX11,EGR1,FOS,FEZF1,SFRP1,PITX2,FANK1, KLF4,HEXB,SSBP4,HIST1H1C,NKX6-2,NKX2-1,ST18
GO:BP	positive regulation of macromolecule biosynthetic process	GO:0010557	3.880	SOX11,PAIP1,EGR1,FOS,FEZF1,SFRP1,PITX2, FANK1,KLF4,HEXB,SSBP4,HIST1H1C,NKX6-2,NKX2-1,ST18
GO:BP	positive regulation of nucleic acid-templated transcription	GO:1903508	3.851	SOX11,EGR1,FOS,FEZF1,SFRP1,PITX2,FANK1, KLF4,HEXB,SSBP4,HIST1H1C,NKX6-2,NKX2-1,ST18
GO:BP	positive regulation of RNA biosynthetic process	GO:1902680	3.848	SOX11,EGR1,FOS,FEZF1,SFRP1,PITX2,FANK1, KLF4,HEXB,SSBP4,HIST1H1C,NKX6-2,NKX2-1,ST18
GO:BP	positive regulation of cellular biosynthetic process	GO:0031328	3.575	SOX11,PAIP1,EGR1,FOS,FEZF1,SFRP1,PITX2, FANK1,KLF4,HEXB,SSBP4,HIST1H1C,NKX6-2,NKX2-1,ST18
GO:BP	positive regulation of RNA metabolic process	GO:0051254	3.520	SOX11,EGR1,FOS,FEZF1,SFRP1,PITX2,FANK1, KLF4,HEXB,SSBP4,HIST1H1C,NKX6-2,NKX2-1,ST18
GO:BP	positive regulation of gene expression	GO:0010628	3.456	SOX11,PAIP1,EGR1,FOS,FEZF1,SFRP1,PITX2, FANK1,KLF4,HEXB,SSBP4,HIST1H1C,NKX6-2,NKX2-1,ST18
GO:BP	positive regulation of nucleobase-containing compound metabolic process	GO:0045935	3.023	SOX11,EGR1,FOS,FEZF1,SFRP1,PITX2,FANK1, KLF4,HEXB,SSBP4,HIST1H1C,NKX6-2,NKX2-1,ST18,CEBPB,HNRNPA2B1,BLM
GO:BP	regulation of transcription by RNA polymerase II	GO:0006357	2.642	SOX11,EGR1,FOS,FEZF1,SFRP1,TXNIP,PITX2, KLF4,HEXB,SSBP4,HIST1H1C,NKX6-2,NKX2-1,ST18
GO:CC	chromosomal region	GO:0098687	1.686	ITGB3BP,ZWINT,CEBPB,PPP1CA,HNRNPA2B1, BLM
GO:CC	transcription factor complex	GO:0005667	1.662	SOX11,FOS,PITX2,KLF4,NKX2-1,CEBPB
GO:CC	NuA4 histone acetyltransferase complex	GO:0035267	2.046	ACTL6B,ACTB
GO:CC	H4/H2A histone acetyltransferase complex	GO:0043189	2.046	ACTL6B,ACTB
GO:CC	histone acetyltransferase complex	GO:0000123	1.460	ACTL6B,ACTB,TAF6

GO:CC	H4 histone acetyltransferase complex	GO:1902562	1.332	ACTL6B,ACTB
GO:CC	protein acetyltransferase complex	GO:0031248	1.302	ACTL6B,ACTB,TAF6
GO:CC	acetyltransferase complex	GO:1902493	1.302	ACTL6B,ACTB,TAF6

Table 12. List of Top GO Enriched Gene Sets in COX-2$^{-/-}$ Females on GD 15:

Source	Term Name	Term ID	-Log$_{10}$(P$_{adj}$)	Intersection (Entrez IDs)
GO:MF	snoRNA binding	GO:0030515	1.723	IMP4,NOP58
GO:MF	endoplasmic reticulum signal peptide binding	GO:0030942	1.353	SRP68,SRP54A
GO:MF	oligopeptidase activity	GO:0070012	1.302	PREP,NDEL1
GO:MF	ligase activity	GO:0016874	1.995	ACSL3,ASNSD1,FARSB,DARS,ADSS
GO:MF	NEDD8-specific protease activity	GO:0019784	1.479	COPS5,USP21
GO:BP	oxoacid metabolic process	GO:0043436	1.913	ACSL3,ASNSD1,IDH1,ACADL,CHPF,FARSB, DSEL,DBI,DARS
GO:BP	organic acid metabolic process	GO:0006082	1.807	ACSL3,ASNSD1,IDH1,ACADL,CHPF,FARSB, DSEL,DBI,DARS
GO:CC	ciliary transition zone	GO:0035869	1.817	KIFAP3,B9D1,TMEM107,CFAP36
GO:CC	small-subunit processome	GO:0032040	1.798	IMP4,NOP58
GO:CC	central region of growth cone	GO:0090724	1.337	YWHAE,NDEL1
GO:CC	GABA-ergic synapse	GO:0098982	2.302	GIT1,GABRA1,C1QBP
GO:CC	distal dendrite	GO:0150002	1.576	MAP2
GO:CC	apical distal dendrite	GO:0150014	1.576	MAP2

Table 13. List of Top GO Enriched Gene Sets in COX-2$^{-/-}$ Females on GD 18:

Source	Term Name	Term ID	-Log$_{10}$(P$_{adj}$)	Intersection (Entrez IDs)
GO:MF	structural constituent of ribosome	GO:0003735	12.868	RPL27A,RPL5,RPSA,RPL39,RPL4,RPL37,RPS8, RPS3,RPL21,RPL30,RPL23A,RPS2,RPL18A, RPL32,RPLP2,RPS13,RPS12
GO:MF	rRNA binding	GO:0019843	5.204	RPL5,PTCD3,RPL4,RPL37,RPS3,RPL23A
GO:MF	structural molecule activity	GO:0005198	4.504	RPL27A,RPL5,RPSA,RPL39,RPL4,RPL37,RPS8, RPS3,RPL21,RPL30,RPL23A,RPS2,RPL18A, RPL32,RPLP2,RPS13,RPS12
GO:MF	RNA binding	GO:0003723	2.640	RPL5,PTCD3,HSPA8,EIF5,RPL4,RPL37,EIF4A1, NOP58,GNL3,RPS3,RPL30,RPL23A,EIF4A2, RPS2
GO:MF	5S rRNA binding	GO:0008097	1.449	RPL5,RPL4
GO:BP	translation	GO:0006412	8.463	RPL27A,RPL5,PTCD3,RPSA,RPL39,EIF5,RPL4, RPL37,EIF4A1,RPS8,RPS3,RPL21,RPL30, RPL23A,EIF4A2,RPS2,CNOT1,RPL18A,RPL32, RACK1,RPLP2,RPS13,RPS12
GO:BP	peptide biosynthetic process	GO:0043043	8.190	RPL27A,RPL5,PTCD3,RPSA,RPL39,EIF5,RPL4, RPL37,EIF4A1,RPS8,RPS3,RPL21,RPL30, RPL23A,EIF4A2,RPS2,CNOT1,RPL18A,RPL32, RACK1,RPLP2,RPS13,RPS12
GO:BP	peptide metabolic process	GO:0006518	7.328	RPL27A,RPL5,PTCD3,RPSA,RPL39,EIF5,RPL4, RPL37,EIF4A1,RPS8,RPS3,RPL21,RPL30, RPL23A,EIF4A2,RPS2,CNOT1,IDE,RPL18A, RPL32,RACK1,RPLP2,RPS13,RPS12
GO:BP	amide biosynthetic process	GO:0043604	6.804	RPL27A,RPL5,PTCD3,RPSA,RPL39,EIF5,RPL4, RPL37,EIF4A1,RPS8,RPS3,RPL21,RPL30, RPL23A,EIF4A2,RPS2,CNOT1,RPL18A,RPL32, RACK1,RPLP2,RPS13,RPS12
GO:BP	cytoplasmic translation	GO:0002181	6.107	RPSA,RPL39,EIF5,EIF4A1,RPL30,EIF4A2,RPS2, RPL18A,RPL32
GO:BP	cellular amide metabolic process	GO:0043603	5.150	RPL27A,RPL5,PTCD3,RPSA,RPL39,EIF5,RPL4, RPL37,EIF4A1,RPS8,RPS3,RPL21,RPL30, RPL23A,EIF4A2,RPS2,CNOT1,IDE,RPL18A, RPL32,RACK1,RPLP2
GO:BP	cytoplasmic translational initiation	GO:0002183	2.462	EIF5,EIF4A1,EIF4A2,RPS2
GO:BP	organonitrogen compound biosynthetic process	GO:1901566	2.264	RPL27A,RPL5,PTCD3,RPSA,RPL39,EIF5,RPL4, RPL37,EIF4A1,RPS8,RPS3,RPL21,RPL30, RPL23A,EIF4A2,RPS2,CNOT1,ATP5E,RPL18A, RPL32,RACK1,RPLP2,RPS13,RPS12,NDUFC2
GO:BP	ribosome assembly	GO:0042255	1.318	RPL5,RPSA,RPL23A,RPS2
GO:CC	cytosolic ribosome	GO:0022626	16.465	RPL27A,RPL5,RPSA,RPL39,RPL4,RPL37,RPS8, RPS3,RPL30,RPL23A,RPS2,RPL18A,RPL32, RACK1,RPLP2,RPS13,RPS12
GO:CC	ribosome	GO:0005840	13.210	RPL27A,RPL5,PTCD3,RPSA,RPL39,RPL4,RPL37, RPS8,RPS3,RPL21,RPL30,RPL23A,RPS2, RPL18A,RPL32,RACK1,RPLP2,RPS13,RPS12
GO:CC	ribosomal subunit	GO:0044391	12.070	RPL27A,RPL5,RPSA,RPL39,RPL4,RPL37,RPS8, RPS3,RPL30,RPL23A,RPS2,RPL18A,RPL32, RACK1,RPLP2,RPS13,RPS12
GO:CC	cytosolic large ribosomal subunit	GO:0022625	9.729	RPL27A,RPL5,RPL39,RPL4,RPL37,RPL30, RPL23A,RPL18A,RPL32,RPLP2
GO:CC	large ribosomal subunit	GO:0015934	6.702	RPL27A,RPL5,RPL39,RPL4,RPL37,RPL30, RPL23A,RPL18A,RPL32,RPLP2
GO:CC	ribonucleoprotein complex	GO:1990904	6.606	RPL27A,RPL5,RPSA,RPL39,HSPA8,RPL4,RPL37, NOP58,RPS8,RPS3,TOP2A,RPL30,RPL23A, RPS2,NOP56,RPL18A,RPL32,RACK1,RPLP2, RPS13,RPS12

GO:CC	cytosolic small ribosomal subunit	GO:0022627	5.664	RPSA,RPS8,RPS3,RPS2,RACK1,RPS13,RPS12
GO:CC	small ribosomal subunit	GO:0015935	3.924	RPSA,RPS8,RPS3,RPS2,RACK1,RPS13,RPS12
GO:CC	polysomal ribosome	GO:0042788	2.540	RPL39,RPL30,RPL18A,RPL32
GO:CC	polysome	GO:0005844	2.425	RPL39,RPS3,RPL30,RPL18A,RPL32

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Figure 03 Citation:

Wong, C.T., Wais, J. and Crawford, D.A. (2015), Prenatal exposure to common environmental factors affects brain lipids and increases risk of developing autism spectrum disorders. Eur J Neurosci, 42: 2742-2760. doi:10.1111/ejn.13028

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Licensed Content Author	Christine T. Wong, Joshua Wais, Dorota A. Crawford
Licensed Content Date	Sep 19, 2015
Licensed Content Volume	42
Licensed Content Issue	10
Licensed Content Pages	19
Type of Use	Dissertation/Thesis
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Portion	Figure/table
Number of figures/tables	1
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Figure 05 Citation:

McCarthy, M., Nugent, B. & Lenz, K. Neuroimmunology and neuroepigenetics in the establishment of sex differences in the brain. *Nat Rev Neurosci* **18,** 471–484 (2017). https://doi.org/10.1038/nrn.2017.61

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