THE ROLE OF PROSTAGLANDIN E2 IN THE EXPRESSION OF HEMOGLOBIN DURING PRENATAL AND POSTNATAL BRAIN DEVELOPMENT - POTENTIAL CONNECTION TO AUTISM SPECTRUM DISORDERS

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Abstract

Abnormal levels of the fetal hemoglobin isoform Hbb-γ and the adult isoforms Hbb-β and Hba-α have been found in the prenatal male brain of our mouse model lacking prostaglandin E2 (PGE2) producing enzyme cyclooxygenase-2 (COX2-knockout). Dysregulations of the COX2/PGE2 pathway has also been linked to autism. In this study, we use quantitative Real-Time PCR, western blots, and immunohistochemistry to investigate the expression of Hba-α, Hbb-γ, and Hbb-β in the brain of COX2-knockin (COX2-KI) mice. Overall, gene expression was affected in a sex-dependent manner and by deficits of the COX2/PGE2 pathway. Protein expression analysis also revealed sex-dependent changes and differences for the COX2-KI animals. Interestingly, regional expression analysis showed a distinct pattern of Hbb-β in the white matter, whereas Hba-α and Hbb-γ were expressed in the grey matter. These results add new knowledge about the involvement of COX2/PGE2 pathway in the regulation of hemoglobin in the brain with implications for autism.

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List of Abbreviations

AA: Arachidonic acid

ACC: Animal Care Committee

ADHD: Attention deficit-hyperactivity disorder

ANOVA: Analysis of variance

ASDs: Autism spectrum disorders

BCL11A: BAF Chromatin Remodeling Complex Subunit 11A

CDC: Centers for Disease Control and Prevention

cDNA: Complementary deoxyribonucleic acid

COX1: Cyclooxygenase-1 enzyme

COX2: Cyclooxygenase-2 enzyme

COX2-KI: Cyclooxygenase-2 knockin

DNA: Deoxyribonucleic Acid

dNTP: Deoxyribonucleotide triphosphate

E1, E11, E16, E19: Embryonic day 1, 11, 16 respectively

EP receptor: E-prostanoid receptor

GABA: Gamma-aminobutyric acid

Gapdh: Glyceraldehyde 3-phosphate dehydrogenase

HSC: hematopoietic stem cells

Hba-α: hemoglobin alpha globin type alpha chain

Hbb-β: hemoglobin beta globin type beta chain

Hbb-γ: hemoglobin beta globin type gamma chain

HbH: homotetramer formed by beta globin

Hprt: Hypoxanthine Phosphoribosyltransferase gene

LA: Omega-6 linoleic acid

NE-4C: Neuroectodermal

NO: nitric oxide

NOS: nitric oxide synthetase

NSAIDs: Nonsteroidal anti-inflammatory drugs

P0, P8, P25: Postnatal day 0, 8, 25 respectively

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PGE2: Prostaglandin E2

PGG2: Prostaglandin G2

PGH2: Prostaglandin H2

Pgk1: Phosphoglycerate kinase 1 gene

PLA2: Phospholipase A2

Ptgs2: Prostaglandin-Endoperoxide Synthase 2 gene

qRT-PCR: Quantitative real-time polymerase chain reaction

RNA: Ribonucleic acid

RQ: Relative Quantification

RT: Reverse Transcriptase

SOX-6: Sex Determining Region Y-Box 6

SCD: Sickle cell disease

SEM: Standard error of the mean

Tris-HCL: Trisaminomethane Hydrochloride

Wnt: Wingless-related integration site

WT: Wild-type

1. Introduction

1.1. Prostaglandin E2 in the heathy nervous system

Lipids play a critical role in the healthy development of the human brain (C. Wong & Crawford, 2014; C. T. Wong, Wais, & Crawford, 2015). These macromolecules account for 50-60% of the total brain's dry weight (Bascoul-Colombo et al., 2016; Lauritzen, Hansen, Jørgensen, & Michaelsen, 2001), and play a critical role in a number of functions crucial for healthy neurodevelopment (Calderon & Kim, 2004; Hoffman, Boettcher, & Diersen-Schade, 2009). One of the major mediators of lipid signaling in the brain is prostaglandin E2 (PGE2). PGE2 is a molecule derived from phospholipids which are found in the cell membrane (Park, Pillinger, & Abramson, 2006). This lipid signaling pathway regulates expression of genes crucial in brain development (Rai-Bhogal, Wong, et al., 2018; C. T. Wong, Ahmad, Li, & Crawford, 2014; C. T. Wong et al., 2016) and it affects essential functions such as synaptic activity, plasticity regulation, formation of dendritic spines and brain maturation (Burks, Wright, & McCarthy, 2007; Chen & Bazan, 2005).

In the brain, PGE2 synthesis occurs following the liberation of arachidonic acid (AA) from phospholipid membranes in response to various stimuli such as inflammatory cytokines (Farooqui, Horrocks, & Farooqui, 2006). The primary enzymes responsible for PGE2 production are cyclooxygenases-1 and -2 (COX1 and COX2). These enzymes synthesize PGE2 in a stepwise fashion. First AA is converted to the intermediate molecule, PGG2, which is subsequently reduced to PGH2 (Wong et al., 2014). Once synthesized, PGH2 is then further metabolized by the prostaglandin synthases into lipid signaling messengers called eicosanoids such as PGE2 (Boie et al., 1997; Breyer, Bagdassarian, Myers, & Breyer, 2001) (Figure 1). COX1 is expressed constitutively in the majority of tissues, whereas COX2 is typically considered an inducible

enzyme and is thought to be increased during inflammatory responses (O'Neill & Ford-Hutchinson, 1993). Interestingly, COX enzymes exhibit cell type-specific expression patterns in the brain. COX1 is expressed in microglial cells, and COX2 is expressed mainly in neuronal cells (Hoozemans et al., 2001). Furthermore, in the brain, COX2 is constitutively expressed in neurons (Kirkby et al., 2016), but can also be induced by cytokines (Herschman, 1996), and released by microglia during inflammatory events.

PGE2 has been shown to mediate several physiological responses in the brain such as pro-inflammatory responses, pain and microglia activation (Wong et al., 2014; Cantaut-Belarif et al., 2017; Cruz Duarte, St-Jacques, & Ma, 2012). Additionally, PGE2 can bind to four specific membrane-bound G-coupled prostanoid EP-receptors (Kitaoka et al., 2007; Chen & Bazan, 2005). Activation of a particular EP-receptor can result in either activation or inhibition of a wide variety of signaling cascades, ultimately leading to alterations in both kinase activity and regulation of gene transcription (Carlson, 2003). Furthermore, research has shown that during prenatal stages, there are increased levels of COX2 and PG-synthases (Calderon & Kim, 2004) and that these increases are accompanied by elevated levels of EP receptor transcripts (Tamiji & Crawford, 2010), indicating that PGE2 is elevated in early development. Thus, PGE2 plays a critical role in a number of functions critical to healthy brain development (Wong et al., 2014), has highly specific responses depending on the signaling pathway it activates (Carlson, 2003), and is prenatally upregulated compared to later stages of development (Calderon & Kim, 2004). Collectively, these facts stress the importance of PGE2 signaling during development and suggest that factors that alter normal PGE2 concentrations may adversely impact a wide variety of functions critical to healthy development of the prenatal brain (Wong et al., 2014).

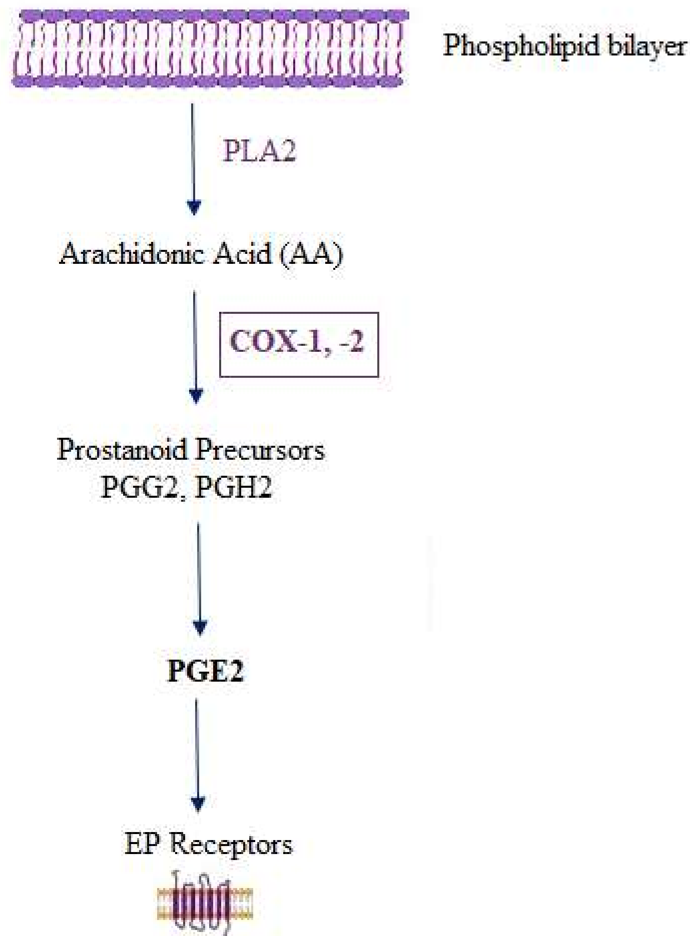


Figure 1. Simplified Prostaglandin E2 (PGE2) synthesis pathway. Phospholipase A2 (PLA2) can release arachidonic acid (AA) from phospholipids found in the cell membrane. Cyclooxygenase-1 (COX1) and cyclooxygenase-2 (COX2) are the PGE2 producing enzymes since they convert AA to PGG2 and then to the stable prostanoid precursor PGH2. Prostaglandin (PG) synthase can metabolize PGH2 into PGE2.

1.2. Abnormal signaling of the Prostaglandin E2 pathway during neurodevelopment

Epidemiological and clinical studies have found a positive correlation between dysregulation of the PGE2 signaling pathway and certain neurodevelopmental disorders. For example, prenatal exposure to a synthetic PGE2 analogue (misoprostol) has been associated with

an increased risk of developing autism and Möbius syndrome (Tamiji and Crawford, 2010; Dufour-Rainfray et al., 2011; Wong & Crawford, 2014). Misoprostol is commonly used for stomach ulcers treatment (Graham et al., 2002), as well as to induce labor or to terminate pregnancies under medically controlled conditions (Faúndes, 2011). For the purpose of pregnancy termination, proper use of misoprostol requires it to be taken in conjunction with a synthetic steroidal drug known as mifepristone (Faúndes, 2011). Previous clinical studies indicate that misuse of misoprostol to terminate pregnancies without the use of mifepristone lead to a higher incidence of autism-related disorders and Möbius syndrome (Marques-Dias, Gonzalez, & Rosemberg, 2003; Pastuszak et al., 1998; Schüler et al., 1999). Notably, exposure to misoprostol affects the embryo more highly in early stages of pregnancy than later in development, suggesting that this drug can be toxic to early neurodevelopment of the fetus (Genest, Di Salvo, Rosenblatt, & Holmes, 1999).

Recently, our lab has shown that abnormal levels of PGE2 in mice can lead to behaviours analogous to those characteristics of autism spectrum disorder (ASD) cases. For example, mice with deficient COX2 activity (COX2-knockin or COX2-KI mice) showed altered social interactions, increased anxiety levels and deficits in motor function (C. T. Wong, Bestard-lorigados, & Crawford, 2018). Interestingly, one single injection of 16,16-dimethyl-PGE2 (a stable derivative of PGE2) during the start of neurogenesis at embryonic day 11 can also lead to similar behavioural deficits (Wong et al., unpublished). Molecular work conducted in our lab in protein and gene expression for these two models have found abnormal expression of specific genes and proteins also found to be dysregulated among some individuals with ASD (Wong et al., 2018; Wong et al., unpublished). More specifically, deficits of the COX2 enzyme in mice models lead to abnormal gene expression of ASD-risk genes (Rai-Bhogal, Ahmad, Li, & Crawford, 2018), as

well as dysregulation of genes associated with pathways and functions impaired in some individuals with ASD, such as cell proliferation, migration and Wnt regulation (Rai-Bohgal et al., 2018; Wong et al., 2018; unpublished). Notably, Wnt signaling cascades are a major pathway found to play an essential role during neurodevelopment. This pathway has also been previously associated with ASD (Lin et al., 2012; Marui et al., 2010). Studies conducted in our lab have shown for the first time crosstalk between PGE2 and Wnt pathways in neuronal cell types (neuroectodermal NE-4C stem cells) (Wong et al., 2014). Additionally, the expression of Wnt-target genes that have been previously associated with ASDs was significantly upregulated in Wnt-induced cells in response to PGE2 treatment (Wong et al., 2014). Recent *in vivo* studies in our lab also showed abnormal expression of Wnt-related genes in mice with deficient COX2 enzymatic activity at embryonic day 16 and 19 (E16 and E19) (Rai-Bhogal et al., 2018) as well as at postnatal day 8 (P8) (Wong et al., unpublished). These results indicate that these two major pathways are interconnected and normal crosstalk between these pathways may play an important role in facilitating an environment which supports healthy neurodevelopment.

Furthermore, *in vitro* studies in our lab using misoprostol or PGE2 have also shown that PGE2 can increase levels of calcium in neuronal cells (Tamiji and Crawford, 2010; Davidson, Wong, Rai-Bhogal, Li, & Crawford, 2016). These results are relevant because dysfunctional calcium regulation has been identified in autism cases, and it is thought to be involved in the etiology of ASD (Krey & Dolmetsch, 2007). These chemicals can also decrease the number and length of neurite extension in a dosage-dependent manner (Tamiji and Crawford, 2010). Overall, these studies suggest that alterations in the PGE2 signaling pathway, due to its abnormal level, might result in profound changes in neurodevelopment that could contribute to the onset of disorders such as autism.

1.2.1. Etiology of Autism Spectrum Disorders and link between environmental risk factors and PGE2 levels

Autism Spectrum Disorders (ASDs) are neurodevelopmental conditions characterized by repetitive behaviours, deficits in social interactions, and impaired communication (Frith and Happé, 2005). ASD, since it was first discovered, is diagnosed through these behavioural problems (Folstein and Rosen-Sheidley, 2001). This disorder affected 1 in 68 individuals by 2014 (CDC, 2012) with a predicted increase to 1 in 59 by 2018 (CDC, 2014). ASD is also four times more common in males than females (Fombonne, 2003; Rivet & Matson, 2011) hence, the importance of studying sex differences when researching ASD. The symptoms and severity of patients with ASD are very diverse and varies from individual to individual. There is substantial evidence that indicates ASDs are caused by a combination of genetic and environmental factors (Schaefer et al., 2013; Karimi, Kamali, Mousavi, & Karahmadi, 2017; Modabbernia, Velthorst, & Reichenberg, 2017). For instance, there is a higher concordance rate for ASD in monozygotic twins when compared to dizygotic twins, indicating that genetics are an important component in the prevalence of autism (Hu, Frank, Heine, Lee, & Quackenbush, 2006). Furthermore, studies have also shown there is a higher risk of displaying autistic behaviours or having ASD for siblings and relatives of individuals with autism (Bailey, Palferman, Heavey, & Le Couteur, 1998; Constantino, Zhang, Frazier, Abbacchi, & Law, 2010). However, there is no single gene that characterizes the etiology of autism, but rather several genes are thought to be involved in ASD (Guerra, 2011; Muhle, Trentacoste, & Rapin, 2004).

As mentioned previously, twin studies have helped to understand the genetic component behind ASD better; yet, they have also reported that for monozygotic twins who have the same genetic material, the concordance rate for ASD varies from 43-88% (Hallmayer et al., 2011;

Ronald & Hoekstra, 2014; Rosenberg et al., 2009; Tick, Bolton, Happé, Rutter, & Rijsdijk, 2016). This research shows that there is more to the etiology of ASD than just genetic dysregulations. Interestingly, dizygotic twins are also twice as likely to both develop ASD compared to non-twin siblings (Hallmayer et al., 2011), indicating that shared prenatal environment might also be important to consider when researching ASD. On top of twin and familial studies, a large body of evidence supports the claim that the environment plays a very significant role in the etiology of autism (Landrigan, Lambertini, & Birnbaum, 2012; Raz et al., 2015; Rossignol, Genuis, & Frye, 2014; Woodruff, Zota, & Schwartz, 2011). Some of the notable environmental risk factors include maternal infection during pregnancy (Goines et al., 2011; Brucato et al., 2017), and various hazardous airborne pollutants (Roberts et al., 2013).

Several environmental risk factors linked to ASD have been found to affect PGE2 levels in the brain (Figure 2) (Tamiji & Crawford, 2010; Wong & Crawford, 2014; Wong, Wais, & Crawford, 2015). For instance, exposure to air pollution has been shown to induce high levels of inflammation and oxidative stress across different ages in the human brain (Calderon-Garciduenas et al., 2007, 2014) and this increases the levels of PGE2 in neurons (Sang et al., 2011). In humans, the level of PGE2 could also be influenced by a wide variety of other environmental risk factors such as dietary imbalances of omega-3 and -6, or exposure to different drugs such as misoprostol and nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin and acetaminophen (Wong & Crawford, 2014; Wong et al., 2015). Pesticides are another environmental risk factor with links to both PGE2 signaling and the pathology of autism. Many pesticides have been found to induce oxidative stress (Abdollahi et al., 2004), which can then elevate levels of PGE2 during critical periods of neurodevelopment. The three major groups of pesticides (Organochlorine Pesticides, Organophosphate pesticides, and Pyrethrins and Pyrethroids) can lead to abnormal

neurodevelopment and a higher chance of developing ASD (Roberts et al., 2007; Rauh et al., 2012). These chemicals can pass the placental barrier and the blood-brain barrier, which can result in abnormal prenatal development (Kojima et al., 2004). Additionally, many common consumer products have been shown to exhibit endocrine-disrupting properties that could affect the healthy development of a fetus. These include plastic containers, lubricants, lotions, cosmetics, and fragrances that are used daily by most of the population. They have been found to induce abnormal PGE2 signaling, hormone activity, and calcium function, which can lead to developmental issues (Wang et al., 2008). Furthermore, chemicals found in these products can disrupt healthy neuronal development (Kimura-Kuroda et al., 2007) and result in behavioural deficits observed in ASD patients (Eskenazi et al., 2013).

In summary, a growing body of evidence seems to implicate that the etiology of autism can be tied to both genetic and environmental factors (Rossignol et al., 2014). Furthermore, many of these risk factors have been found to be capable of adversely impacting PGE2 levels in the developing brain (Wong, Wais, & Crawford, 2015). Collectively, these studies suggest that several environmental risk factors linked to ASD can lead to abnormal PGE2 signaling and can affect the lipid metabolism balance (Chauhan et al., 2004; Liew et al., 2015). Furthermore, the interaction between genetic and environmental risk factors suggests that certain genetic mutations may lower an individual's ability to tolerate prenatal exposure to certain environmental toxicants, making them more susceptible to developing ASD-related symptoms during development.

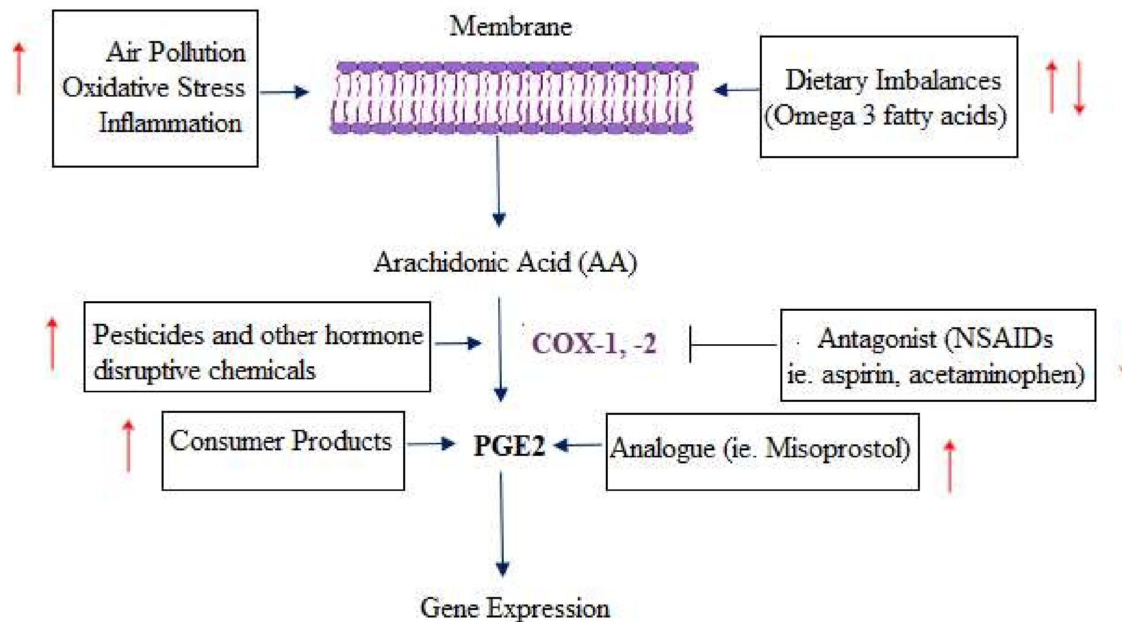


Figure 2. Environmental factors linked to ASD that can affect levels of Prostaglandin E2 in the brain. These compounds can reduce or increase the levels of PGE2, causing problems with lipid signaling in the brain via changes in gene and protein expression. Boxes represent the environmental factors, and red arrows indicate an increase or decrease of PGE2 levels due to that specific environmental factor. *Adapted from Wong et al., 2015*.

1.3. PGE2 and Wnt in the hematopoietic system

Previous research has linked PGE2 with the vertebrate hematopoietic stem cell system (HSC). More specifically, previous research suggests PGE2 may play a regulatory role during the initiation of vertebrate HSC development (North et al., 2007). For instance, the stable derivative of PGE2, 16,16-dimethyl-PGE2, was found to increase the number of embryonic stem cell hematopoietic colonies formed (North et al., 2007). Moreover, PGE2 has been known to play a regulatory role during erythropoiesis in murine bone marrow (Fisher and Hagiwara, 1984). Interestingly, mice with deficiencies of COX2 have been found to exhibit diminished regeneration of hematopoietic lineage (Lorenz et al., 1999). These results indicate that PGE2 is crucial in HCS

induction, maintenance, and function in vertebrates. Moreover, whole genome microarray analysis conducted in our lab using COX2-knockout mice model showed upregulation in gene expression for two hemoglobin isoforms (hemoglobin gamma-*Hbb-γ,* and hemoglobin beta-*Hbb-β*) at prenatal stages E16 and 19 in males but not in females (Rai-Bohgal et al., 2018; unpublished).

An association between Wnt signaling and HSC regulation has also been found in research investigating HSC regulation in adult bone marrow (Reya et al., 2003). Wnt activation is required for the maintenance of HSC (Congdon et al., 2008) and previous research has shown that Wnt signaling can regulate erythroid cell specificity in mouse models (Baron, Isern, & Fraser, 2012). Studies conducted in developing mouse erythroid cells found expression of genes associated with the Wnt pathway (Isern et al., 2011). As mentioned earlier, associations between Wnt and PGE2 pathways during development has been well established (Wong et al., 2014). Collectively, these findings could suggest that Wnt and PGE2 pathways might also be working together in HSC formation and hematopoietic regeneration.

1.4. Hemoglobin structure, expression through development and main functions

Structurally, the most common form of hemoglobin found in erythrocytes is a tetramer (Schechter, 2008) which typically consists of two alpha chains and two beta chains ($\alpha 2\beta 2$). In humans, expression of the hemoglobin protein is regulated in part by the β-globin locus, which is found on chromosome 11 (Sankaran et al., 2010; Mcgann et al., 2013). This locus is regulated through development in the erythrocyte lineage (Cantú & Philipsen, 2014; Mcgann et al., 2013) (Table 1). The embryonic form of β-globin molecule (ε-globin) is expressed only during the first trimester for humans (Sankaran et al., 2010). This form is derived from the yolk sac and corresponds to the primitive erythropoiesis stage (Cantú & Philipsen, 2014; Mcgann et al., 2013). After this developmental stage, the main β-globin molecule produced is now called the fetal γ-

globin, which is derived from fetal liver (Cantú & Philipsen, 2014). Two γ-globin chains and two adult α-globin chains (2α2γ) form the stable fetal hemoglobin, which is the main hemoglobin during gestation (Manning et al., 2007). Around birth, there is the fetal to adult genetic switch between the fetal and adult hemoglobin, where the γ-globin gets silenced and the adult β-globin expression increases (Manning et al., 2007) (Figure 3). The α-globin locus is found on chromosome 16, and it has one genetic switch between the embryonic alpha (denoted as ζ) and the adult alpha which happens at fetal stages (Cantú & Philipsen, 2014) (Table 1). Interestingly, fetal hemoglobin has a higher affinity for oxygen than the adult form (Walker & Turnbull, 1954), which is crucial during development. It should be noted that in mice the genetic switch between hemoglobin isoforms is slightly different than the one described previously for humans and is believed to occur around embryonic day 11 (Sankaran et al., 2010). Additionally, since hemoglobin is primarily studied in erythrocytes, our understanding of both its structure and developmental expression pattern largely reflects its role in these cells (Schechter, 2008).

Table 1. Different subunits of hemoglobin, their locus, and developmental expression. Adult hemoglobin is a tetramer form, commonly two alpha and two beta subunits (α2β2), while fetal hemoglobin consists of two alpha and two gamma subunits (α2γ2) and embryonic hemoglobin is formed by two ζ-globin and two ε-globin.

Subunit	Chromosome	Expression
ζ-globin	16	Embryonic
α-globin	16	Fetal and Adult
ε-globin	11	Embryonic
γ-globin	11	Fetal
β-globin	11	Adult

There are several proteins thought to be involved in the genetic switchover that occurs during development for hemoglobin. BAF Chromatin Remodeling Complex Subunit 11A (BCL11A) regulates the expression of β-globin molecules at different stages of development. Specifically, this zinc-finger transcription factor switches and silences the expression of fetal γ-globin (Hbb- γ) (Sankaran et al., 2008, 2010). Moreover, abnormalities in the *BCL11A* gene have been found in individuals with ASD (Basak et al., 2015) and they have also been found to cause intellectual disabilities in mice models (Dias et al., 2016). Krueppel-like factor 1 (KPL1) is another transcription factor that can activate both the adult β-globin (Hbb-β) and BCL11A; therefore, repressing fetal γ-globin (Vinjamur et al., 2016). Similarly, MYB is a transcription factor that can regulate this switch. For instance, the knockdown MYB in primary adult erythroid cells caused significant increases in γ-globin production (Sankaran et al., 2011). In mice, the transcription factor

SOX-6 has also been found to be a repressor of the fetal γ-globin in collaboration with BCL11-A (Sankaran et al., 2011). Interestingly, in the COX2-knockout model, we found downregulation of SOX-6 at prenatal stages, which could lead to increase expression of γ-globin (Rai-Bohgal et al., 2018).

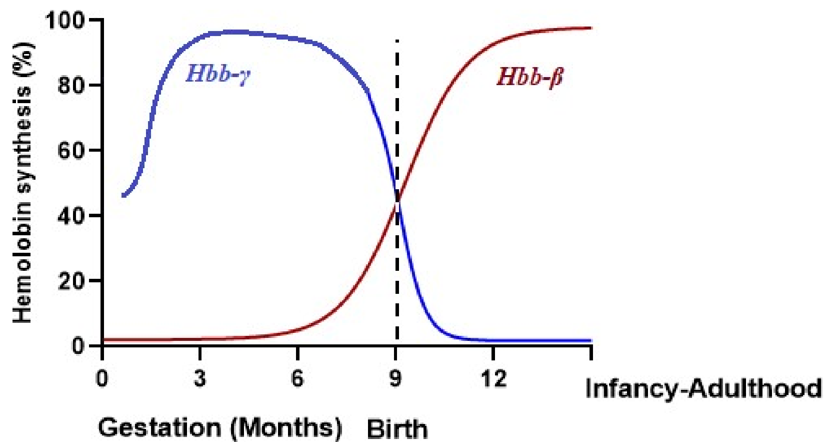


Figure 3. The switch for β-like globin molecules across human developmental stages for fetal and adult isoforms. At the end of the first trimester, fetal globin (Hbb-γ) is highly expressed. At birth, the switch to adult globin (Hbb-β) occurs. *Modified from data from Mcgann et al., 2013.*

Much of our understanding regarding the role of hemoglobin in the body came from studies investigating its association with erythrocytes in blood. The main function of hemoglobin in red blood cells is to transport oxygen and to regulate erythrocyte metabolism. In addition, hemoglobin plays a role in oxidation and erythrocyte senescence (Giardina et al., 1995). Hemoglobin has also been shown to be expressed in non-vascular tissues where it can have a different role. For instance, in vaginal epithelial cells, hemoglobin was described to have a role in recognition of pathogens, possibly associating hemoglobin to antimicrobial and antioxidative functions with a potential role in inflammation (Saha et al., 2014). Hemoglobin has also been found in neurons in the brain

(Biagioli et al., 2009; Ritcher et al., 2009; Mitsunaga et al., 2016), but the role of hemoglobin in neuronal function is still largely unknown. We will discuss this further in the next section.

1.5. Hemoglobin in neuronal cells

As mentioned previously, recent research has shown that hemoglobin can be found in avascular tissues and nonerythroid cells. For example, hemoglobin has been found in macrophages (Liu et al., 1999), epithelial cells of the vagina (Saha et al., 2014), the eye lens (Wride et al., 2003), myelin (Setton-Avruj et al., 2007) and most recently in neuronal cells (Biagioli et al., 2009; Mitsunaga et al., 2016).

Some of the research which identified that hemoglobin was expressed in the human brain was conducted in post-mortem cortical samples obtained from the substantia nigra, a region known to be rich in dopaminergic neurons (Biagioli et al., 2009). Microarray analyses of dopaminergic cells overexpressing hemoglobin identified that these cells exhibited altered expression profiles of hemoglobin genes involved in oxygen homeostasis and oxidative phosphorylation, making a connection between hemoglobin and mitochondrial function (Biagioli et al., 2009). Treatments with mitochondria inhibitors reduced the expression of hemoglobin isoforms in nigral, striatal and cortical neurons (Ritcher et al., 2009) This further demonstrates that hemoglobin might play a role in mitochondrial functions in the brain, and it may be necessary for neuronal function since these cells have high aerobic metabolism. Hemoglobin has also been localized in striatal GABAergic neurons and cortical pyramidal neurons in the brain (Ritcher et al., 2009).

Moreover, hemoglobin can also bind to nitric oxide (NO) in the brain and can regulate the activity of nitric oxide synthase, thereby exerting a direct impact on the production of NO in the brain (Lourençoa et al., 2017). This could be physiologically important for brain function as NO

has been found to have functions in several aspects of neurodevelopment and brain function. Among its main functions in the brain, NO is an unconventional messenger crucial for learning and memory (Haley, Wilcox, & Chapman, 1992) and it also regulates the neurotoxicity of glutamate (Dawson et al., 1991). It has been described in the literature that hemoglobin might form a complex with NO and therefore, trap this molecule (Gunasekar et al., 1995). For example, hemoglobin has been used in experiments to determine the function that nitric oxide might have in the nervous system. To identify if nitric oxide was involved in NMDA neurotoxicity, hemoglobin was added to primary cortical cultures due to its binding to NO (Dawson et al., 1991). Another experiment to test if NO has a function in long-term potentiation in the hippocampus relied on the ability of hemoglobin to bind to extracellular nitric oxide (Haley, Wilcox, & Chapman, 1992). Thus, abnormal levels of hemoglobin might potentially affect these processes and affect brain function through its effects on NO.

1.6. Hemoglobin and pathologies

Hemoglobin has been very well studied for its involvement in sickle cell disease (SCD). Individuals with SCD have atypical hemoglobin molecules with a distorted shape that changes the function of erythrocytes. Additionally, the developmental genetic switch in hemoglobin expression has been studied due to its potential applications for treatments of SCD. More specifically, it has been found that clinical induction of fetal hemoglobin leads to decrease symptoms for SCD (Platt et al., 1984; Akinsheye et al., 2011) due to its high affinity for oxygen.

Associations between hemoglobin levels in the brain and neurodegenerative diseases, such as Alzheimer's Disease (Vanni et al., 2018; Ferrer et al., 2011), Prion Disease (Vanni et al., 2018; Barbisin et al., 2014), Multiple System Atrophy (J.D. Mills, Ward, Kim, Halliday, & Janitz, 2016), Multiple Sclerosis (Broadwater et al., 2011; Brown et al., 2016), Parkinson's Disease (Ferrer et

al., 2011; Shephard, Greville-Heygate, Marsh, Anderson, & Chakrabarti, 2014) and dementia (Ferrer et al., 2011), have also been previously established. For example, increased hemoglobin expression in the brain has been detected in Parkinson's disease patients (Shephard et al., 2014). Moreover, upregulation of the adult hemoglobin forms (hemoglobin beta -Hbb-β, and hemoglobin alpha-Hba-α) has also been found in Alzheimer's disease (Chuang et al., 2012; Vanni et al., 2018).

Hemoglobin genes have also been studied as potential markers of chronic social stress in mice model (Stankiewicz et al., 2014). For instance, mice submitted to social stress had increased expression of hemoglobin genes in the prefrontal cortex (Stankiewicz et al., 2014). Furthermore, hemoglobin expression in the hippocampus and cerebellum of newborn macaques was found to increase after maternal exposure of nanomaterials such as diesel exhaust particles, titanium dioxide, and carbon black which can be found in the environment (Mitsunaga, Umezawa, Takeda, & Nakamura, 2016). Maternal exposure to nanomaterials has been associated with an increased risk for neurodevelopmental problems (Mitsunaga et al., 2016). Moreover, a study in ASD patients found increased levels of the fetal hemoglobin *Hbb-γ* gene expression in blood samples (Basak et al., 2015). Consequently, hemoglobin could play a role in neurodevelopmental disorder pathologies.

Recent research in our lab showed that both *Hbb-β* and *Hbb-γ* gene expressions were significantly upregulated in the prenatal stages E16 and E19 of the COX2 homozygous knockout mice, but only for males (Rai-Bohgal et al., 2018; unpublished). Interestingly, the gene expression of the transcription factor SOX-6 was found to be downregulated in our model for the males, which might explain the high levels of adult *Hbb-β* (Rai-Bohgal et al., 2018). We have found altered expression of ASD-related genes using this COX2-Knockout model as well as abnormal expression of the Wnt pathway (Rai-Bohgal et al., 2018). This could suggest a connection between

abnormal COX2 pathway, hemoglobin levels, and neurodevelopmental issues, and indicates that abnormal expression of hemoglobin may play a role in the male bias of certain neurodevelopmental disorders.

2. Rationale

Studies in our lab focus on the effects of altered levels of PGE2 in neurodevelopment. We have shown for the first time that PGE2 can disturb neuronal function *in vitro* and *in vivo*. In neuroectodermal stem cells (NE-4C), our *in vitro* cell model, results have shown that increased levels of PGE2 affect migration of neuronal stem cells (Wong et al., 2014), accelerate neuronal proliferation and differentiation (Wong et al., 2016), increase the level of calcium in the cells (Davidson et al., 2016), and affect the expression of various genes associated with ASD (Wong et al., 2016). In our *in vivo* mouse model, maternal injection of PGE2 during embryonic day 11 (E11), has been found to disrupt the expression of genes linked to ASD at E16, E19 (Rai-Bhogal et al., 2018), and P8 (Wong et al., unpublished) and cause abnormal neural cell proliferation and microglia activation at P8 (Wong et al., unpublished) in the offspring. For both PGE2-exposed and COX2-knockin deficient mice model (COX2-KI), ASD-linked behaviours such as repetitive behaviours, anxiety, and impaired sociability were shown to be altered (Wong et al., 2018; Wong et al., unpublished). Moreover, in another COX2-deficient mice model involving COX2-knockout mice, we have shown changes in expression of genes associated with ASD, which can lead to disrupted biological pathways that are crucial in brain development, such as synaptic transmission and immune response regulation (Rai-Bhogal et al., 2018). Interestingly, the genes with the most significant upregulation in the microarray study using COX2-knockout animals were *Hbb-β* and *Hbb-γ* for the prenatal brain of males (Rai-Bhogal et al., 2018). These effects were not observed in females (unpublished).

Thus, in this study, we will use our candidate autism mouse model COX2-KI, that has a targeted mutation that inhibits the activity of COX2 in neuronal cells. Brain samples at four different stages will be used, including two prenatal (E16 and E19) and two postnatal stages (P8

and P25). This is a novel study since we will evaluate the genetic profile of these three hemoglobin isoforms throughout brain development as well as their protein expression. We will also analyze potential sex differences and how abnormal PGE2/COX2 pathway affects the expression of the hemoglobin isoforms.

3. Research Plan:

3.1. General Objective

The primary objective of this research is to determine if abnormal COX2/PGE2 signaling affects the expression of the hemoglobin isoforms in the brain of the COX2-KI mouse at different neurodevelopmental stages. Based on the preliminary findings from E16 and E19 in the COX2-knockout model, we hypothesize that the hemoglobin expression level will be affected in the COX2-KI mice and that the differences will be sex-specific. To investigate this, we propose two studies:

3.2. Study 1: Gene and protein expression profile of hemoglobin isoforms in the brain.

3.2.1. Expression of the hemoglobin genes throughout prenatal and postnatal brain development.

Objective: In this study, I aim to quantify the hemoglobin gene expressions for *Hbb-γ, Hbb-β,* and *Hba-α* in the developing mouse brain lacking neuronally expressed PGE2 producing enzyme COX2. I will use male and female homozygous COX2-KI offspring to determine the expression of hemoglobin prenatally at E16 and E19 and postnatally at P8 and P25 compared to the 129S6 wild-type.

Methodology: I will use quantitative Real-Time PCR to quantify the gene expression level of *Hbb-γ, Hbb-β,* and *Hba-α* across neurodevelopment to assess the genetic switch that occurs in the brain and how it compares to the erythrocyte lineage. Furthermore, I will determine if there are any sex-dependent differences for the hemoglobin isoform expression and if deficits in the COX2/PGE2 pathway affect their expression.

Hypothesis: I expect that the ratio between adult and fetal hemoglobin in the healthy brain will follow what was previously described in the literature for the erythrocyte lineage which is that starting at E16 there will be a decrease in *Hbb-γ,* and an exponential increase in *Hbb-β* expression (Sankaran et al., 2010). I also expect to find differences in the gene expression of hemoglobin isoforms between COX2-KI model and the control based on previous research conducted in our lab that found upregulation of *Hbb-γ,* and *Hbb-β* in prenatal stages for the COX2-knockout model (Rai-Bohgal et al., 2018).

3.2.2. Effect of COX2-KI on protein expression for postnatal stage P25.

Objective: In this part of the study, I aim to identify the protein expression of Hba-α, Hbb-β, and Hbb-γ in the postnatal brain at P25. I will be using male and female wild-type as well as COX2-KI animals to determine the effects of abnormal COX2/PGE2 pathway on the hemoglobin expression.

Methodology: In order to quantify the protein expression, I will be conducting western blot analysis using specific antibodies for the isoforms. Measurements for 129S6 wild-types will provide us with a better understanding of how the hemoglobin isoforms are expressed postnatally. Using the COX2-KI, I will be able to evaluate the effect of abnormal levels of PGE2 on the protein expression of hemoglobin isoforms. I will also analyze any potential sex differences.

Hypothesis: Similarly to the above section, I am expecting changes in protein expression between the COX2-KI model and the 129S6 wild-type (Rai-Bohgal et al., 2018). Moreover, I expect that potential dimerization between the isoforms might be detected based on previous research conducted in neuronal cells (Russo et al., 2013).

3.3. Study 2: Regional localization of the three hemoglobin isoforms in the mouse brain

Objective: This study aims to localize the hemoglobin proteins in different brain regions to investigate their specific expression for 129S6 wild-type males at P25.

Methodology: I will use immunohistochemical techniques with specific antibodies against the different forms of hemoglobin (Hbb-γ, Hbb-β, and Hba-α) to identify the brain regions that express the various types of hemoglobin. Subcellular localization of each isoform will also be analyzed in this study.

Hypothesis: I hypothesize that hemoglobin expression in the mouse brain for the specific isoforms will be similar between them since they are known to form dimer and tetramer structures with each other (Russo et al., 2013). I also expect the cerebellum to have high expression of hemoglobin isoforms based on the literature (Schelshorn et al., 2008).

3.5. Model System

I will be using an animal model system already established in our lab: homozygous knockin mice lacking the neuronally expressed PGE2 producing enzyme, COX2-KI. Homozygous COX2-KI mice were chosen due to the constitutive neuronal expression of this enzyme in the brain and that only the PGE2 synthase activity is disrupted, while the peroxidase activity of this model is still intact. Furthermore, this model has been previously linked to ASD due to the behavioural and molecular characteristics found in the COX2-KI animals that reflect what has been described in ASD (Wong et al., 2018).

4. Materials and Methods

4.1. Animal models

129S6 wild-type mice known as WT 129S6/SvEvTac were obtained from the Taconic Laboratory and were used as controls for these studies. Our COX2-knockin (COX2-KI) mice model, also known as Ptgs2Y385F (B6.129S6FVB-Ptgs2tm1.1Fun/J mice, 008101; Queen's University, laboratory of C. Funk) (Yu et al., 2006) were purchased from Jackson Laboratories. These mice have a targeted point mutation in the *Ptgs2* gene that results in an amino acid substitution that inactivates the cyclooxygenase activity of the enzyme but leaves the peroxidase activity unchanged. They were backcrossed with the 129S6 WT mice for at least five generations before the experiments. For our studies, we needed COX2-KI homozygous mice offspring. Due to infertility of the homozygous COX2-KI females, heterozygous females were bred with homozygous or heterozygous males to obtain COX2-KI homozygous offspring that were used for this research. Breeding was conducted by Ph.D. students in the lab and myself. All animals were maintained in group housing at York University under the same controlled conditions that included 12-hours light/dark cycle. Research Ethics Board of York University approved all the protocols and experiments and they followed the York University Animal Care Committee ethics guidelines.

4.2. Genotype Analysis

The genotype of the COX2-KI offspring was identified by PCR analysis of the COX2 gene (*Ptgs2*), and primer sequence was obtained from Jackson Laboratories (Table 2). For prenatal stages, sex was determined using PCR for the *SRY* gene (sex-determining region Y) (Table 2). DNA was extracted from ear tissue for Ptgs2 analysis and tail samples for sex identification. Tissue samples were homogenized in alkaline lysis buffer (25mM NaOH) followed by neutralization buffer (Tris-HCl). PCR reaction was composed of PCR master mix (5 units/µL Taq DNA

polymerase Biolabs #M0480S, 2.5 mM dNTP, 20mm MgSO4, 10x Taq buffer BioLabs #M04481S), 10µM of primers (Table 2), 4µl of DNA and ddH2O. It was performed in an Eppendorf Mastercycler. Electrophoresis was conducted on a 1.2% agarose gel with SafeView (Abm, G108) and 6x-BB was used as loading buffer.

4.3. Brain Extraction

Sedation and decapitation of mice were followed by brain extractions and occurred on E16, E19, and P8 and P25. Extractions were conducted by Ph.D. students in the lab and myself. E16 represents the peak of neurogenesis in the cerebral cortex (Poluch & Juliano, 2015) and is also a critical period for environmentally induced abnormal learning and memory and hyperactivity (Ardalan, Chumak, Vexler, & Mallard, 2019; Berger-Sweeney & Hohmann, 1997), whereas E19 is the last prenatal stage that we can collect before birth where the brain is still developing. P8 in mice corresponds to infant stages in humans (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013) where early signs of ASD-related behaviours have been detected (Robins, Fein, Barton, & Green, 2001; Zwaigenbaum, Bryson, & Garon, 2013). On the other hand, P25 mice have a more mature brain with fully formed brain regions such as the cerebellum.

The n value for all stages consisted of at least three independent animals for each condition from independent litters. For E16, n values were as followed: n=11 for 129S6 males, n=11 for 129S6 females, n=5 for COX2-KI males, n=7 for COX2-KI females. For E19, n values varied between n=14 for 129S6 males, n=8 for 129S6 females, n=4 for COX2-KI males, n=4 for COX2-KI females. For P8, the number of animals consisted of n=11 for 129S6 males, n=6 for 129S6 female, n=6 for COX2-KI males, n=8 for COX2-KI females. For P25, n values were n=5 for 129S6 males, n=8 for 129S6 female, n=5 for COX2-KI males, n=4 for COX2-KI females.

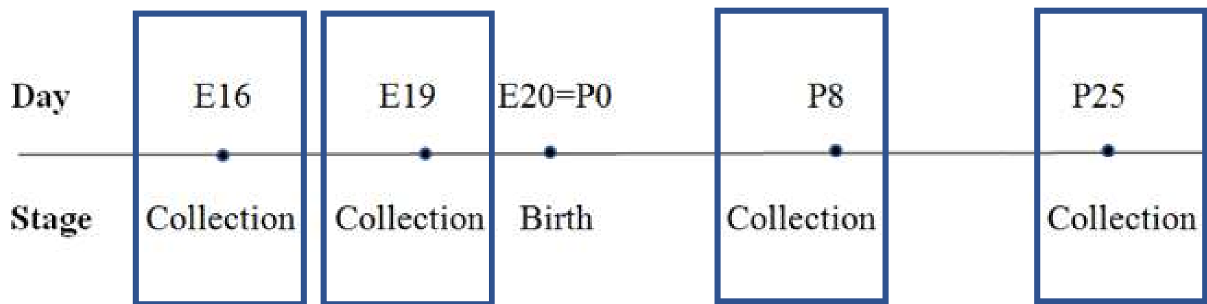


Figure 4. Timeline including brain extractions used in our experiments. "E" stands for embryonic day and "P" for postnatal day. A plug is formed in females after fertilization, which marks the embryonic day 1 and the start of the embryonic stage. E20 or P0 represents birth and the start of postnatal stages.

4.4. RNA and Protein Isolation

To determine gene expression changes, RNA isolation was conducted, and then RNA was converted to cDNA. The standard Trizol method (Invitrogen) was used to isolate RNA from brain samples where 1 mL of trizol was used per tissue sample and immediately followed by tissue homogenization using Polytron power homogenizer. RNA phase separation was done using chloroform. RNA was then precipitated with isopropyl alcohol and dissolved in RNase-free water. RNA samples underwent DNase treatment (New England Biolabs, Ipswich, MA). To convert RNA to cDNA, 6 µg of RNA samples were then reverse-transcribed using MMuLV reverse transcriptase (New England Biolabs, Ipswich, MA). Following RT (Reverse Transcriptase), PCR for the *Gapdh* gene (Table 2) was conducted to check for the presence of cDNA. Protein extraction was conducted simultaneously with RNA isolation following the standard Trizol method (Invitrogen) indications. 0.3M Guanidine HCl was used to wash the protein pellet, followed by 100% ethanol wash. The protein pellet was dissolved using 1% SDS, constant agitation and the heating block at 50^0C for no longer than 3 minutes at a time.

4.5. Quantitative Real-Time PCR

Primer design for the hemoglobin isoforms was done using Primer 3 Express program (Thermo Fisher Scientific) (Table 3). qRT-PCR (quantitative real-time polymerase chain reaction) was conducted to determine the expression of the three hemoglobin isoforms (*Hbb-γ, Hba-α, Hbb-β*) using Syber Green. 7500 Fast RT-PCR system (Applied Biosystems) was used to run qRT-PCR. Animal samples were pooled and divided by condition and sex (129S6 wild-type male, 129S6 female, COX2-knockin male, COX2-knockin female) including at least three animals for each condition.

4.6. Analysis of qRT-PCR

The ΔΔCt method was used as described by Tamiji and Crawford, 2010, and Wong et al., 2014 to calculate gene expression. Two housekeeping genes were used as endogenous controls: hypoxanthine phosphoribosyl transferase (*Hprt*) and phosphoglycerate kinase 1 (*Pgk1*). Raw C_t (Threshold cycle) values for COX2-KI animals were normalized using the genomic means of the endogenous controls to obtain ΔCt values. Relative quantities (RQ) represented the fold change expression of each gene compared to the reference sample and were calculated using the formula 2^ΔCt-ΔCt of the baseline or wild-type control. All reference and target genes were measured in triplicates, and the mean value for each gene was used to determine the expression changes. The 129S6 wild-types males were used as the baseline (RQ=1). COX2-knockin animals were then compared to the 129S6 wild-types. Sex differences were also determined for each condition. To plot the RQ values of the different genes together in the line graphs, all RQ values were calculated using 2^ΔCt-ΔCt of the 129S6 male for *Hbb-γ* at P25. The logarithm of these expressions was then calculated in order to plot the RQ values of each gene together. For the mouse erythrocyte line graph, values were first taken from Sankaran et al., 2010 and Mcgann et al., 2013, and then we

proceeded to calculate the logarithmic expression of each gene so we could compare to the mouse brain line graph from our data.

4.7. Western blots

To determine protein expression changes, western blots were conducted on the protein samples corresponding to 129S6 wild-type male and female, and COX2-knockin male and female for P25. 25 ug whole protein lysates were boiled for 2 minutes after addition of loading buffer (1xLaemmli sample buffer from BioRad with beta-mercaptoethanol) and were separated in a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were then transferred to a supported nitrocellulose membrane (0.2 um). 5% Milk in TBS-T (20Mm Tris ph+7.5, 137 mM NaCl, 1% Tween-20, and 5% non-fat dry milk) was used as blocking buffer for the samples for 1 hour. Washes in between steps consisted of 1xTBS-T. Primary antibodies used in the experiments were Hba-α (Abcam ab102758, rabbit polyclonal 1:1000 diluted in 5% milk), Hbb-β (Abcam ab214049, rabbit monoclonal 1:5000 diluted in 5% milk), Hbb-γ (Invitrogen PA5-49336, rabbit polyclonal 1:1000 diluted in 5 % milk) and GAPDH (Abcam ab8245, mouse 1:10000 diluted in 2% milk). Primary antibody incubation for the hemoglobin isoforms was overnight at 4^{0}C, while GAPDH was incubated for 1 hour. Secondary antibodies were incubated for 1 hour and included anti-rabbit (Abcam, ab6721) for the hemoglobin expression and anti-mouse (Abcam, ab97040) horseradish peroxidase-conjugated for the GAPDH expressions. The membranes were then incubated in ECL substrate (BioRad) and visualized using Geliance 600 Imaging System (Perkin Elmer).

4.8. Immunohistochemistry

P25 slides corresponding to 129S6 males were used to identify regional localization of the different hemoglobin isoforms in the brain. Slides were cut sagittally and using the left side of the brain. Slide-mounted tissue sections by the Centre of Phylogenetics were de-paraffinated using

100% xylene and dehydrated in ethanol dilutions. For antigen retrieval, sodium citrate (10mM citric acid, pH=6) was used in a glass chamber covering the slides and boiled in a microwave at 10% power for 20 minutes. 1xPBS was used for all the intermediate washes at room temperature for 5 minutes between steps. Slides were then incubated with 0.3% H_2O_2 in methanol for 30 minutes to inactivate endogenous peroxidase activity. Subsequently, slides were incubated for 30 minutes in blocking solution (5% goat serum and 0.1% TritonX-100 diluted in 1xPBS). Primary antibodies were incubated overnight at 4^0C Hba-α (Abcam ab102758, rabbit 1:100 diluted in 1xPBS), Hbb-β (Abcam ab214049, rabbit 1:100 diluted in 1XPBS), Hbb-γ (ThermoFisher, rabbit 1:100 diluted in 1xPBS). For secondary antibody, slides were incubated for 1 hour at room temperature using biotinylated anti-rabbit IgG antibody (Vector Laboratories, 1:2000). Immediately after, slides were incubated for 1 more hour at room temperature with Elite Reagent (Vectastain Avidin Biotin Complex kit from Vector Laboratories). To finish the staining 3,3'-Diaminobenzidine (DAB) substrate solution (Sigma fast tablet) was used in 0.3% ammonium nickel sulfate hexahydrate and Mili Q water. Images were taken using Zeiss spinning disk confocal microscope (Axio Observer. Z1 Microscope) with high resolution imaging camera Axiocam MRm in Life Science Building at York University. Images were then visualized with the ZEN 2.1 blue/black edition program.

4.9. Statistics

Numerical data are reported in bar graphs as mean±standard error of the mean (SEM), with the mean representing at least three individuals from independent litters for each condition. Statistical analyses using GraphPad Prism were completed through two-way ANOVA and followed by Bonferroni’s pairwise comparison test to determine specific differences. Significance is determined at $p<0.05$.

Table 2. PCR primer sequences.

Primer	Name	Length (bp)	Sequence (3’-5’)
Forward	Mus *Gapdh*	20	TTGTGATGGGTGTGAACCAC
Reverse	Mus *Gapdh*	20	GTCTTCTGGGTGGCAGTGAT
Forward	Mus *Sry*	28	TCCCAGCATGCAAAATACAGAGATCAGC
Reverse	Mus *Sry*	25	TTGGAGTACAGGTGTGCAGCTCTAC
Forward	Mus *Ptgs2*	20	ACCAGTCTCTCAATGAGTAC
Reverse	Mus *Ptgs2*	22	AGAATGGTGCTCCAAGCTCTAC

Table 3. Quantitative Real Time PCR primer sequences.

Primer	Name	Length (bp)	Sequence (3’-5’)
Forward	Mus *Hbb-γ*	27	TGAGAACTTCAAACTCTTGGGTAATGT
Reverse	Mus *Hbb-γ*	22	TGAATTCATTGCCGAAGTGACT
Forward	Mus *Hbb-β*	21	TGTGACAAGCTGCATGTGGAT
Reverse	Mus *Hbb-β*	18	CCCAGCACAATCACGATC
Forward	Mus *Hba-α*	23	CCTGGAAAGGATGTTTGCTAGCT
Reverse	Mus *Hba-α*	22	GAGCCGTGGCTTACATCAAAGT
Forward	Mus *Pgk1*	24	CAGTTGCTGCTGAACTCAAATCTC
Reverse	Mus *Pgk1*	21	GCCCACACAATCCTTCAAGAA
Forward	Mus *Hprt*	29	TCCATTCCTATGACTGTAGATTTTATCAG
Reverse	Mus *Hprt*	23	AACTTTTATGTCCCCCGTTGACT

5. Results

5.1. Study 1. Expression profile of hemoglobin isoforms in the brain

5.1.1. Gene expression of the hemoglobin isoforms within prenatal and postnatal brain development

To date, previous studies in mice and humans only reported the expression of hemoglobin genes through development in the erythrocyte lineage. It is well documented that a genetic switch between fetal (Hbb-γ) and adult (Hbb-β) hemoglobin is developmentally regulated in the blood (Sankaran et al., 2010). During early prenatal stages in erythrocytes, *Hbb-γ* is highly expressed due to its high affinity for oxygen required for healthy development of the fetus. Around E11 in mice, *Hbb-γ* expression starts to be silenced, and *Hbb-β* expression begins to exponentially increase until it reaches maximum expression throughout postnatal development and adulthood (Sankaran et al., 2010). *Hba-α* expression in erythrocytes remains constant during fetal and adult stages in the erythrocyte lineage (Sankaran et al., 2010).

However, the expression of different hemoglobin isoforms has not been well studied in the nervous system and across neurodevelopment. Therefore, in this study, we aim to determine the expression of hemoglobin *Hbb-γ, Hbb-β*, and *Hba-α* in the brain and determine if their pattern of expression follows what has been found in the blood. Moreover, we will also examine whether the expression is affected by the lack of neuronally expressed PGE2 producing enzyme COX2. We will use brain samples from four developmental stages: E16, E19, P8, and P25 from the wild-type 129S6 mice and COX2-knockin (COX2-KI) mice. Our previous whole genome microarray analysis in COX2-knockout animals showed upregulation of fetal *Hbb-γ* and adult *Hbb-β* hemoglobin expressions at prenatal stages in the male (Rai-Bohgal et al., 2018) but not in the

female (unpublished). This study aims to test these results in the COX2-KI model and determine the expression of the hemoglobin isoforms at the four previously mentioned developmental stages. For all gene expression experiments shown below, the values for each experimental group were standardized against the wild-type male results (Relative Quantity or RQ for 129S6 wild-type male, referred to as 129S6-M, equals 1) as shown in the figures. RQ represents the fold change for the samples. In all the comparisons of hemoglobin expression, we will also compare sex differences.

5.1.2. Expression of *Hbb-γ* at four neurodevelopmental stages

Our previous whole genome microarray study showed upregulation of *Hbb-γ* at E16 in the COX2-knockout male mice when compared to wild type and no change at E19 (Rai-Bohgal et al., 2018) with no changes in COX2-knockout females at either E16 or E19 (unpublished). In this study, we aim to determine the gene expression levels of *Hbb-γ* using SYBR-green quantitative Real-Time PCR (qRT-PCR) in the COX2-KI male and female brains compared to the 129S6 wild-type at E16, E19, P8, and P25.

First, a two-way ANOVA analysis was completed for sex differences ($F_{(1,8)}$=290.8, p<0.0001), condition (comparison between COX2-KI and 129S6 animals; $F_{(1, 8)}$=10.73, p=0.011), and the interaction between these two factors ($F_{(1, 8)}$=0.02911, p=0.8688) . Since there was no interaction effect no additional pairwise comparisons were necessary. The results showed sex-dependent differences in *Hbb-γ* expression at E16 in the wild-type mice, which was maintained in the COX2-KI while COX2 deficiency affected the expression of *Hbb-γ* at this stage.

A two-way ANOVA was completed for sex differences ($F_{(1, 8)}$=32.32, p=0.0005), condition ($F_{(1, 8)}$=54.86, p<0.0001) and the interaction between these two factors ($F_{(1, 8)}$=25.43, p=0.001) at E19 for *Hbb-γ* expression. Similarly to E16, the expression of *Hbb-γ* at E19 was also increased in

the 129S6 female (RQ=2.25, p=0.0004) compared to the 129S6 male (RQ=1) (Figure 5B). However, sex differences were not observed in COX2-KI animals (RQ=0.8 for COX2-KI-F, and RQ=0.725 for COX2-KI-M; p=0.97). We found that the *Hbb-γ* expression in COX2-KI-female was significantly downregulated (RQ=0.8, p=0.0001) compared to 129S6-female (RQ=2.25). No difference was observed between the COX2-KI-male (RQ=0.725; p=0.7994) and the 129S6 male (RQ=1). The results showed sex-dependent differences of *Hbb-γ* expression in the 129S6 wild-type mice and downregulated expression in the COX2-KI female.

Previous research from blood samples shows that *Hbb-γ* expression is very low after birth (Sankaran et al., 2010). Here, we evaluated the expression of *Hbb-γ* in the brain at postnatal stages. A two-way ANOVA was conducted for sex differences ($F_{(1, 8)}$=3.011, p=0.12), condition ($F_{(1, 8)}$=20.97, p=0.0018) and their interaction ($F_{(1, 8)}$=16.60, p=0.0036) at P8 for *Hbb-γ* expression. We observed no sex differences between the 129S6-M (RQ=1) and 129S6-F at P8 (RQ=2.84; p=0.8203) (Figure 5C). However, the COX2-KI males were found to have a significantly increased *Hbb-γ* expression (RQ=7.805; p=0.0204) when compared to the COX2-KI females (RQ=3.24). Interestingly, *Hbb-γ* expression was significantly upregulated in the COX2-KI males by nearly 8 fold (RQ=7.8, p=0.0017) compared to 129S6 males (RQ=1), whereas no differences were found between the COX2-KI females (RQ=3.24; p>0.99) and the 129S6 females (RQ=2.84). Overall, at P8 no sex differences in *Hbb-γ* expression were detected in the wild-type, but we found significantly increased expression in COX2-KI males compared to COX2-KI females. Moreover, COX2 deficient males had significant upregulation of *Hbb-γ* expression in comparison to 129S6 males.

As development progresses, the postnatal expression of *Hbb-γ* in blood samples has been shown to be at very low levels (Sankaran et al., 2010). Here, we investigated the expression of the

fetal *Hbb-γ* in the mature brain at P25. A two-way ANOVA was conducted on *Hbb-γ* expression at P25 for sex differences ($F_{(1, 8)}$=29.34, *p*=0.0006), condition ($F_{(1, 8)}$=172.3, *p*<0.0001) and their interaction ($F_{(1, 8)}$=13.51, *p*=0.0063). Additional pairwise comparisons were conducted where the 129S6 animals did not show sex-dependent changes in gene expression for *Hbb-γ* (RQ=1.68 for 129S6-F, RQ=1 for 129S6-M; *p*>0.9999). However, the COX2-KI females had significantly increased expression (RQ=14.21; *p*=0.0012) relative to the COX2-KI males (RQ=7.0). Surprisingly, *Hbb-γ* was almost 8 fold higher for the COX2-KI males (RQ=7.7, *p*=0.0009) when compared to the wild-type 129S6 males (RQ=1) (Figure 5D). Moreover, the expression for COX2-KI females was also significantly upregulated (RQ=14.19, *p*=0.0001) compared to 129S6 females (RQ=7.73). These results showed that at P25, there were no sex differences between 129S6 animals for the level of *Hbb-γ*. However, the lack of COX2 enzyme resulted in increased expression of *Hbb-γ* for both COX2-KI males and females.

Overall, we showed that the expression of the fetal *Hbb-γ* at prenatal stages E16 and E19 was differentially expressed between males and females with downregulation of *Hbb-γ* expression in COX2-KI females observed only at E19. Interestingly, in the two postnatal stages, P8 and P25, we did not find sex-dependent differences in the 129S6 wild types. However, the expression of *Hbb-γ* in COX2-KI animals was higher than in the corresponding 129S6 controls with upregulation in COX2-KI males at P8 and upregulation in both COX2-KI males and females at P25.

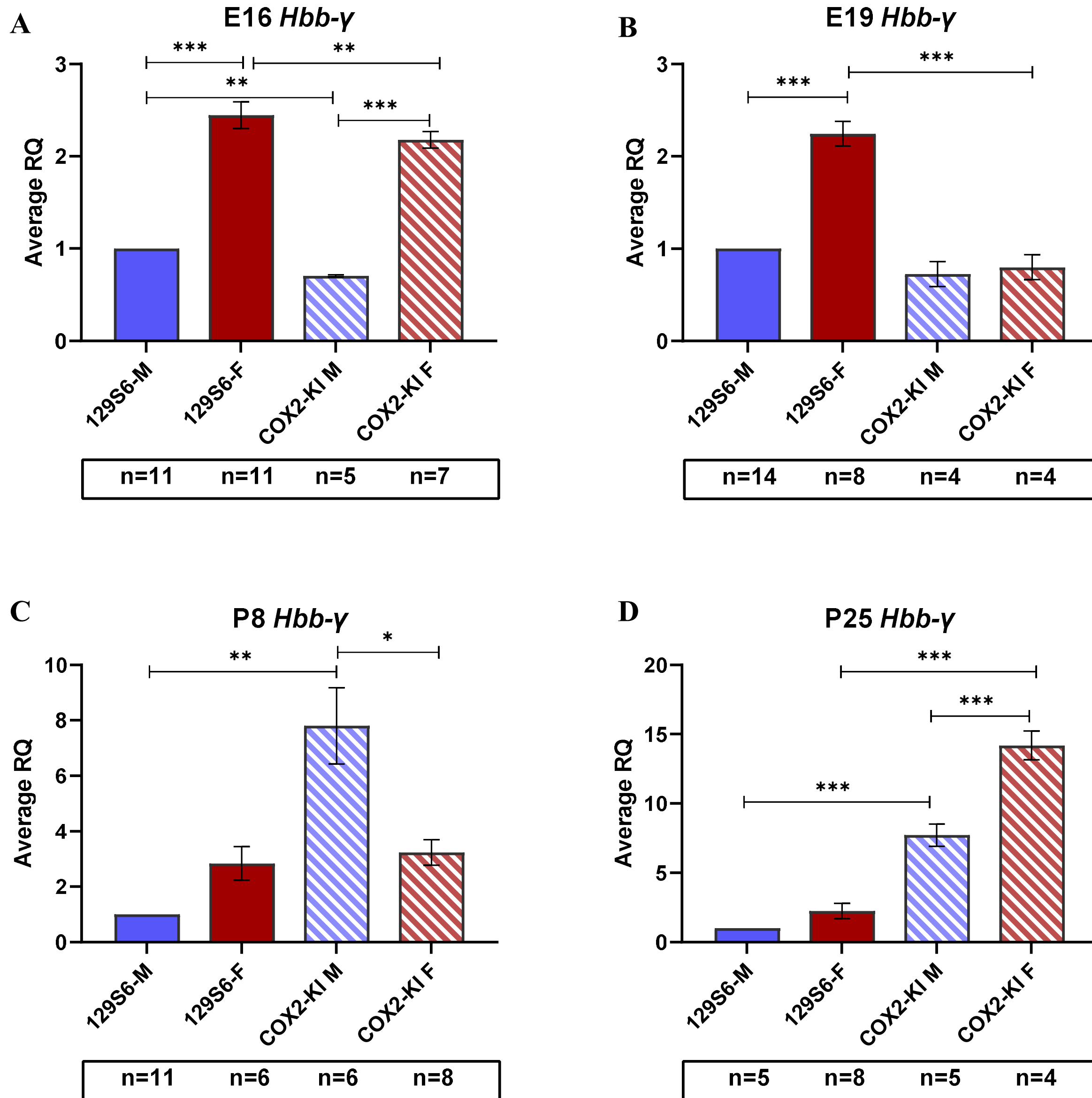


Figure 5. Changes in the gene expression of hemoglobin gamma (*Hbb-γ*) at four neurodevelopmental stages. COX2-knockin animals were compared to 129S6 wild-type mice, and sex-dependent differences were also considered. 129S6 males were used as the baseline (RQ=1). Animals were pooled by sex and conditions. We conducted three independent experiments on these samples to represent three technical replicates. Animals were taken from at least two litters, and n value was between 4-14 mice per condition; specific n values are given in the figure. Averages of the RQ values were calculated to determine statistical differences. Statistical significance is marked '*' for $p<0.05$, '**' for $p<0.01$ and '***' for $p<0.001$. Error bars represent ±SEM values. *Hbb-γ* expression at **A)** Embryonic day 16, **B)** Embryonic day 19, **C)** Postnatal day 8, and **D)** Postnatal day 25.

5.1.3. Expression of *Hbb-β* at four neurodevelopmental stages

Our previous whole genome microarray study also showed upregulation of another hemoglobin gene, called the adult hemoglobin *Hbb-β* at E16 and E19 in the COX2-knockout male mice when compared to wild type (Rai-Bohgal et al., 2018). The follow-up microarray analysis shows no changes in *Hbb-β* expression in COX2-knockout females at either E16 or E19 (unpublished). In this study, we aim to quantify the expression level of *Hbb-β* using real-time qRT-PCR in the COX2-KI males and females brain compared to the 129S6 wild-type at E16, E19, P8, and P25.

A two-way ANOVA was conducted on *Hbb-β* expression at E16 for sex differences ($F_{(1, 8)}$=284.7, p<0.0001), condition ($F_{(1, 8)}$=0.8858, p=0.3742) and their interaction ($F_{(1, 8)}$=7.756, p=0.0237). The expression of *Hbb-β* at E16 showed significant sex differences for both the 129S6 wild-type and COX2-KI animals (Figure 6A). The expression of *Hbb-β* was three times higher for the 129S6 females when compared to the 129S6 males (RQ=2.93, p<0.0001). The same trend was observed in the COX2-KI mice where the females showed higher expression (RQ=2.64; p<0.0001) than the COX2-KI males (RQ=1.36). No significant differences were found between COX2-KI male and female animals and the corresponding wild-type counterparts (RQ=1.36 for COX2-KI-M, RQ=2.64 for COX2-KI-F, RQ= 2.93 for 129S6-F, RQ=1 for 129S6-M; p=0.1797 for COX2-KI-M and 129S6-M comparison, p>0.9999 for COX2-KI-F and 129S6-F comparison). The results showed that at E16, the expression level of *Hbb-β* in the 129S6 wild-type was sex-dependent and it remained unaffected in the COX2-KI.

A two-way ANOVA was completed on *Hbb-β* expression at E19 for sex differences ($F_{(1, 8)}$=0.3647, p=0.5627), condition ($F_{(1, 8)}$=0.2857, p=0.6075) and their interaction ($F_{(1, 8)}$=3.889, p=0.0841) showing no statistical differences for each factor (Figure 6B). No additional pairwise

comparisons were required. Thus, neither the sex of the animal nor the COX2-KI condition affected the expression of *Hbb-β* at E19.

Two-way ANOVA analysis was conducted for sex differences ($F_{(1, 8)}$=113.8, p<0.0001), condition ($F_{(1, 8)}$= 76.23, p<0.0001) and their interaction ($F_{(1, 8)}$=221.8, p<0.0001) on *Hbb-β* expression at P8. Additional pairwise analysis showed no significant sex-dependent differences for the 129S6 animals (RQ=1.25 for 129S6-F, RQ=1 for 129S6-M; p= 0.1040) (Figure 6C), whereas the COX2-KI males had significantly increased expression of *Hbb-β* (RQ=2.41, p<0.0001) in comparison to the COX-KI females (RQ=0.88). There is also a significant upregulation of this gene for the COX2-KI males (RQ=2.41, p<0.0001) compared to the 129S6 wild-type males (RQ=1). Moreover, COX2-KI females showed downregulation of *Hbb-β* (RQ=0.88, p=0.0145) as compared to the wild-type females (RQ=1.25). At this stage, we found that defects in the COX2 pathway affected the expression of *Hbb-β* for both males and females having increased expression in COX2-KI males and decreased expression in COX2-KI females.

A two-way ANOVA was completed at P25 and no significant differences were found for sex-dependent differences ($F_{(1, 8)}$=0.4967, p=0.501), condition ($F_{(1, 8)}$=1.437, p=0.2650) or the interaction between these two factors ($F_{(1, 8)}$=13.55, p=0.006) (Figure 6D). These results showed that at P25 *Hbb-β* expression was not affected by sex or by COX2 deficits in females.

Overall, *Hbb-β* expression across neurodevelopment in the wild-type 129S6 animals shows no sex differences except for E16 with females having a higher level of this gene than males. In COX2-KI animals, we observed an increased expression level only in males at P8.

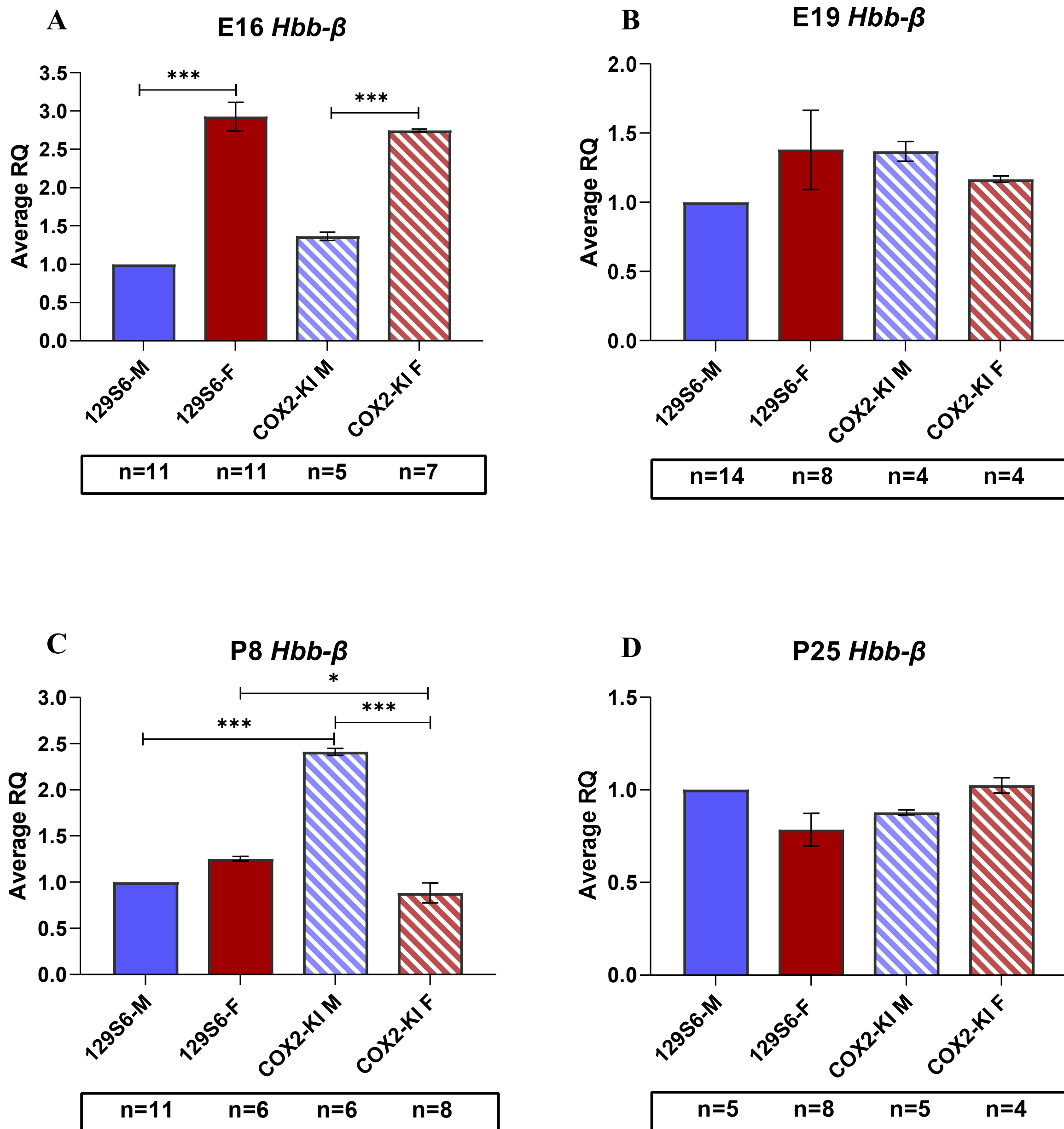


Figure 6. Changes in the gene expression of hemoglobin beta (*Hbb-β*) at four neurodevelopmental stages. COX2-knockin (COX2-KI) animals were compared to 129S6 wild-type mice, and sex-dependent differences were also considered. 129S6 males were used as the baseline (RQ=1). Animals were pooled by sex and conditions. There were three independent experiments conducted which represent three technical replicates. Animals were taken from at least two litters, and n value was between 4-14 mice per condition; specific n values are given in the figure. Averages of the RQ values were calculated to determine statistical differences. Statistical significance is marked '*' for $p<0.05$, '**' for $p<0.01$ and '***' for $p<0.001$. Error bars represent ±SEM values. *Hbb-β* expression at **A)** Embryonic day 16, **B)** Embryonic day 19, **C)** Postnatal day 8, and **D)** Postnatal day 25.

5.1.4. Expression of *Hba-α* at four neurodevelopmental stages

Our previous microarray analysis showed upregulation of hemoglobin alpha (*Hba-α*) expression in COX2-knockout males during neurogenesis at E16 with normal levels at E19 (Rai-Bohgal et al., 2018). No difference was observed in COX2-knockout females for these two prenatal stages (unpublished). In this study, we used q-RT-PCR to test the expression of *Hba-α* at E16 and E19 as well as two postnatal stages at P8 and P25 using COX2-KI animals.

Two-way ANOVA was completed at E16 for sex ($F_{(1, 8)}$=1.08, p=0.3286), condition ($F_{(1, 8)}$=1.717, p=0.2264) and the interaction between these two factors ($F_{(1, 8)}$=17.26, p=0.0032). *Hba-α* expression at E16 showed sex-dependent differences between the 129S6 animals with the female having significantly increased expression (RQ=2.094, p=0.0371) when compared to the male (RQ=1) (Figure 7A). However, the COX2-KI animals did not have any significant sex-dependent difference (RQ=2.151 for COX2-KI-M, RQ=1.495 for COX2-KI-F; p=0.3048). There was a significant increase in the expression of COX2-KI males (RQ=2.151, p=0.0283) in comparison with the 129S6 wild-type male (RQ=1). No statistical difference was found between the COX2-KI females (RQ=1.495, p=0.3902) and the 129S6 wild-type females (RQ=2.094). These results showed that in the wild-type at E16, there were sex differences in the expression of *Hba-α* and that the expression was only affected in COX2-KI males.

A two-way ANOVA was completed using E19 samples for *Hba-α* expression, and no differences were found for sex differences ($F_{(1, 8)}$=0.1741, p=0.6874), condition (comparison between 129S6 animals and COX2-KI; $F_{(1, 8)}$=2.014, p=0.1936) or the interaction between these two factors ($F_{(1, 8)}$=5.098, p=0.0539) (Figure 7B). Therefore, no follow-up with additional pairwise comparisons was required.

Two-way ANOVA was also conducted at P8 for sex ($F_{(1, 8)}$=15.05, *p*=0.0047), condition ($F_{(1, 8)}$=26.14, *p*=0.009) and the interaction between these two factors ($F_{(1, 8)}$=8.291, *p*=0.0205). *Hba-α* expression at P8 showed sex-dependent differences between the 129S6 animals with the female having significantly less expression (RQ=0.7, *p*=0.0084) when compared to the male (RQ=1) (Figure 7C). However, the COX2-KI animals did not have any significant sex-dependent difference (RQ=0.648 for COX2-KI-M, RQ=0.603 for COX2-KI-F; *p*=0.89). There was a significant decrease in *Hba-α* expression in COX2-KI males (RQ=0.648, *p*=0.003) in comparison with the 129S6 male (RQ=1). No statistical difference was found between the COX2-KI females (RQ=0.603, *p*=0.9172) and the 129S6 females (RQ=0.702). These results showed that in the wild-type at P8, there were sex differences in the expression level of *Hba-α* and that the expression was affected in COX2-KI males.

Two-way ANOVA analysis was also conducted at P25 for sex ($F_{(1, 8)}$=12.28, *p*=0.008), condition ($F_{(1, 8)}$=9.91, *p*=0.0136) and the interaction between these two factors ($F_{(1, 8)}$=21.92, *p*=0.0016). Similarly to P8, additional pairwise comparisons showed sex-dependent differences in the 129S6 animals with the females having significantly less expression of *Hba-α* (RQ=0.74, *p*=0.002) than the males (RQ=1) (Figure 7D). No sex-dependent differences were found for the COX2-KI model (RQ=0.75 for the COX2-KI-M, RQ=0.79 for the COX2-KI-F; *p* >0.9999). This gene was found to be significantly decreased for COX2-KI males (RQ=0.75, *p*=0.0033) when compared to the 129S6 male (RQ=1), whereas the COX2-KI females did not show any statistical differences (RQ=0.79, *p*>0.9999) compared to 129S6 females (RQ=0.74). The downregulation of *Hba-α* for the COX2-KI males observed at P8 was maintained at P25.

Overall, we observed that the expression of *Hba-α* showed sex differences in the 129S6 mice with females having higher expression prenatally at E16 and lower postnatally. However, in the

COX2-KI animals, we did not find sex differences in *Hba-α* expression due to its prenatal upregulation at E16 and postnatal downregulation in males.

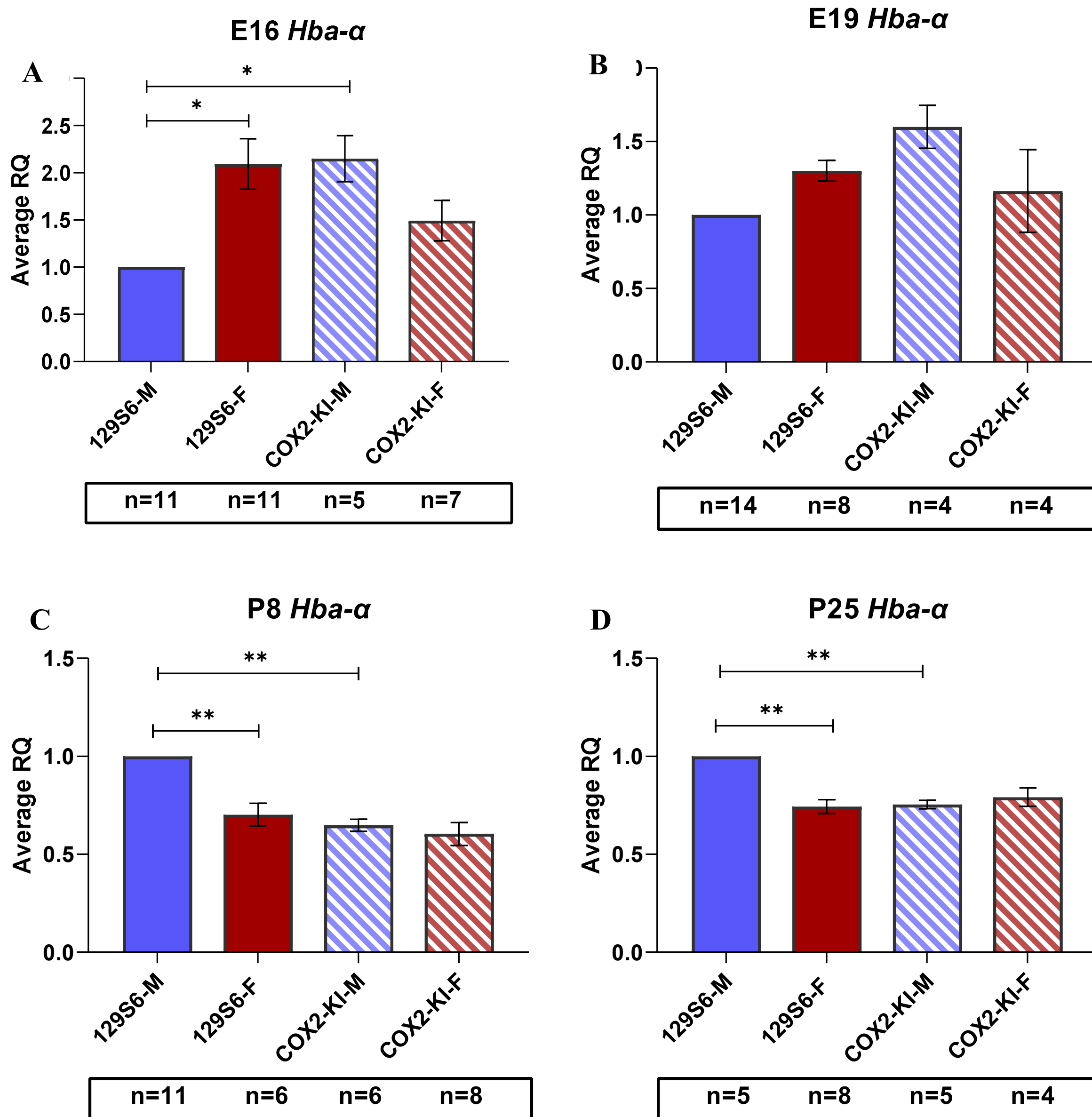


Figure 7. Changes in the gene expression of hemoglobin alpha (*Hba-α*) at four neurodevelopmental stages. COX2-knockin (COX2-KI) animals were compared to 129S6 wild-type mice, and sex-dependent differences were also considered. 129S6 males were used as the baseline (RQ=1). Animals were pooled by sex and conditions. There were three independent experiments which represent three technical replicates. Animals were taken from at least two litters, and n value was between 4-11 mice per condition; specific n values are given in the figure. Averages of the RQ values were calculated to determine statistical differences. Statistical

significance is marked ‘*’ for $p<0.05$, ‘**’ for $p<0.01$ and ‘***’ for $p<0.001$. Error bars represent ±SEM values. Hba-α expression at **A)** Embryonic day 16, **B)** Embryonic day 19, **C)** Postnatal day 8, and **D)** Postnatal day 25.

5.1.5. Expression profile in the developing healthy brain compared to the erythrocyte lineage.

Current literature shows that the expression of fetal *Hbb-γ* and adult *Hbb-β* in the erythrocyte lineage undergoes a genetic switch at prenatal stages (approximately around E11 in the mouse) with *Hbb-γ* becoming silenced and *Hbb-β* beginning its exponential expression (Sankaran et al., 2010) (Figure 8A). By E16 and E19, *Hbb-β* is on the rise, while *Hbb-γ* is gradually decreasing. Beginning at P8, there are very low levels of *Hbb-γ* expression, and *Hbb-β* is predominantly expressed.

To date, there is very little evidence showing the expression of *Hbb-γ* and *Hbb-β* in the developing brain. In this study, we showed the expression pattern of these hemoglobin isoforms in the healthy developing mouse brain between E16 and P25 developmental points. Using the data presented in section 5.1.2. and 5.1.3. above, we first examined the expression pattern of *Hbb-γ* and *Hbb-β* across development. We observed that the pattern of expression follows a different trajectory compared to the erythrocyte lineage. Figure 8B summarizes the results from averaging 129S6 males and females together at the four stages mentioned above using the logarithmic expression in order to show changes between the RQ of *Hbb-γ* and *Hbb-β* in the healthy brain.

First, we observed that the expression of *Hbb-β* was much higher than *Hbb-γ* across the developmental stages tested. The gradual decrease in the pattern of expression of the fetal *Hbb-γ* with higher levels prenatally declining in postnatal stages appears to be similar to what was found in the blood. Surprisingly, in contrast to the erythrocyte lineage, the expression pattern of the adult *Hbb-β* was high during prenatal stages and declined slightly postnatally between E16 and P25.

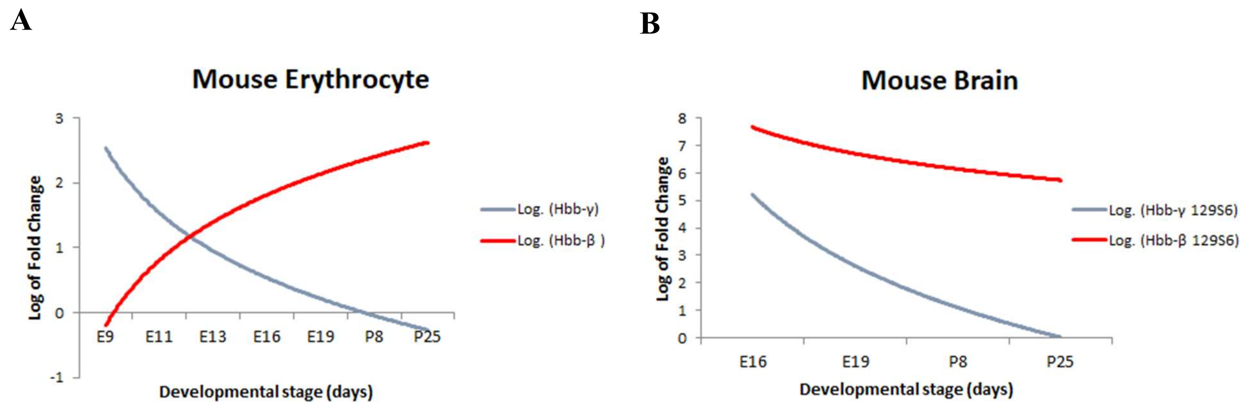


Figure 8. Comparison between gene expression profiles of *Hbb-γ* and *Hbb-β* in the mouse erythrocyte lineage and the brain. All expressions were standardized against P25 *Hbb-γ* expression, and then the logarithmic expression was plotted in order to observe changes between *Hbb-γ* and *Hbb-β* expression. **A)** Expression of *Hbb-γ* and *Hbb-β* across development in the erythrocyte lineage, **B)** Expression of *Hbb-γ* and *Hbb-β* across brain development. The starting point for *Hbb-β* was relatively higher for the brain than erythrocytes at the same stage. *Hbb-γ* showed a similar trend across neurodevelopment and erythropoiesis. Mouse erythrocyte data is adapted from Sankaran and colleagues, 2010. Mouse brain data correspond to control 129S6 samples that represent at least two independent litters, and n is between 5-14 for each stage.

5.1.6. The effect of COX2 deficiency on the expression of hemoglobin genes

To evaluate the effect of COX2 deficiency on the expression of *Hbb-γ, Hbb-β* and *Hba-α* we also plotted the logarithm value from COX2-KI and 129S6 mice from each developmental time point and separated them by sex (Figure 9). The expression of the fetal *Hbb-γ* in the 129S6 wild type male and female showed a trend with relatively higher levels in prenatal stages, which tended to decline postnatally. The trend for COX2-KI males showed that the pattern of *Hbb-γ* expression across the prenatal development was very similar to the 129S6 males (Figure 9A). We observed that this pattern changed postnatally in the COX2-KI males for postnatal stages (Figure 9A). In the COX2-KI females, the expression pattern of *Hbb-γ* prenatally was very similar to the 129S6 females with an increased in the trend for COX2-KI females in P25 (Figure 9B). The expression

pattern of the adult *Hbb-β* in the COX2-KI animals follows the same pattern across development with a slight increase in the trend for COX2-KI males at P8 (Figure 9A). *Hba-α* expression seemed to very similar to *Hbb-β* expression levels throughout development with a slight decline in the expression pattern at postnatal stages (Figure 9).

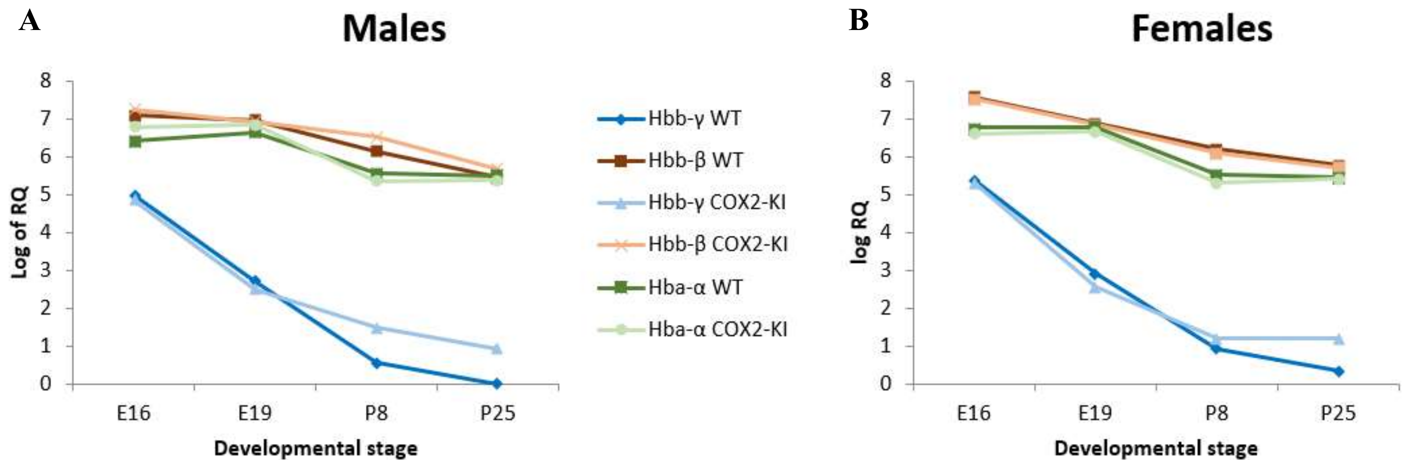


Figure 9. Abnormal COX2/PGE2 pathway affects the expression of hemoglobin isoforms (*Hbb-γ, Hbb-β,* and *Hba-α*) across neurodevelopment. All expressions were standardized against the expression of *Hbb-γ* 129S6 wild-type (WT) at P25. The logarithm of the expressions was plotted in these graphs to observe overall changes between the WT and the COX2-KI for each isoform divided by sex: **A)** Male data, **B)** Female data. *Hbb-γ* levels were lower compared to the other two isoforms. *Hbb-β* and *Hba-α* seemed to have similar expression levels. COX2 deficiencies affected *Hbb-γ* mainly postnatally, while Hbb-β expression was only affected by dysregulations of COX2 at P8. *Hba-α* expression was more affected in males by COX2 deficiencies prenatally and postnatally.

5.2.1. Protein expression for the hemoglobin isoforms at postnatal stage P25

Our next objective was to determine the expression of Hbb-γ, Hbb-β, and Hba-α in the brain at the protein level. We also investigated potential sex-dependent effects in the healthy brain and mice lacking the COX2 enzyme. Western blots were conducted in mouse brain samples in order to quantify the changes in protein expression for each hemoglobin isoform. Expression of GAPDH, which is a housekeeping protein, was determined and used as a control for this study. The values were standardized against the 129S6 male results (fold change for 129S6 male is 1). This study will focus on one postnatal developmental stage: P25. Based on previous studies conducted in erythrocyte lineage and neuronal cell cultures, the protein expression of Hba-α and Hbb-β can be detected as a monomer, dimer, or tetramer. Previous studies in dopaminergic cells overexpressing tagged Hba-α and Hbb-β chains found a tetramer band for both isoforms at 64 kDa as well as a dimer (at 34 kDa) (Russo et al., 2013). Another study conducted western blot analysis using brain and whole hemoglobin antiserum that recognizes all hemoglobin isoforms found that hemoglobin can be recognized as monomer bands (16kDa), dimer bands (32kDa) and tetramer bands (64kDa) in the brain (Ritcher et al., 2009). To our knowledge, Hbb-γ protein levels had not been previously studied in the brain. In this study, we evaluated the protein expression for all three separate hemoglobin isoforms in male and female wild-type and COX2 deficient mice at P25.

5.2.2. Protein expression of Hbb-γ at P25

We characterized the expression levels of Hbb-γ in the brain at postnatal stage P25. Our western blot analysis showed two distinct bands corresponding to the Hbb-γ dimer and tetramer structures (Figure 10A). A two-way ANOVA was conducted for Hbb-γ dimer level for sex ($F_{(1, 8)}$=17.64, p=0.003), condition ($F_{(1, 8)}$=0.7064, p=0.4251) and their interaction ($F_{(1, 8)}$=14.59, p=0.0051). The Hbb-γ dimer level showed no sex-dependent significant differences for 129S6

animals (Fold change=0.96 for 129S6-F, Fold change=1 for 129S6-M, $p>0.999$). However, the COX2-KI males had significantly higher expression than the COX2-KI females (Fold change=1.46 for COX2-KI-M, Fold change=0.66 for COX2-KI-F; $p=0.0028$). No significant differences were found between the COX2-KI males and the 129S6 males (Fold change=1.46 for COX2-KI-M, Fold change=1 for 129S6-M; $p=0.0657$) or the COX2-KI females and the 129S6 females (Fold change=0.66 for COX2-KI-F, Fold change=0.96 for 129S6-F; p=0.4096) (Figure 10A and B).

A two-way ANOVA was completed for Hbb-γ tetramer level and no significant difference was found for sex ($F_{(1, 8)}=4.067$, $p=0.0785$), condition ($F_{(1, 8)}=2.73$, $p=0.1371$) or the interaction between these two factors ($F_{(1, 8)}=1.003$, $p=0.3459$) (Figure 10A and C). Thus, no follow-up with additional pairwise comparisons was required.

Overall, there is a sex-dependent upregulation only for the COX2-KI males for the Hbb-γ dimer at P25 with no differences in the tetramer level.

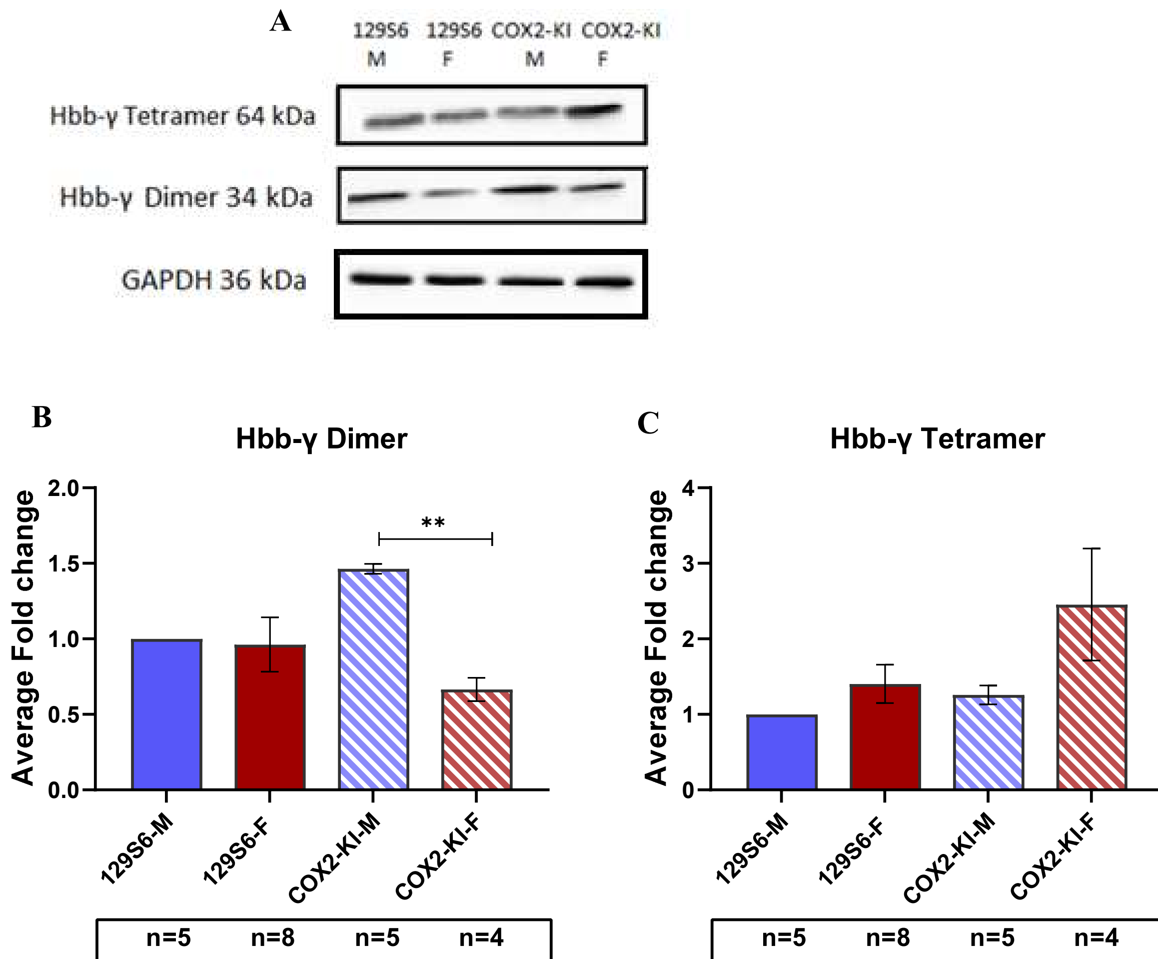


Figure 10. Protein expression analysis for hemoglobin gamma (Hbb-γ) at P25. Results were standardized against the 129S6 wild-type male (129S6-M Fold change=1). GAPDH was used as a control to determine changes in protein levels. There were three independent experiments which represent three technical replicates. Animals were taken from at least three litters, and n value was between 4-11 mice per condition; specific n values are given in the figure. Statistically significant differences are marked '**' for $p<0.01$ and '***' for $p<0.001$. Error bars represent ±SEM values. A) Western blot membrane for Hbb-γ dimer, and tetramer and GAPDH. Fold changes for the different conditions for B) Hbb-γ dimer, and C) Hbb-γ tetramer.

5.2.3. Protein expression of Hbb-β at P25

Previous studies in dopaminergic cell lines found expression of Hbb-β as a monomer, dimer, and tetramer (Russo et al., 2013). Here, we used western blot analysis to assess the Hbb-β

expression in the whole brain at P25 in the 129S6 and COX2-KI males and females. We confirmed that Hbb-β expression showed the presence of monomer, dimer, and tetramer structures (Figure 11A).

A two-way ANOVA was completed for Hbb-β monomer level for sex ($F_{(1, 8)}$=1.545, *p*=0.2491), condition ($F_{(1, 8)}$=83.76, *p*<0.0001) and the interaction between these two factors ($F_{(1, 8)}$=0.2486, *p*=0.6315). These results showed that the level of Hbb-β monomer was downregulated in the COX2-KI animals, but it was not sex-dependent.

A two-way ANOVA was conducted for Hbb-β dimer level for sex ($F_{(1, 8)}$=82.67, *p*<0.0001), condition ($F_{(1, 8)}$=26.14, *p*<0.0001) and their interaction ($F_{(1, 8)}$=0.0439, *p*=0.84). These results showed that the COX1-KI animals had an overall significant reduction of the Hbb-β dimer, with significant differences between sex for both 129S6 and COX2-KI animals.

A two-way ANOVA was completed for Hbb-β tetramer level for sex ($F_{(1, 8)}$=19.05, *p*=0.0024), condition ($F_{(1, 8)}$=12.70, *p*=0.0074) and their interaction ($F_{(1, 8)}$=21.03, *p*=0.0018). The Hbb-β tetramer level did not show significant sex-dependent differences between the 129S6 female and male mice (Fold change=1.132 for 129S6-F, Fold change=1 for 129S6-M, *p*>0.999) (Figure 11A and D). However, sex differences were found for the COX2-KI mice with the females having a higher level than the males (Fold change=5.879 for COX2-KI-F, Fold change=0.5206 for COX2-KI-M; *p*=0.0014). We found no significant difference btween the Hbb-β tetramer level in the COX2-KI males and the 129S6 males (Fold change=0.5206 for COX2-KI-M, Fold change=1 for 129S6-M; *p*>0.999). On the other hand, the level in COX2-KI female mice was significantly upregulated in comparison to the 129S6 females (Fold change=05.879 for COX2-KI-F, Fold change=1.132 for 129S6-F; *p*=0.0030). These results showed that COX2 deficiency affected the Hbb-β tetramer level in COX2-KI.

In summary, we found that in the brain at P25 Hbb-β appeared as a monomer, dimer, and tetramer structure. We observed that in COX2 deficient animals, the levels of monomer and dimer were significantly reduced, but the tetramer level was increased in COX2-KI females only.

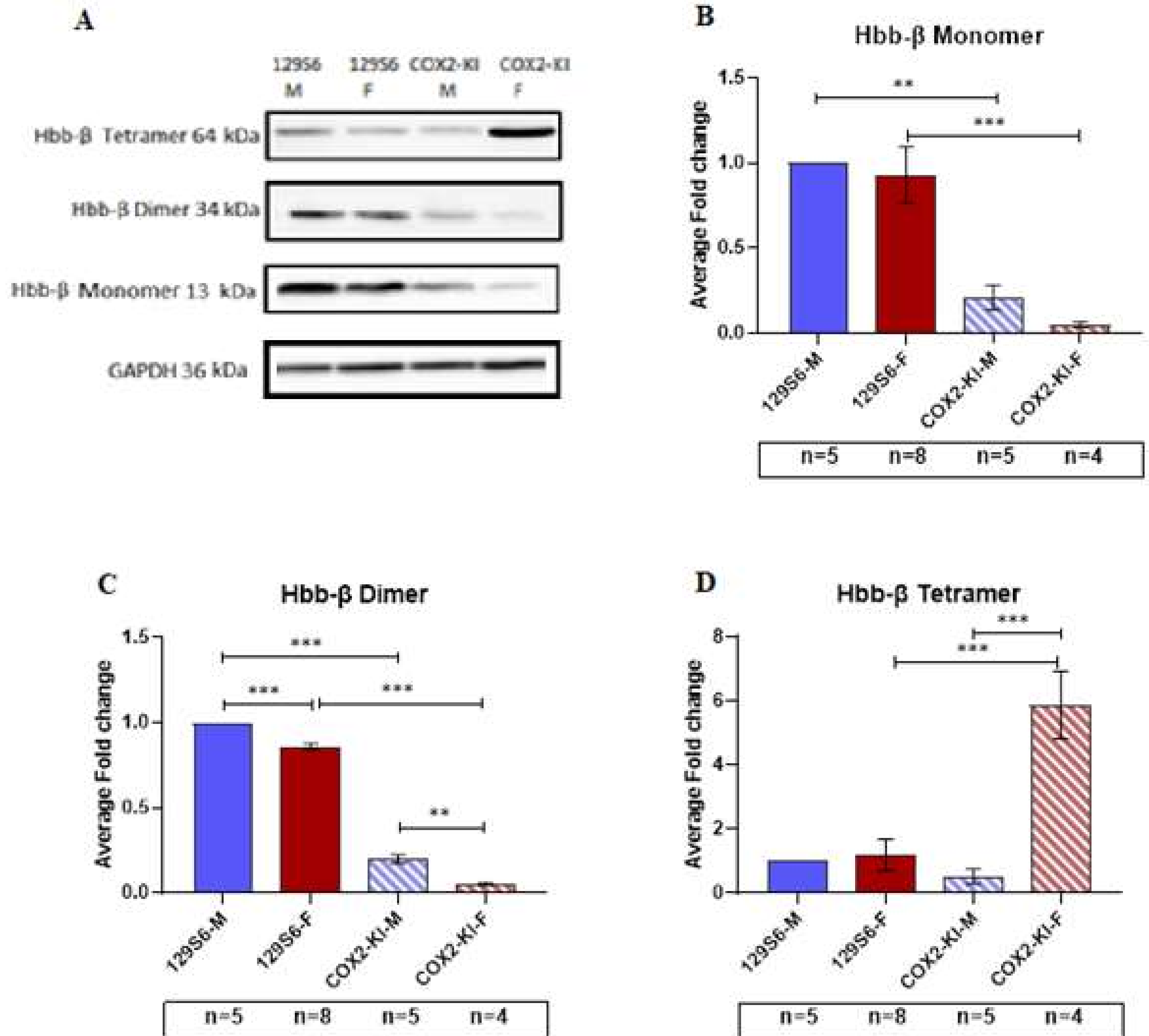


Figure 11. Protein expression analysis for hemoglobin beta (Hbb-β) at P25. Results were standardized against the 129S6 wild-type male (129S6 Male Fold change=1). GAPDH was used as the control to determine changes in protein levels. There were three independent experiments which represent three technical replicates. Animals were taken from at least three litters, and n value was between 4-11 mice per condition; specific n values are given in the figure. Statistically significant differences are marked '**' for $p<0.01$ and '***' for $p<0.001$. Error bars represent ±SEM values. A) Western blot membrane for Hbb-β monomer, dimer, and tetramer and GAPDH,

and Fold changes for the different conditions for B) Hbb-β monomer, C) Hbb-β dimer, and D) Hbb-β tetramer.

5.2.4. Protein expression of Hba-α at P25

Similarly to Hbb- β, Hba-α has been analyzed in dopaminergic cell cultures where a tetramer and monomer structure was identified (Russo et al, 2013). In this study, Hba-α showed two distinct bands in the brain at P25 corresponding to the size of monomers and tetramers (Figure 12A).

A two-way ANOVA for Hba-α monomer level demonstrated no significant differences for sex ($F_{(1, 8)}=0.3053$, $p=0.5957$), condition ($F_{(1, 8)}=0.1652$, $p=0.6950$) or their interaction ($F_{(1, 8)}=2.496$, $p=0.1528$) (Figure 12A and B). Consequently, no additional pairwise comparisons were conducted for the level of Hba-α monomer.

A two-way ANOVA was also conducted for Hba-α tetramer level for sex ($F_{(1, 8)}=10.78$, $p=0.0111$), condition ($F_{(1, 8)}=4.818$, $p=0.0595$) or their interaction ($F_{(1, 8)}=11.85$, $p=0.0088$). Additional pairwise comparisons for the Hba-α tetramer showed no sex-dependent significant differences in the 129S6 animals (Fold change=0.9797 for 129S6-F, Fold change=1 for 129S6-M, $p>0.999$) (Figure 12A and C). However, there was a significant difference between COX2-KI males and females with males showing lower levels than females (Fold change=0.2794 for COX2-KI-M, Fold change=1.139 for COX2-KI-F; $p=0.0086$). We also observed that the COX2-KI male has a significantly lower level than the 129S6 male (Fold change=0.2794 for COX2-KI-M, Fold change=1 for 129S6-M, $p=0.0242$) with no difference between the COX2-KI females when compared to the 129S6 females (Fold change=1.139 for COX2-KI-F, Fold change=0.9797 for 129S6-F, $p=0.0242$).

Overall, we observed the presence of Hba-α monomer, and tertamer in the mature brain. There was no change in the Hba-α monomer level in all animals tested. However, we found sex-dependent differences in Hba-α tertamer with the COX2-KI males having significantly reduced level.

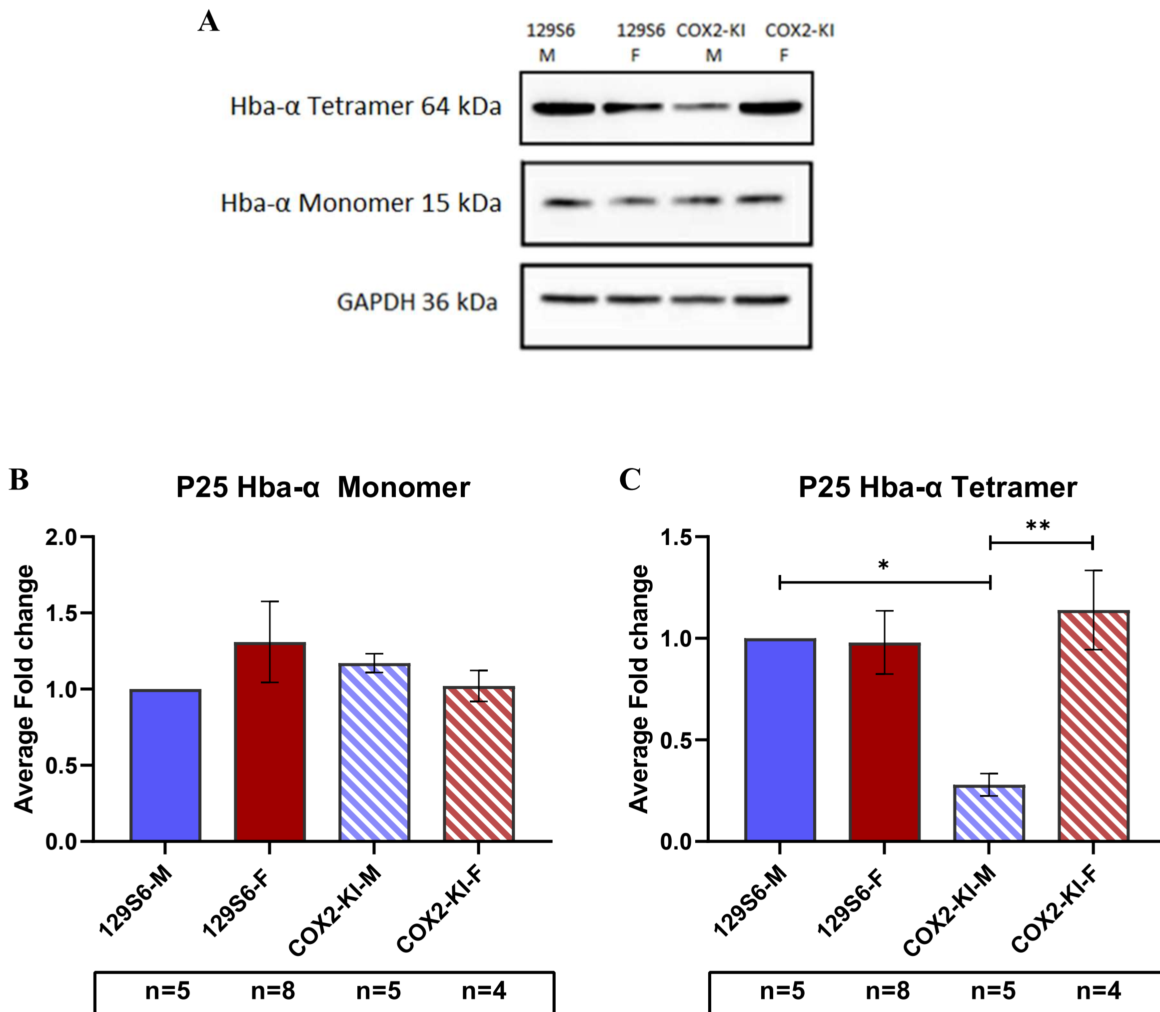


Figure 12. Protein expression analysis for hemoglobin alpha (Hba-α) at P25. Results were standardized against the 129S6 male (129S6-M Fold change=1). GAPDH was used as the control to determine changes in protein levels. There were three independent experiments which represent three technical replicates. Animals were taken from at least three litters, and n value was between 4-11 mice per condition; specific n values are given in the figure. Statistically significant differences are marked '**' for $p<0.01$ and '***' for $p<0.001$. Error bars represent ±SEM

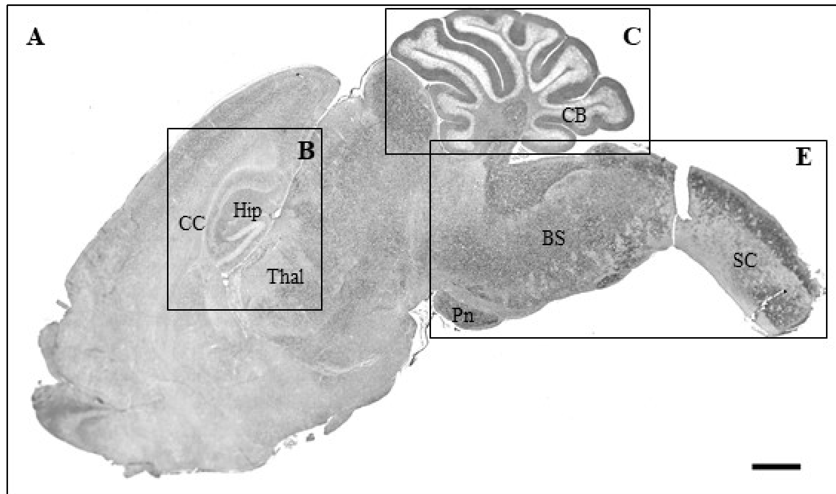
values. A) Western blot membrane for hemoglobin alpha monomer, and tetramer and GAPDH. Fold changes for the different conditions for B) Hba-α monomer, and C) Hba-α tetramer.

5.3.1 Study 2. Regional expression of hemoglobin isoforms in the mouse brain

The expression analysis of the three individual isoforms Hbb-β, Hbb-γ, and Hba-α in the healthy brain has not been very well studied before. In this study, we aimed to determine the expression of individual hemoglobin isoforms in the mouse brain using immunohistochemical techniques. With confocal microscopy, we evaluated the regional and subcellular localization of all three isoforms in the mature mouse brain at P25.

5.3.2. Regional localization of Hbb-γ in the mouse brain at P25

Our western blot analysis surprisingly showed a detectable level of the fetal Hbb-γ in the mature brain at the P25 stage. Therefore, we aimed to determine the specific regional expression of this protein in the brain. Using immunohistochemistry analysis, we observed that Hbb-γ was expressed ubiquitously in different regions of the brain with similar patterns as seen in Hba-α (see below in section 5.3.4.). We found specific localization in the cerebellum, brain stem, pontine nuclei, and spinal cord (Figure 13A, C, E). Specifically in the cerebellum, Hbb-γ appeared to be expressed in the molecular layer, Purkinje cell layer, and within the cytosol of Purkinje cells (Figure 13C, D). No expression was observed in the corpus callosum (Figure 13B). Control with no primary antibody was shown in Figure 13 F.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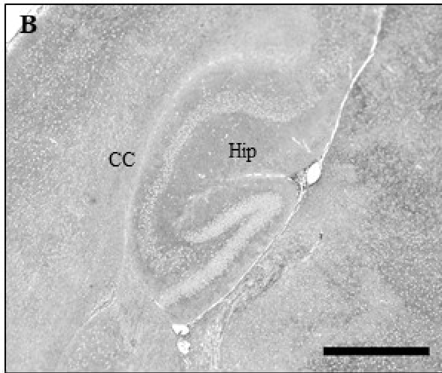
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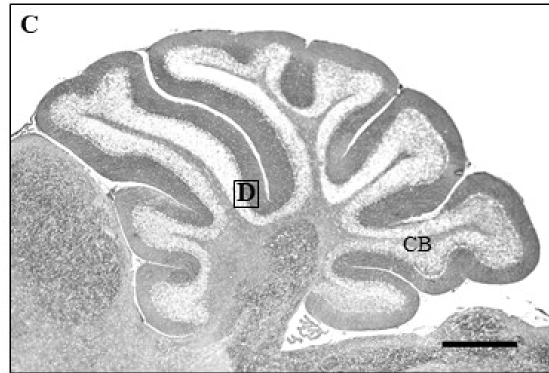
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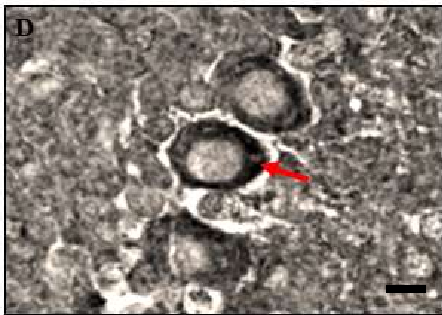
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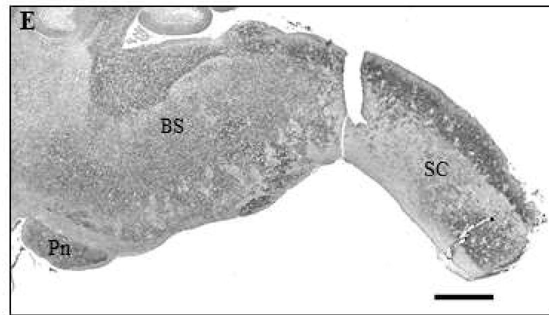
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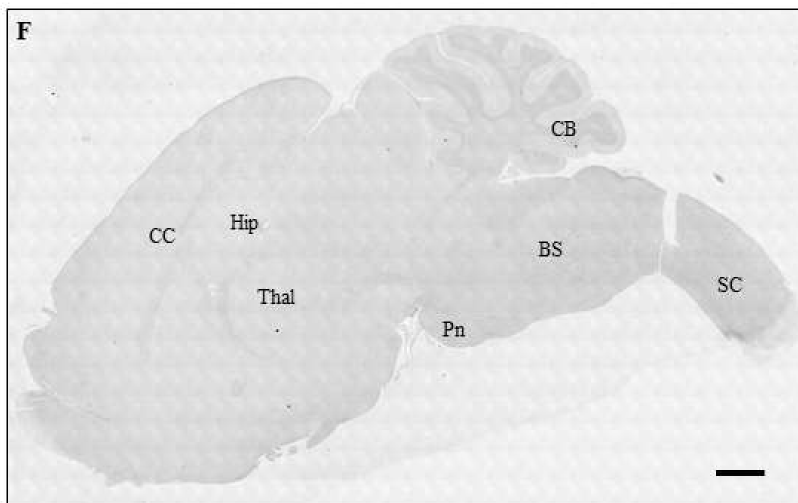
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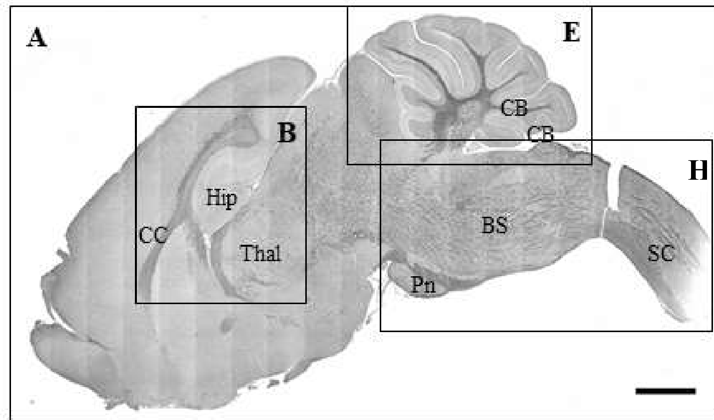
Figure 13. Immunohistochemical detection of Hbb-γ expression in the mouse brain. Brain sections corresponding to 129S6 males were cut sagittally. Hbb-γ expression was found in different cells of the brain across various regions. **A)** Whole brain staining showing the different regions with hippocampus marked as Hip, thalamus marked as Thal, corpus callosum marked as CC, cerebellum marked as CB, brain stem marked as BS, pontine nuclei marked as Pn, and spinal cord marked as SC, **B)** No expression was observed in corpus callosum **C)** Expression in the cerebellum, **D)** Purkinje cells of the cerebellum stained for Hbb-γ showing its expression in the cytosol. Cytosolic localization of Hbb-γ is marked by a red arrow in the Purkinje cells of the cerebellum. **E**) Expression in the brain stem and spinal cord, **F)** Negative controls with the absence of primary antibodies for the whole brain. Scale bars are shown in each image and correspond to 1000 µm (A, F), 500 µm (B, C, E) and 10 µm (D).

5.3.3. Regional localization of Hbb-β in the mouse brain at P25

The expression of Hbb-β was also observed in various regions of the P25 mouse brain (Figure 14A). We found a distinct pattern of expression compared to Hba-α and Hbb-γ, where Hbb-β seemed to be expressed mainly in white matter (Figure 14). However, similarly to Hba-α and Hbb-γ, Hbb-β staining was also observed in the Purkinje cell layer (Figure 14E). Interestingly, no expression was observed in Purkinje cells (Figure 14F). In addition, we observed a distinct pattern of expression in fibers of the corpus callosum (Figure 14B), white matter layer and Purkinje cell layer of the cerebellum (Figure 14E), brain stem (observed expression in longitudinal tracks) (Figure 14J), spinal cord (Figure 14H), pontine nuclei (longitudinal fasciculus) (Figure 14I), mesencephalon (Figure 14D), and thalamus (stria medullaris) (Figure 14C). Control with no primary antibody was shown in Figure 14K.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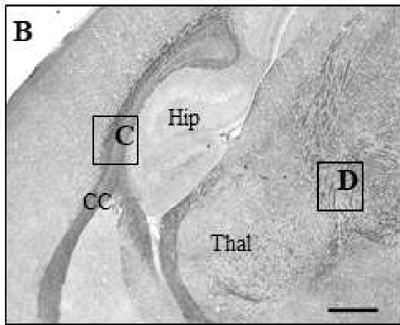
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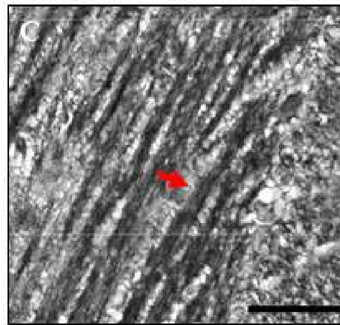
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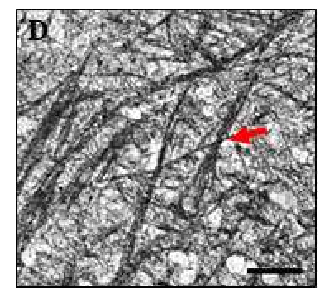
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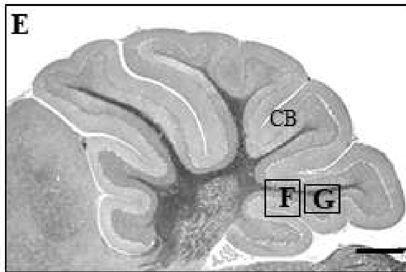
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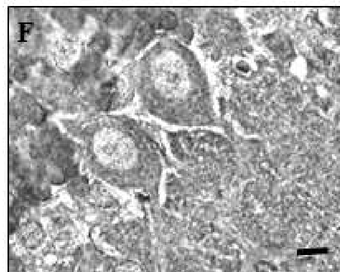
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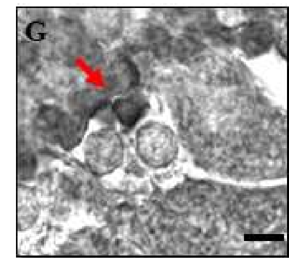
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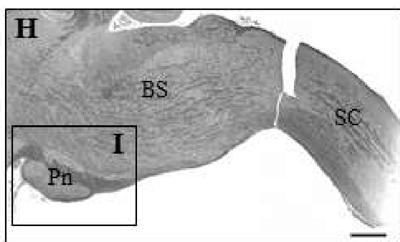
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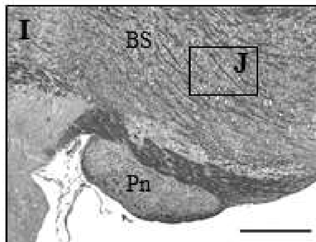
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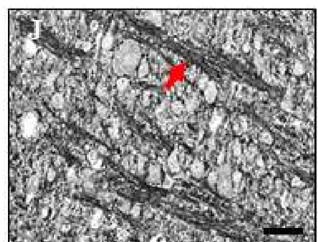
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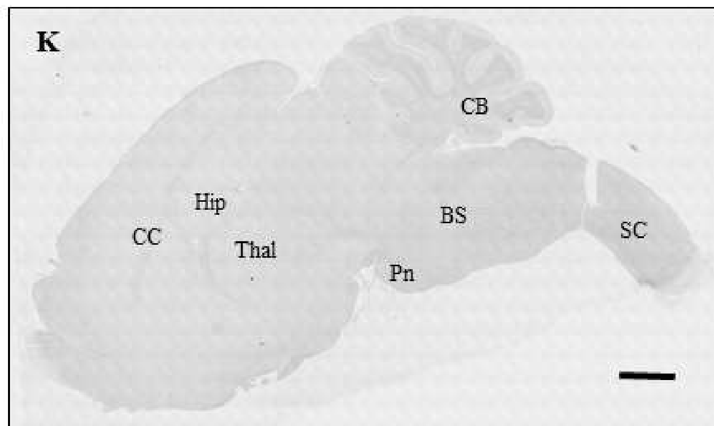
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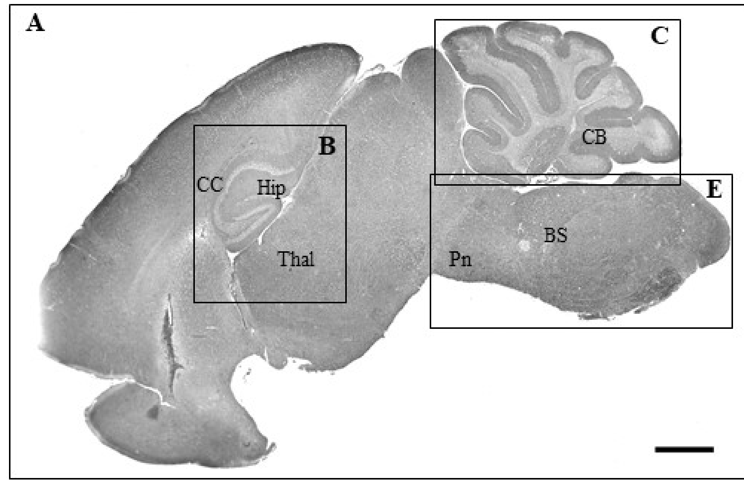
Figure 14. Immunohistochemical detection of Hbb-β expression in the mouse brain. Brain sections taken from 129S6 males were cut sagittally. Hbb-β expression was found in different regions. **A)** Whole brain staining showing the different regions with hippocampus marked as Hip, thalamus marked as Thal, corpus callosum marked as CC, cerebellum marked as CB, brain stem marked as BS, pontine nuclei marked as Pn, and spinal cord marked as SC, **B)** Expression in the hippocampus, thalamus and corpus callosum, **C)** Specific expression in the corpus callosum, **D)** Specific expression in the mesencephalon; red arrows were used to indicate the fibers, **E)** Expression in the Purkinje cell layer and white matter of the cerebellum**, F)** Purkinje cells of the cerebellum did not express Hbb-β either in the cytosol or axon, **G)** Expression in the Purkinje cell layer of the cerebellum, **H)** Expression in the brain stem and spinal cord, **I)** Expression in the longitudinal fasciculus of the pontine nuclei and in fibers of brain stem, **J)** Specific expression in the fibers of the brain stem; red arrow was used to indicate the fiber expression, **K**) Negative controls with the absence of primary antibodies for the whole brain. Scale bars are shown in each image and correspond to 1000 μm (A, K), 500 μm (B, E, H, I) and 10 μm (C, D, F, G, J).

5.3.4. Regional localization of Hba-α in the mouse brain at P25

We observed that Hba-α was ubiquitously expressed across various regions of the P25 mouse brain similarly to what we found for Hbb-γ (Figure 15A). We found that Hba-α expression was abundant in cells of the cerebellum, particularly Purkinje cells, brain stem, and spinal cord (Figure 15C, D and E). Within the cerebellum, Hba-α appeared to be expressed in the molecular layer, Purkinje cell layer and within the cytosol and axon of Purkinje cells (Figure 15C, D). In contrast to Hbb-β, no expression was observed in the corpus callosum (Figure 15B). Control with no primary antibody was shown in Figure 15 F.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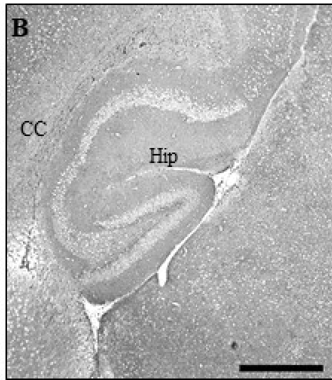
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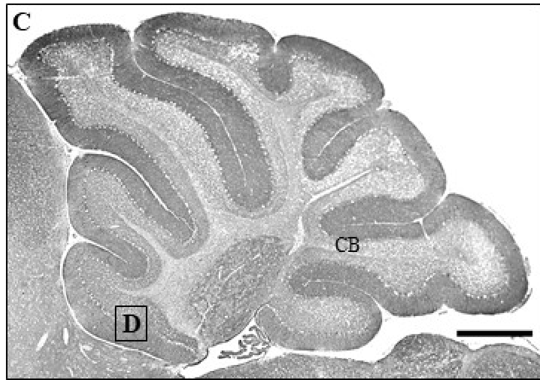
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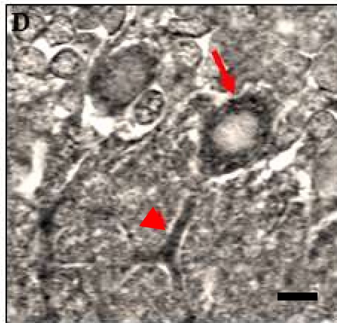
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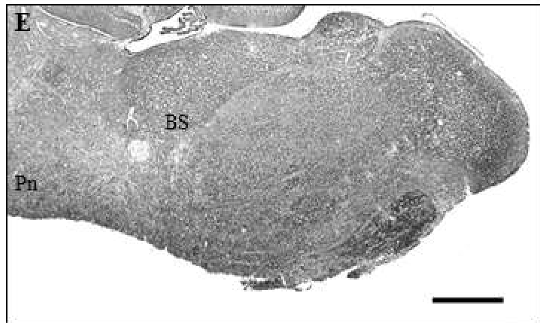
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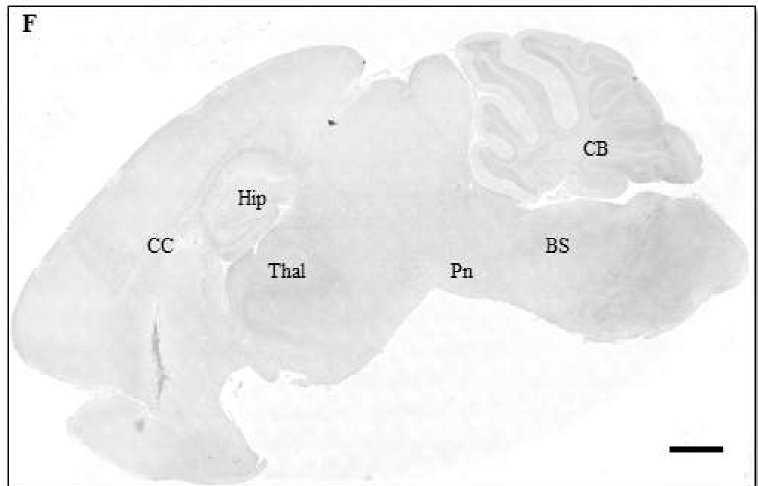
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Figure 15. Immunohistochemical detection of Hba-α expression in the mouse brain. Brain sections corresponding to 129S6 males were cut sagittally. Hba-α is widely expressed in different cells of the brain, mainly in the cytosol. **A)** Whole brain staining showing the different regions with hippocampus marked as Hip, thalamus marked as Thal, corpus callosum marked as CC, cerebellum marked as CB, brain stem marked as BS, pontine nuclei marked as Pn, and spinal cord marked as SC, **B)** No expression was observed in the corpus callosum, **C)** Expression in the cerebellum, **D)** Purkinje cells of the cerebellum stained for Hba-α showing its expression in the cytosol and axon. Cytosolic localization is marked by a red arrow, and axonal localization is marked by an arrowhead in the Purkinje cells of the cerebellum. **E**) Expression in the brain stem and spinal cord, **F)** Negative control with the absence of primary antibodies for the whole brain. Scale bars are shown in each image and correspond to 1000 μm (A, F), 500 μm (B, C, E) and 10 μm (D).

6. Discussion

In this study, we investigated the gene expression profiling of three hemoglobin isoforms (*Hba-α, Hbb-β*, and *Hbb-γ)* across four neurodevelopmental stages of the mouse brain. Overall, we found that there are sex differences for the hemoglobin expression of these isoforms in several developmental stages and that deficits in the COX2/PGE2 pathway lead to changes in the expression of these isoforms. We also conducted protein expression analysis on mature postnatal brain samples for Hba-α, Hbb-γ, as well as Hbb-β. We observed differential expression of monomer, dimer, and tetramer structures between the three isoforms. Hba-α showed expression of monomer and tetramer, Hbb-γ showed expression of dimer and tetramer, whereas Hbb-β showed all three structures. Finally, we found that these proteins are also localized differently across several brain regions at P25.

Our lab has been investigating the link between abnormalities in the COX2/PGE2 signaling pathway and Autism Spectrum Disorders (ASDs). The link has been previously documented in clinical studies and has been attributed to various genetic and environmental factors impacting the level of PGE2. For example, many environmental risk factors such as air pollution, pesticides, consumer products, and drugs like aspirin and misoprostol can dysregulate PGE2 levels and have been observed in many ASD cases (Calderon-Garciduenas et al., 2007, 2014; Wong & Crawford, 2014; Wong et al., 2015; Roberts et al., 2007; Rauh et al., 2012). We have already provided molecular and behavioural evidence in mice exposed to higher levels of PGE2 or lacking PGE2 producing enzyme (COX2-KI) for the possible mechanisms that might lead to brain pathology that results in ASD. This study stems from our previous whole genome microarray study done in COX2-knockout mice that discovered upregulation of hemoglobin genes (*Hbb-β* and *Hbb-γ*) in COX2-Knockout males but not females in prenatal stages (Rai-Bohgal et al., 2018). In the

following sections, I will discuss the importance of my findings and what it means for brain pathologies such as ASD.

6.1. Gene expression profile of hemoglobin isoforms across brain development

We conducted a gene expression profiling of the three hemoglobin isoforms across mouse neurodevelopment. Overall, sex differences in gene expression were found between male and female wild-type animals for these isoforms, primarily during prenatal stages of development. Additionally, compared to wild-type controls, effects of PGE2 deficits in the COX2-KI animals were detected for the three hemoglobin isoforms, mainly during postnatal stages of development. More specifically, we found significant upregulation of the fetal hemoglobin, *Hbb-γ,* at postnatal stages in the COX2-KI animals, whereas for *Hba-α* there was only downregulation in males postnatally, and *Hbb-β* had upregulation and downregulation depending on the sex of the COX2-KI.

6.1.1. Expression of *Hbb-γ* across neurodevelopment

Through our gene expression profiling study, we found significant sex differences only at prenatal stages E16 and E19 for *Hbb-γ* where 129S6 females had higher expression than the males. This sex difference was maintained for COX2-KI mice but only at E16, whereas at E19 there were no sex-dependent differences for the COX2-KI animals. This was likely due to the significant downregulation found in COX2-KI females when compared to the 129S6 control females. Collectively, these results suggest that the COX2/PGE2 pathway might play an important role in regulating the sex-dependent expression of *Hbb-γ* in the prenatal brain and that deficits in the COX2/ PGE2 pathway may affect *Hbb-γ* level at E19 in females.

The main hemoglobin tetramer structure during prenatal stages in the blood is formed by two alpha chains (2x Hba-α) with two gamma chains (2x Hbb-γ) or α2γ2 (Cantú & Philipsen, 2014; Manning et al., 2007) and has a stronger affinity for oxygen compare to its postnatal adult form (Hbb-β) (Walker & Turnbull, 1954). The adult hemoglobin structure is mainly formed by two alpha chains (2x Hba-α) and two beta chains (2x Hbb-β) (α2β2) (Manning et al., 2007). In humans, high levels of the fetal hemoglobin structure α2γ2 have been found in the blood during prenatal stages, due to the high expression of *Hbb-γ* (Stamatoyannopoulos, 2005). Although the role of hemoglobin in brain cells is still not known, downregulation of *Hbb-γ* in prenatal stages as seen in our COX2-KI females could affect the oxygen level in the fetal brain.

Interestingly, the most unexpected result in this study was the postnatal expression of the fetal *Hbb-γ* found in brain samples from our mouse model. *Hbb-γ* is typically found at high levels in the blood prenatally, and its postnatal expression declines to very low levels (Cantú & Philipsen, 2014; Manning et al., 2007) resulting from the fetal-to-adult genetic switch which occurs around E11 in mice (Mcgann et al., 2013). The expression of *Hbb-γ* in erythrocytes is silenced before birth, followed by an exponential increase in the expression of the adult hemoglobin form *Hbb-β* (Xu, Hong, & Wang, 2009). During adulthood, the expression of *Hbb-γ* gene has been found to account for less than 1% of the relative expression of all hemoglobin isoforms in blood samples obtained from both adult humans and mice (Xu et al., 2009; Mcgann et al., 2013).

To our knowledge, this is the first study that shows the expression of *Hbb-γ* across neurodevelopment, and in particular, its expression in the postnatal mouse brain. Moreover, we also show that the lack of neuronally expressed COX2 results in age- and sex-specific upregulation of *Hbb-γ* expression. We observed an upregulation of *Hbb-γ* expression in the COX2-KI males at P8 and both COX2-KI males and females at P25. These result show that the COX2/PGE2 pathway

may play an important role in regulating *Hbb-γ* during postnatal brain development. It should be noted that some brain regions, such as the cerebellum are still undergoing development at P8 (Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013). P8 in mice corresponds to infant stages in humans where early signs of ASD-related behaviours have been detected (Koterba, Leezenbaum, & Iverson, 2014; Pressler & Auvin, 2013). Furthermore, there is a relationship between PGE2/COX2 pathway and the masculinization of the brain (Amateau & McCarthy, 2004; Wright, Burks, & McCarthy, 2008). Upregulation of COX2 and COX1 which results in high levels of PGE2 has been found in the developing rat brain for males as a response to increased levels of the major masculinization hormone in the brain called estradiol (Amateau & McCarthy, 2004). Moreover, COX-inhibitors have been found to impair male sexual behaviour in rats, whereas exposure of PGE2 to newborn female rats can cause male sexual behaviours (Amateau & McCarthy, 2004). Additionally, as COX2-KI males were found to exhibit changes in *Hbb-γ* expression during a developmental stage that correlates with the onset of ASD-related behaviours in humans (Koterba et al., 2014; Pressler & Auvin, 2013; Semple et al., 2013), it is possible these sex-dependent differences in Hbb-γ expression may relate to the increased susceptibility of males to various neurodevelopmental disorders (Fombonne, 2003; Rivet & Matson, 2011). Furthermore, this suggests that *Hbb-γ* may have the potential to serve as a useful biomarker which may aid in the early detection of certain neurodevelopmental disorders.

Overall, these results show that in the mouse brain *Hbb-γ* is normally expressed at high levels prenatally, and its expression is also present at detectable levels during postnatal development. Moreover, deficiency of the PGE2 producing enzyme leads to upregulations of this gene at specific prenatal and postnatal stages, potentially affecting the male and female brain differently.

Upregulation of *Hbb-γ* expression in postnatal stages of the erythrocyte lineage has been found in pathological conditions such as sickle cell anemia patients were it modulates the severity of this disease (Edoh, Antwi-Bosaiko, & Amuzu, 2006; Metaxotou-Mavromati et al., 1982). This disease is characterized by abnormalities in hemoglobin shape caused by a single base pair substitution in the gene encoding the human β-globin subunit, which affects their oxygen-carrying function (Bunn, 1997). Moreover, treatments for sickle cell anemia have been focused on increasing the levels of normal *Hbb-γ* in order to raise the oxygen levels for the different cells (Platt et al., 1984; Akinsheye et al., 2011). Thus, maybe one of the reasons that we observed high levels of *Hbb-γ* in our COX2-KI animals might be to increase oxygen levels in the cells.

One possible explanation for alterations in the expression of the various hemoglobin isoforms observed in our COX2-KI model may relate to the transcription factor BCL11A. BCL11A is expressed across a range of different tissues and its role in erythropoiesis is to regulate *Hbb-γ* expression by silencing it during the fetal to adult genetic switch (Sankaran et al., 2008). The persistent high expression of *Hbb-γ* in adulthood in blood has been associated with modification of BCL11A (Sankaran et al., 2008). Furthermore, BCL11A is not only found in erythrocytes but also in the central nervous system, where it can form a complex with CASK (calcium/calmodulin-dependent serine protein kinase) to regulate axon outgrowth and branching (Kuo, Hong, Chien, & Hsueh, 2010). Interestingly, it has been shown in mouse models that *BCL11A* haploinsufficiency (one allele of the gene has a variation and the gene is not able to function normally) affected cognition, caused deficits in social behavior, and resulted in microcephaly (Dias et al., 2016). *De novo* mutations of this gene have also been found in ASD cases (De Rubeis et al., 2014; Iossifov et al., 2012). *BCL11A* dysregulation has also been implicated in other neurodevelopmental disorders such as developmental delay, schizophrenia, and

attention deficit hyperactivity disorder (ADHD) which suggests that *BCL11A* might be a critical neurodevelopmental gene as well as an important erythrocyte developmental regulator (Basak et al., 2015). Furthermore, a study in ASD patients with BCL11A haploinsufficiency found increased levels of *Hbb-γ* gene expression in blood samples (Basak et al., 2015). This is of particular interest to our study since we also found upregulated levels of *Hbb-γ* in the COX2-KI mice. Future studies should focus on investigating the regulation of *BCL11A* expression in the COX2-KI animals to explain a mechanism for the upregulation of *Hbb-γ* in our model.

6.1.2. Expression of *Hbb-β* and *Hba-α* across neurodevelopment

In this study, the expression of the adult hemoglobin isoform Hbb-β was found to exhibit an unexpected trend throughout development. More specifically, Hbb-β was found to be expressed at high levels in the healthy brain of 129S6 wild-type mice during prenatal stages (E16 and E19). Furthermore, its expression was found to decline slightly throughout development, with lower levels being detected during postnatal stages (P8 and P25). This pattern of expression is different from what was previously observed in the mice erythrocyte lineage where *Hbb-β* expression begins to increase at prenatal stages and reaches a plateau state by E19, which continues through adulthood (Mcgann et al., 2013).

We also show that the lack of COX2 affects the expression of *Hbb-β* only in males in an age-specific fashion with downregulation at E16 and upregulation at P8. Moreover, similarly to *Hbb-γ* at that stage, both 129S6 and COX2-KI females had generally higher expression when compared to males. The sex-dependent expression of *Hbb-β* and *Hbb-γ* in the brain requires further investigation to understand how it is differentially regulated in males and females. However, a potential explanation as mentioned previously could be through the connection between sex-hormones and PGE2 levels (Amateau & McCarthy, 2004; Wright et al., 2008) and how increased

or decreased levels of PGE2 can affect the masculinization of the brain (Amateau & McCarthy, 2004). Furthermore, sex hormones have been found to regulate hemoglobin protein levels (Jelkmann W., 2011; Shahani et al., 2009) which could present a link between PGE2 levels, sex hormones, and hemoglobin expression as we will discuss in the protein section 6.2.

At P8, we did not observe sex differences in *Hbb-β* expression in the healthy brain, but it was found in COX2-KI animals. This is likely due to the significant upregulation of this gene in the COX2-KI males and the downregulation of COX2-KI females. At this stage, we found that defects in the COX2 pathway affected the expression of *Hbb-β* in an opposing manner between the COX2-KI males (increased expression) and COX2-KI females (decreased expression). At P25, we did not see any effects in gene expression. These results show that *Hbb-β* expression postnatally is affected by deficits in the COX2/PGE2 pathway only at P8. Dysregulation of this gene in pathologies would be discussed below together with *Hba-α* dysregulation as they have been both investigated together.

In our studies, *Hba-α* expression was showed to remain mostly constant throughout neurodevelopment, which follows what has been shown in the erythrocyte lineage (Sankaran et al., 2010). Moreover, *Hba-α* expression in the prenatal brain has sex difference only at E16 when comparing between the wild-types. This sex difference was lost in the COX2-KI animals at this stage likely due to the upregulation of this gene in the COX2-KI males. Furthermore, at P8 and P25, this gene showed sex-dependent differences between the 129S6 animals with females having less expression of *Hba-α* when compared to the male. The lack of sex-dependent differences in our COX2-KI model as opposed to what was found in wild-types might be due to the significant downregulation observed in the COX2-KI males when compared to 129S6 wild-type males for both stages. These results showed that the expression of *Hba-α* is affected differently in males than

females for the wild-types and that the COX2/PGE2 might play a role in regulating the expression of this gene only for males.

Altered expression of *Hbb-β* and *Hba-α* have been previously reported in neurodegenerative diseases, including Alzheimer's Disease (Vanni et al., 2018; Ferrer et al., 2011), prion disease (Vanni et al., 2018; Barbisin et al., 2014), Multiple System Atrophy (J.D. Mills, Ward, Kim, Halliday, & Janitz, 2016), Multiple Sclerosis (Broadwater et al., 2011; Brown et al., 2016), Parkinson's Disease (Ferrer et al., 2011; Shephard, Greville-Heygate, Marsh, Anderson, & Chakrabarti, 2014) and dementia (Ferrer et al., 2011). In particular, downregulation of *Hbb-β* and *Hba-α* expression was found in macaques during late stages of prion disease, which are defined as transmissible encephalopathies that constitute progressive neurodegenerative disorders (Barbisin et al., 2014). Different variants of prion disease in humans have shown either upregulation or downregulation of *Hbb-β* expression (Vanni et al., 2018). Furthermore, downregulation of *Hbb-β* and *Hba-α* in the brain has also been shown in Alzheimer's disease patients (Vanni et al., 2018; Singh, 2014). It has been hypothesized that these downregulations can lead to an increased amount of free iron that can be toxic to the cell via the generation of reactive oxygen species (Singh, 2014).

Concerning how dysregulation of *Hbb-β* expression might affect the normal brain function, it has been found that overexpression of α and *β*-chains can cause impairments in motor skill learning in mouse (Codrich et al., 2017), suggesting a possible connection between hemoglobin levels and Parkinson's disease (Codrich et al., 2017). Moreover, upregulation of *Hbb-β, Hba-α* has also been associated with aging in mice and humans (Blalock et al., 2003; Chuang et al., 2012). Low levels of blood flow are often found in aged brains and *Hbb-β, Hba-α* expressions have been shown to be regulated by factors that are induced by hypoxia (Iadecola, 2004; Wang & Semenza, 1993), hinting at a connection between upregulation of hemoglobin expression and a response to

the elevated hypoxic environment characteristic of aging brains. Previous research has suggested that the hypoperfusion seen in human aged brains might increase the production of hemoglobin, which could serve as an oxygen reservoir during hypoxia states (Chuang et al., 2012). If this is the case, upregulation of the *Hbb-β* or *Hba-α* expression in the COX2-KI animals could reflect an elevated hypoxic environment in the brain of these animals. Hypoxic states have also been linked to ASD (Gardener, Spiegelman, & Buka, 2009; Van Tilborg et al., 2018). Furthermore, recent studies in rats have associated fetal inflammation and postnatal hypoxia with autism-like behaviours and myelin deficits (Van Tilborg et al., 2018). Collectively, these studies support a link between neurodevelopmental disorders such as autism and hemoglobin dysregulation and suggest that the impact of hemoglobin expression on hypoxic states may underlie their relationship.

6.1.3. Mechanisms by which COX2/PGE2 pathway can affect hemoglobin gene expression

Previous research has linked PGE2 with the vertebrate hematopoietic stem cell system (HSC) (Fisher and Hagiwara, 1984; Lorenz et al., 1999; North et al., 2007). For example, studies conducted on zebrafish found that the stable derivative of PGE2, 16,16-dimethyl-PGE2, increased the number of embryonic stem cell hematopoietic colonies formed (North et al., 2007). Interestingly, the opposite trend was found in studies involving mice with deficiencies of COX2, which found less regeneration of hematopoietic lineage (Lorenz et al., 1999). One possible mechanism by which dysregulation of the COX/PGE2 pathway may impact hemoglobin expression might be through its effect on various transcription factors and kinases that regulate hemoglobin expression. For instance, a whole genome microarray study in our lab using COX2-knockout mice showed changes in gene expression for SOX6, a transcriptional factor that regulates hemoglobin expression (Rai-Bohgal et al., 2018). PGE2 is also known to affect gene expression through PKA and CREB in mice and cell models (Wong et al., 2014) which can potentially affect

the expression of other hemoglobin regulatory genes like BCL11A. Thus, the changes in hemoglobin expression observed in our COX2-KI model may reflect various indirect downstream effects resulting from dysregulation of this pathway. However, the impact of PGE2 on SOX-6 and BCL11A and the encoded proteins still needs to be investigated and confirmed.

Wnt signaling is another crucial pathway during neurodevelopment, and the expression of genes related to this pathway has been found by our lab to be altered by PGE2 using *in vivo* and *in vitro* models (Rai-Bhogal, Wong, et al., 2018; Wong et al., 2014, 2016). Wnt signaling has been linked to HSC regulation in adult bone marrow. Wnt activation is also required for the maintenance of HSC (Congdon et al., 2008). Our previous research has already shown that COX2/PGE2 signaling can crosstalk with the Wnt pathway and regulate expression of its developmental genes (Wong et al., 2016; Bohgal et al., 2017). Therefore, another mechanism by which the COX2/PGE2 pathway is affecting hemoglobin expression could be through the interaction with the Wnt signaling pathway.

6.2. Protein expression for the hemoglobin isoforms at postnatal stage P25

We investigated sex-dependent expression of three hemoglobin isoforms (Hbb-γ, Hbb-β, and Hba-α) at the protein level in the mature healthy mouse brain, as well as in mice lacking COX2. Overall, we found expression of monomer, dimer, or tetramer structures for these isoforms. Hbb-β was the only isoform that had the three structures, whereas Hbb-γ had only dimer and tetramer structures and Hba-α only had monomer and tetramer structures. Among these hemoglobin isoforms, we also found that deficits of COX2/PGE2 pathway exerted the most profound effects in Hbb-β at the protein level. This will be discussed in this section.

In this study, we quantified the sex-dependent expression of Hbb-γ in the postnatal healthy mouse brain at P25, as well as mice lacking COX2. At this stage, we showed that the fetal Hbb-γ protein was expressed as a dimer and tetramer. The expression of the tetramer did not show any sex differences in the wild type or the COX-KI animals. A sex-dependent difference in expression of the dimer was however found between COX2-KI animals, although this effect was not observed in comparisons between male and female wild-type controls. This result indicates that for P25, Hbb-γ dimer level has an interaction effect between sex and deficit in the COX2 pathway that creates that statistical difference between the COX2-KI animals. It is well established that Hbb-γ can form dimers and tetramers with Hba-α in the erythrocyte lineage (Cantú & Philipsen, 2014; Manning et al., 2007). However, it is not yet known whether Hbb-γ dimerizes with Hba-α or if it homodimerizes in the brain. To determine which type of dimers are being formed, further studies using techniques such as co-immunoprecipitation are required.

Hbb-β was previously found to be expressed in dopaminergic cell lines and erythrocytes, where it has been found as a dimer, and tetramer (Russo et al., 2013; Cantú & Philipsen, 2014; Manning et al., 2007). Similarly, we also found Hbb-β dimer and tetramer expression in neuroectodermal NE-4C cells (Appendix A). In the present study, we detected Hbb-β as a monomer, dimer, and tetramer structures in the mouse brain at P25. Notably, Hbb-β was the only hemoglobin out of the three tested to have all three forms in the postnatal brain. Moreover, we found that deficits in the COX2 pathway greatly affected the levels of each structure. More specifically, the monomer and dimer were significantly reduced in both COX2-KI males and females, whereas the tetramer was expressed at a higher level in the COX2-KI female. Sex differences in Hbb-β protein levels were also found, with the wild-types exhibiting sex differences only for the dimer form, whereas the COX2-KI animal had sex differences for the dimer and

tetramer. These findings are significant as our research is the first to identify sex-dependent expression of the adult hemoglobin Hbb-β isoforms and their dimerization patterns in the healthy mouse brain, as well as in the brains of COX2-KI animals.

However, it is still not clear what the functional implications of this differential expression is for males and females in the developing brain. In the blood, sex differences in the expression of the adult tetramer hemoglobin (α2β2) have been previously investigated at the protein level in different species of mammals (including humans and mice), birds, and reptiles with females having lower levels of hemoglobin than males (Williamson, 1916; Murphy, W. G., Tong, E., & Murphy, C., 2010). Interestingly, the opposite trend has been found for the fetal tetramer structure of α2γ2 in erythrocytes where females have higher levels when compared to males (Chang et al., 1997; Dover et al., 1992; Steinberg et al., 1995). It is thought that the sex-dependent differences found in the erythrocyte lineage are due to sex hormone levels and their effect on erythropoiesis (Jelkmann W., 2011; Shahani et al., 2009). Likewise, sex-hormones also affect sexual dimorphism in the brain. Testosterone in the brain during prenatal stages is aromatized to the major masculinizing hormone in neurodevelopment known as estradiol (McCarthy, 2008; Lenz et al., 2013). As mentioned previously, there is a connection between estradiol levels, COX2, and PGE2 production, which leads to masculinization of the brain (Amateau & McCarthy, 2004). This link can explain the sex-dependent differences observed in 129S6 as well as COX2-KI animals for hemoglobin expression.

Hba-α protein has been shown to form different structures in neuronal cells such as monomer, dimer, or tetramer (Russo et al., 2013; Appendix A). In our research, Hba-α was found to be expressed as a monomer and tetramer structure at P25. The monomer did not have any significant changes, whereas the tetramer was found to be significantly downregulated in the

COX2-KI male compared to the wild-type. Hba-α monomer has been shown to be able to function independently, and it can regulate nitric oxide (NO) release in vascular endothelial cells (Straub et al., 2012). Also, Hba-α is known to form very weakly associated homodimers when it is isolated and in high concentrations (Bellelli, Brunori, Miele, Panetta, & Vallone, 2006). These studies give evidence of the potential role of the Hba-α monomer and they also suggest that Hba-α might be able to form homodimers in other tissues such as the brain.

As mentioned above, the vast majority of what is known about hemoglobin comes primarily from research investigating its role in the blood. The main form of adult hemoglobin in erythrocytes are two alpha chains binding with two beta chains ($\alpha_2\beta_2$) with the known role of transporting oxygen (Sankaran et al., 2010). There is also evidence that Hba-α might act independently from Hbb-β protein. For instance, as mentioned previously Hba-α monomers can regulate NO release in vascular endothelial cells independently of Hbb-β (Straub et al., 2012). Hba-α does this function by regulating nitric oxide synthetase (NOS) in endothelial cells (Straub et al., 2012). This is of particular importance since NO acts as a secondary messenger in the brain, which is crucial for learning and memory (Haley, Wilcox, & Chapman, 1992). Thus, abnormal levels of hemoglobin might affect this process due to its connection with nitric oxide. It has also been described in the literature that the hemoglobin tetramer structure can form a complex with nitric oxide and therefore, trap this molecule (Gunasekar et al., 1995). For instance, hemoglobin has been used in experiments to determine the function that nitric oxide might have in the nervous system. It should be noted that NO can also be released in excess as a result of imbalances in oxidative stress and antioxidant capacity (Yui, Kawasaki, Yamada, & Ogawa, 2016). A connection between oxidative stress, imbalances of antioxidants and ASD have been shown in previous research (Yui et al., 2016), as well as a link between NO levels and ASD (Pagalan et al., 2019;

Sweeten, Posey, Shankar, & McDougle, 2004). The connection between hemoglobin and NO could also provide a mechanism of the potential role of hemoglobin in ASD.

As mentioned previously, hemoglobin isoforms have been shown to act independently as it is the case of Hba-α. Additionally, hemoglobin isoforms have also been found to bind to themselves, as it is the case of Hbb-β, which can form homotetramers called HbH (Forget & Bunn, 2013). This tetramer has been found to have a stronger binding affinity for O_2 than the well known $\alpha_2\beta_2$ heterotetramers (Bellelli et al., 2006). The different expression of hemoglobin monomers, dimers, and tetramers found in this study within the three isoforms, might be explained by the independent functions that they might adopt in different brain regions or cell populations (James D. Mills, Kim, Halliday, & Janitz, 2015; Vanni et al., 2018). In fact, isolated Hbb-β has been shown to be able to form unstable homodimers in the blood (Bellelli et al., 2006). These studies suggest that the Hbb-β dimer observed in our study may be binding to a different type of hemoglobin, or alternatively, it could be capable of forming stable homodimers in the brain as opposed to the blood. Further analysis needs to be conducted to determine the proteins that these hemoglobin isoforms are binding to create the different structures.

Even though the function of hemoglobin in the central nervous system remains mostly unclear, one of the potential roles that it could have is in mitochondrial function in the brain. For instance, treatments with mitochondria inhibitors reduced the expression of hemoglobin isoforms in nigral, striatal, and cortical neurons (Ritcher et al., 2009). Furthermore, abnormal overexpression of α- and β-chains in mouse dopaminergic neuroblastoma iMN9D cells altered the expression of genes that are important for mitochondrial activity (Biagioli et al., 2009). Total hemoglobin has also been found in high levels in the mitochondria of substantia nigra neurons and cortex of the brain of Parkinson disease (Shephard, Greville-Heygate, Marsh, Anderson, &

Chakrabarti, 2014; Shephard, Greville-Heygate, Liddell, Emes, & Chakrabarti, 2016), and Multiple Sclerosis patients (Brown et al., 2016). Interestingly, there is considerable evidence showing mitochondrial dysfunction in ASD patients (Griffiths & Levy, 2017; Siddiqui, Elwell, & Johnson, 2016; N. Á. Varga et al., 2018). Given the connection between hemoglobin and mitochondrial function and the fact that there is mitochondrial dysfunction in ASD, it could indicate that abnormalities in hemoglobin expression might be affecting proper mitochondrial function and could be associated to neurodevelopmental disorders such as ASD.

6.3. Regional expression of hemoglobin isoforms in the mouse brain

In this study, we detected differential expression of hemoglobin isoforms in the brain. We were expecting overlapping expressions of these isoforms since they tend to bind and form dimers and tetramers with each other. However, we observed a marked difference in the localization of Hbb-β when compared to the other two isoforms. There seems to be a localization pattern for the isoforms where Hbb-β tends to be highly expressed in the white matter while Hbb-γ and Hba-α seemed to be highly expressed in the grey matter. One notable exception is that all three hemoglobin isoforms appeared to be localized in some overlapping cells found in the Purkinje cell layer of the cerebellum. Analysis with cell-specific markers needs to be conducted to identify these cells as this would provide further insights into regions where all isoforms may be expressed.

To our knowledge, this is the first time that Hbb-γ expression has been studied in the different regions of the postnatal brain. Hbb-γ at P25 was ubiquitously expressed in the grey matter, staining mostly cell bodies. We observed high expression in specific regions such as the pontine nuclei, spinal cord, brain stem, and the cerebellum. In the cerebellum, we found high expression in the molecular layer and the Purkinje cell layer. Specifically, cell bodies of Purkinje cells were stained. Expression in the molecular layer likely indicates that stellate cells or some

Purkinje cells were stained (Kirsch, Liscovitch, & Chechik, 2012). The expression in Purkinje cells was very similar to what was seen in Hba-α, indicating that they might be localized together, which could potentially suggest that they may be binding together to form tetramers. Additionally, the Purkinje cell layer is characterized mainly by astrocytes and neurons, which could indicate that Hbb-γ at postnatal stages is present in those cell types (Kirsch et al., 2012).

Hbb-β expression was found almost exclusively in white matter areas of the brain, suggesting its expression may be localized to oligodendrocytes. Regions with high expression of Hbb-β were the fiber tracks in the corpus callosum (anterior commissure, genu, and splenium), pons nuclei (longitudinal fasciculus), thalamus, spinal cord, and the brain stem. It was also expressed in the white matter of the cerebellum and the Purkinje cell layer where it seemed to stain the cell membrane particularly. Since Hbb-γ and Hba-α are also expressed in the Purkinje cell layer, it might suggest that some of these isoforms might be forming dimer and tetramer structures there. However, Purkinje cells did not appear to have a high expression of Hbb-β as opposed to what was seen in Hbb-γ and Hba-α. The white matter layer of the cerebellum is characterized mainly by the presence of oligodendrocytes (Kirsch et al., 2012) which support the expression that we see throughout the brain, which leads to believe that Hbb-β might be staining oligodendrocytes in the corpus callosum, thalamus, spinal cord, and brain stem. Also, in line with our results, *Hbb-β* gene has been found in high levels in the white matter of patients with multiple system atrophy (James D. Mills, Kim, Halliday, & Janitz, 2015). There is currently a controversy on whether hemoglobin is present in oligodendrocytes and astrocytes. Some researchers have found their expression to be in these glia cells, whereas other studies have found no expression in these cell types (Biagioli et al., 2009; He et al., 2009; Schelshorn et al., 2009). However, our data seem to

support that at least Hbb-β is expressed in oligodendrocytes. To confirm these results, colocalization of this isoform with oligodendrocyte markers should be conducted in brain samples.

Hba-α expression appeared to be highly expressed in the grey matter, staining mostly cell bodies, similarly to Hbb-γ. Hba-α at P25 was found to be highly expressed in specific regions such as the spinal cord, brain stem, cortex, molecular layer of the cerebellum and the Purkinje cell layer of the cerebellum. Within the Purkinje cell layer, Hba-α is highly expressed in the axon and cell body of Purkinje cells, and it also appeared to stain the plasma membrane of the same cells that Hbb-β did. This result could potentially indicate that Hba-α might colocalize with Hbb-β and Hbb-γ in the Purkinje cell layer, specifically in the cell bodies of Purkinje cells with Hbb-γ and in the plasma membrane of cells with Hbb-β. Expression of Hba-α in the molecular layer of the cerebellum also suggests that it might bind to Hbb-γ since this isoform is also expressed there. Furthermore, previously, Hba-α has been found in parallel fibers, which are part of the molecular layer of the cerebellum (Schelshorn et al., 2009).

Overall, the expression of hemoglobin in the grey and white matter is very interesting since it is not well-known what functions they might have there. It has been hypothesized that the observed potential expression in oligodendrocytes indicates that hemoglobin might act as oxygen storage due to the localization of oligodendrocytes adjacent to neurons (Biagioli et al., 2009). Under hypoxic conditions, oxygen could be released from olygodendrocytes and provide the adjacent neurons with the oxygen needed to maintain their aerobic metabolism (Biagioli et al., 2009).

As mentioned previously, hemoglobin is thought to play a role in oxidative stress and mitochondrial function in the brain. Research suggests that mitochondrial function failures and oxidative stress might be involved in neurodegeneration in white and grey matter lesions

(Vercellino et al., 2009). Oxidative stress is also affected by iron, which can accumulate with age and is released by oligodendrocytes and myelin (Haider et al., 2014; Hametner et al., 2013). Since there is also a connection between hemoglobin levels and iron, potential dysregulation of hemoglobin given its localization in the white and grey matter might be one of the causes behind the relationship between mitochondrial deficits, oxidative stress and neurodegeneration.

6.5. Significance

The results of this study provide evidence that abnormalities in the COX2/PGE2 pathway can lead to dysregulation in the hemoglobin gene and protein expression in the developing brain. These expressions also had significant sex-dependent differences across neurodevelopment, which stresses the importance of taking sex differences into account when researching brain development. Our lab has previously shown that deficits in the COX2 pathway can lead to altered expression of genes associated with ASD, which can result in disrupted biological pathways that are crucial in brain development (Rai-Bhogal et al., 2018; Wong et al., 2018). Furthermore, in the COX2-KI mice model, we have shown ASD-linked behaviours such as repetitive behaviours, anxiety, and impaired sociability (Wong et al., 2018). In support of the present study, previous research has also found abnormal expression of hemoglobin isoforms in the brain of ASD patients suggesting that hemoglobin might be associated to this disorder (Basak et al., 2015). Now, with this study, we found altered gene and protein hemoglobin levels across neurodevelopment, which offers validation to the potential link between hemoglobin expression and neurodevelopmental disorders such as ASD.

The mechanism to explain the connection between hemoglobin levels and neurodevelopmental disorders such as ASD has not been well studied. Through this research we found that there are abnormalities in expression of hemoglobin in the brain of our ASD model

mice across different neurodevelopmental stages and we showed a differential formation of dimers and tetramers from what was previously found in blood. The results presented here show that the function of the hemoglobin proteins in the developing brain can be very specific and different from the erythrocyte lineage which could have an impact on the function of the hemoglobin proteins in the brain. For example, homodimerization of Hbb-β has been shown to have higher affinity for oxygen than the canonical heterodimer (Bellelli et al., 2006). This can be related to the higher need for oxygenation in the brain, especially for neuronal cells during neurodevelopment (Ortega et al., 2017; Biagioli et al., 2009). The effect of dysregulation of COX2 in the formation of these dimers and tetramers can then affect the ability of the hemoglobin proteins to function properly in the COX2-KI model.

Related to oxygen levels, abnormal Hba-α and Hbb-β have been associated to hypoxia states previously (Iadecola, 2004; Wang & Semenza, 1993). Research shows that hypoxia inducible factors can increase hemoglobin levels in the blood (Iadecola et al., 2004) and we showed in this study that there is upregulation of hemoglobin in the brain samples of our COX2-KI animals. There is also strong evidence indicating that there is hypoxia in the brain of ASD individuals (Van Tilborg et al., 2018). These results suggest that one potential mechanism by which dysregulation of COX2 increases hemoglobin levels could be by elevating the expression of hypoxia factors hinting at a connection between hemoglobin expression and neurodevelopmental disorders that present hypoxia states. Furthermore, upregulation of Hba-α and Hbb- β can lead to increased free iron in the cells which is linked to more production of reactive oxygen species (Singh, 2014). The disbalance between reactive oxygen species and antioxidants have been connected to mitochondrial dysfunction which is found in many ASD cases (Griffiths & Levy, 2017; N. Á. Varga et al., 2018). It should be noted that there is a strong link between

hemoglobin levels and proper mitochondrial function in neuronal cells (Ritcher et al., 2013; Biagioli et al., 2009); therefore, the dysregulation of hemoglobin proteins as seen in our study could be indicating that there is mitochondrial dysfunction in the brain of our COX2-KI model and a connection to neurodevelopmental disorders.

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Appendix A

A.1. Study 3: Expression of hemoglobin for differentiated and undifferentiated NE-4C cells

Rationale & Objectives: Studies in our lab focus on the effects of altered levels of PGE2 in neurodevelopment. We have previously demonstrated that abnormal PGE2 concentrations can disturb neuronal function *in vitro*. More specifically, results from previous experiments using Neuroectodermal stem cells (NE-4C) showed that increased levels of PGE2 affect migration of neuronal stem cells (Wong et al., 2014), accelerate neuronal proliferation and differentiation (Wong et al., 2016), increase the level of calcium in the cells (Davidson et al., 2016), and affect the expression of various genes associated with ASD (Wong et al., 2016). To build off our previous results, I will also use both differentiated and undifferentiated NE-4C cell cultures to determine how the expression of the various hemoglobin isoforms changes throughout the process of differentiation in this neuronal cell line.

Methodology: I will quantify protein expressions of Hba-α and Hbb-β using western blots, as explained previously. Heterodimerization will also be studied for the first time using this model, and I will detect potential changes between differentiated and undifferentiated cells for hemoglobin expression.

Hypothesis: I expect hemoglobin to be expressed in neuronal stem cells for both differentiated and undifferentiated cells based on previous research (Ritcher et al., 2009). Heterodimerization had also been described in neuronal cells previously (Russo et al., 2013); thus, I expect to see those forms in our model.

A.2. Materials and Methods

A.2.1. Neuronal cells NE-4C

In vitro studies were conducted on NE-4C cells which were purchased from American Tissue Culture Collection (ATCC). These cells are derived from Embryonic day 9 or E9 primary brain cell cultures corresponding to forebrain and midbrain mouse embryos. This cell line was chosen due to its ability to divide and differentiate into distinct neural cell types after induction, thereafter, resulting in the formation of classic morphological structures such as neurospheres or clusters of neural stem cells. NE-4C cells were grown by one Ph.D. and one MSc. student in our lab in minimum essential medium, and supplemented with 10% fetal bovine serum, 2 mM glutamine, 1X penicillin-streptomycin mixture (Invitrogen). Protein was subsequently isolated from day 0, 2, and 4 (before differentiation) and day 6,8,10, and 12 (during and after differentiation) by the previously mentioned students. Differentiation was induced with serum deprivation (Wong et al., 2016) with the addition of 1xB-27 instead of FBS. Replacement of the supplemented differentiating media was done every 2 days. Disruption of neurospheres occurred on day 6, and cells were seeded onto culture plates with poly-L-lysine coated coverslips.

A.2.2. Protein isolation and western blots

Total protein was isolated from NE-4C cells by one Ph.D. and one MSc. students in the lab at several differentiation days (Day 0, 2, 4, 6, 8, 12) using the NucleoSpin®RNA/Protein-Kit (Macherey-Nagel). Protein from mouse brain lysate (Abcam ab4022) was used as a positive control. To determine protein expression changes, western blots were conducted on the previously mentioned protein samples. Loading buffer (1xLaemmli sample buffer from BioRad with beta-mercaptoethanol) was added to 25 ug whole protein lysates and were separated in a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were then transferred to a supported

nitrocellulose membrane (0.2 um). 5% Milk in TBS-T (20Mm Tris ph+7.5, 137 mM NaCl, 1% Tween-20, and 5% non-fat dry milk) was used as blocking buffer for the samples for 1 hour. Washes in between steps consisted of 1xTBS-T. Primary antibodies used in the experiments were Hba-α (Abcam ab102758, rabbit polyclonal 1:1000 diluted in 5% milk), Hbb-β (Abcam ab214049, rabbit monoclonal 1:5000 diluted in 5% milk), Hbb-γ (Invitrogen PA5-49336, rabbit polyclonal 1:1000 diluted in 5 % milk) and GAPDH (Abcam ab8245, mouse monoclonal 1:10000 diluted in 2% milk). Primary antibody incubation for the hemoglobin isoforms was overnight at 4^0C, while GAPDH was incubated for 1 hour. Secondary antibodies included anti-rabbit (Abcam ab6721) and anti-mouse (Abcam ab97040) horseradish peroxidase-conjugated. Nitrocellulose membranes were incubated in ECL substrate (BioRad) and visualized using Geliance 600 Imaging System (Perkin Elmer) immediately following incubation.

A.3. Preliminary results for expression of hemoglobin in differentiating NE-4C cell cultures

Our previous study shows that the expression of hemoglobin isoforms changes in the developing brain. In this study, we used differentiating NE-4C stem cells to determine if the expression of hemoglobin isoforms (Hba-α, and Hbb-β) is regulated during the process of neuronal differentiation. The NE-4C cells were differentiated for 12 days, as described in Wong et al., 2016.

A.3.1. Expression of Hbb-β in NE-4C cells

To examine changes in Hbb-β protein expression in NE4C cells, western blots using the Hbb-β antibody at different stages across differentiation (Day 0 – Day 12) were conducted. Hbb-β was found to be structurally expressed both as a dimer and tetramer in this model. Interestingly, an opposing trend was observed between the tetramer and dimer forms for Hbb-β. More specifically, although expression of the tetramer form of Hbb-β was found in NE-4C stem cells at Day 0, its expression progressively declined following differentiation to neuronal cells (Figure

16). Conversely, the Hbb-β dimer form was detected in NE-4C cells on day 0, and the appearance of progressively stronger bands at each day suggested its expression may be higher as differentiation progressed. Further experiments need to be conducted to determine if these changes are statistically significant as well as to identify effects of PGE on Hbb-β expression in NE-4C cells.

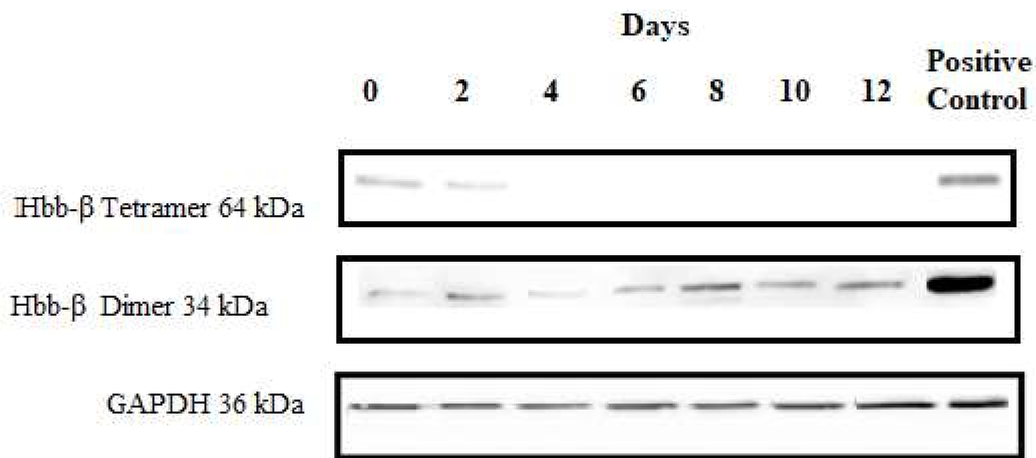

Figure 16. Protein expression of hemoglobin beta (Hbb-β) and GAPDH for NE-4C cells at different stages of differentiation. A positive control corresponding to mouse brain lysate was used in the last lane. A band corresponding to the dimer and tetramer forms was observed for Hbb-β. The bands for the dimer form appeared to be stronger with the progression of differentiation. The opposite trend was observed for the tetramer form, where it seems to have a higher expression for the stem cells compared to differentiated cells. GAPDH was used as a control.

A.3.2. Expression of Hba-α in NE-4C cells

Similarly to what was observed in Hbb-β, western blot analyses detected Hba-α as both a dimer and tetramer structure in NE-4C cells (Figure 17). However, when compared to the pattern of heterodimer expression observed with Hbb-β throughout differentiation, an opposing trend was observed between the tetramer and dimer forms for Hba-α. More specifically, we found that the

expression of the Hba-α dimer appeared to have stronger bands with the progression of differentiation, whereas the tetramer seemed to be more consistent throughout cell differentiation. When comparing the Hba-α dimer to the tetramer, the dimer structure seems to have weaker bands, suggesting it may be expressed at lower levels compared to the tetramer structure at least during early stages of differentiation. Further analysis needs to be conducted to determine if the difference in expressions is statistically significant as well as to identify if PGE2 affects the expression of Hba-α in NE-4C cells

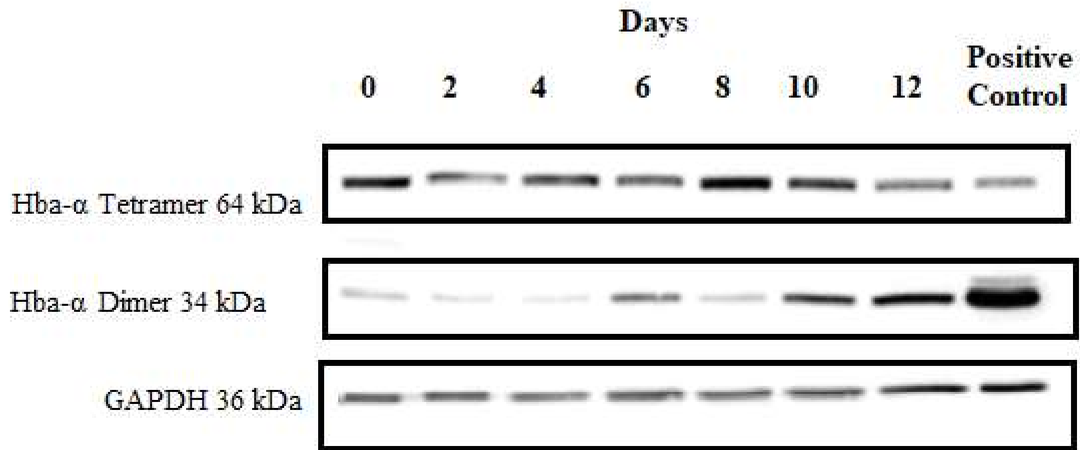

Figure 17. Protein expression of hemoglobin alpha (Hba-α) and GAPDH for NE-4C cells at different stages of differentiation. A positive control corresponding to mouse brain lysate was used in the last lane. A band corresponding to the dimer and tetramer forms was observed for Hba-α. The bands for the dimer form appeared to get stronger with the progression of differentiation, whereas the tetramer form seemed to remain constant. GAPDH was used as a control.

A.4. Summary

A.4.1. Expression of Hbb-β and Hba-α for NE-4C cells throughout differentiation

In this study, we aimed to identify hemoglobin expression in NE-4C cells across differentiation from day 0 (stem cells) to day 12 (fully differentiated cells). We found that Hbb-β

and Hba-α both have expression of the dimer and tetramer in NE4C cells. These cells also exhibited opposing trends with respect to their overall expression of the isoforms. More specifically, we observed a progressive reduction in the tetramer form of Hbb-β throughout differentiation. Conversely, the dimer form of Hba-α appeared to progressively get higher in expression during this process. Further analysis needs to be conducted to determine if the trends are statistically significant and to determine if Hbb-γ is expressed in NE-4C cells.

NE-4C cells can divide and differentiate into distinct neural cell types after induction. Following induction, these cells can form morphological structures such as neurospheres - clusters of neural stem cells. Subsequently, they can divide and differentiate into either GABAergic or glutamatergic neurons (B. V Varga et al., 2008). In our lab, we have shown that exposure to PGE2 affects the migration and proliferation of the cells, as well as the expression of Wnt-related genes (Wong et al., 2014; Wong et al., 2016; Davidson et al., 2016). Hbb-β protein expression in NE-4C cells was detected as a dimer and tetramer form. The dimer structure appeared to exhibit progressively stronger bands with differentiation, while the tetramer seemed to have the opposite expression with weaker bands as differentiation progresses. Similarly, Hba-α was also detected as a dimer and tetramer structure. Its expression pattern for the dimer form seemed to reflect what was found for the dimer of Hbb-β since it also appears to have stronger bands later in differentiation, whereas the tetramer showed more consistent bands throughout cell differentiation. Collectively, these results reflect the possible existence of a developmentally regulated change in neuronal expression of hemoglobin isoforms, whereby Hba-α expression may progressively increase during later stages of brain development as the relative quantity of differentiated neuronal cells increases.

The expression of Hba-α and Hbb-β genes has previously been found in research conducted in dopaminergic A9 cell lines (Biagioli et al., 2009). Additionally, the ability of Hba-α and Hbb-β to form dimer and tetramer structures has also been demonstrated in previous studies using mouse dopaminergic neuroblastoma iMN9D cells (Russo et al., 2013). In these cells, they showed that Hba-α and Hbb-β were binding together to form the canonical tetramer form (α2β2). However, analysis throughout the differentiation of neuronal cells has not been conducted for hemoglobin isoforms until now. Future experiments in our lab are going to focus on determining if PGE2 exposure affects expression of hemoglobin in NE-4C cells across differentiation, as well as whether the dimer and tetramer structures detected in this model reflects the binding of Hba-α and Hbb-β, or perhaps the formation of a novel heterodimer or homodimer structure.