Oxidative Stress and Neuronal Changes Associated with Prenatal Ethanol Exposure in Human and Monkey Brains

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ABSTRACT

Background: Prenatal ethanol exposure (PNEE) causes irreversible intellectual and behavioral disabilities, clinically known as fetal alcohol spectrum disorder. Few neuropathologic studies of human brain exist. **Hypotheses:** First, markers of oxidative stress persist following PNEE. Second, PNEE is associated with inhibitory and excitatory neuron changes. **Methods:** Human brain autopsies (153) with known PNEE were reviewed; 18 cases (fetus to adult) and controls were selected. Oxidative stress and neuronal differentiation markers were used for immunohistochemistry. **Results:** There were no obvious differences between control and PNEE brains using oxidative stress markers. In human PNEE brains, glutamatergic neurons were reduced 15.96 % and 18.03% in dentate gyrus and temporal cortex, respectively. GABAergic neurons reactive for parvalbumin were reduced in all hippocampal regions (CA1= 57.86%, CA3= 65.15%, and DG= 53.39%) and temporal cortex (44.13%) in all age groups. **Conclusion:** GABAergic neuron reduction in human following PNEE could explain motor and behavior distractibility in FASD individuals.

DEDICATION

For the four pillars in my life; GOD, parents, family, and friends.

GOD, I am very speechless for all the joys you give me in my life.

Mom and Dad, I am strongly proud to dedicate this work to both of you whose sincere praying and tremendous help throughout my study.

Brothers, sisters and whole family, I am pleased to call you Unknown Soldiers because without your unconditional love, help and support this would not be done.

For my friends, who stand shoulder-to-shoulder, support me, and absolutely love me.

In addition, for all the children who are mentally suffering in the world, this is a very modest work to support you.

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“Let us put our minds together and see what life

We can make for our children”.

Sitting Bull

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ABBREVIATIONS

4HNE	4 Hydroxy-nonenal
8OHdG	8-Hydroxy-deoxyguan-osine
ADH	Alcohol dehydrogenase
ARND	Alcohol-related neurodevelopmental disorder
BAC	Blood alcohol concentration
BSA	Bovine serum albumin
CALB1	Calbindin1
CALB2	Calbindin 2
CDC	The Centers for Disease Control
CYPs	Cytochrome P450 2E1
DAB	Diaminobenzidine
EAAC1	Excitatory amino acid transporter 1
EAAT3	Excitatory amino acid transporter 3
FAE	Fetal alcohol effect
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorder
GABA	γ-Amino butyric acid
GAD1/GAD67	Glutamate decarboxylase
GCLC	Glutamate cysteine ligase catalytic
GD	Gestational day
GPx	Glutathione peroxidase
GR	Glutathione reductase

H2O2	Hydrogen peroxide
HO•	Hydroxyl radical
IP	Intraperitoneally
IQ	Intelligence quotient
MDA	Malondialdehyde
MRI	Magnetic resonance imaging
NIAAA	The National Institute on Alcohol Abuse and Alcoholism
NO•	Nitric oxide
O2-•	Superoxide anion
ONOO-	Peroxinitrite
PBS	Phosphate buffered saline
pFAS	Partial FAS
PND	Postnatal day
PNEE	Perinatal ethanol exposure
PVALB	Parvalbumin
QFT	Quantity, Frequency, Timing
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SES	Socioeconomic status
TBARS	Thiobarbituric acid reactive substances

Chapter 1: Introduction

1. Background

1.1 Alcoholism

Alcohol consumption is one of the leading causes of death worldwide [1]. Alcohol-related mortality has been studied in many countries. In the United States, over 100 metabolic and non-metabolic diseases are attributed to alcohol. Cardiovascular disease prevails in alcoholic compared to non-alcoholic individuals [2]. In the United States, 60–90% of cirrhosis-related deaths have been attributed to alcohol consumption [3]. Several types of upper and lower gastrointestinal tract cancer have been attributed to alcoholism [1,4]. Tumors can also arise in the respiratory tract following excessive alcohol consumption [5]. Alcohol consumption is a risk factor for serious injuries. According to National Trauma Data Bank (NTDB), alcohol is responsible for one fifth of all trauma among 10,611 persons [6]. In addition, heavy alcohol consumption is associated with approximately 42% of injuries [7]. Reportedly, one third of death related accidents are admitted to alcohol intoxication [8]. Consequently, 5% of alcoholics develop lifelong disabilities [1]. Therefore, alcohol consumption is a major public health issues in western countries and generates enormous economic pressure in these countries. Increased awareness of the risks of alcohol intoxication appears to decrease the alcohol-related mortality rate, but despite this, the mortality rate has increased 3-fold between 2004 and 2005. In Canada, 3,958 deaths were directly attributed to alcohol in 2005 [9].

Alcohol consumption during pregnancy is a substantial risk factor for teratogenic effects on the fetus, which include growth deficiency, developmental delay, mental retardation, and

facial anomalies. Prenatal deficits following alcohol use is detected frequently in western countries where alcohol is commonly consumed [10]. Not only in western countries, but also China [11], Africa [12], Australia [13], and India [14] have reported high prevalence of birth anomalies following *in utero* ethanol exposure. The prevalence of fetal abnormalities is substantially higher in countries where alcohol consumption is common among parturient women than in countries where alcohol consumption is uncommon [15].

In some communities, more than 70% of pregnant women consume alcohol during the first trimester before becoming aware of the pregnancy [16]. As a result, over 20% of preterm births and 8% of prenatal deaths are associated with maternal ethanol exposure [17]. In order to understand the etiology of alcohol induced fetal anomalies, the patterns of alcohol consumption should be considered. The section below defines the different patterns of alcohol drinking.

1.2 Patterns of alcohol consumption

The Centers for Disease Control (CDC) and National Institute on Alcohol Abuse and Alcoholism (NIAAA) have defined the standard for alcohol consumption to induce severe harm to a biological system. Levels of consumption have been identified as binge drinking, and heavy drinking [18].

Heavy episodic drinking or binge drinking is defined as four drinks per occasion for a woman, and five drinks per occasion for a man. A single drink for a women per day and double drinks for a man per day are considered as a heavy drinking in U.S [19]. This is equivalent to 10 UK units (1.5 US units is equivalent to 1 UK unit) weekly and eight UK in a single occasion in United Kingdom [20].

Although these measures are slightly different in some literature, researchers have been using these indications to evaluate the magnitude of alcohol toxicity and the amount of alcohol exposure in individuals with known ethanol abuse [4].

1.3 Fetal alcohol spectrum disorder (FASD)

1.3.1 Defining FASD

Fetal alcohol syndrome (FAS) was first described in 1973 as an intellectual disability with growth deficiency and facial dysmorphology [21,22]. Fetal alcohol spectrum disorder (FASD), the currently accepted clinical term, is defined as intellectual disability and cognitive impairment caused by ethanol exposure during fetal development [10]. This exposure causes biological malfunction of the brain. FAS, partial FAS (pFAS), and alcohol-related neurodevelopmental disorder (ARND) are all considered forms of FASD [10]. Multiple approaches have been employed to clinically differentiate the various forms of FASD.

Individuals with FASD have cognitive and behavioral deficits and are less productive in society, school and family, therefore FASD is not only a health concern, but is also a complex interplay of social, legal, and educational factors that influence people in multiple communities worldwide [15]. Early intervention is urgently needed to prevent the most devastating complications of FASD, namely lifelong cognitive, behavioral, and physical disabilities.

1.3.2 FASD Prevalence

Numerous studies have correlated the consumption pattern with the prevalence of the different FASD forms. FASD may go undetected in children who do not exhibit the characteristic facial features of FAS; therefore, it can be difficult to accurately estimate the FASD prevalence rather than FAS [23]. The FAS prevalence diagnosed by pediatricians in

Australia was reportedly 0.58 per 100,000 school-aged children. By contrast, the highest FASD prevalence occurred in South African communities, with 9% of school-aged children affected [24]. In South Africa, which has the highest incidence of binge alcohol consumption, the FAS prevalence was estimated as higher than the pFAS prevalence. By contrast, in Italy, which has a lower binge-drinking incidence, the FAS prevalence was reportedly lower [25].

Comprehensive studies in the United States have estimated the FAS prevalence at 2.0 per 1,000 live births [26] and the FASD prevalence at 9.1 per 1,000 live births [27]. A population study in North Dakota found that 1 of 7 childhood deaths were in children diagnosed with FASD (Burd et al. 2008). The FASD status was unknown in 1 of 5 childhood deaths in siblings of FASD children [3]. Potentially, these children may have died before definitive diagnosis or may not have exhibited the characteristic features of FASD.

Although the FASD prevalence has increased in Canadian subpopulations, the prevalence in the general population has not yet been established in Canada [10]. In northern Manitoba, a reported 0.7% of all live neonates had FASD (Williams et al. 1999). This incidence may be higher in select communities. The First Nations community reported FASD in 19% of live births in British Columbia and 10% in Manitoba [28]. Another study of the same ethnic group estimated the FAS and pFAS rate at 55–101 per 1000 live births [29].

This high prevalence corresponds to high mortality rate in cases of FASD. A population study performed by Li et al. (2012) [3] found that 87% of women with FASD children died at less than 50 years of age. Considering the 9.1 per 1,000 live births FASD incidence in England [30], FASD diagnosis can play a vital role in determining the risk of preterm death in women with an older FASD child. In North Dakota, the risk of stillbirth was increased in mothers who consumed alcohol during pregnancy. The mortality rate of FASD children was up to 44-times higher than in

normal children. This finding highlights the importance of encouraging pregnant mothers with FASD children to enroll in an abuse treatment program [3].

1.3.3 Risk factors for FASD

The risk factors of FASD are categorized into primary and secondary factors. Studies have investigated these factors using questionnaires, reported alcohol consumption, and population studies [31]. The magnitude of alcohol exposure, frequency of consumption, and the time of exposure during pregnancy (i.e. quantity / frequency / timing or QFT) are the most important factors. Secondary factors include maternal nutrition, weight, socioeconomic status, education level, primary caregivers, genetic factors, and other drugs consumption. Accuracy, honesty, and specificity are three considerations during data collection from mothers and fathers in maternal clinics [32]. The following sections describe the risk factors that may increase the FASD incidence followed by the secondary factors influencing the variation in FASD characteristics.

1.3.3.1 Primary risk factors for FASD

1.3.3.1.1 Quantity of alcohol consumption during pregnancy

One of the most important risk factors of FASD is the quantity of maternal alcohol consumption. The quantity of alcohol toxicity is estimated with maternal blood alcohol concentration (BAC). Correlation studies have shown higher incidence of intellectual disability in offspring of mothers who reported higher amount of alcohol consumption [33]. In addition, a retrospective study showed an increase of FASD rate with prenatal binge alcohol consumption [25]

1.3.3.1.2 Frequency of alcohol exposure during pregnancy

Another critical risk factor is the frequency of consumption during pregnancy. The rate of alcohol consumption occurrence of a pregnant mother directly affects cognitive and behavioral abilities on a fetus [34]. Studies of several subpopulation in the United States reveal that the normal alcohol consumption pattern differs along cultural and ethnic lines i.e. heavy consumption in one group may be considered normal in other groups [25].

1.3.3.1.3 Time of alcohol consumption during pregnancy

The timing of prenatal ethanol exposure has the most significant correlation to FAS anomalies. In particular, facial dysmorphology and cardiac malformations have been correlated with alcohol exposure during the first trimester. Ethanol induced apoptosis and neural crest migration disturbances were speculated to be responsible for facial anomalies in FASD individuals [35,36]. In addition, cerebellar, hippocampal, and cortical malformations have been associated with ethanol exposure during the third trimester.

Brain cells proliferate and migrate during almost the entire 9 months of pregnancy, and every developmental stage has its own criteria. When the pregnant woman is exposed to any chemical agent such as ethanol during pregnancy, FASD can result. Studies in the United States have conclusively shown the relationship between prenatal alcohol exposure during the first trimester and the specific feature of facial dysmorphology in FASD children, which affects the migration and proliferation of neurons in the fetal brain during the first trimester [37,38]. The characteristic facial dysmorphology of FASD observed in humans has also be replicated in animal models of FASD [39]. In addition to facial abnormalities, cardiac anomalies in FASD individuals mainly occur following prenatal alcohol exposure during the first trimester [40].

The third trimester is one of the most critical stages of fetal brain development. Studies continue to strengthen the link between the distinct neuroanatomical features of FASD and ethanol exposure during the third trimester. The cerebellum, hippocampus, and frontal cortex are the most vulnerable to maternal alcohol consumption during the third trimester [41-43]. During weeks 32 to 36 of gestation, the areas that are responsible for cognitive and behavioral function are developing. As a result, continued alcohol consumption through the third trimester will produce a child with the clinical features of FASD as well as behavioral and cognitive deficits [44,45]. The severity of disease depends on the magnitude, frequency, and time of maternal alcohol consumption during pregnancy.

1.3.3.2 Secondary risk factors for FASD

While studies continue to employ the QFT factors to predict the characteristic birth defects, other studies have explored additional risk factors that may be associated with the FAS incidence, such as the maternal weight, gravidity (number of previous pregnancies), parity (number of previous births), age, socioeconomic status, education level, primary caregivers, genetic factors, and maternal sensitivity to alcohol [46].

1.3.3.2.1 Maternal nutrition

One of the major maternal variables that influence the incidence of FASD is the nutritional status of the mothers throughout their pregnancies. Inadequate nutrient intake during pregnancy has been found to interact with the metabolism of ethanol which may intensify the alcohol toxicity in fetuses known of *in utero* alcohol exposure [47]. In this respect, iron and zinc are two major examples of nutrient deficiencies which have been widely reported to be connected with developing neurodevelopment anomalies in FASD children [48].

Inadequate iron intake of pregnant women; which is seen commonly in alcoholic mothers, raises the chances of destruction of brain neuroanatomical features of fetuses and body developmental delay in childhood. Moreover, binge drinker mothers in U.S. who have low iron concentration delivered children with somatic growth retardation; that is one of the diagnostic criteria for FASD children [49].

Zinc is another example for inadequate nutrient leads to increase the risk of having classic FASD child. Like iron status, zinc concentration is decreased in alcoholic mothers due to metabolic interactions. Longitudinal studies reported that zinc deficiency occurs highly during prenatal alcohol exposure is proposed the effect of neurodevelopmental damages in offspring's [50].

1.3.3.2.2 Maternal weight, gravidity, parity

Mothers with a higher body mass index have a decreased risk of bearing FASD children because the higher body mass reduces the blood alcohol concentration and thus decreases the risk of ethanol exposure to the fetus [46]. Gravidity, parity, and maternal age in combination play a crucial role in increasing the disease severity. Studies in several countries [24,51-54] have found that an increase in these three variables in childbearing women directly increases the severity of FASD. In addition, maternal age and race have recently added to the childbearing variables in mothers of FASD children. A longitudinal study documented higher incidence of FAS in South African women and higher age mothers in U.S. [46].

1.3.3.2.3 Paternal genetic predisposition

Genetic variability is another factor influencing the incidence of FASD worldwide [29]. In fact, the maternal ability to metabolize alcohol and expose the fetus to ethanol is genetically

determined [26]. Therefore, genetic predisposition and metabolism are essential factors in studies of alcoholism and neonatal deficits following alcohol exposure.

In fact, genetic susceptibilities have highlighted the importance of studying paternal alcohol consumption which have been anticipated to be interconnected with increasing the risk of FASD via epigenetic transmission [46,55].

1.3.3.2.4 Socioeconomic status, education level, primary caregivers

Socioeconomic status (SES) can modify the family from raising normal children to caring for children with long-life disabilities. The most severe FAS cases have been reported in lower SES families [46]. Bignol et al. (1987) found that the FAS incidence in the United States was 16 times greater in lower SES families than in higher SES families [56]. Therefore, evaluation of the SES of mothers with FAS children or children with undiagnosed FASD is very important in determining the rate of FASD in different populations.

1.3.4 Clinical manifestations of FASD

Although FAS has physical characteristics that can be obvious, most FASD is not easily diagnosed. Evaluation by multiple clinical specialties is needed [57]. Access to prenatal records is required to predict the disease severity. The risk of FASD in future siblings must also be considered [3]. Following prenatal alcohol exposure, the infant will have generalized decreased cognitive, behavioral, and motor abilities. Facial dysmorphology and growth restriction are the main manifestations of FASD. The following explicitly explain each manifestation appear in individual diagnosed with FASD.

1.3.4.1 Cognitive and behavioral disabilities

Several studies have associated alcohol-mediated changes in neuron development with functional and behavioral delay in FAS children [58]. These include attention deficits, learning disabilities, tremors, and gait disturbances. Intelligence quotient (IQ) tests show cognitive disability in children known of prenatal ethanol exposure [59]. Behavioral impairments have been evaluated with multiple tasks given to FASD children and sex-matched controls and comprehensive based study of MRI abnormalities in FASD children [60,61].

Along with cognitive and behavioral manifestations, ethanol induced teratogenicity has been linked to motor impairments in FASD children. The mechanism of motor control damage following binge alcohol consumption have been related to ethanol induced aggregation in both central and peripheral nervous systems; especially motor neurons in cerebellum [62,63].

1.3.4.2 Executive function deficits

Executive functions is a set of advanced cognitive abilities of decision-making, flexible thinking, working memory, planning, organizing, and prioritizing information [64]. Longitudinal studies revealed that children diagnosed with FASD manifested with deficient in problem solving, strategy planning, thought organizing, time management, and information processing [59,65]. As reported via teachers, parents, and caregiver, FASD children are slower compared with normal children at the same school age [66].

1.3.4.3 Facial dysmorphology

Facial abnormality is the main visible feature of FASD and can include microcephaly, a smooth philtrum, thin upper lip, dropped eyelid, micropthalmia, and short palpebral fissures [26]. However, the facial features of FAS are not observed in all affected children. Depending on the

severity and the time of alcohol exposure, children may not express the characteristic facial features of FAS [10].

1.3.4.4 Somatic growth reduction

Growth restriction and small circumference of the head are other distinct manifestations that may occur in children exposed prenatally to alcohol [67]. The severity of growth impairment may depend on the magnitude of alcohol consumption during fetal developmental; an increased BAC increases the likelihood of physical deficits [68]. Early intervention is recommended prevent lifelong disability in the estimated one million at-risk children [29,57].

1.3.5 Diagnosis of FASD

Diagnosis of FAS may be delayed or overlooked because mothers often do not admit alcohol consumption until after delivery; therefore, the magnitude and time of consumption may be inaccurate [69]. Facial morphology is key in recognizing FASD, but it is not exclusive to FASD. Other diseases such as Williams syndrome and Cornelia de Lange Syndrome exhibit similar facial dysmorphology [29]. Therefore, FASD diagnosis depends on multiple assessments, including physical, dysmorphological, and neurobehavioral examinations in addition to confirming maternal alcohol consumption [9]. Physical assessment can determine the growth rate by measuring the patient height, weight, and head circumference.

Facial assessment for morphological abnormalities is another tool to diagnose the severity of maternal alcohol exposure. Cognitive, executive, and communicative functions in children should be assessed to determine their neurobehavioral abilities [57] (Figure 1). Then, FASD child undergo in one of five diagnostic criteria from Institute of Medicine's (IOM) for alcohol-induced abnormalities, (Figure 2).

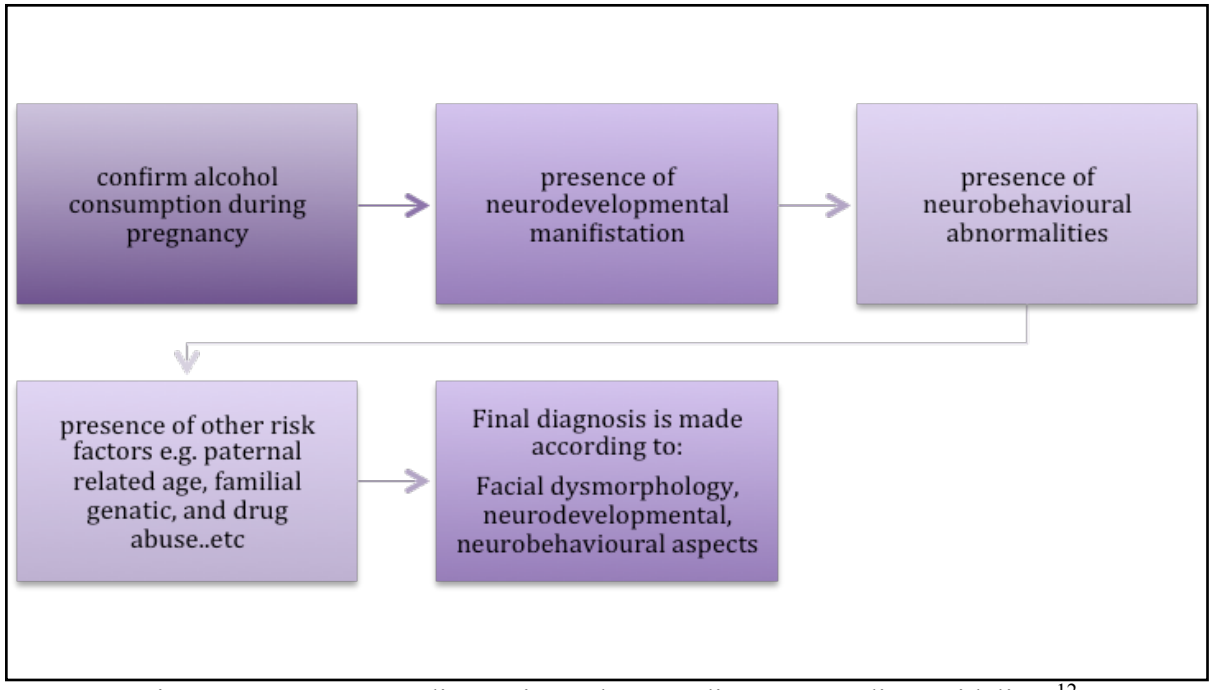

Figure 1: How FASD diagnosis made according to Canadian guidelines[12]

FAS with confirmed prenatal alcohol exposure	• facial dysmorphology • neurodevelopmental abnormalities. • Growth retardation
FAS without confirmed prenatal alcohol exposure	• facial dysmorphology • neurodevelopmental abnormalities. • Growth retardation
pFAS with confirmed prenatal alcohol exposure	• some facial dysmorpholgy • growth retardation or neurodevelopmental abnormalities or behavioural/ cognitive abnormalities
FAS with confirmed prenatal alcohol exposure and *ARBD*	• Conginital deformitiesrelated; cardiac, renal, ocular and other abnormalities
FAS with confirmed prenatal alcohol exposure and *ARND*	• neurodevelopmental abnormalities or a complex pattern of behavioural/ cognitive abnormalities

Figure 2: Distinct diagnostic criteria of FASD anomalies in Institute of Medicine (IOM)

1.3.6 Pathogenesis of FASD

1.3.6.1 Oxidative stress

Metabolic reactions within each cell are controlled via enzymes. Imbalance in these enzymes can disrupt the cellular processes [70]. Reactive oxygen species (ROS), which include superoxide anion (O2-•) and hydroxyl radical (HO•), hydrogen peroxide (H2O2) along with reactive nitrogen species (RNS) which include nitric oxide (NO•) and peroxinitrite (ONOO-) are the main byproducts of cellular reactions [71].

Under normal physiological state antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase) detoxify and maintain the proper redox potential in the cells [72]. External risk factors such as alcohol consumption, cigarette smoking, and radiation exposure can lead to an increase in the level of ROS [73]. Imbalance between ROS/RNS and antioxidants causes cellular disturbances, which is called oxidative stress [72]. Increased oxidative stress leads to apoptosis due to mitochondrial membrane depolarization and involves caspase and cytochrome C pathways [74]. Mitochondrial membrane disturbance causes damage to macromolecules such as DNA, proteins, and lipids [75]. In other words, protein, lipid, and DNA damages are consequences of persistent oxidative stress in a cell [76].

Mitochondria utilize less than 5% of the oxygen to produce O2-• [77]. O2-• reacts with NO• and produce ONOO- that play a vital role in protein, lipid, and DNA oxidations [78,79]. O2-• can also reacts with superoxide dismutase to produce hydrogen peroxide (H_2O_2). Under normal conditions H_2O_2 is detoxified by glutathione peroxidase (GPx) or catalase (CAT). A failure in this process may produce HO• that later on activates the lipid peroxidation cascade [80].

1.3.6.2 Alcohol metabolism

Ethanol metabolism takes place in the liver (microsomes and mitochondria). Reactive oxygen species (ROS) including superoxide (O2−•), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•) are the main byproducts of ethanol metabolism [81]. Ethanol metabolism follows two steps (See figure 3). First, alcohol is metabolized into acetaldehyde via enzymatic reactions [82]. Three different enzymatic reactions may take place depending on the location of the metabolization. In the liver, alcohol dehydrogenase (ADH) or cytochrome P450 2E1 (CYPs), which is a heme-cofactor protein, convert alcohol to acetaldehyde with reduction of NADPH/NADP+ ratio [83]. In the brain catalase (CAT), which is a heme-containing enzyme, and/ or cytochrome P450 2E1 metabolize ethanol to produce acetaldehyde, followed by another conversion to acetate by the enzyme acetaldehyde dehydrogenase [84].

These reactions stimulate the respiratory chain reaction to produce ROS in the mitochondria [82]. Chronic alcohol exposure leads to defective mitochondria which increases cellular oxidative stress mediated damage [85] and possibly neural developmental delay [86].

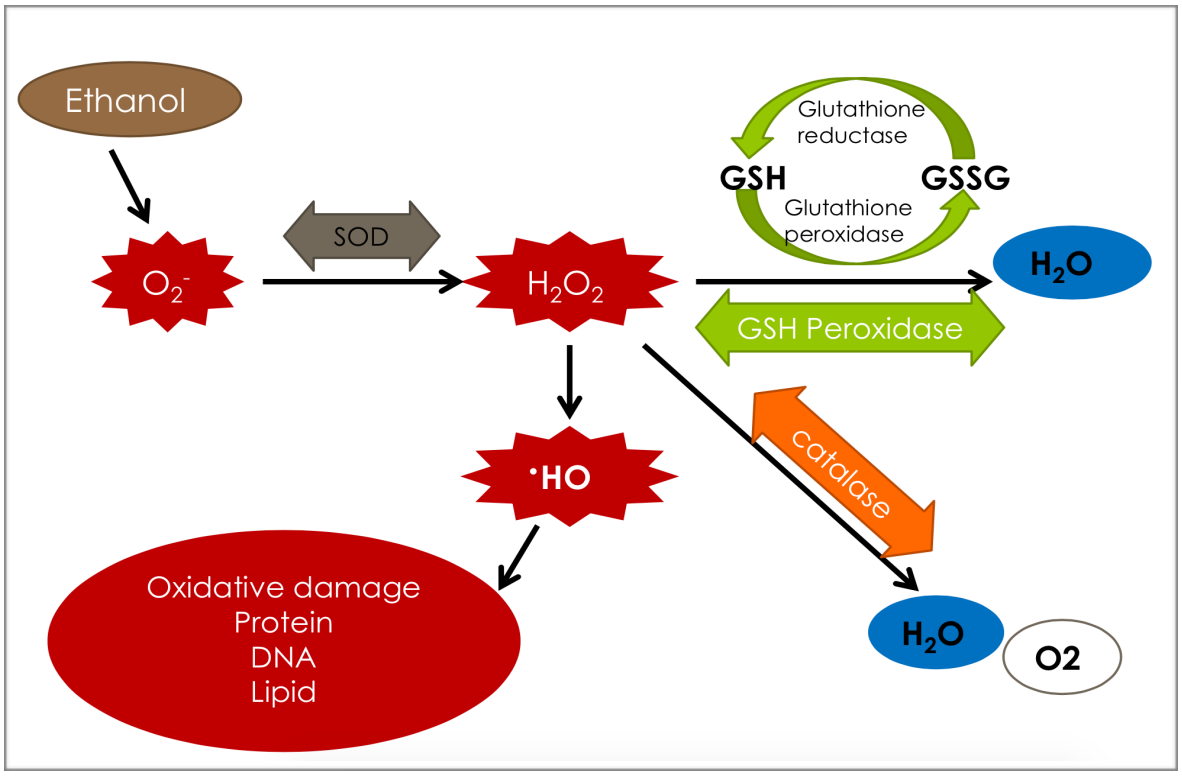

Figure 3: Ethanol metabolism

Immunohistochemistry can be used to detect byproducts of hypoxic and oxidative metabolism. Malondialdehyde (MDA) and 4-hydroxy-trans-2-nonenal (HNE), 4-oxo-trans-2-nonenal (4-ONE) are markers for lipid peroxidation. Following PNEE, they have been found in rat brain tissue at GD17-18 as well as in rat cerebellum, hippocampus, and cerebral cortex at postnatal day (PND) 4 to 9 [87,88]. Following PNEE at GD6-12, 8-hydroxydeoxyguanosine (8OHdG), a marker for DNA modification, is found in high concentration in rat cerebellum [86].

1.3.7 Oxidative stress and FASD corroborations

Cellular peroxidation has significant impacts on various organs such as liver, pancreas, kidney and brain. Since brain only utilizes 20% of oxygen during metabolic reactions, it is can be affected with minimal ROS/RNS imbalance [71]. A vast number of immature cells in the brain are

results from intracellular ROS/RNS imbalance. In addition to oxidative damage resulted from unsaturated fatty acids [89]. In the brain, oxidative stress damage causes disturbance to the blood brain barrier (BBB) leading to neural cells apoptosis, glutamatergic excitotoxicity, disruption in GABA neurons and restriction in synapses [90-94].

Oxidative stress damage during early fetal developmental of brain leads to alterations of neurogenesis and neural cell migration, causing changes in brain structures because fetal brain is where the antioxidant defense mechanism has not been well developed [95].

Antioxidants have been associated with behavioral deficits in animal models treated with prenatal ethanol exposure. Intracellular disturbances can be used as a marker for consuming alcohol during gestational period, regardless of the amount consumed [96]. The degree of cellular damage appears to be correlated with the time, quantity, and frequency of ethanol exposure [83].

1.3.8 Neuroimaging studies of human FASD

Whole-brain imaging technique has been used to assess the abnormalities of brain cortices in children diagnosed with FASD [97]. Sowell reported severe reduction in the frontal cortex volume in children with history of PNEE. This reduction was also associated with cognitive deficits and behavioral impairments [97].

Trying to understand the effect of PNEE on offspring was one of the topics that has intrigued a number of authors worldwide. Structural MRI is an approach that helps specify the changes among all brain areas [98]. Following cognitive function assessments, functional MRI demonstrated stimulation of frontal and prefrontal cortex of children with FASD [99].

Magnetic resonance imaging (MRI) studies of FASD children reported reduced volume of cerebral cortex, hypothalamus, cerebellum, and other parts of brain [100]. The parietal lobe can be negatively affected by PNEE compared to the occipital and temporal lobes [101]. Specifically,

binge drinking causes damage to white matter more than gray matter [102]. Hippocampus has also been damaged following alcohol exposure [103]. These reductions perhaps depend on the sensitivity of neuronal cells to ethanol exposure during gestation and the time (i.e. prenatal and/or postnatal) of the exposure [104].

Motor function is affected by PNEE and cerebellar damage has been demonstrated during the third trimester in human and first two weeks of PN in rat following alcohol consumption [104]. Another study reported that basal ganglia, cerebellum, anterior vermis, and corpus callosum were changed following excessive alcohol exposure during gestation [83].

MR imaging of youths with FASD showed significant reduction in the white matter (10.7%), cortical gray matter (9.5%), intracranial volume (8.7%), hippocampus (11.3%), thalamus (14.0%), and putamen (12.6%) [105].

1.3.9 Human brain autopsies for FASD

It is important to note that only 25 autopsy cases of FAS have been described in the literature; these are likely the most severely affected cases. The reported abnormalities include microencephaly, hydrocephaly, agenesis of corpus callosum, agenesis of anterior commissure, periventricular and leptomeningeal heterotopias, cerebral dysgenesis, and brainstem dysgenesis [63,101,106,107] (Figure 4).

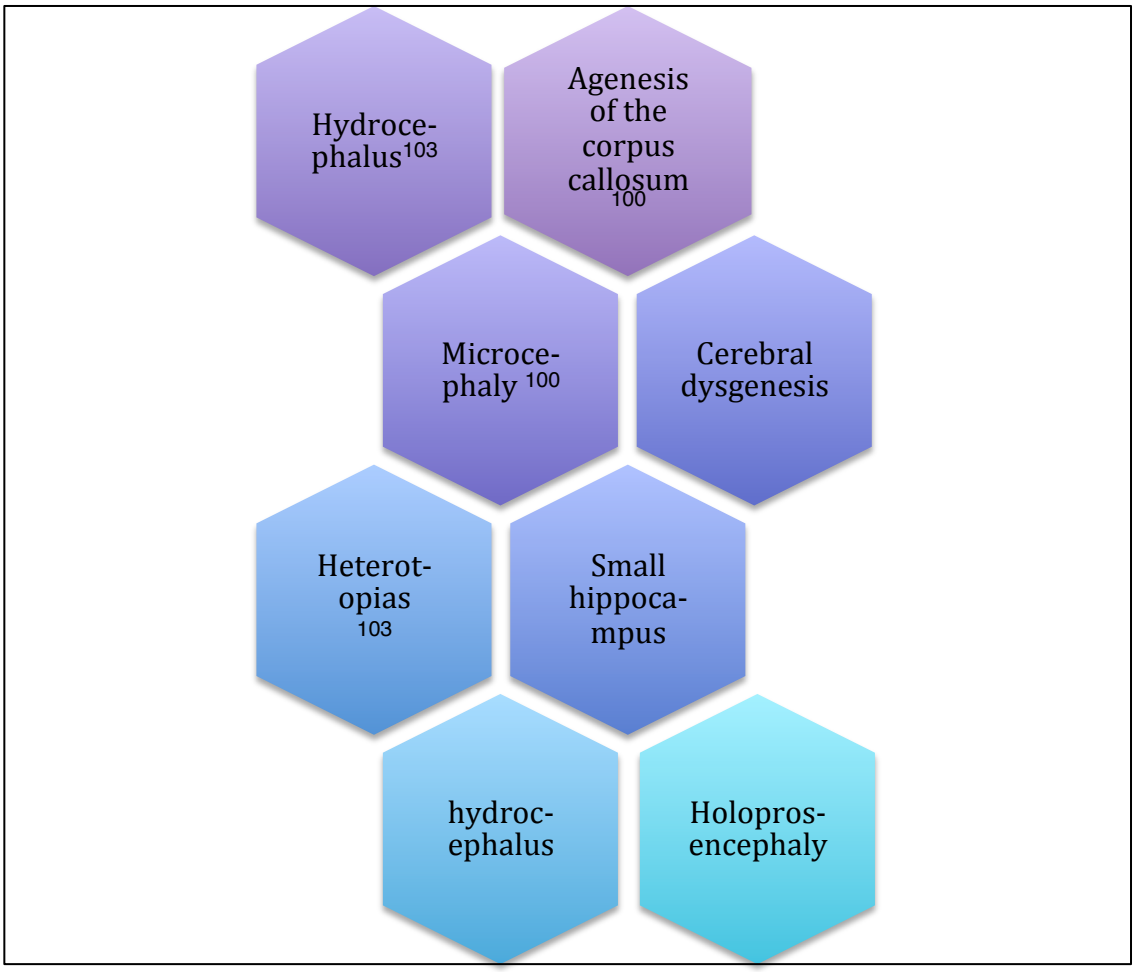

Figure 4: Neuropathological anomalies of FASD individual [15,108,109]

1.3.10 Neurogenesis and neuron migration in human developmental brains

Neurogenesis and neuron migration are two critical processes during brain development. Neocortex neurons are mainly generated in the ventricular zone (VZ) and migrate using long "radial glial guides" [110]. These long excitatory projections are generated during 8-10 weeks gestational phase in humans [111]. Excitatory neurons use glutamate as a neurotransmitter during brain development. Inhibitory cortical interneurons develop later in the developmental process, most being generated in the subventricular zone (SVZ) by 32 weeks of gestation [110,112]. These

interneurons migrate transversely into the neocortex and deep gray matter structures. At the end of the migration process, the six-layer neocortex will be formed [113].

Two main classes of inhibitory neurons exist in the central nervous system: glycine and gamma-aminobutyric acid (GABA). About 90% of brain inhibitory neurotransmitter is GABA. The biochemical synthesis of GABA begins with glutamate precursor which is decarboxylated to GABA via glutamic acid decarboxylase (GAD) [114,115]. In humans, calcium-binding proteins that play a vital role in cell signaling, differentiation, generation, and migration. They are especially abundant in GABAergic neurons [116]. Three predominant subgroups of calcium binding proteins can be detected; parvalbumin (PVALB), calbindin 1 (CALB1), and calbindin 2 (CALB2).

Exposure to alcohol during the periods of embryonic and fetal development might disturb neurogenesis and neuron migration phenomena [83]. An early study in mice correlated the craniofacial manifestations with impairment in neurogenesis [117]. Human autopsies [22] showed reduction in the head size and brain volume (microcephaly and microcephaly respectively) [21,61].

1.3.11 Animal model studies of FASD

Animal models demonstrate abnormalities similar to those in humans following alcohol exposure. Animals can be exposed to defined amounts of alcohol for a specific period of time. Nutritional aspects and other risk factors can be controlled in these experiments. The route of administration however, was varied; intraperitoneal injection, gas inhalation, and/or liquid based food [118]. Studies using animal models help researcher to understand the wide-range of FASD associated signs and symptoms, and to potentially formulate a better outcome in humans. Animal (mostly rodents) experiments have helped to explain different domains such as facial characteristics, behavioral imbalance, and neuropathological deformities.

1.3.11.1 Facial dysmorphism

Facial dysmorphism appears in FAS animal models [39,119]. Kotch and Sulik (1992) have explored discrete facial anomalies in C57Bl/6J mouse embryos when the exposure to ethanol was done during a time analogous to the first trimester in human pregnancy [39]. C57Bl/6J female mice were exposed to alcohol at day 7 of gestation and have shown to mimic facial appearance of children diagnosed with FAS [120].

Non-human primates have also been studied in order to understand the mechanism of the spectrum of FASD. Dr. Sterling Clarren and coworkers conducted a series of experiments on 54 pregnant pigtailed macaque monkeys (*Macaca nemestrina*). These monkeys were administered different doses of alcohol orally once a week throughout their gestation period. Thirty-three offspring were born and studies were conducted when they were at 6 months of ages [121]. The major craniofacial malformations of FAS appeared in three out of twenty four offspring. In addition to one of those offsprings had flattened in the midline and one had flattened maxilla with dental disruption [122].

Other report found a correlation between the craniofacial malformation and the amount of alcohol consumption by the pregnant monkeys [60]. The major finding of this paper was that the offspring that had been prenatally exposed to a high percentage of alcohol (1.8g/kg) showed severe craniofacial abnormalities. However, these anomalies were expressed along the midline, palpebral fissure, upper lip, and smooth philtrum. Authors concluded that the frequency of alcohol exposure correlates with the severity of craniofacial dysmorphology [123].

Table 1 Published studies on non-human primate

Species	Time exposure	Age at conducting	Rout of exposure	Quantity of alcohol	Tissue	Malformation	Reference
M. Nemestria	1-24 GW	6 month	Nasogastric	0.0, 0.3, 0.6, 1.2, 1.8, 2.5, 3.3, & 4.1 g/kg	Brain	No facial malformation Microcephaly	Sheller et al., 1988
M. Nemestria						12% of had seizers	Clarren et al., 1988
M. Nemestria						3/26 Microphthalmia Ganglion cell loss	Clarren et al., 1990
M. Nemestria		133 days				1 had holoprosencephaly & exencephaly	Clarren et al., 1992
M. Nemestria		6 month			Cerebe-llum	Diminished Purkinje cell counts	Bonthius et al., 1996
M.vervet	150	2year	Voluntary drink	3-5 drinks equivalent	Frontal lobe	Loss Neuron counts Increase interstitial neurons	Burke et al., 2009
M. Fascicularis	G1-55	Immediately after exposure	Gavage	8h	Cortex, caudate putamen	Neuroapoptosis	Farber et al., 2010

1.3.11.2 Functional and behavioral impairments

In addition to the facial dysmorphism, functional and behavioral disturbance were also associated with FAS animal models. Rats showed impairment of spatial ability following PNEE (PN20-30) [124]. Another study showed similar deficits in neonatal rats with earlier alcohol exposure period (PN7-9) [125]. These functional impairments perhaps developed from hippocampal and/or frontal cortex damage following neonatal alcohol exposure [126]. Furthermore, socio-behavior disabilities similar to human were also reported in rodents following maternal alcohol consumption [127].

Macaque nemestrina offspring with PNEE demonstrated functional delay when cognitive assessments were done [128]. Some of them had significant delay in playroom assessment as well as in motor ability [128]. Hyperactivity of young primates exposed to alcohol during gestation was recorded to be 2 to 3 times higher than the offspring who were not exposed to alcohol [128]. Several studies associated the early ethanol exposure during critical developmental stage with teratogenic damage in Macaque brain function [121-123].

1.3.11.3 Neuropathological distortions

Animal models have similar brain structure deformities seen in human FAS. For example, mouse embryos exposed to alcohol on gestational age 7 days exhibit agenesis of the corpus callosum, defects in development of the cerebral cortex, hippocampus, and basal ganglia, and enlargement of the lateral ventricle [120,129]. Rat offspring with PNEE (at gestational day GD10 and GD 21) demonstrates leptomeningeal heterotopias over the cerebral cortex [130].

Rodents demonstrated microglial loss following 3-5 PND in the cerebellum [131]. Other authors reported neuronal changes in the hippocampus of ethanol-exposed mice [132].

Godin et al. identified anomalies related to ganglionic eminence, corpus callosum agenesis, hippocampus, lateral ventricles, cerebral cortex, and basal ganglia in mouse offspring prenatally exposed to alcohol at day seven of gestation [120]. A later study using rodent models of alcohol exposure (at 7 days of gestation) revealed size reduction in the facial and forebrain area (holoprosencephaly), one of the critical features noted in FASD children [109]. In addition cerebral heterotopia and cortical dysplasia also can be present in children born with FASD [120]. However, exposure to ethanol at a later stage of development showed global volumetric reduction in the brain [109].

1.3.11.4 Neuroimaging studies of FASD animal models

Several imaging approaches such as scanning electron microscopy and magnetic resonance imaging (MRI) have been used to assess the neuronal changes in offspring following exposure to alcohol in animal models [110,133]. PNEE on day 7 of gestation in mice caused a reduction in neural plate size that indeed can induce brain deformities. Scanning electron microscope was used to measure the overall brain size, forebrain in particular [45]. MRI of mouse embryos prenatally exposed to alcohol at later developmental stage (gestation day10) revealed that a global reduction in all brain regions in addition to ventricle enlargement [134]. MRI show reduced volume of deep brain structures [105], which are postulated to be related to loss of neurons.

Chapter 2. Research objectives

Overall, my main objective is to verify some of the rodent PNEE experimental data-using human and monkey brain specimens with known PNEE.

2.1 Rationale

2.1.1 Oxidative stress markers are persistent following *in utero* alcohol exposure

Brain tissue from rat offspring of PNEE (GD17-18) via intragastric gavage had increased levels of 4-HNE and DNA damage measured by Western blot [87]. MDA, a marker for protein peroxidation assessed with colorimetric assay specific for lipid oxidation, was increased in cerebellar neurons of rat offspring following 6 g/kg/day liquid diet ethanol at postnatal days (PD 4-9) [88]. Moreover, intraperitoneal ethanol injection of pregnant mice at GD9 led to DNA damage detected via the level of 8-OH deoxyguanosine (8-OHdG) using DNeasy Tissue kit [96]. These studies and others in rodents showed that it is possible to detect oxidative stress markers soon after PNEE.

My study is constructed to evaluate the persistence of oxidative stress markers in human and non-human primate brain autopsies following *in utero* alcohol exposure (figure 5).

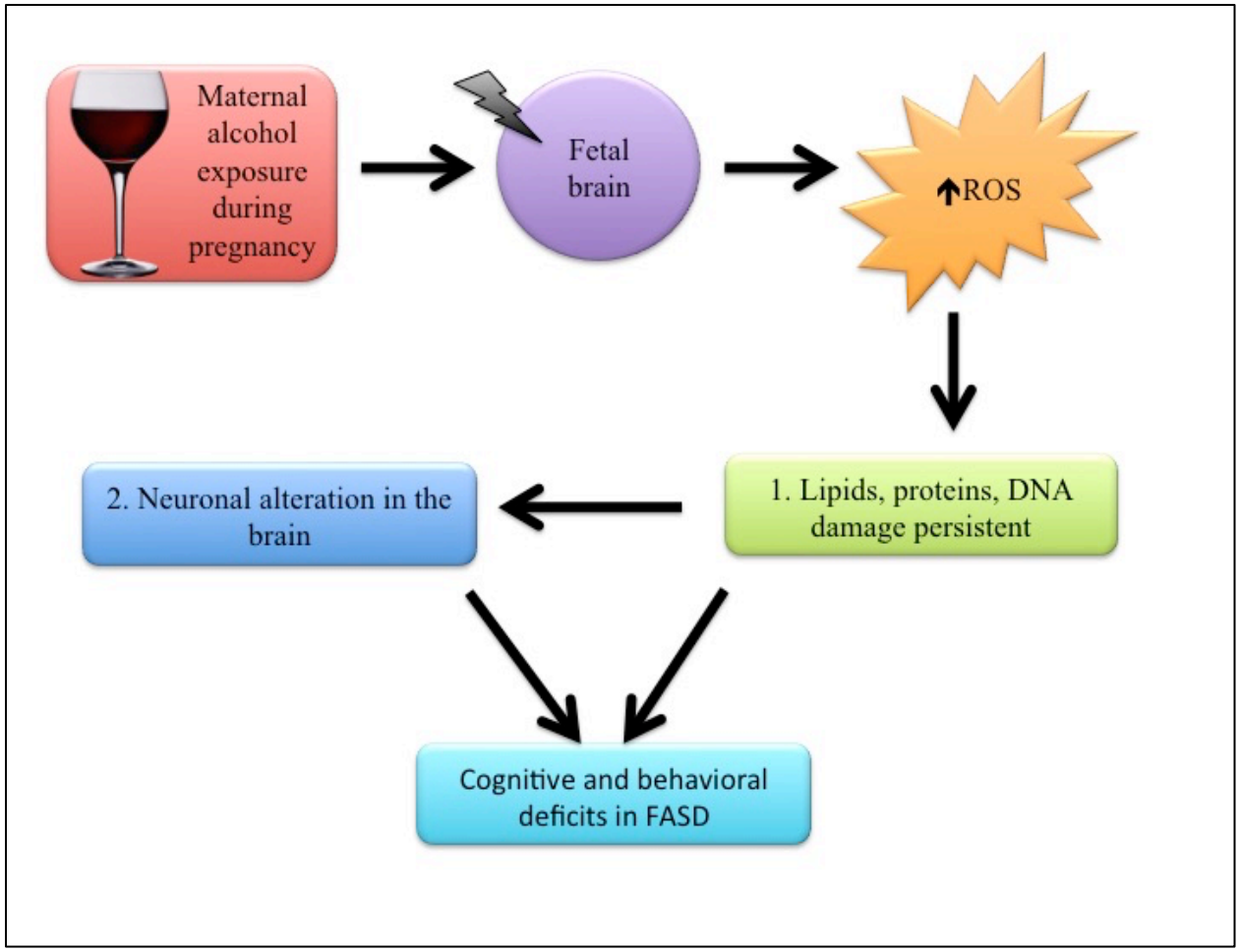

Figure 5: Diagram depicting the experimental hypothesis

2.1.2 Hypothesis 1

I hypothesize that markers of oxidative stress will be detectable in the youngest age groups of humans (fetuses or neonates) with PNEE and in macaque brains, which are comparable to the infant age group in the humans. If there are no differences, there may be several possible explanations: 1) Oxidative changes in the brain might be a species-specific effect found in rodent but not found in primates, 2) The *in utero* alcohol exposure might not be sufficient, 3) Postnatal life circumstances in monkeys and humans might gradually eliminate

markers of *in utero* hypoxic stress, or 4) Death circumstances in humans cause oxidative changes that obscure the *in utero* changes due to PNEE.

2.1.3 Neuron distribution changes in individuals with PNEE or diagnosed with FASD

Guinea pig offspring (PND 12) whose mothers had been administered 4g/kg ethanol during pregnancy (GD 2-67) via intragastric gavage showed loss of CA1 hippocampal pyramidal neurons stained with cresyl violet stain [135]. A similar study with the same circumstances reported dying cerebellar Purkinje neurons at PND1 and PND5 as assessed by Terminal deoxynucleotidyl transferase (TUNEL), which labels nucleic acid terminals in order to detect DNA damage [136]. Microscopic neuron counting of brain tissue of PND10 rats exposed to ethanol (GD1- GD20) showed neuron reduction in hippocampus regions (CA1, CA3, DG) [42].

2.1.4 Hypothesis 2

I hypothesize that quantitative neuron changes will be identified in children with a clinically verified neurological disorder (i.e. FASD). The selective (i.e. relative) reduction of excitatory vs. inhibitory neurons might be dependent on the timing of the exposure. Monkeys exposed intermittently to alcohol throughout pregnancy might have a reduction in both neuron types (relative to the total brain cell density).

2.2 Experimental Goals

2.2.1 Goal #1: To determine if markers of oxidative damage persist in human and non-human primate brain following *in utero* alcohol exposure.

Immunohistochemistry will be performed to detect the oxidative stress markers of DNA and lipids in specific areas from human and non-human primate brain autopsies. Cells will be quantified and appropriate statistics will be applied.

2.2.2 Goa1# 2: To determine if human and monkey *in utero* alcohol exposure is associated with altered balance of excitatory and inhibitory neurons.

Immunohistochemistry will be used to demonstrate markers of glutamatergic and GABAergic neurons in selected hippocampus and temporal lobe of human and non-human primate. Controls will be compared to individuals known to have *in utero* alcohol exposure. An appropriate statistical analysis will be performed to calculate the alterations in neuron counts.

Chapter 3. Experimental methodology

3.1 Materials

3.1.1 **Human autopsy brain tissue**

The brains of 153 humans of various ages (22 week fetuses to 49 years) who were reportedly exposed to alcohol *in utero* (as indicated in the autopsy report history) were obtained at autopsy at the Health Sciences Centre in Winnipeg, Manitoba, Canada, from 1980 until 2014. Permission to conduct the study was acquired from the University of Manitoba Health Research Ethics Board (HREB Ethics protocol # H2011: 213). The study is retrospective. Five different neuropathologists had performed brain examinations during the study period, so sampling was not identical. In general, autopsies were performed 1-2 days after death, the brains were fixed in 10% formalin for approximately 2 weeks after autopsy, and then cut into ~1 cm thick coronal slabs. Brain samples were taken from similar regions (frontal lobe, basal nuclei, hippocampus / medial temporal lobe, posterior cerebellum, et al.) and then embedded in paraffin wax. Sections were cut at 5µm thickness, applied to glass microscope slides, and stained with hematoxylin and eosin.

The slides were carefully reviewed by Dr. Marc R. Del Bigio, an experienced neuropathologist at the University of Manitoba, to document neuropathological abnormalities and to verify tissue integrity. Selected cases were harmonized into pairs of alcohol exposed and age/sex matched controls in the fetal, infantile (newborn to 1 year), child (1 to 12 years), and teenage (12 to 18 years) age groupings.

Table 2: Human controls and FASD cases (sex/age) matched

Group	Control #	Age	FASD #	Age	Sex
Fetuses	1	22 GW	26	23 GW	M
	2	37+ GW	27	36+ GW	M
	3	38 GW	28	36+ GW	F
	4	38 GW	29	40 GW	F
	5	38 GW	30	40+ GW	F
	6	40 GW	31	40+ GW	M
Infants	7	24 d	32	23 d	F
	8	2 m	33	2 m	M
	9	3 m	34	3 m	M
	10	5 m	35	5 m	F
	11	7 m	36	7 m	M
	12	9 m	37	11m	F
Children	13	9 y	38	9.5 y	M
	14	8 y	39	8 y	F
	15	7.5 y	40	7 y	F
	16	3.5 y	41	3 y	F
	17	22 m	42	22 m	M
	18	15.5 m	43	16 m	F
Teens	19	17 y	44	17 y	M
	20	16 y	45	16 y	M
	21	13.5 y	46	15.5 y	F
	22	15.5 y	47	15.5 y	M
	23	12 y	48	15 y	F
	24	14 y	49	14 y	M
	25	12 y	50	13 y	M

Notes: a) d – days, m - months, y – years, GW- Gestational weeks

b) M - Male, F - Female,

3.1.2 Macaque brain tissue

Dr. Sterling Clarren and co-workers conducted several experiments on 54 pregnant gravid pigtailed macaques (*M. nemestrina*) that were orally administered different doses of alcohol once a week during their gestation (1st week; 0.0, 0.3, 0.6, 1.2, 1.8, 2.5 or 4.1 gm./kg and 5th week; 2.5, 3.3 or 4.1 gm./kg). Macaques were monitored during gestation for any unexpected complications. Thirty-three offspring were born with no major structural deficits in the brain. Macaque brain autopsies were collected at 6 months postnatal age. Tissues were placed in 10% neutral buffered formalin (NOTE: detailed information about the duration of fixation is not available) and then embedded in paraffin for neuropathological studies [121,122,128,137-139].

The major findings of the Clarren et al. studies were as follows:

- Only one animal, a macaque that was administered the highest dose of alcohol, 4.1g/kg, had microcephalic brain and growth delay.
- The majority of alcohol exposed monkeys showed bilateral ganglion loss from the eyes [122].
- Purkinje cells loss in the cerebellum was alcohol dose dependent [140].
- The cranial area demonstrated a 1.7% difference in animals exposed to ethanol [121].
- Ethanol exposure during the G1-G24 weeks produced more severe cognitive and behavioral aberration than late gestational (G6-G24 weeks) exposure [121,128].

Dr. Clarren kindly provided us with the paraffin embedded tissue from the posterior cerebrum of these monkeys. This included the posterior frontal lobe, hippocampus, temporal lobe, thalamus, posterior striatum, parietal and occipital lobe. The tissue blocks, including the hippocampus, were heated at 60˚C for 30 minutes to melt the old wax. Therefore, I re-embedded in new paraffin, which is easier to section with a Finesse microtome

(#77510250GB). The blocks were cut into 5µm thickness to proceed with the immunohistochemistry.

3.1.3 Antibodies and optimization

I conducted two optimization steps to ensure the immunoreactivity of each antibody. The first step for optimization used human brain samples with acute (3 days) and chronic (months) infarcts and Alzheimer disease. This would ensure that the antibodies were capable of detecting oxidative markers and different neuron types. The second step used a four-tissue sample array block (Figure 6), which includes two macaque occipital brain sections (control with 0 mg/ml *in utero* alcohol and affected with 0.6 mg/ml *in utero* alcohol) and two human brain sections (normal cerebral cortices from 8h and 39h postnatal autopsies 21 and 23 years old, respectively). All paraffin-embedded samples were sectioned at 5-µm thickness.

Immunohistochemical testing was carried out (by the author) using four commercially available markers of oxidative damage (Glutamate cysteine ligase catalytic (GCLC), Malondialdehyde (MDA), 4-Hydroxyno-nenal (4HNE), and 8-Hydroxydeoxyguanosine (8OHDG) (Table 3). These antibodies have been used to show oxidative stress changes in a wide range of brain disorders including FASD in animal models. GCLC is expressed highly in astrocyte cells of human optic neuropathy brains [141]. MDA is displayed in cytoplasmic granules in neurons, astrocytes, and macrophages of human brains with multiple sclerosis lesions [142]. Similarly, oxidized lipids in 4HNE exhibited cytoplasmic in hippocampal neurons in rats during neurogenesis [143]. Nuclear DNA has been immunostaiend with 8OHDG in human hippocampus diagnosed with preclinical Alzheimer's disease (PCAD) [144].

I used five commercially available markers of excitatory or inhibitory neurons. For excitatory glutamergic neurons, anti-glutamate transporter (EAAC1) for human brains, and

excitatory amino acid transporter 3 (EAAT3) for macaque brains have been selected (by the author) in this study. EAAC1 and EAAT3 have been found immunohistochemically in neurons and dendrites of patients with schizophrenia [145], Alzheimer disease, and Huntington disease [146].

I chose parvalbumin (PVALB), and calbindin1 (CALB1) for inhibitory GABAergic neurons in human brains, and GAD1/GAD67 for macaque brains in this experiments (see Table 4). Parvalbumin (PVALB) and calbindin1 (CALB1) have been detected in adult mice cerebellar Purkinje neurons, temporal cortex, and hippocampus following alcohol exposure [147]. Similarly, GAD1/GAD67 has been identified immunohistochemically labeled pyramidal neurons dorsal hippocampus of adult schizophrenic rats [148].

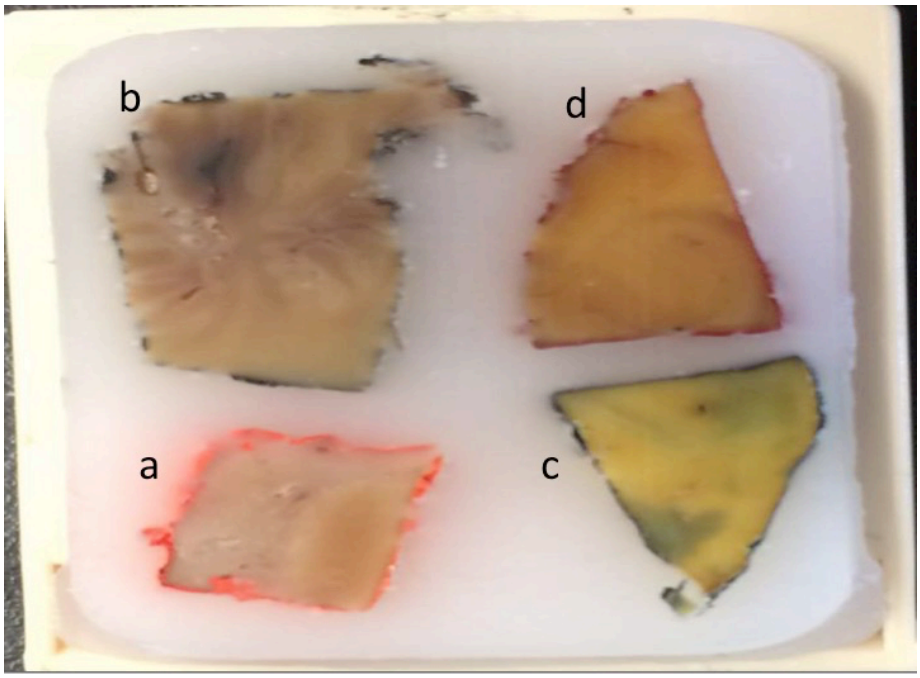

Figure 6: Four-tissue sample array block

a. Section from a 12 year old normal human brain cortex 8h post-mortem delay autopsy. b. Section from a cerebellum of a human Alzheimer's brain. c. Occipital cortex section from a 6-month macaque with 0% ethanol exposure. d. Occipital cortex section from a 6-month macaque prenatally exposed to 0.6gm/kg ethanol.

3.2 Methods

3.2.1 Immunohistochemistry

Immunohistochemistry (IHC) has been widely used as the gold standard method to detect the presence of an antigen (protein) in a specific tissue. In this project, I used a standard protocol to investigate the presence of a targeted protein in the brain tissue. Slides were deparaffinised and rehydrated in xylene. The antigen-unmasking step was adjusted (by the author) differently for each antibody. This was followed by a distilled water wash (Tables 3 and 4). Then, I treated

the slides with a mixture of 30% hydrogen peroxide and 100% methanol for 10 minutes. This was followed by two washes of 1x Phosphate buffered saline (PBS).

I blocked the tissues for 30 minutes for non-specific binding with a 1.5% serum blocking; this was performed according to the species the secondary antibody was raised for. Then, I applied the primary antibodies with a specific concentration and incubation time, adjusted also by the author (Tables 3 and 4). The secondary antibodies that arose against the primary antibodies were diluted in BSA and added on the tissue sections for 60 minutes. Then, I applied a mixture of 1:400 streptavidin, diluted in 1x PBS, for 30 minutes. DAB has been used as a chromogenic substrate. Dehydration, mounting, and cover slipping followed a hematoxylin counterstain.

3.2.1.1 Detection of oxidative stress damage

Nineteen human FASD cases were selected according to their tissue qualities (6 fetuses, 6 infants, and 6 children), age-matched controls and sex-matched controls. I examined these cases for the presence of markers of cell death and oxidation. I optimized all antibodies using positive control tissues to verify that the abnormalities can be detected. Then, I evaluated cell types microscopically at 100x and 400x by morphology, which, in some circumstances, can be unambiguous (e.g. large neurons, endothelial cells). I used paired blinded evaluation to determine if the oxidative marker was similar or differed between the controls and exposed subjects (cell distribution, signal intensity) (Table 3).

Table 3: Oxidative stress antibody conditions for immunohistochemistry

Name	Antibody type	Catalogue #	Antibody concentration (mg / mL)	Incubation time	Antigen retrieval	Secondary detection
Glutamate cysteine ligase catalytic (GCLC)	Rabbit polyclonal	Abcam (ab40929)	0.00125	90min	PH6	Goat Anti-Rabbit
Malondial-dehyde (MDA)	Rabbit polyclonal	Abcam (ab6463)	0.0006667	90min	PH6	Goat Anti-Rabbit
4 Hydroxy-nonenal (4HNE)	Rabbit polyclonal	Calbiochem (393207)	0.0006667	90min	PH6	Goat Anti-Rabbit
8-Hydroxy-deoxyguan-osine (8OHDG)	Mouse monoclonal	Abcam (ab48508)	0.02	90min	PH6	Goat Anti-Mouse

3.2.1.2 Neuronal cell distribution detection

The excitatory amino acid transporter 3 (EAAT3) and glutamate transporter EAAC1 are expressed in glutamatergic (excitatory) neurons in the brain. I used Anti-parvalbumin (PVALB) antibody, Anti-calbindin1 (CALB1) antibody, and Anti-GAD1/GAD67 antibody to identify the GABAergic (inhibitory) neurons. IHC with diaminobenzidine (DAB) labeling was used (by the author) to determine the abundance of excitatory and inhibitory neurons in the hippocampus and temporal cortex areas (Table 4).

Table 4: Neuron immunohistochemistry markers

Name	Antibody type	Vendor and Catalogue #	Antibody concentration (mg / ml)	Incubation time	Antigen retrieval	Secondary detection
Anti-Glutamate Transporter (EAAC1)	Rabbit Anti-Rat	Alpha Diagnostic (EAAC11-A)	0.00125	90min	PH6	Goat Anti-Rabbit
Excitatory amino acid transporter 3 (EAAT3)	Rabbit Polyclonal	Santa Cruz Bio. (Sc-25658)	0.005	90min	PH6	Goat Anti-Rabbit
Parvalbumin (PVALB)	Goat Polyclonal	Santa Cruz Bio. (Sc-7447)	0.01	90min	PH6	Rabbit Anti-Goat
Calbindin1 (CALB1)	Rabbit monoclonal	Abcam (ab11426)	0.00125	90min	PH8	Goat Anti-Rabbit
GAD1/GAD67	Rabbit Polyclonal	Novus Biologicals (NB100-56385)	0.005	90min	PH6	Goat Anti-Rabbit

3.2.2 Immunofluorescence

3.2.2.1 Direct immunofluorescence

Following IHC detection, I used immunofluorescence following a standard protocol. The sections were deparaffinised (by the author) in xylene and rehydrated with 100%, 95%, and 70% ethanol. This was followed by a tap water rinse. I used the antigen retrieval conditions according to the datasheet and literature recommendations (Table 5). Then, I immersed the tissue sections completely in 100% methanol at –20°C for 10 minutes. This was followed by a PBS wash. I blocked all my specimens with the appropriate blocking serum for 60 minutes. Then, I drained

the blocking solution and applied a primary antibody with the appropriate concentration diluted in antibody diluents (1xPBS and 1% BSA) (Table 5).

I kept the tissue slides overnight at 3°C. The following day, I washed the slides in PBS three times. Then, I applied the appropriate secondary antibodies to a dark area for 60 minutes. Multiple PBS washes followed. Then, I mounted the slides with the Antifade-mounting medium for fluorescence (#110906).

Table 5: Neuronal changes, antibodies specific conditions for immunofluorescence

Name	Antibody type	Vendor and Catalogue #	Antibody concentration (mg / ml)	Incubation time	Antigen retrieval	Secondary detection
Anti-Glutamate Transporter (EAAC1)	Rabbit Anti-Rat	Alpha Diagnostic (EAAC11-A)	0.00125	90min	pH6	Cy3 Donkey Anti Rabbit
Excitatory amino acid transporter 3 (EAAT3)	Rabbit Polyclonal	Santa Cruz Bio. (sc-25658)	0.005	90min	pH6	Cy3 Donkey Anti Rabbit
Parvalbumin (PVALB)	Goat Polyclonal	Santa Cruz Bio. (Sc-7447)	0.01	90min	pH6	Cy3 Rabbit Anti-Goat
Calbindin1 (CALB1)	Rabbit monoclonal	Abcam (ab11426)	0.00125	90min	pH8	Cy3 Donkey Anti Rabbit
GAD1/GAD67	Rabbit Polyclonal	Novus Biologicals (NB100-56385)	0.005	90min	pH6	Cy3 Donkey Anti Rabbit

3.2.3 Cell counts

I selected anatomical regions including CA1, CA3 and the dentate gyrus sectors of the hippocampus and inferior temporal cortex of human brain (Figure 7), in addition to the posterior frontal cortex in the macaque brains (Figure 8). Then, photographed at 100x for counting and 400x for cell morphology. I used ImageJ software, a stereological technique, for counting the neural cell population. The ratio of excitatory and inhibitory neurons present in the FASD brain tissues was then calculated (by the author).

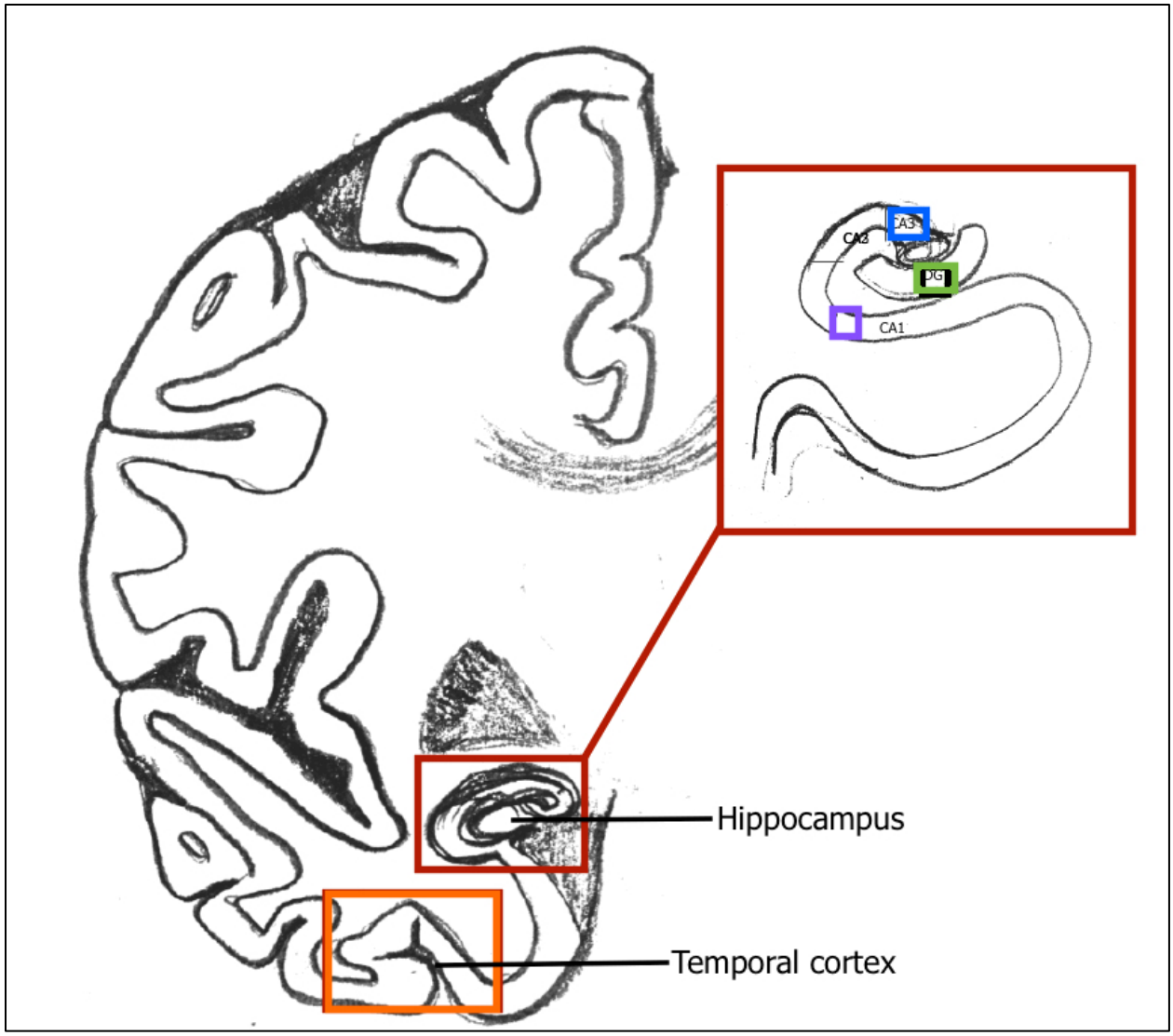

Figure 7: Diagram depicting the specific area of interest in human brain

Hippocampus regions CA1 (purple square), CA3 (blue square), and dentate gyrus (green square).

In addition to, inferior temporal cortex (orange square).

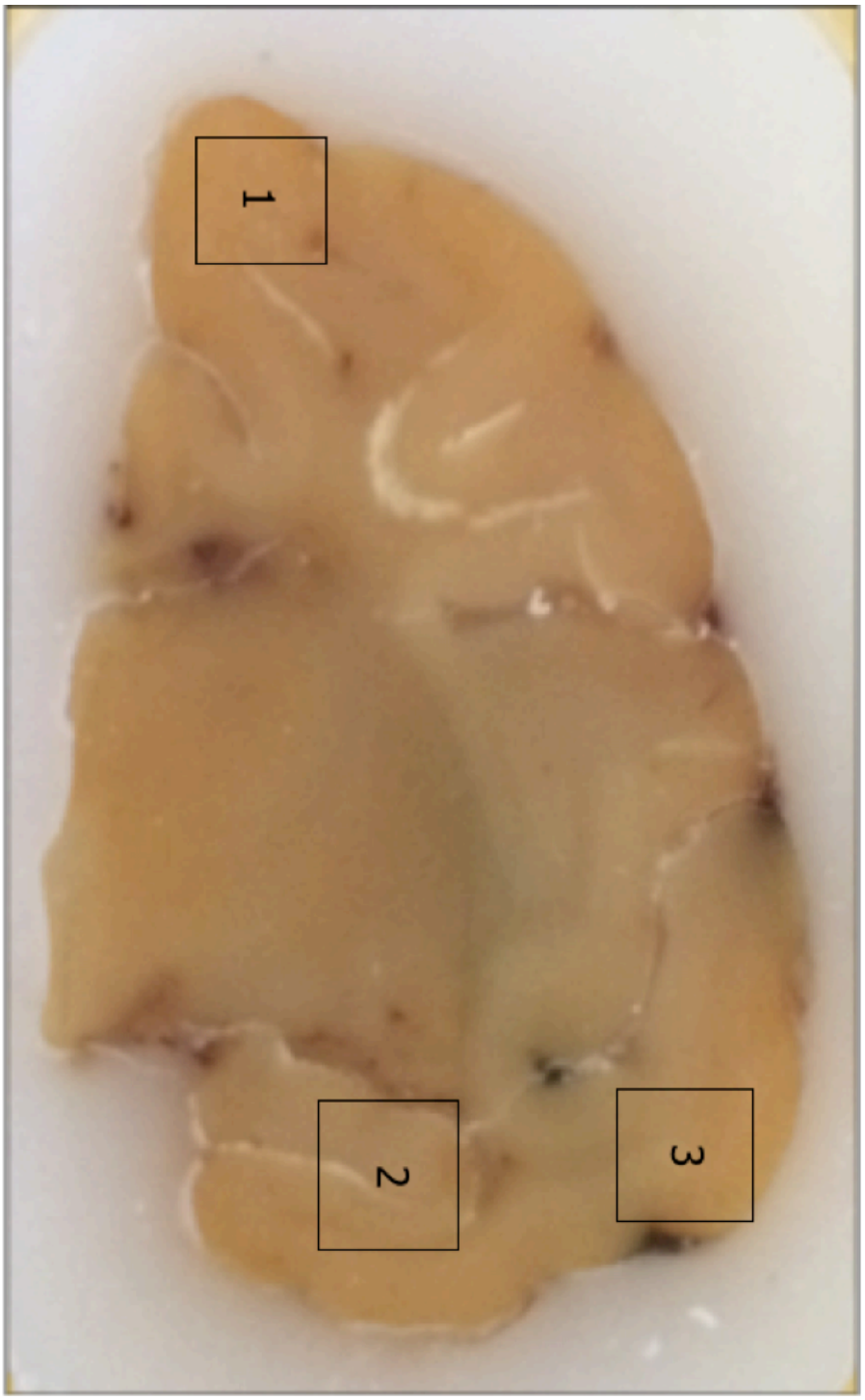

Figure 8: Left hemisphere from M. nemestrina brain tissue

embedded in the paraffin. The circles indicate the interest areas of the study: 1. Posterior frontal cortex. 2. Hippocampus. and 3. Inferior temporal area.

3.3 Data Analysis

The data analysis followed three steps: 1. Photograph the tissue slides. 2. Quantify the targeted neurons. 3. Conduct statistical tests.

After pairing human and macaque brain tissue slides (age/sex) (by the author), I used an upright optical light microscope; Olympus BX51 connected to a camera (MicroPublisher 5.0 RTV) to determine the area of interest; CA1, CA3, dentate gyrus, inferior temporal cortex, and posterior frontal cortex by their histological features. From these areas, I choose specific zones approximately 0.50 mm x 0.50 mm in consistent pattern along all human and macaque cases for cell counting. Images were taken (by the author) at 200x magnification for counting and 400x magnification for cell features in the specific area of interest. Then, I counted the targeted neurons using semi quantitative software (ImageJ). The ratio of positive cells in each region were obtained from the formula:

Number of positive cells in the selected field ÷ Total number of the cells in the field

After that, I conducted appropriate statistical tests translate the raw data from ImageJ to rational results.

3.3.1 Chi-Square analysis

Oxidative stress markers results examined 36 cases, 18 ethanol exposed fetus (6 cases), infants (6 cases), and children (6 cases) and 18 sex-matched controls. I blindly categorized the cases into three ordinal variables according to the degree immunoreactivity based on the darkness of the cells; 0= Absent, 1= Low, 2= Moderate, and 3= High. Chi-square analysis was used (by the author) for the association between nominal variables of oxidative stress reactivity overall age groups.

3.3.2 ANOVA

I performed the analysis of variance to calculate the significant interaction between neuron distribution; glutamergic, and GABAergic *in utero* alcohol exposure and non exposed individuals among different variables including age, sex, and selected brain regions. These variables were besides measured in Paired t test to calculate the percentage different between ethanol-exposed and non-ethanol groups in this study. The confidence interval of the difference was established equal to 95%.

Chapter 4. RESULTS

4.1 Neuropathological findings in human autopsied brains exposed to alcohol *in utero*

Major pathological deformities in the autopsy series (153 cases) included hydrocephalus (4 cases), agenesis of the corpus callosum (2 cases), microencephaly (5 cases), neural tube closure defects (one case each of sacral myelomeningocele with Chiari type 2 malformation, sacral myelomeningocele, anencephaly, exencephaly), and one case each of lissencephaly, holoprosencephaly, and Dandy-Walker malformation. Heterotopic neural tissue was detected in the subarachnoid compartment in 2 cases. Ischemic damage included in-utero cerebral infarct (2 cases), periventricular white matter damage (3 cases), and microscopic evidence of neuronal hypoxia in most stillbirth cases. Cases with these major pathological abnormalities were not used for the immunohistochemical analysis.

4.2 Oxidative stress marker detection

4.2.1 Localization of oxidative stress markers in human and non-human primate

To measure oxidative stress damage I examined the presence of oxidized DNA, and lipids within hippocampal and cortical brain regions using antibodies that recognize GCLC, 8OHdG, 4HNE, and MDA. It is important to report that these antibodies work very well in non-FASD human brains and macaque brain tissues. Then FASD brain tissues were examined using these antibodies.

Glutamate cysteine ligase catalytic (GCLC) is the rate-limiting enzyme that catalyzes glutathione production, which is up regulated during oxidative stress. GCLC displayed strong cytoplasmic positivity in glial cells and less in endothelial cells and macrophages of PNEE human, non-PNEE human, and monkey cerebral cortex layer II-III (figure 9). Nuclear oxidation

of 8OHDG exhibited robust nuclear labeling in the PNEE and non-PNEE human cerebral cortices layer II-III, as well as macaque monkey occipital cortices (figure 10). Both 4HNE (figure 11 a,b) and MDA (figure 11 c,d) showed a high population of oxidized cellular lipids in PNEE human and monkey in lipofuscin of neurons and in perivascular white matter cells presumed to be macrophages or pericytes. Some macrophage labeling appeared individual cases. Immunofluorescence of non-PNEE human brain tissue confirms the presence of 8-OHdG in the brain tissue (figure 12).

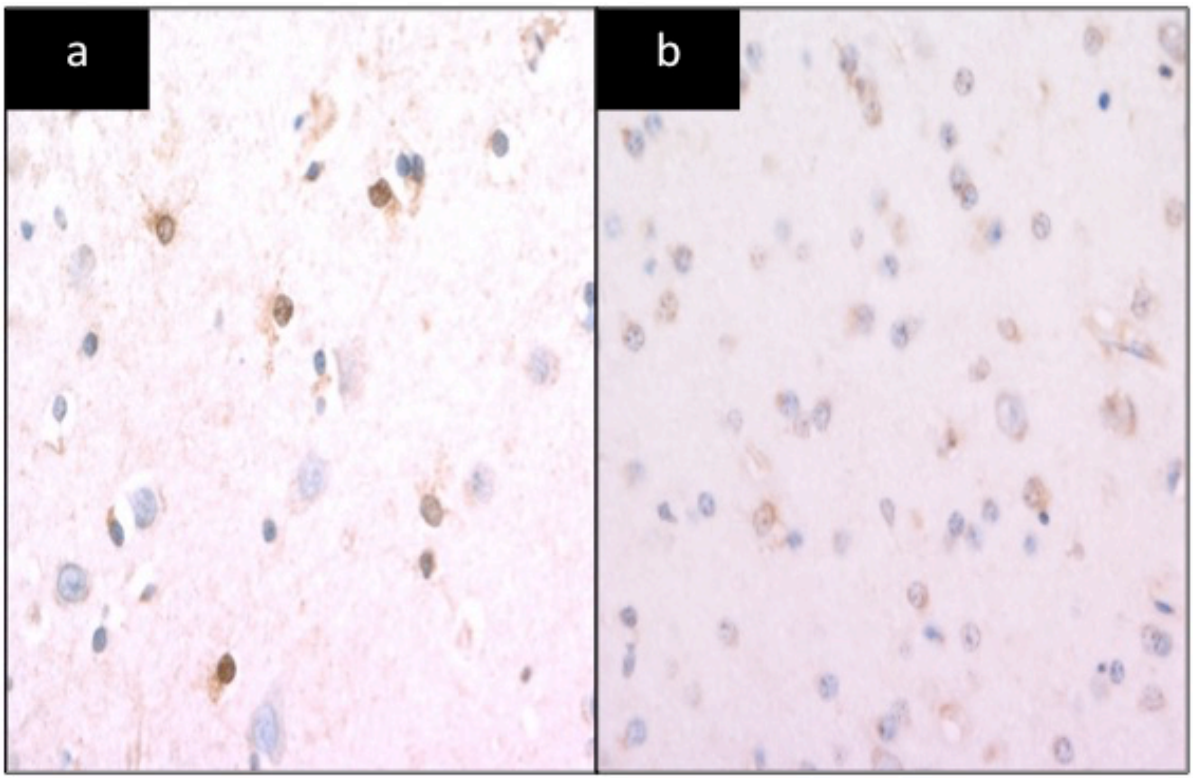

Figure 9: GCLC expressed in deep temporal cortical layer. (IHC with 1/800 diluted anti-GCLC on paraffin-embedded tissue). a. AD human brain b. Macaque monkey brain 6 months age prenatally exposed to ethanol. The human AD and monkey cases show strongly immunoreactive glial cells. Glial cells expressed reactive with rich brown cytoplasmic GCLC. 400x magnification.

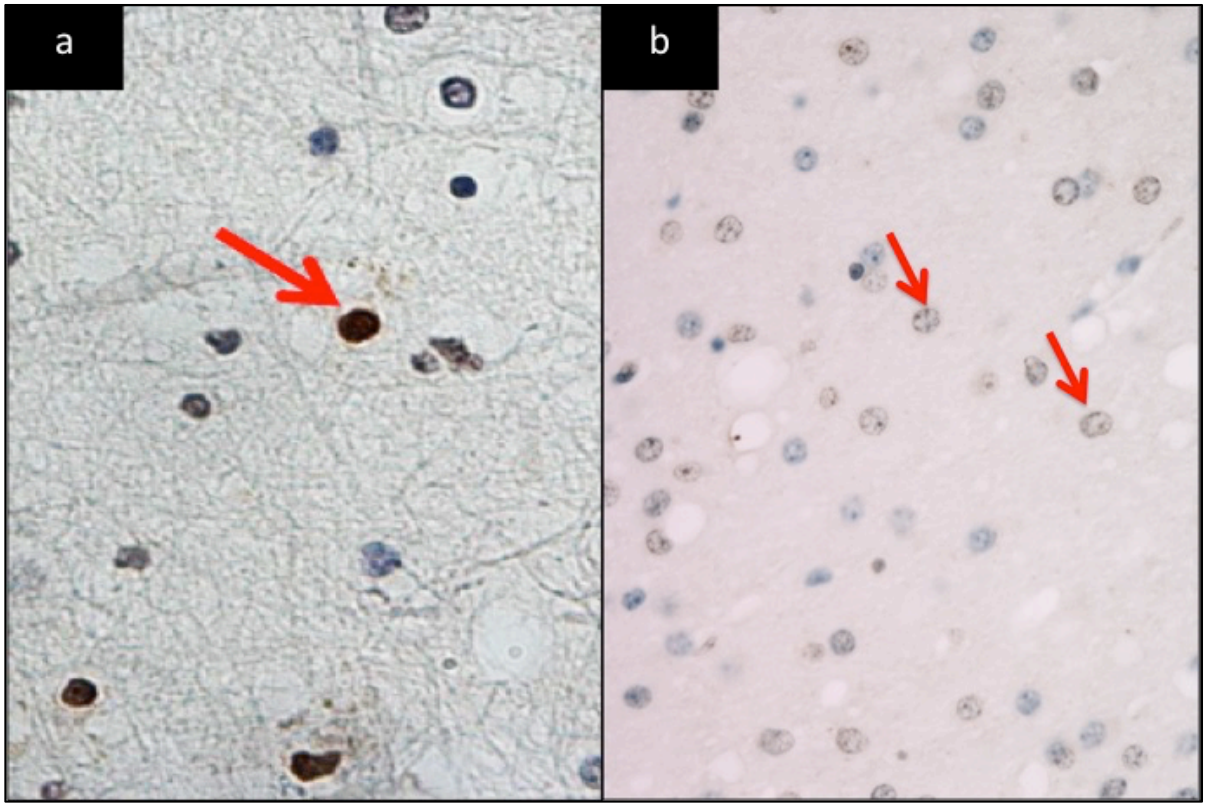

Figure 10: Nuclear oxidation expressed in deep temporal cortical layer. (IHC with 1/50 diluted anti-8OHdG on paraffin-embedded tissue). a. AD human brain b. Six-month-old macaque cortical brain prenatally exposed to ethanol. The human AD and monkey cases show nuclear immunoreactive cells. 400x magnification.

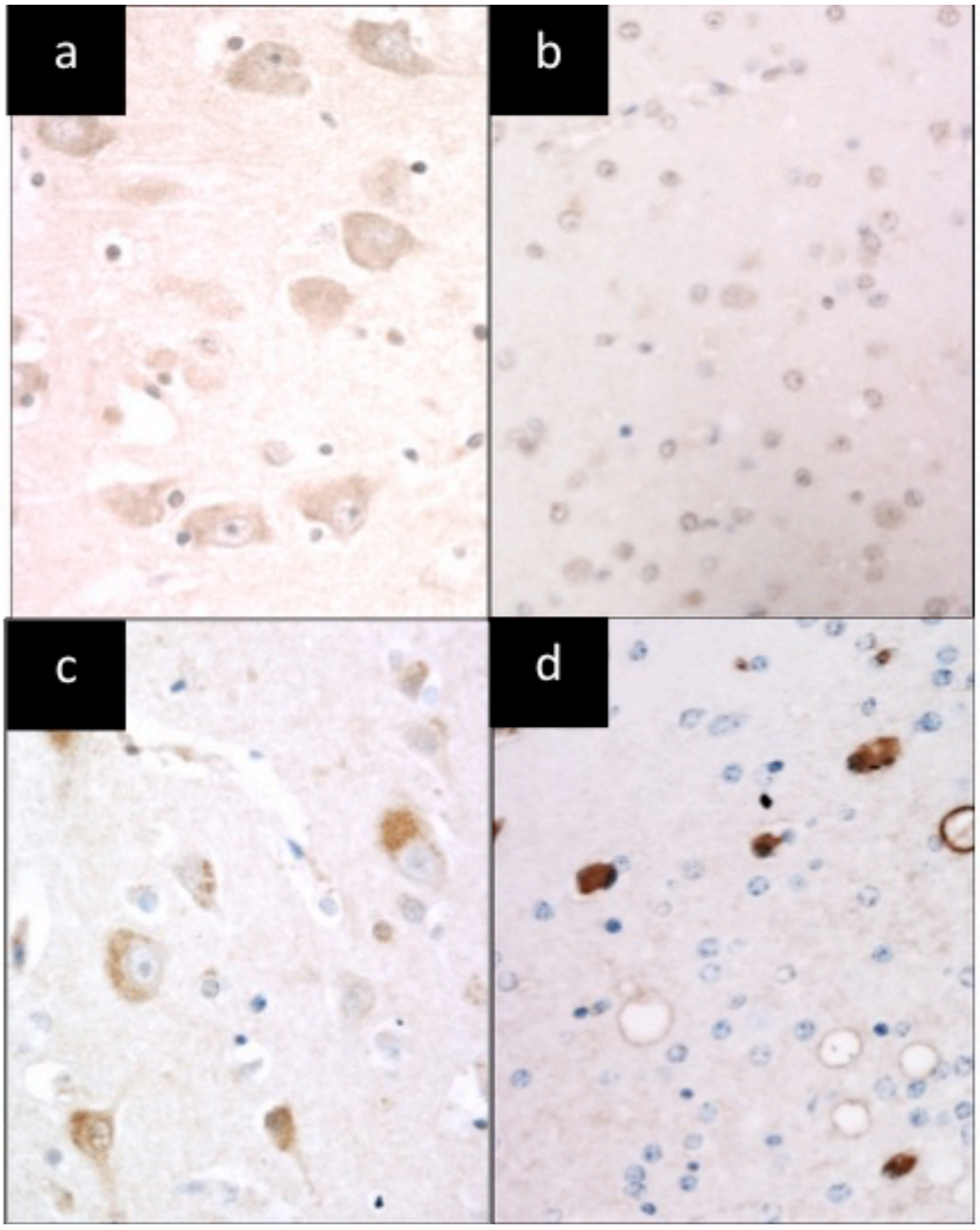

Figure 11: Lipid oxidation expressed in deep temporal cortical layer. a,b. IHC with 1/1500 diluted anti-4HNE on paraffin-embedded tissue. c,d. IHC with 1/1500 diluted anti-MDA on paraffin-embedded tissue. a,c AD human brain. b,d. Macaque monkey cortical brain 6 months age prenatally exposed to ethanol.

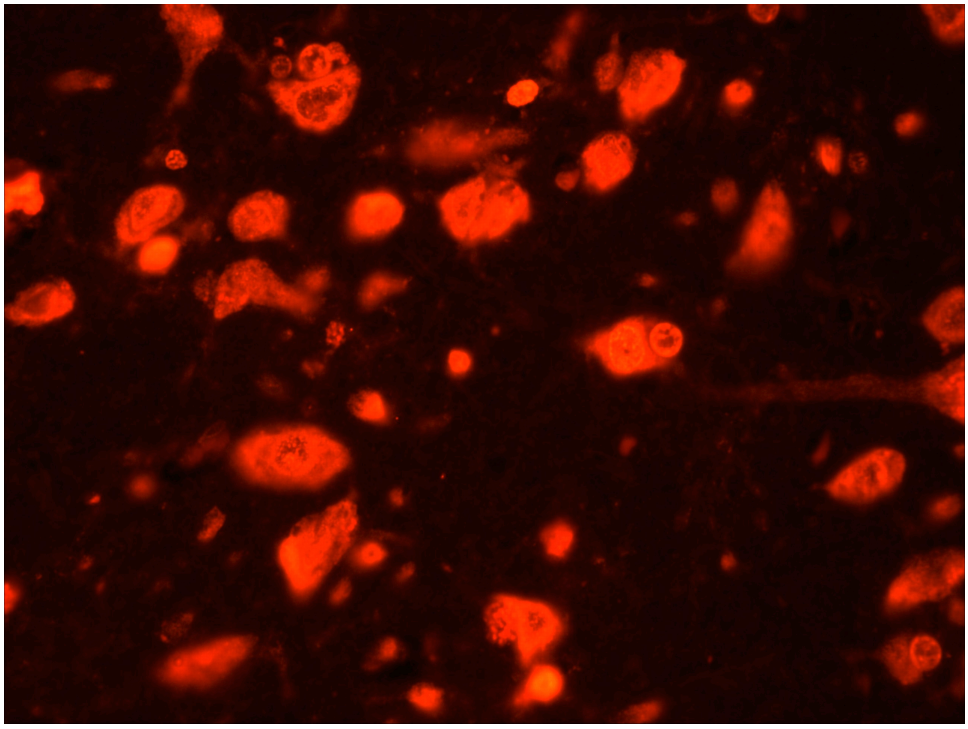

Figure 12: Immunofluorescence staining of formalin-fixed human brain tissue. Paraffin-embedded AD human brain tissue immunostained with 1/50 diluted 8-OHdG antibody followed by cy3 secondary antibody. Red stain exhibited 8-OHdG staining in brain cells. 400X magnification.

4.2.2 Quantitative comparison of oxidative stress markers in human

4.2.2.1 GCLC expression

GCLC was prominent in the majority of cases, however paired comparisons suggested higher expression in control than PNEE cases. When I separated the analysis by age group, it was indicted that the difference was most pronounced in fetuses (figure 13,14).

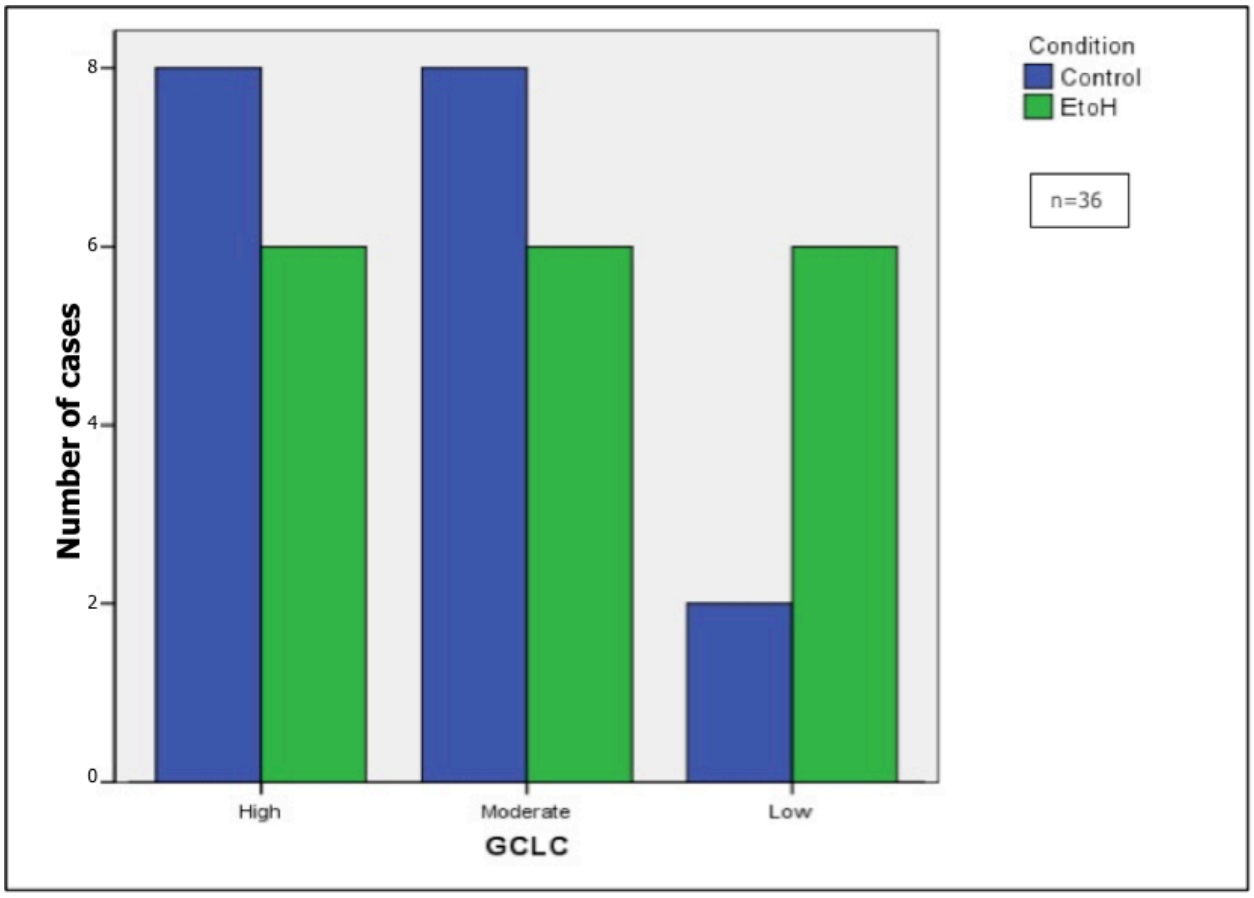

Figure 13: GCLC expression in human brain. Chi square test was performed on ordinal variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. There was no difference in neuronal immunoreactivity to GCLC in human brain tissues (n=36) (Chi-square test value=2.000, df= 2, P= 0.3679).

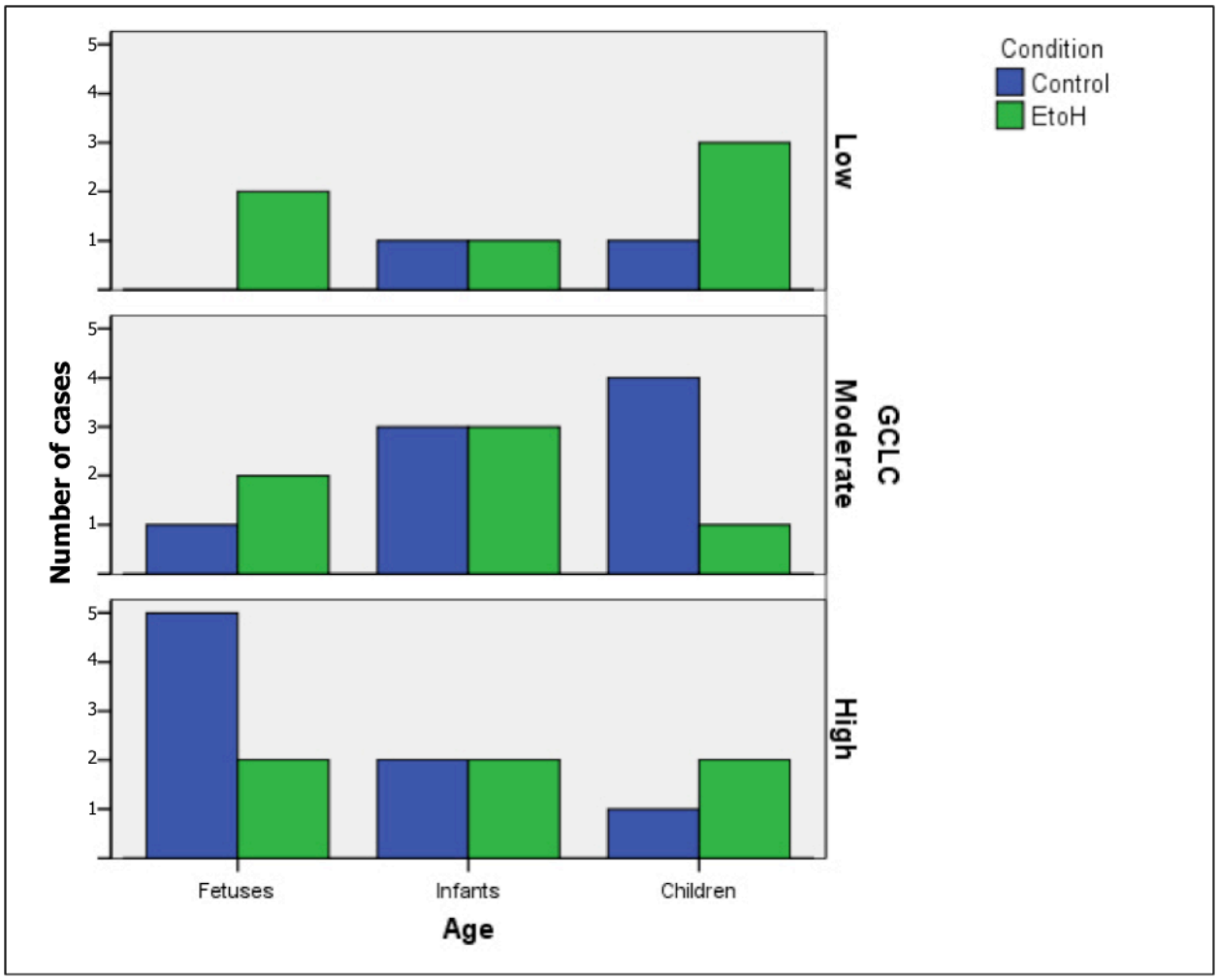

Figure 14: Age-based difference in GCLC expression of human brain. Chi square test was performed on ordinal variables variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. There was no difference in neuronal immunoreactivity to GCLC in human brain tissues (n=36) among all age groups (Chi-square test value=1.667, P= 0.435).

4.2.2.2 Quantitative comparison of DNA oxidation

Nuclear peroxidation demonstrated with anti-8OHdG was not prominent in any age group. Overall there was no statistically significant difference between controls and PNEE (figure 15). Among the three age groups tested, fetuses showed the most variance in nuclear expression of 8OHdG, tending to be higher in controls (figure 16).

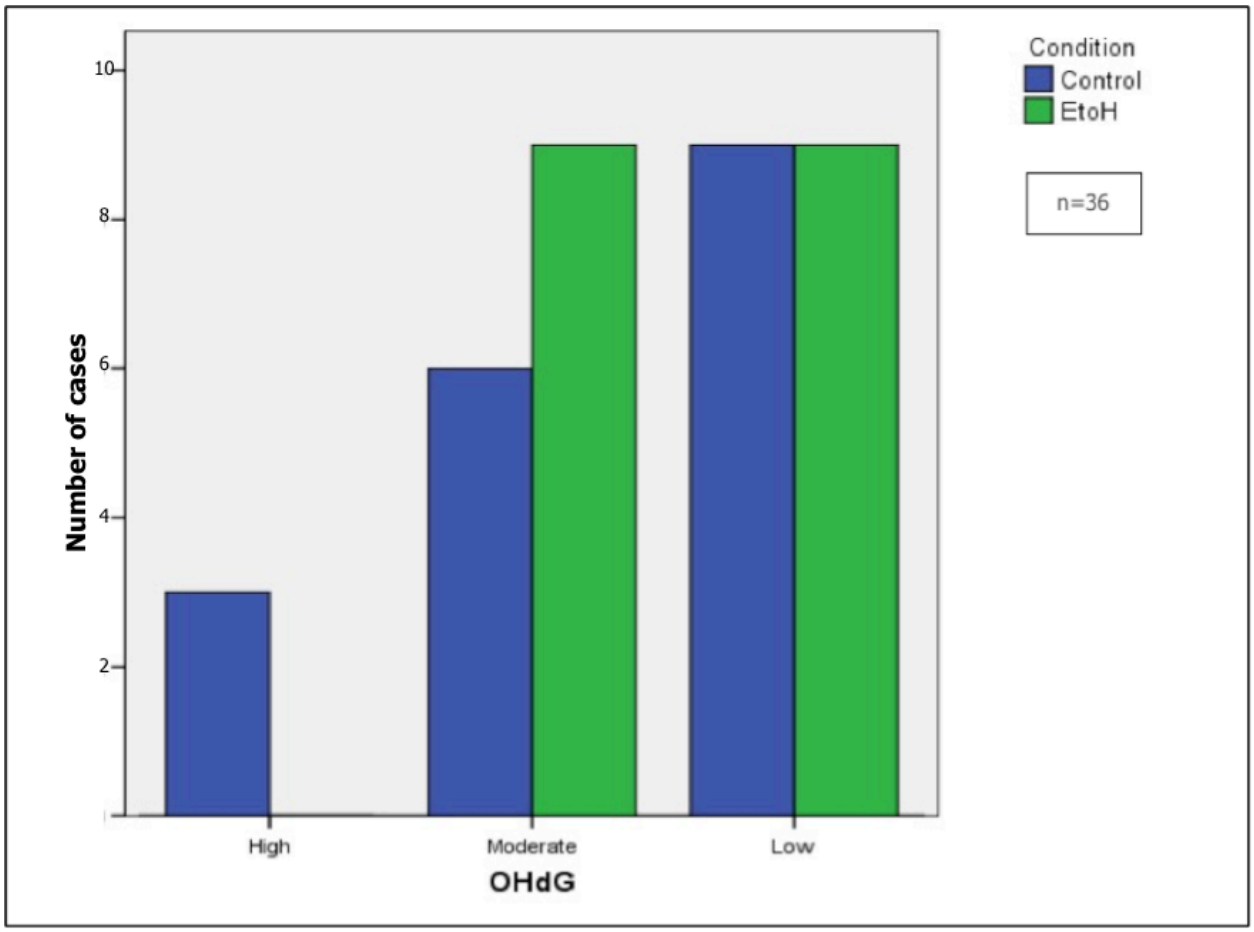

Figure 15: 8OHdG expression in human brains. Chi square test was performed on ordinal variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. There was no difference in neuronal immunoreactivity to 8OHdG in human brain tissues (n=36) (Chi-square test value=10.500, df= 2, P= 0.3052).

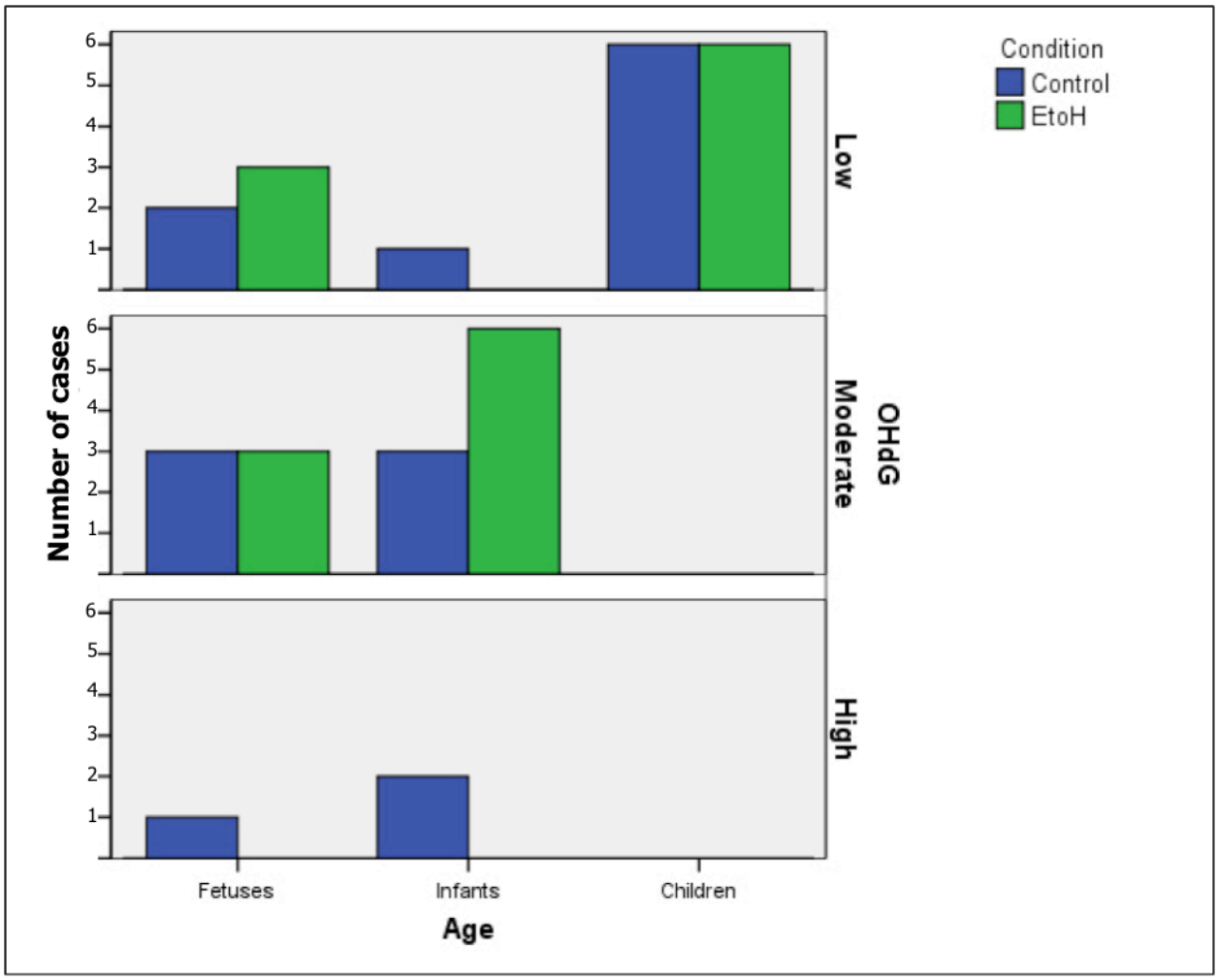

Figure 16: Age-based difference in 8OHdG expression of human brain. Chi square test was performed on ordinal variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. There was no difference in neuronal immunoreactivity to 8OHdG in human brain tissues (n=36) among all age groups (Chi-square test value=1.564, P= 0.345).

4.2.2.3 Quantitative comparison of Lipid oxidation

HNE and MDA were detected in the majority of cases. There were no significant differences between controls and PNEE cases overall or when divided by age, although the control cases tended to have more intense immunoreactivity (figure 17, 18, 19).

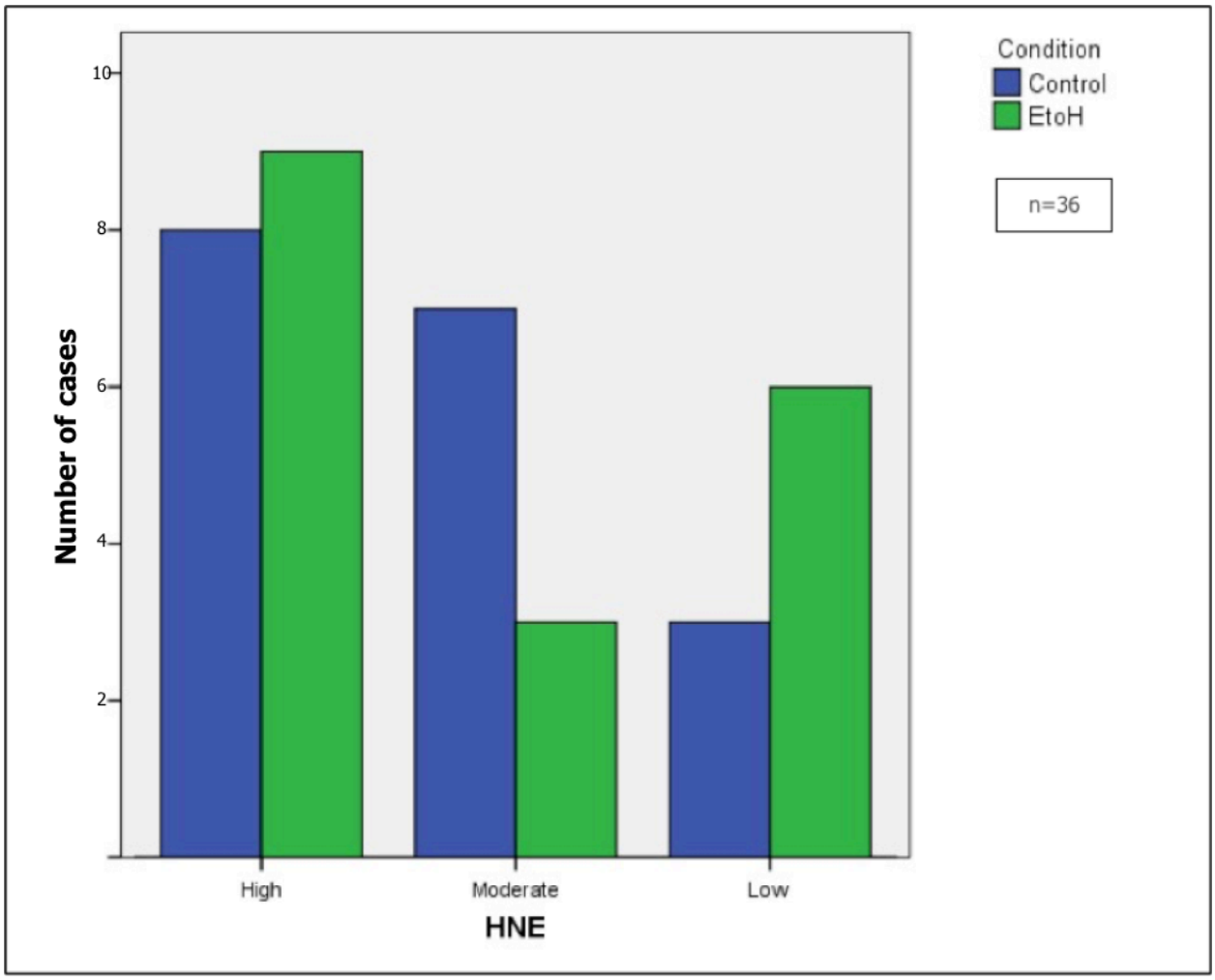

Figure 17: 4HNE expression in human brains. Chi square test was performed on ordinal variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. There was no difference in neuronal immunoreactivity to 4HNE in human brain tissues (n=36) (Chi-square test value=3.167, df= 2, P= 0.2053).

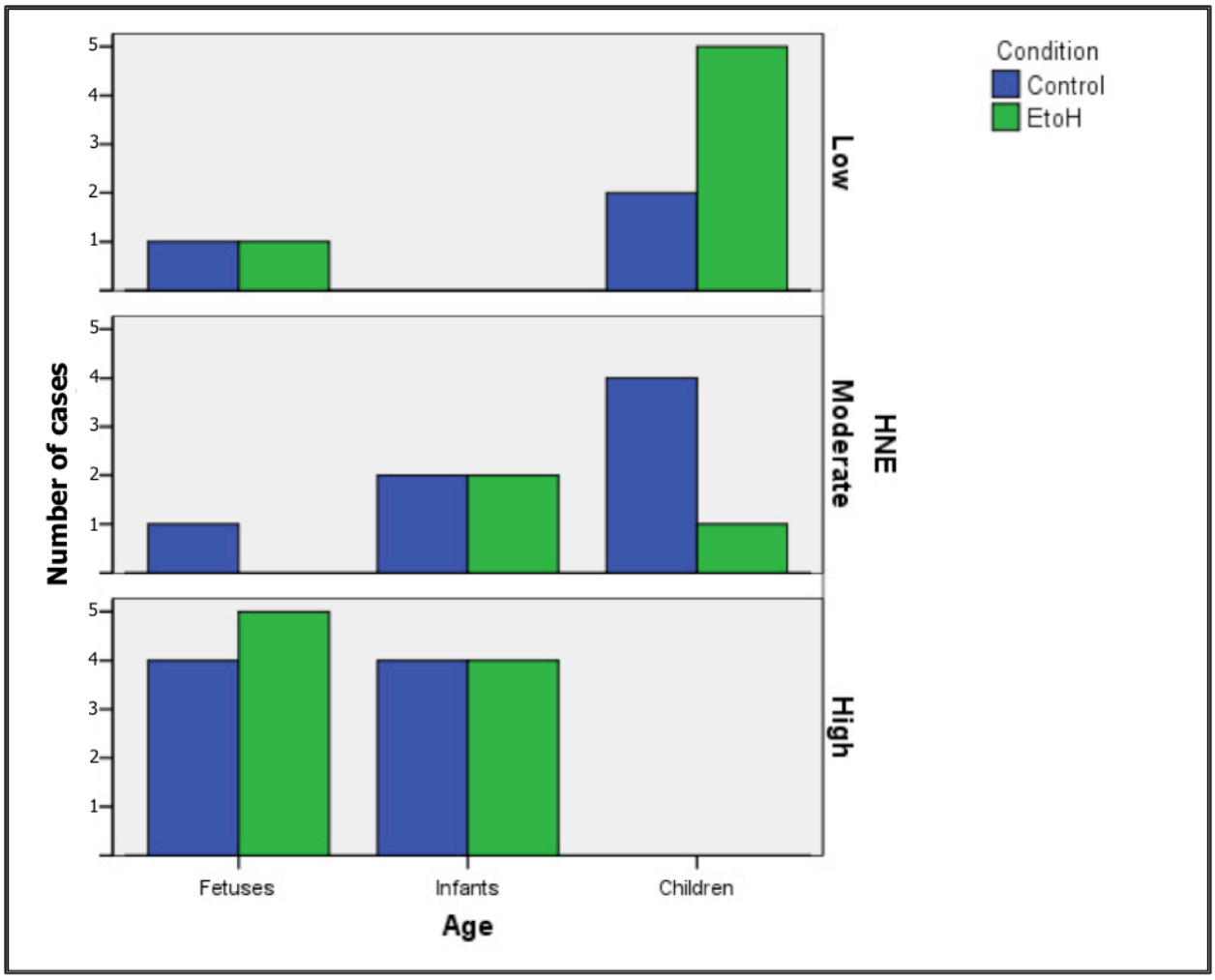

Figure 18: Age-based difference in 4HNE expression of human brain. Chi square test was performed on ordinal variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. There was no difference in neuronal immunoreactivity to 8OHdG in human brain tissues (n=36) among all age groups (Chi-square test value=2.546, P= 0.2000).

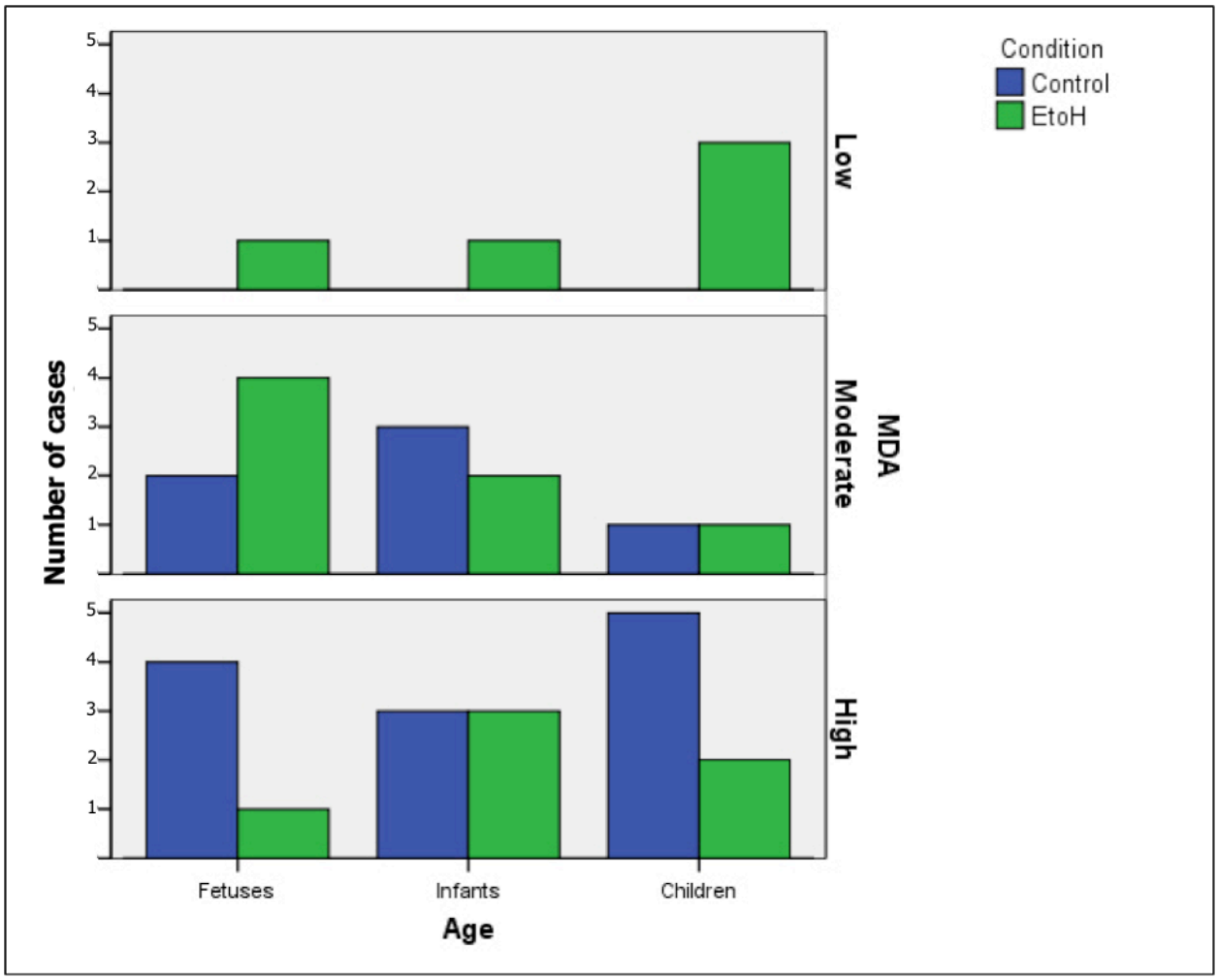

Figure 19: Age-based difference in MDA expression of human brain. Chi square test was performed on ordinal variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. There was no difference in neuronal immunoreactivity to 4HNE in human brain tissues (n=36) (Chi-square test value=1.202, P= 0.3034).

4.2.3 Oxidative stress detection in non-human primate

The macaque monkey controls and PNEE samples were categorized according to the intensity of immunostaining: 0= absent 1= low, 2= moderate, and 3= high. GCLC expressing glial cells were present in the white matter. However, there were no significant differences in GCLC expression between controls and PNEE samples (figure 20). There was no 8OHdG

immunoreactivity in macaque brain tissue from either controls or PNEE samples (data not shown). Both lipid oxidation markers, MDA and 4HNE, were demonstrated in a small number of neurons; there were no significant differences between controls and PNEE samples (figure 21, 22).

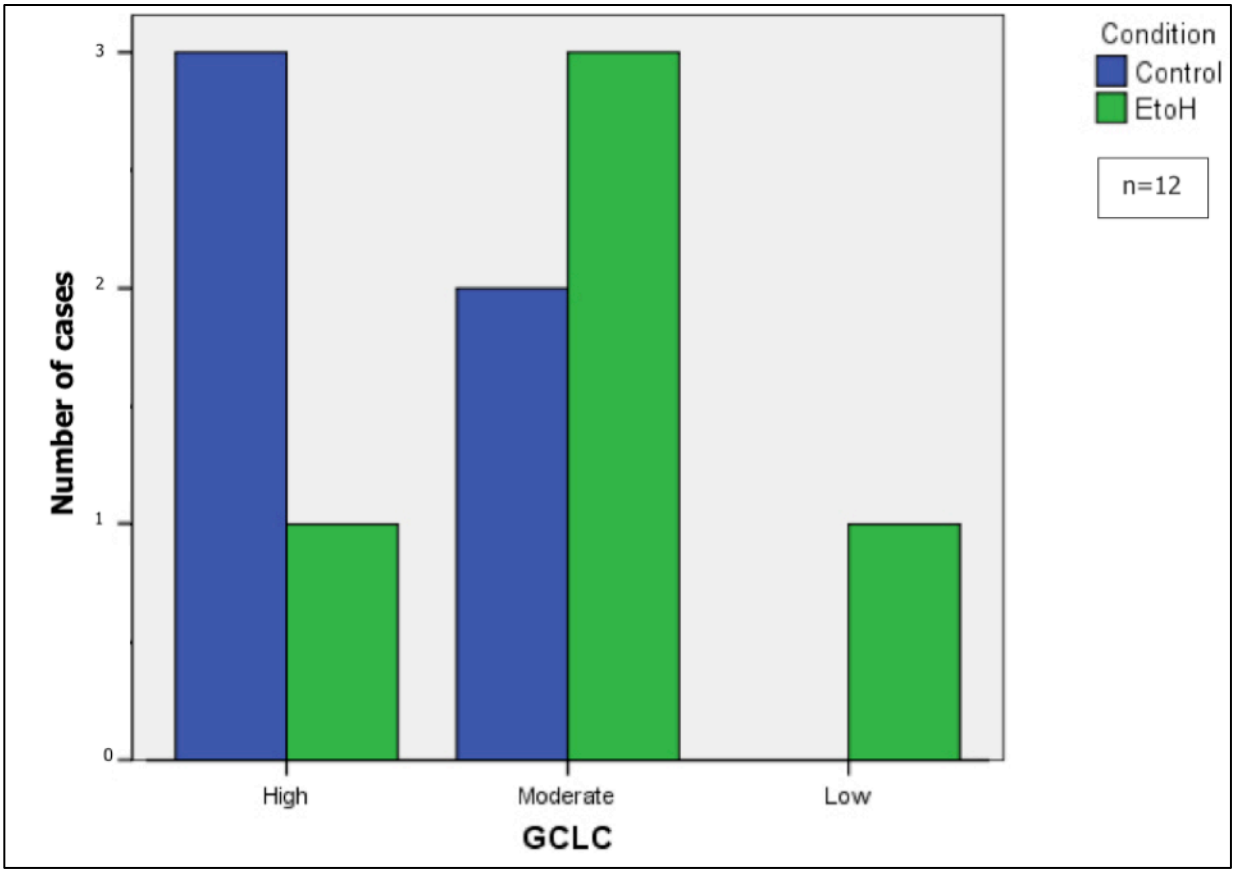

Figure 20: GCLC expression in macaque brains. Chi square test was performed on ordinal variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. (2 cases with no expression are not shown). There was no difference in neuronal immunoreactivity to 4HNE in macaque brain tissues (n=12) (Chi-square test value=2.200, df= 2, P= 0.333).

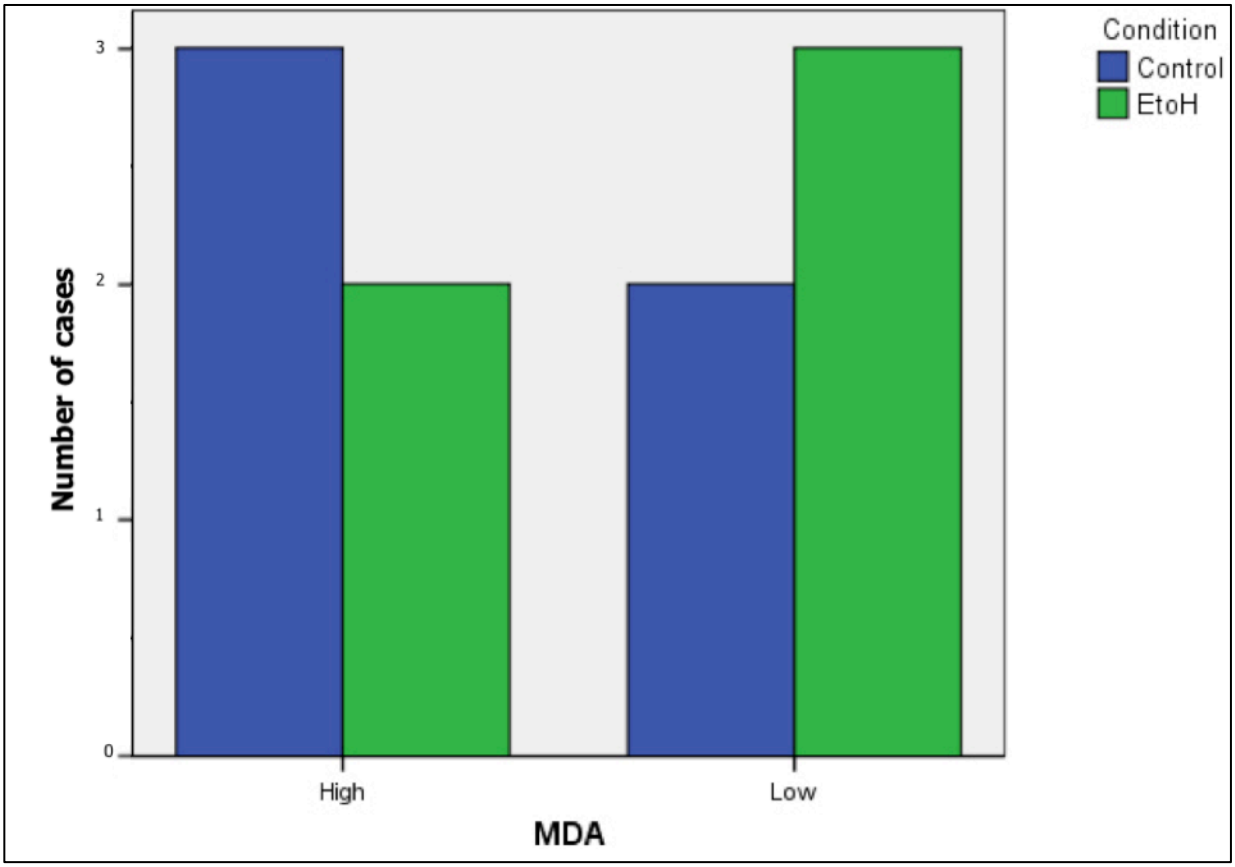

Figure 21: MDA expression in macaque brains. Chi square test was performed on ordinal variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. (2 cases with no expression are not shown). There was no difference in neuronal immunoreactivity to 4HNE in macaque brain tissues (n=12) (Chi-square test value=1.400, df= 2, P= 0.527).

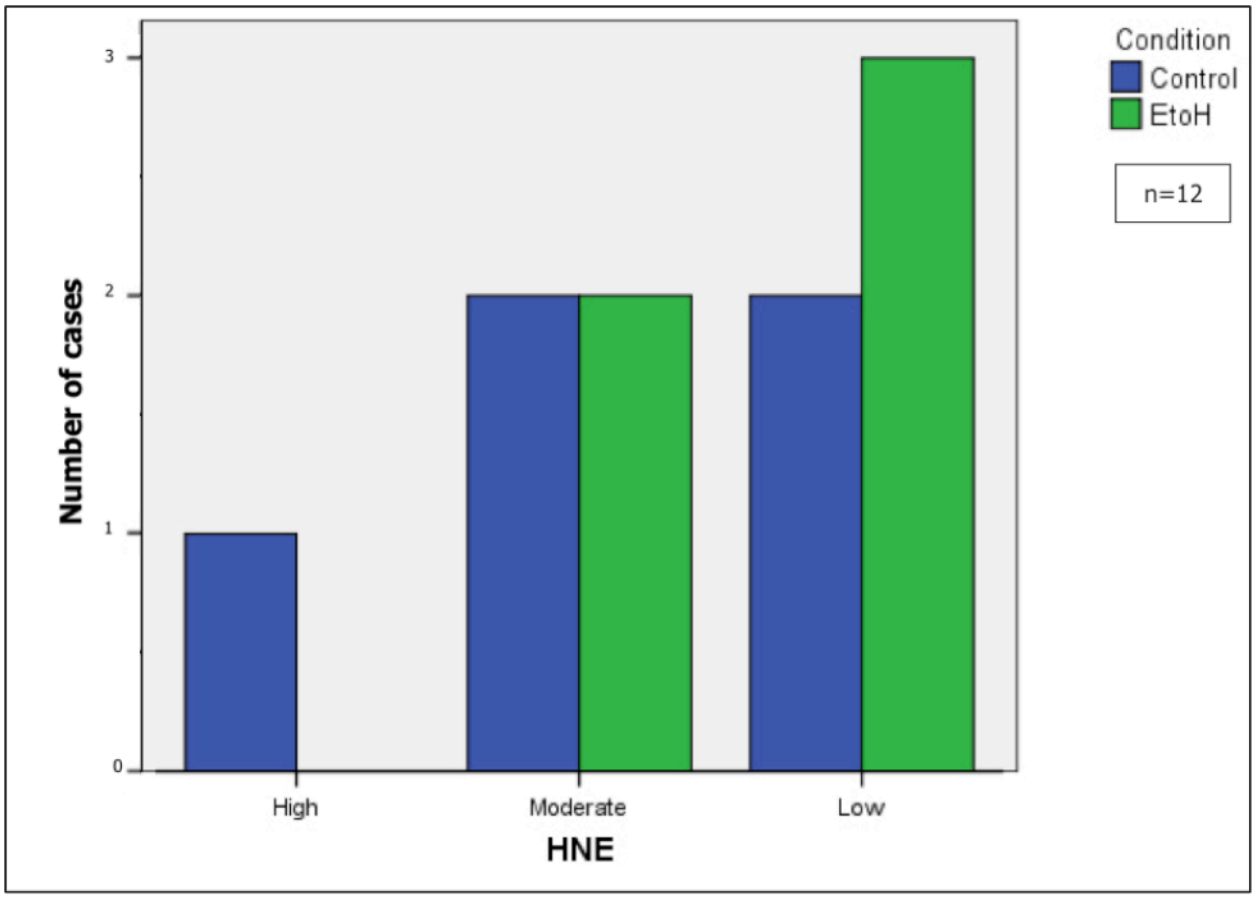

Figure 22: 4HNE expression in macaque brains. Chi square test was performed on ordinal variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. (2 cases with no expression are not shown). There was no difference in neuronal immunoreactivity to 4HNE in macaque brain tissues (n=12) (Chi-square test value=1.200, df= 2, P= 0.549).

4.3 Neuron type markers

4.3.1 Glutamatergic neurons in human brain

Glutamatergic neurons were best demonstrated in human brain samples using indirect immunohistochemistry with avidin-biotin peroxidase detection of Anti-EAAC1 (table 2). EAAC1 is evident as intracellular cytoplasmic labeling and neuropil staining in neurons of hippocampus (CA1, CA3, DG) and cortical layers II-III and V-VI. EAAC1 is expressed similarly in all age groups (infants, children, and teenage) in both controls and PNEE cases with but with different distribution (Figure 23).

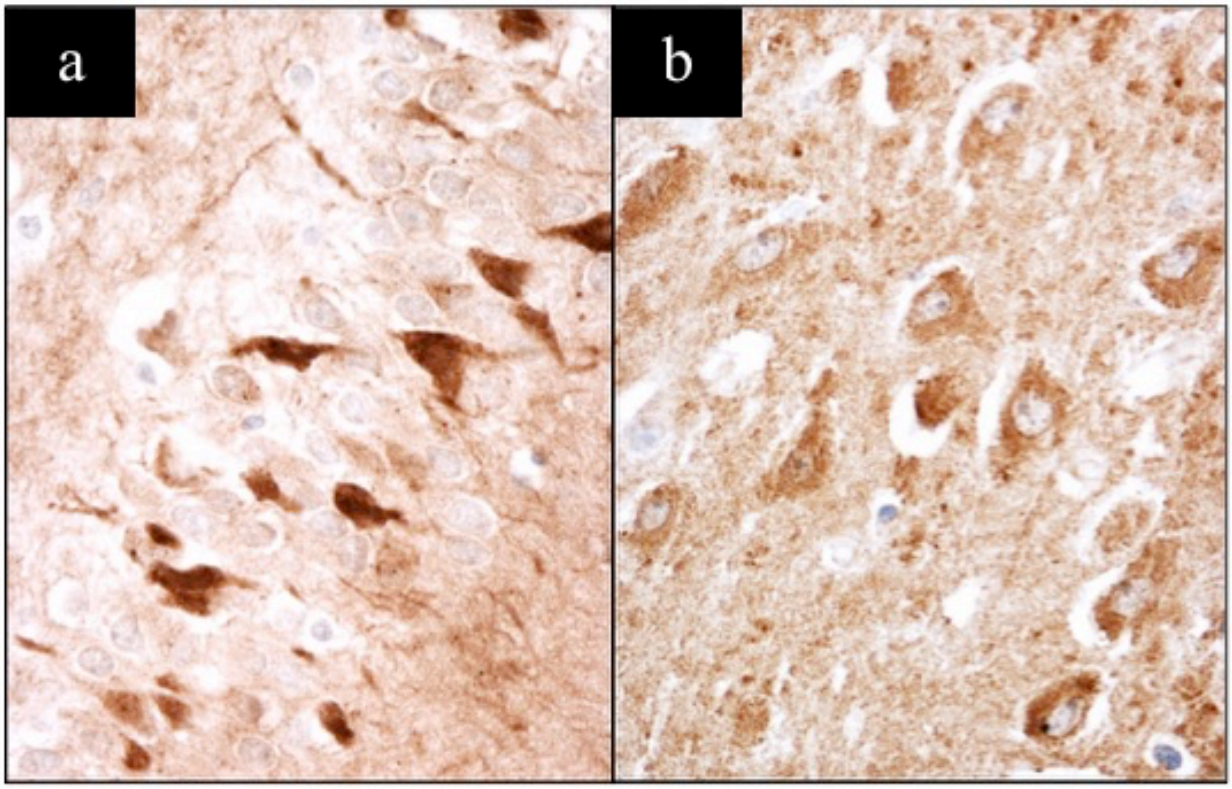

Figure 23: Glutamergic neurons in human deep temporal cortical layer. (IHC with 1/800 diluted anti-EAAC1 on paraffin-embedded tissue). a. Control female 15 months age, b. FASD female 16 months age. Control and FASD cases show strongly immunoreactive neurons. 400x magnification.

4.3.2 The intensity of EAAC1 expression in human brains prenatally exposed to ethanol

EAAC1 immunoreactivity in infants, children, and teenage brains exhibits diffuse staining in neuropil because of expression in cells bodies, dendrites, and axons. This makes it difficult to count the individual neurons in the regions of interest. Therefore, the intensity of Anti-EAAC1 cortex was categorized into three ordinal variables according to immunoreactivity and neuropil intensity; moderate immunoreactivity within neurons with moderate neuropil

staining, high immunoreactivity within neurons with moderate neuropil staining, and high immunoreactive diffuse neurons with intense neuropil staining.

The pattern of high immunoreactive diffuse neurons with intense neuropil staining was significantly increased in ethanol exposed infants, children, and teenage brain compared to age/sex matched control groups (data not shown).

4.3.3 Excitatory neuron changes in human brains with *in utero* ethanol exposure

Excitatory neuron EAAC1 expression was significantly reduced; 15.96 % and 18.03% in dentate gyrus and temporal cortex, respectively. In dentate gyrus, infants and children showed significant reduction 26.46% and 16%, respectively among different group ages. Unlike dentate gyrus and temporal cortex, CA1 or CA3 hippocampal regions did not show a significant difference of ethanol-exposed cases compared with control groups (Figure 24).

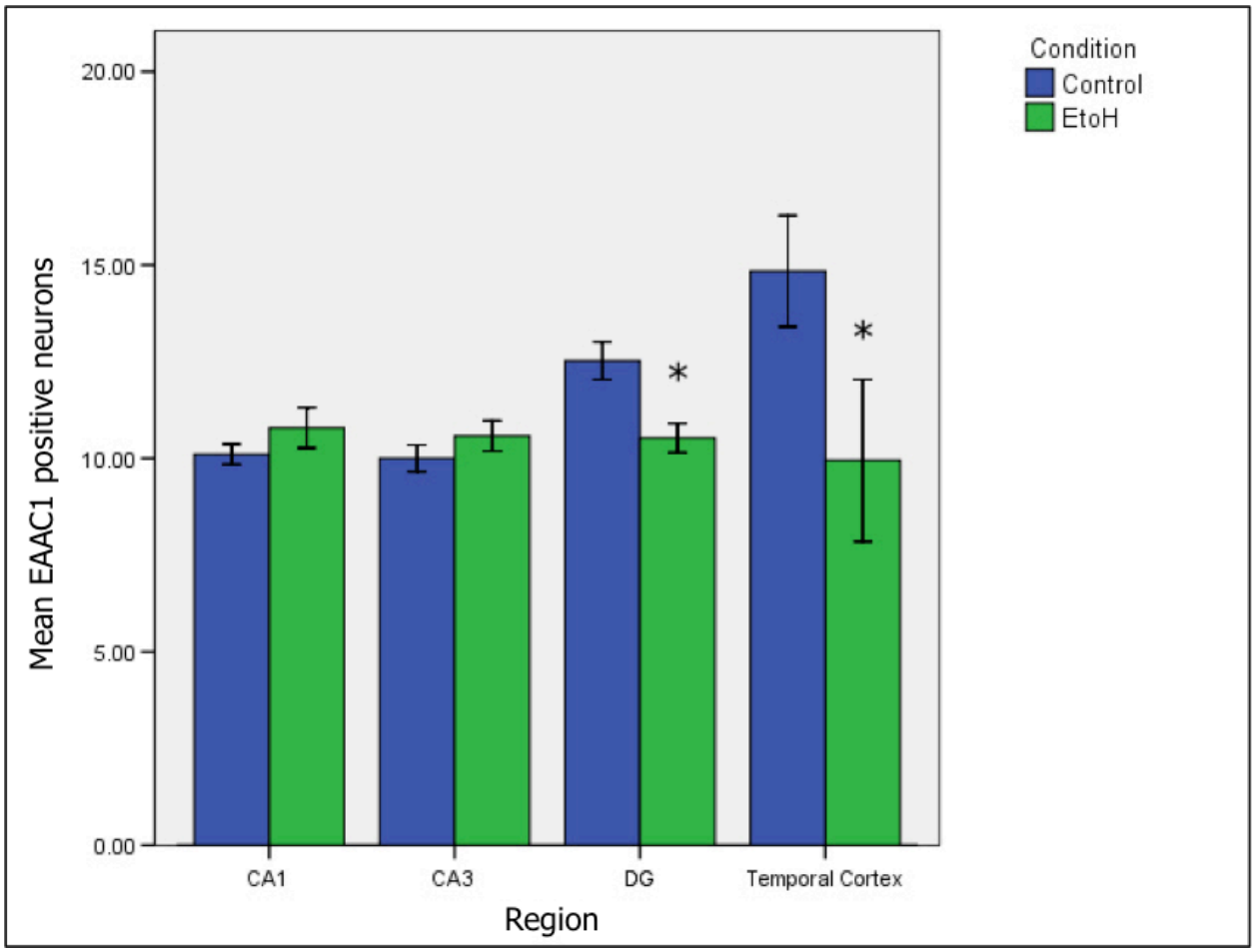

Figure 24: Mean neuron EAAC1 expression in human brain regions (CA1, CA3, DG, and temporal cortex). Asterisks demonstrate significant reductions in the mean neurons count expressed to EAAC1 of human dentate gyrus (P value equals 0.0030, t = 3.4341, df = 18) and temporal cortex (P value equals 0.0437, t = 2.1694, df = 18) between controls (n=19) and Ethanol exposed groups (n=19) among all age groups. Error bars = +/- 1 SE.

4.3.4 Age groups based EAAC1 expression in human following ethanol exposure

Subfield analysis of dentate gyrus and temporal cortex were conducted (by the author) among different group ages (infants, children and teens). Infants and children (but not teens) had a significant reduction, 26.50% and 16.00%, respectively of EAAC1 expression in dentate gyrus

following *in utero* alcohol exposure (figure 25). However, all group ages showed no significant reduction of EAAC1 expression in temporal cortex (figure 26).

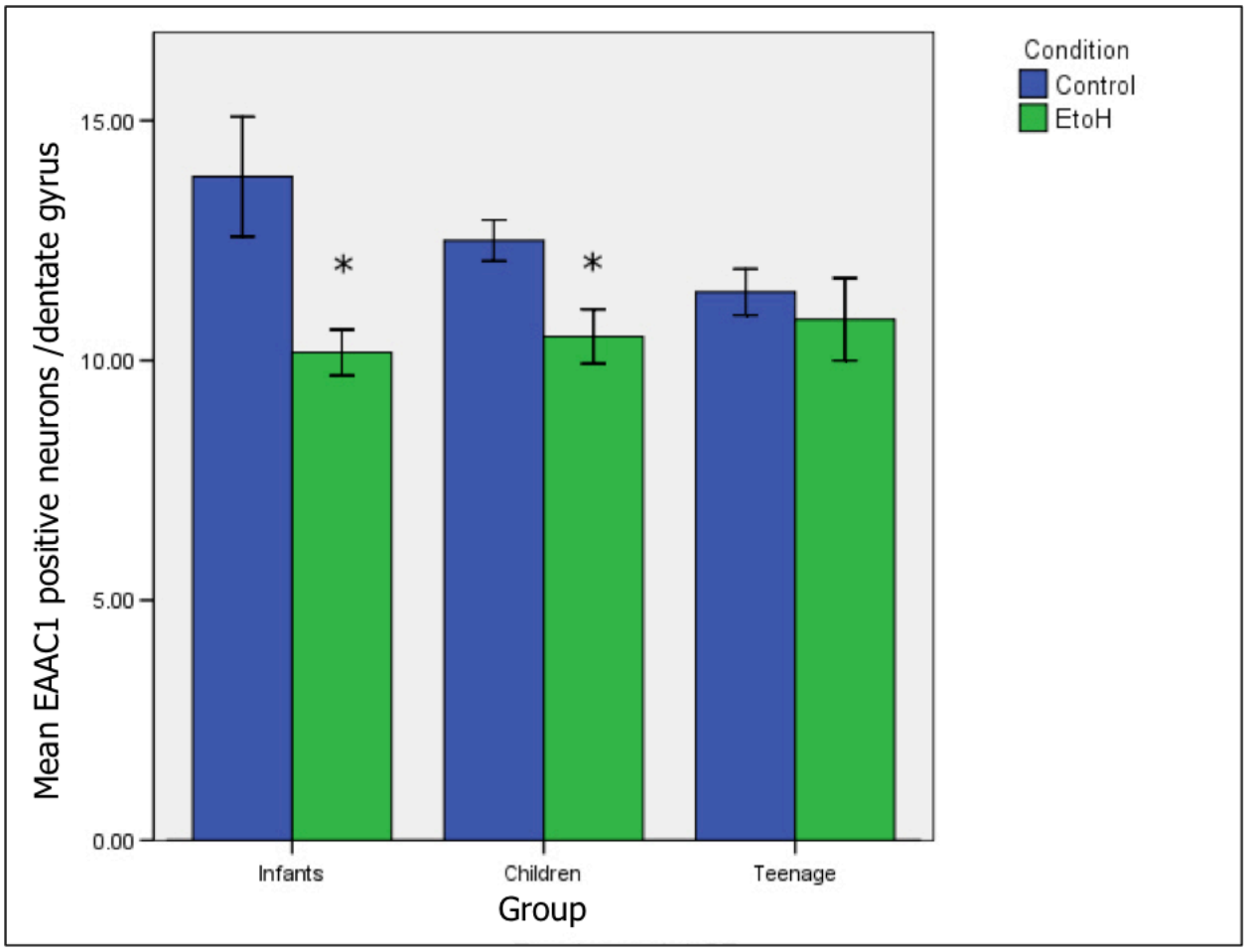

Figure 25: Mean EAAC1 expression in dentate gyrus of human brains in infants, children, and teens groups. Asterisks demonstrate significant reductions in the mean neurons count expressed to EAAC1 of human dentate gyrus on infants (n=6) (P value equals 0.0354, t = 2.8593, df = 5) and children (n=6) (P value equals 0.0117, t = 3.8730, df = 5) between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

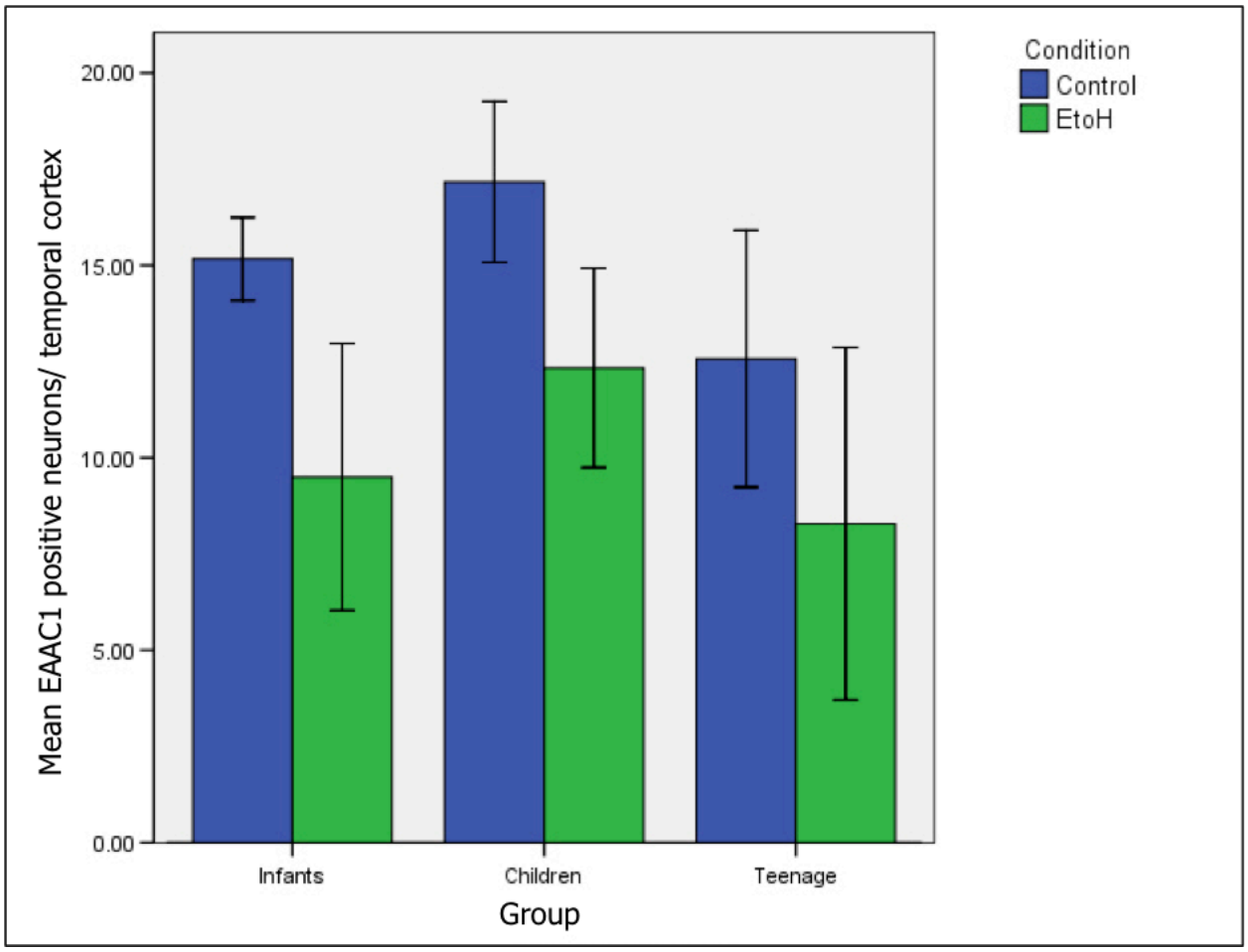

Figure 26: Mean EAAC1 expression in temporal cortex of human brains in infants, children, and teens groups. Within the temporal cortex there was no significant reduction in all-different age groups tested (infants: n=6, children: n=6, and teenage: n=7) between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

4.3.5 Sex difference based EAAC1 expression in human following ethanol exposure

In dentate gyrus, a reduction of glutamatergic neurons was significant in female group (33.33%) PNEE compared with controls (figures 27). However, neither male nor female group showed a significant reduction in human neurons expression to EAAC1 in temporal cortex (figures 28).

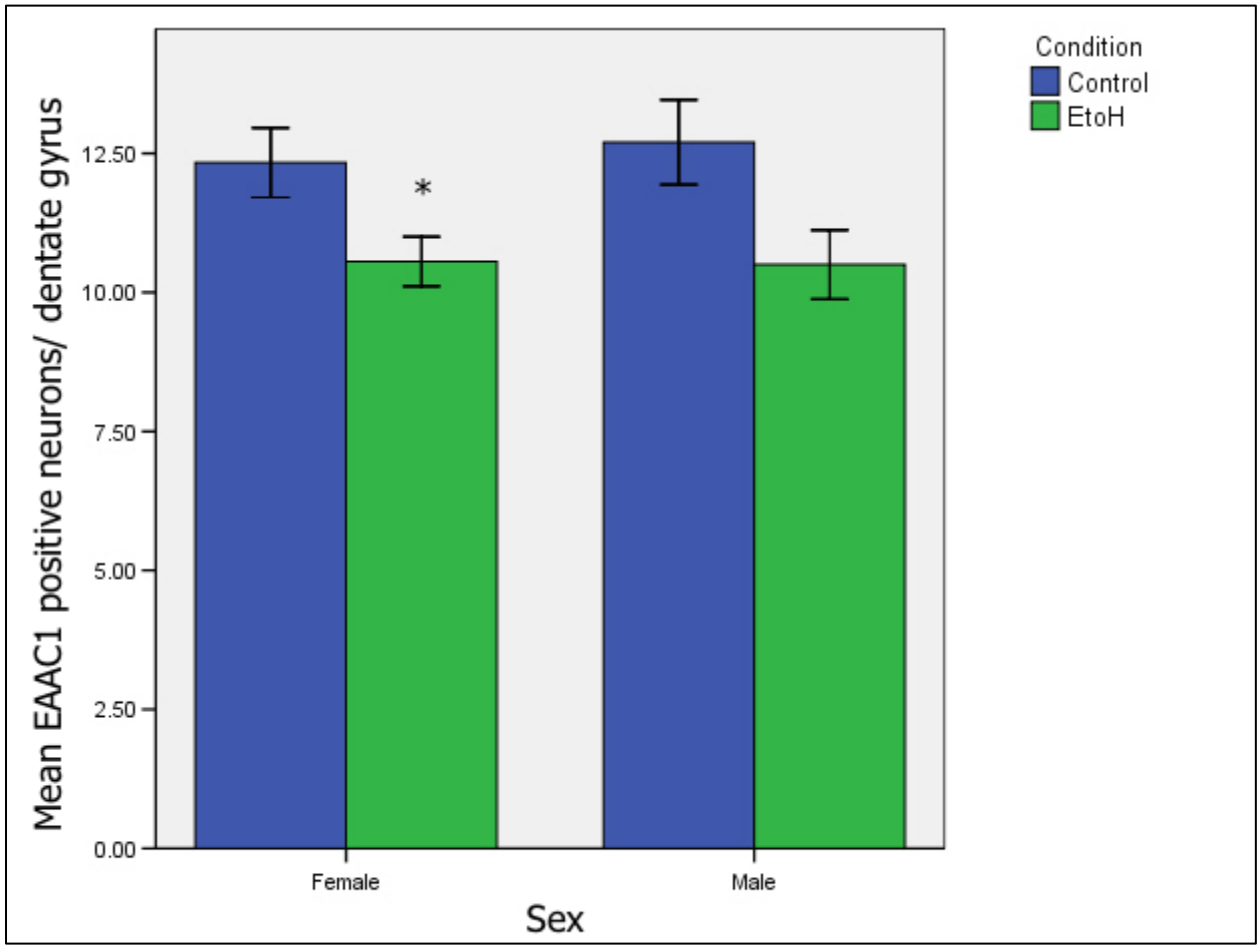

Figure 27: Mean excitatory neuron counts in human dentate gyrus based on sex difference. Within the dentate gyrus, human female (n= 9) (P value equals 0.0092, t = 3.4112, df = 8), not male (n= 10), group showed a significant reduction in the mean neurons count expressed to EAAC1 between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

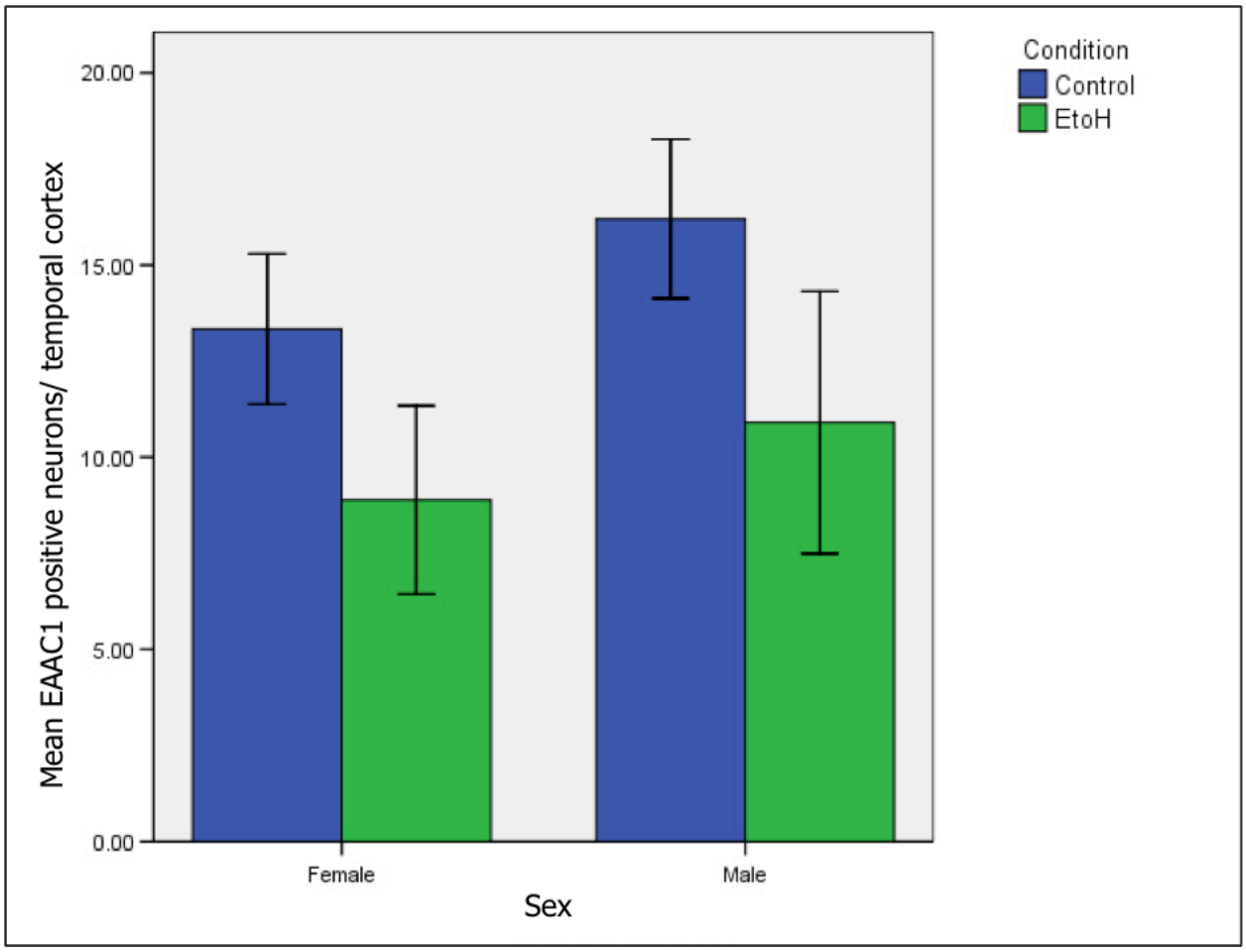

Figure 28: Mean excitatory neuron counts in human temporal cortex based on sex difference. Within the temporal cortex there was no significant reduction in both female (n= 9) and male (n= 10) groups between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

4.3.6 Inhibitory neurons in human brain

Distinct inhibitory neuron populations were labeled in human cerebral cortex with the anti-parvalbumin (PVALB) (figure 29) and anti-calbindin1 (CALB1) antibodies (figure 30), although no labeling was observed when I used this antibody in monkey brain tissue. These proteins were also detected with immunofluorescence (figure 31).

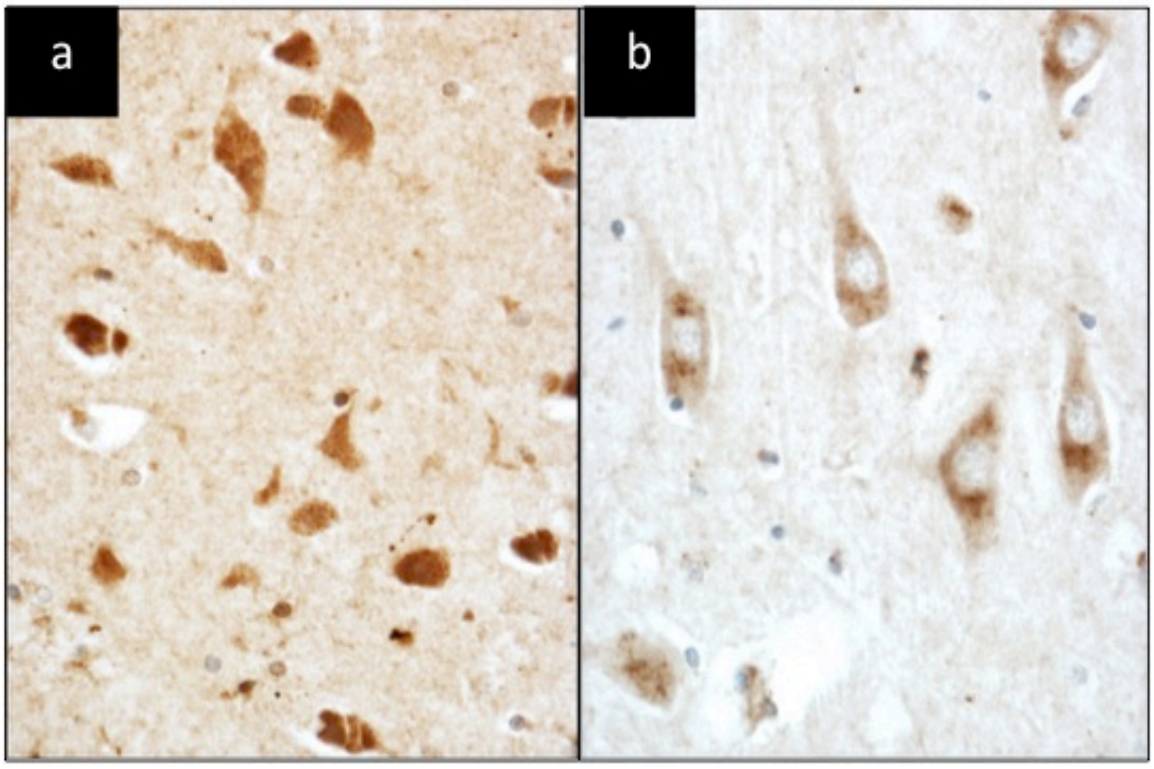

Figure 29: Inhibitory neurons in deep temporal cortical layer

(IHC with 1/100 diluted anti-parvalbumin on paraffin embedded tissue). a. Control female 12 years; b. FASD female 15 years. The control shows many large immunoreactive neurons. Fewer are evident in the FASD case. 400x magnification.

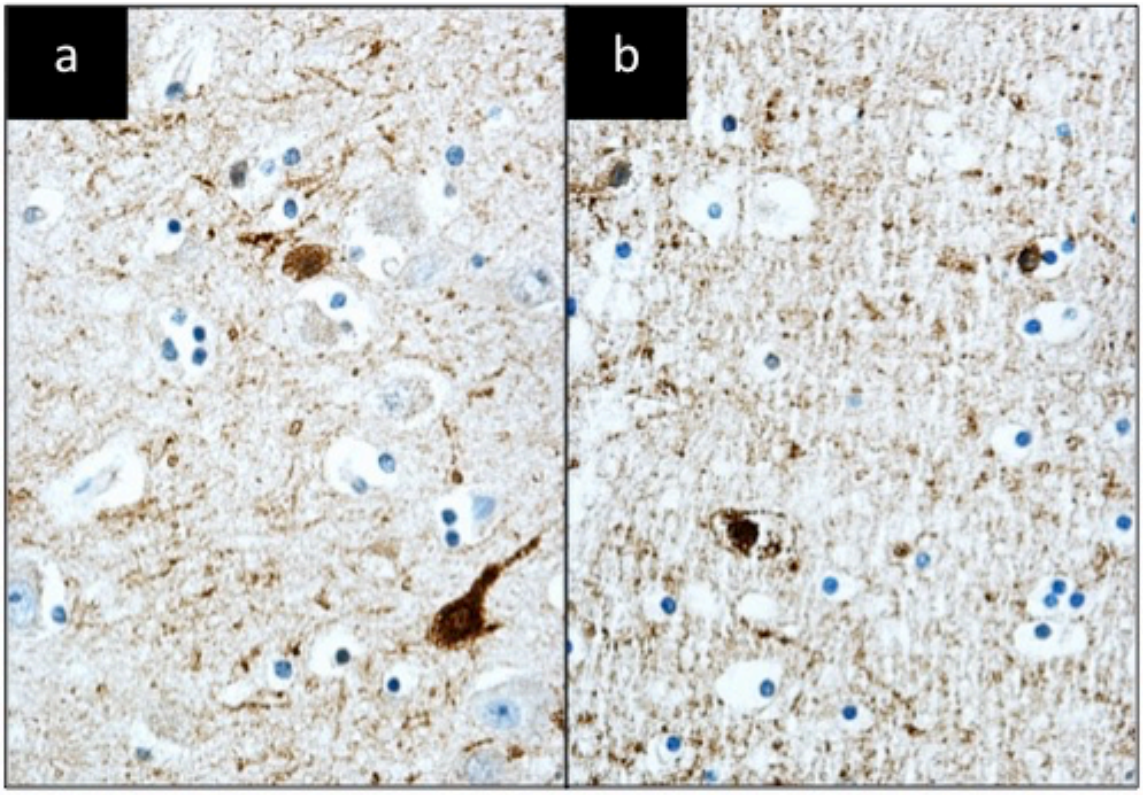

Figure 30: Inhibitory neurons in deep temporal cortical layer

(IHC with anti-calbindin1 (CALB1)). a. Control female 8 years; b. FASD female 8 years. The control shows many immunoreactive large neurons. Fewer are evident in the FASD case. 400x magnification.

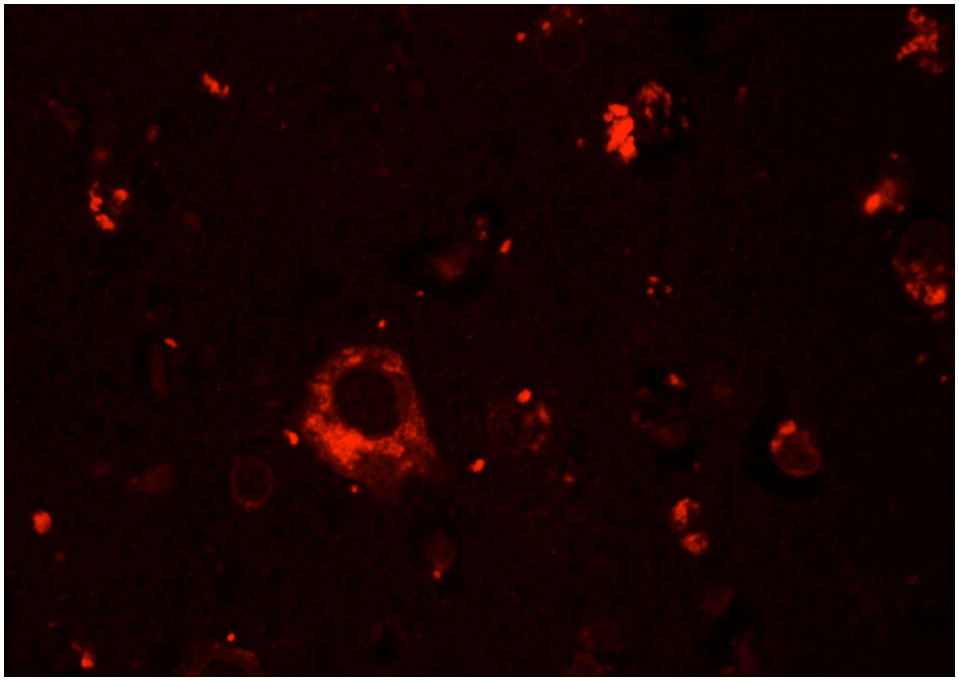

Figure 31: Immunofluorescence labeling of Parvalbumin. Paraffin-embedded AD human brain tissue, immunostained with 1/50 diluted parvalbumin antibody followed by cy3 secondary antibody. Red corresponds to parvalbumin in inhibitory neurons. 400X magnification.

4.3.7 Inhibitory neuron changes in human brains with *in utero* ethanol exposure

I examined tissues from 38 humans in total (infants, children, and teenage; controls n=19, PNEE n= 19) (table 6, table 7). The PNEE cases had a substantial reduction in inhibitory neurons labeled with anti-parvalbumin (PVALB) in all hippocampus sectors (CA1= 57.86%, CA3= 65.15%, and DG= 53.39%) and inferior temporal area 44.13% (figure 32). Similarly, calbindin1 (CALB1) staining of these human cases illustrated a decrease in all tested brain regions (figure 33).

Table 6: Statistical parameters of parvalbumin (PVALB) on human brain regions

Brain regions	Control	Ethanol-exposed
	Mean±SEM	Mean±SEM
CA1	13.74±1.55	5.79±0.41
CA3	17.68±1.87	6.16±0.57
DG	13.11±1.04	6.11±0.59
Temporal Cortex	12.53±0.67	7.00±0.47

Table 7: Statistical parameters of calbindin1 (CALB1) on human brain regions

Brain regions	Control	Ethanol-exposed
	Mean±SEM	Mean±SEM
CA1	12.58±1.64	6.00±0.46
CA3	12.47±1.39	6.89±0.51
DG	10.74±0.66	6.42±0.57
Temporal Cortex	17.32±2.14	8.47±0.65

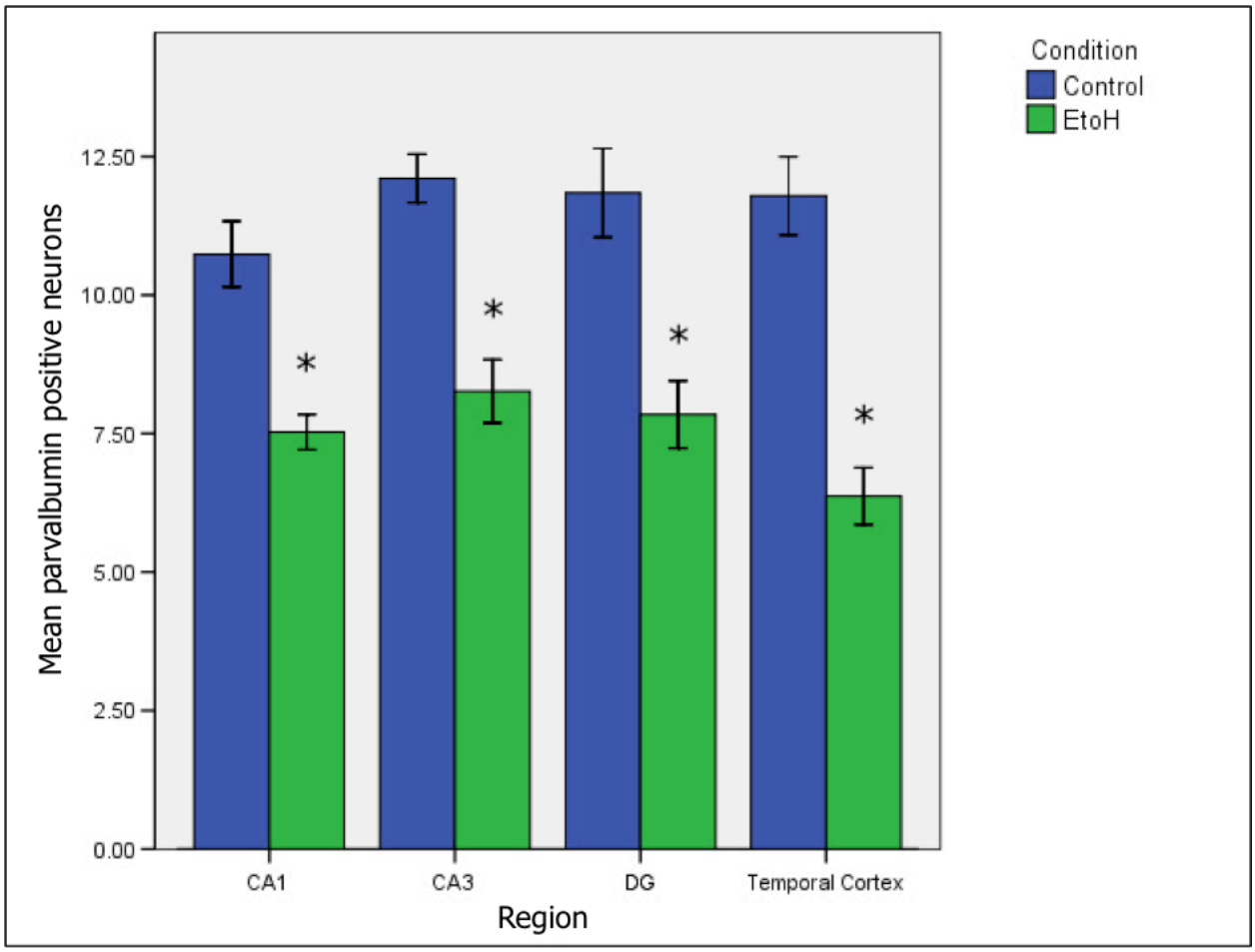

Figure 32: Mean inhibitory neuron counts detected in parvalbumin of human hippocampal and temporal cortex. Asterisks demonstrate significant reductions in the mean neurons count expressed to parvalbumin of human CA1 (P value equals 0.0001, t = 13.5674, df = 18), CA3 (P value equals 0.0437, t = 2.1694, df = 18), dentate gyrus (P value equals 0.0001, t = 17.7034, df = 18), and temporal cortex (P value equals 0.0001, t = 12.5657, df = 18) between controls (n=19) and Ethanol exposed groups (n=19) among all age groups. Error bars = +/- 1 SE.

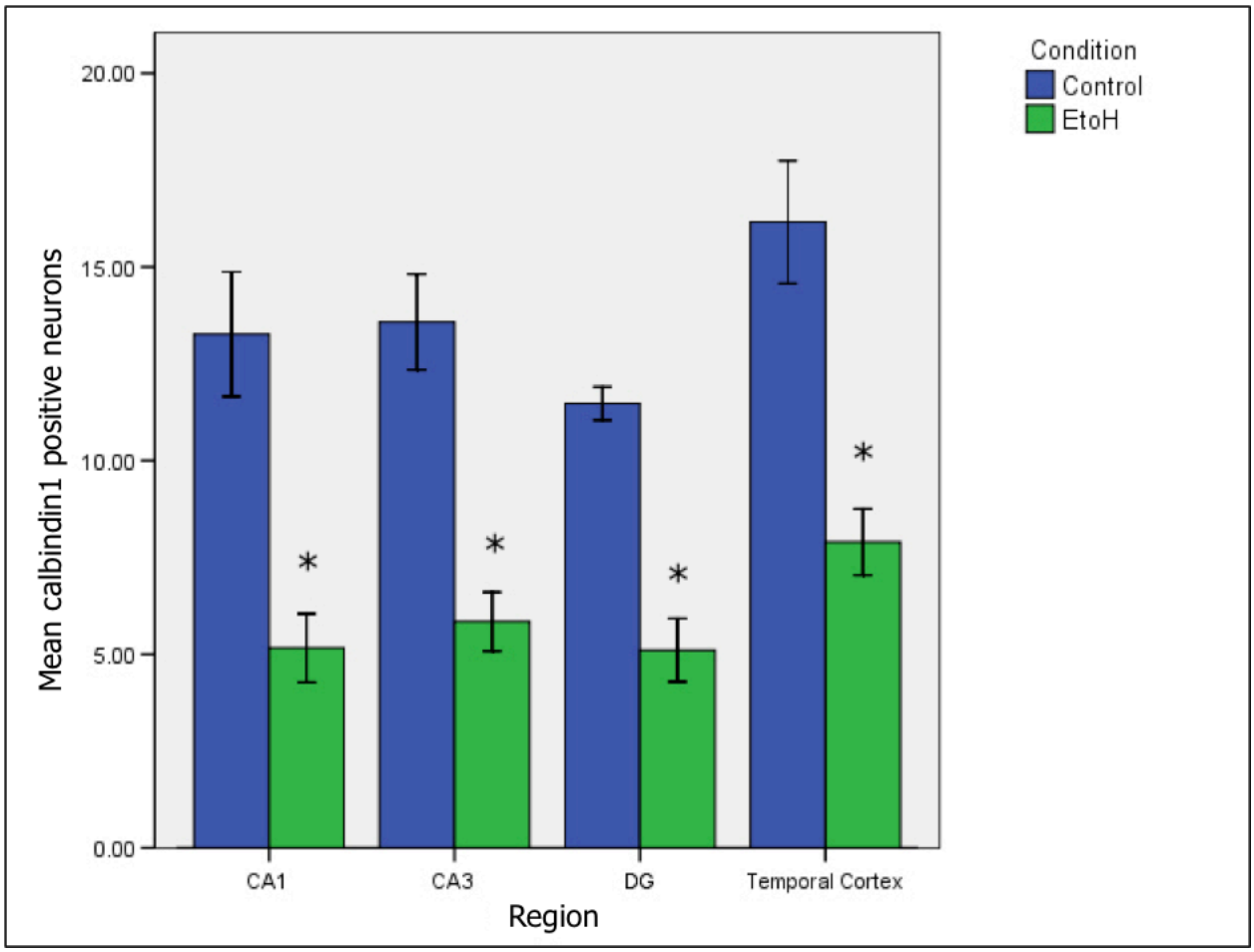

Figure 33: Mean inhibitory neuron counts detected in calbindin1 (CALB1) of human hippocampal and temporal cortex. Asterisks demonstrate significant reductions in the mean neurons count expressed to calbindin1 of human CA1 (P value equals 0.0001, t = 7.1478, df = 18), CA3 (P value equals 0.0001, t = 5.2889, df = 18), dentate gyrus (P value equals 0.0001, t = 7.2607, df = 18), and temporal cortex (P value equals 0.0030, t = 6.9524, df = 18) between controls (n=19) and Ethanol exposed groups (n=19) among all age groups. Error bars = +/- 1 SE.

4.3.8 Age group based GABA expression in human following ethanol exposure

I tested different human age groups for inhibitory neuron quantification of parvalbumin (PVALB) and calbindin1 (CALB1) in each brain region separately. In CA1 region, a significant reduction of parvalbumin (PVALB) labeled neurons was detected in infants 41.77% (figure 34). In CA3 region, the reductions were 33.76% in infants, and 25.33% in children (figure 35). In dentate gyrus, parvalbumin was reduced in infants (44.44%) and teenage (45.68%) groups only (figure 36). In temporal cortex, the reduction appeared substantially in infants (62.67%), children (32.81%), and teenage (41.18%) (figure 37).

Calbindin1 expression also showed significant reduction in all brain regions tested separately among different age groups. Within the CA1 region, 68.18% reduction in infants, , and 67.90% in teenage groups (figure 38). In CA3 region, the reductions were 64.36% in infants, 43.49% in children, and 59.09% in teens (figure 39). In dentate gyrus, the PNEE was associated with significant diminution in infant; 47.37%, in children; 44.26%, and 71.60% in teens (figure 40). Temporal cortices of PNEE cases showed declines among different group ages (infants; 67.83%, and teens 42.86%) compared with the controls (figure 41).

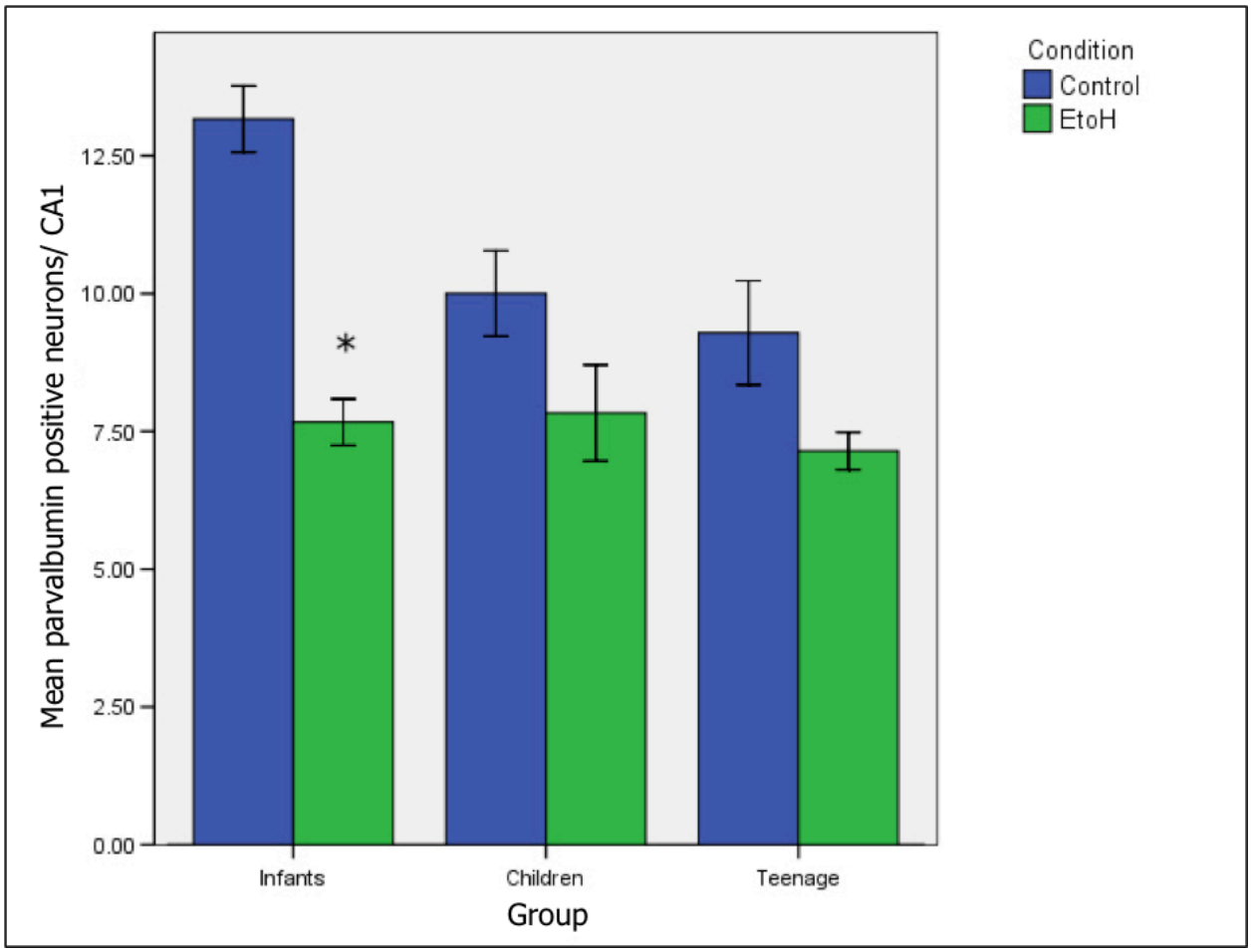

Figure 34: Inhibitory neurons labeled with anti-parvalbumin in human CA1 among infants, children, and teens groups. Within the CA1 region, only human infants (n=6) (P value equals 0.0016, t = 6.2143, df = 5) showed a significant reduction in the mean neurons count expressed to parvalbumin between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

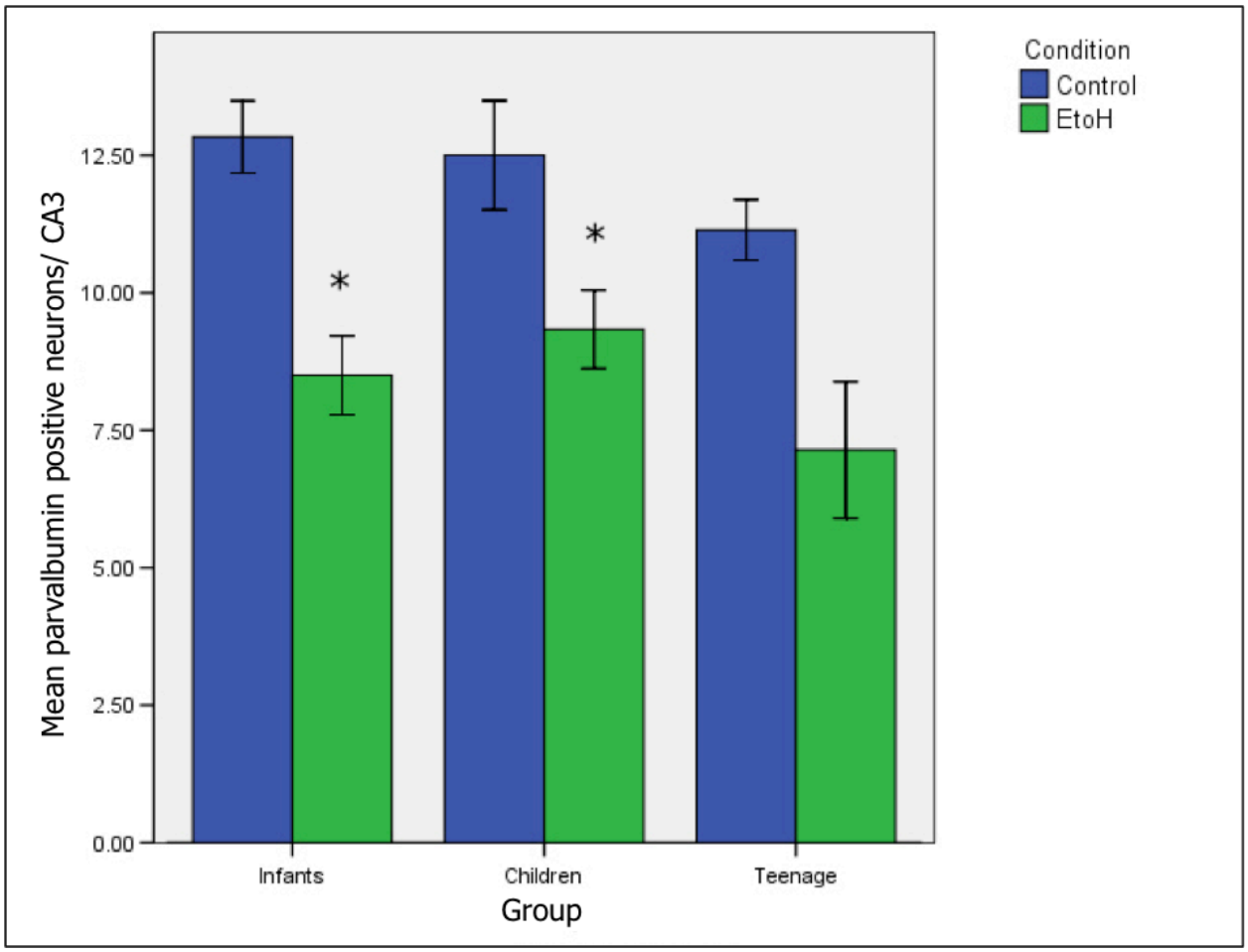

Figure 35: Inhibitory neurons labeled with anti-parvalbumin in human CA3 among infants, children, and teens groups. Within the CA3 region, human infants (n=6) (P value equals 0.0062, t = 4.5398, df = 5) and children (n=6) (P value equals 0.0274, t = 2.8983, df = 5) showed significant reduction in the mean neurons count expressed to parvalbumin between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

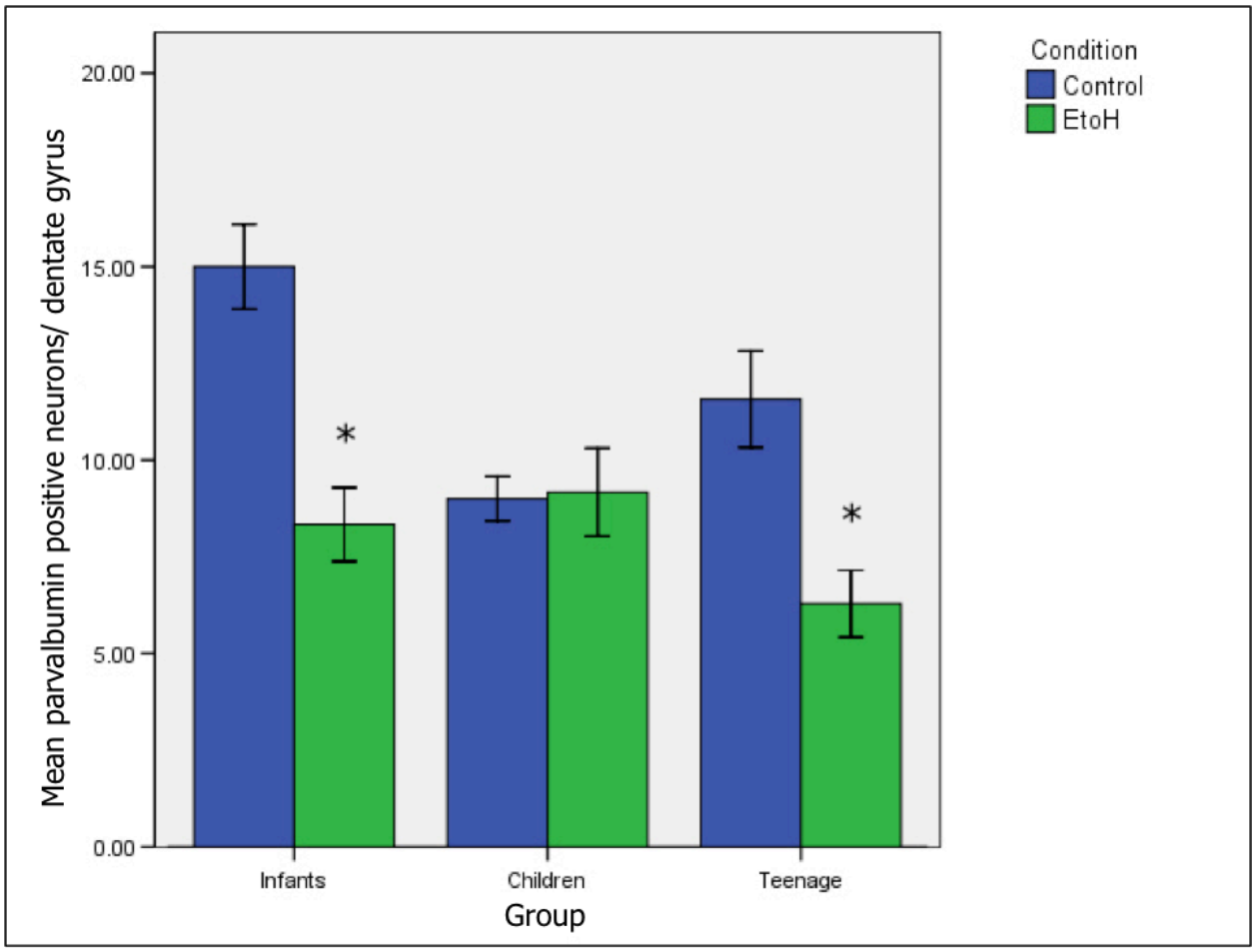

Figure 36: Inhibitory neurons labeled with anti-parvalbumin in dentate gyrus of human infants, children, and teens groups. Within the dentate gyrus, both infants (n=6) (P value equals 0.0199, t = 3.3710, df = 5) and teens (n=7) (P value equals 0.0005, t = 6.7931, df = 6) showed significant reduction in the mean neurons count expressed to parvalbumin between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

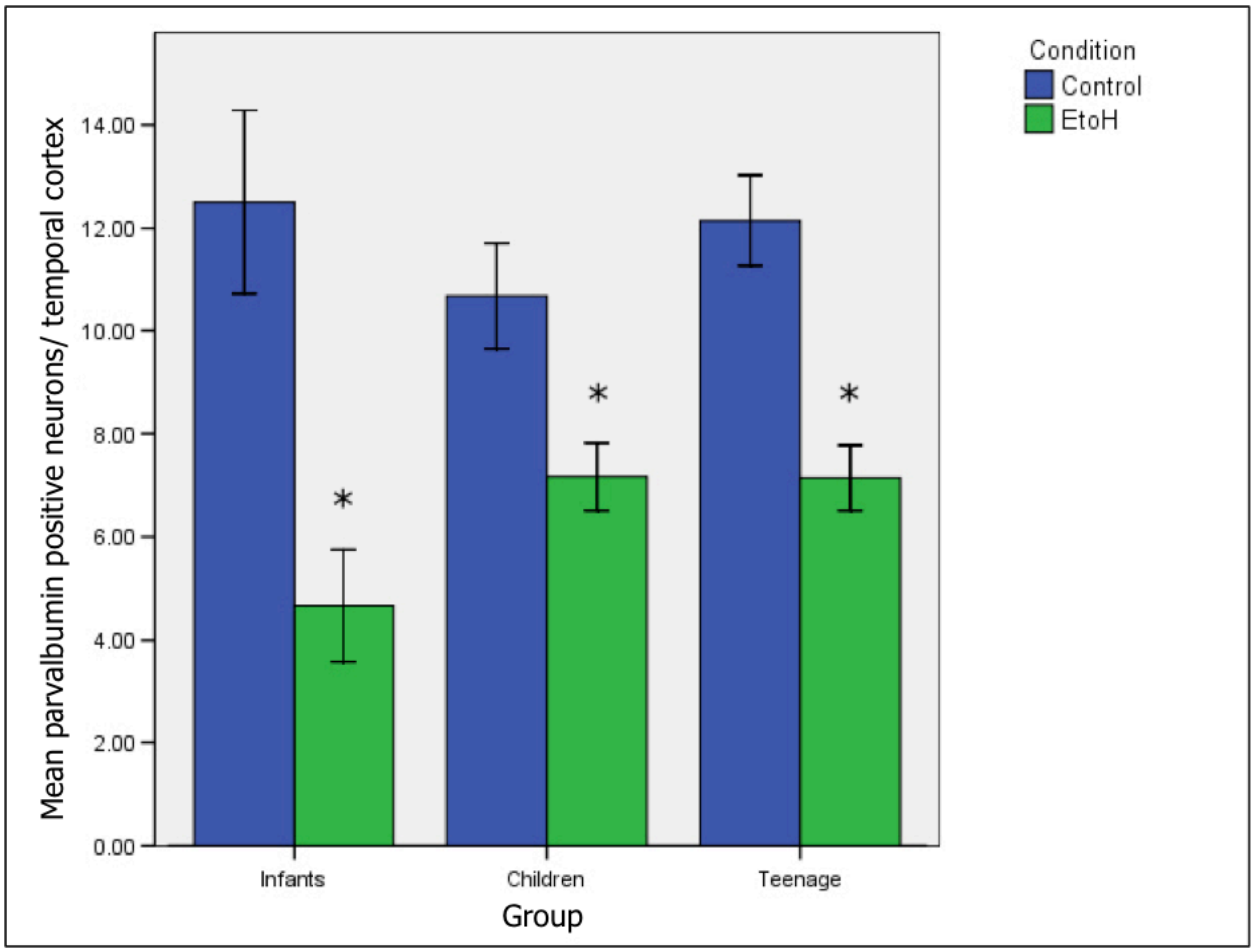

Figure 37: Inhibitory neurons labeled with anti-parvalbumin in temporal cortex of human infants, children, and teens groups. Within the temporal cortex, all age groups; infants (n=6) (P value equals 0.0008, t = 7.2696, df = 5), children (n=6) (P value equals 0.0443, t = 2.6713, df = 5) and teens (n=7) (P value equals 0.0025, t = 5.0000, df = 6) showed significant reduction in the mean neurons count expressed to parvalbumin between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

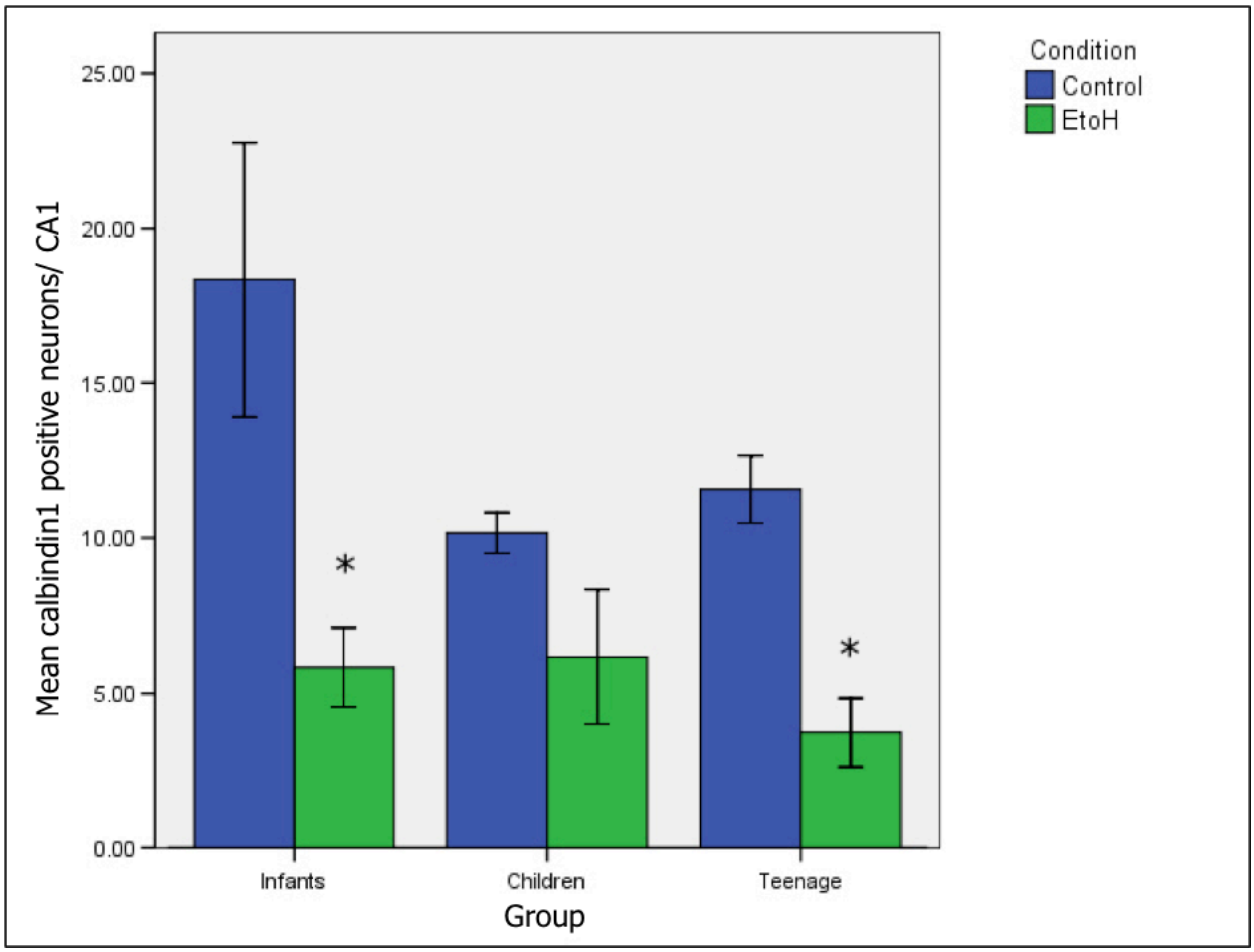

Figure 38: Inhibitory neurons labeled with anti-calbindin1 in CA1 of human infants, children, and teens groups. Within the dentate gyrus, both infants (n=6) (P value equals 0.0439, t = 2.6782, df = 5) and teens (n=7) (P value equals 0.0026, t = 4.9259, df = 6) showed significant reduction in the mean neurons count expressed to calbindin1 between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

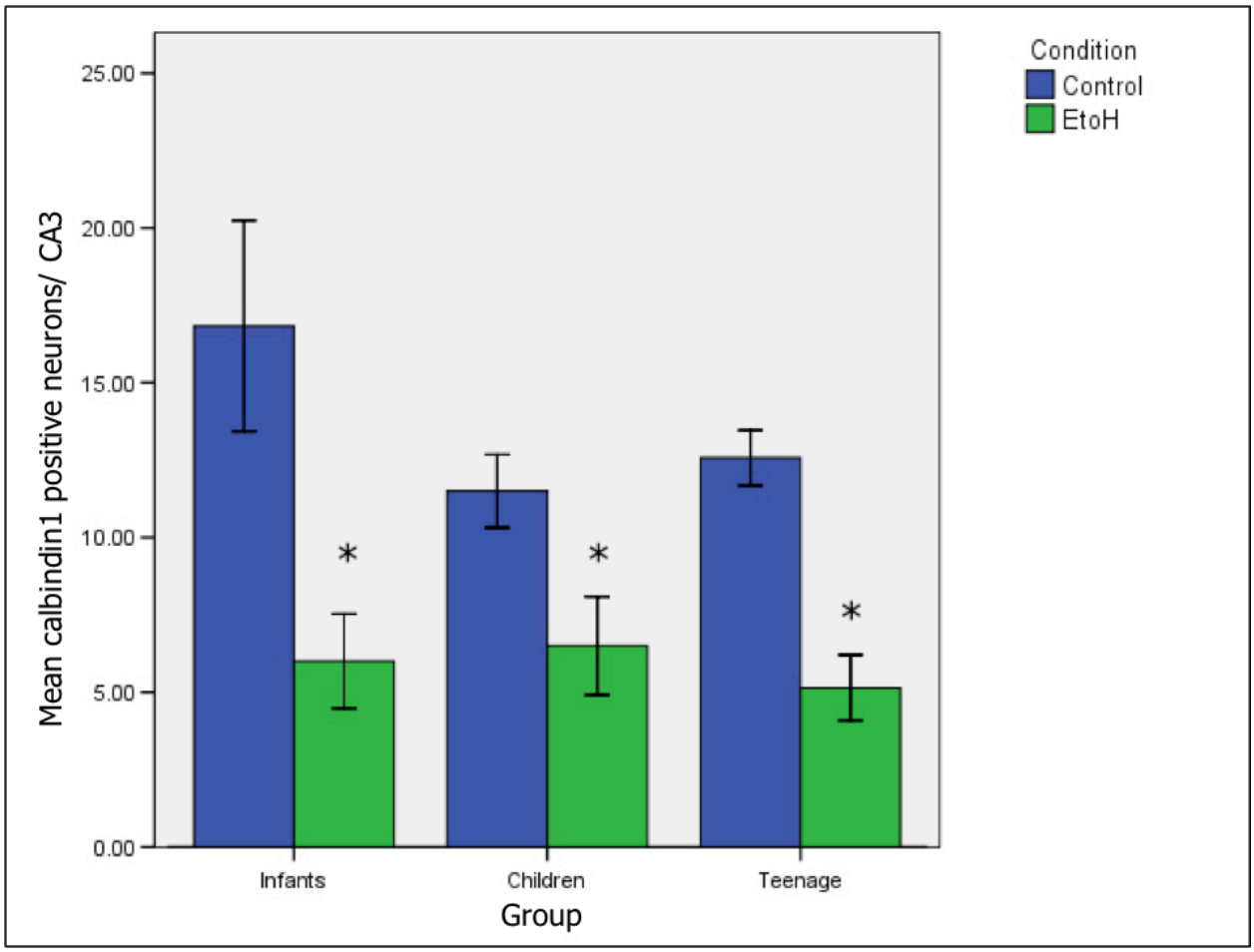

Figure 39: Inhibitory neurons labeled with anti-calbindin1 in CA3 of human infants, children, and teens groups. Within the CA3, all age groups; infants (n=6) (P value equals 0.0485, t = 2.5950, df = 5), children (n=6) (P value equals 0.0462, t = 2.6352, df = 5) and teens (n=7) (P value equals 0.0053, t = 4.2600, df = 6) showed significant reduction in the mean neurons count expressed to calbindin1 between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

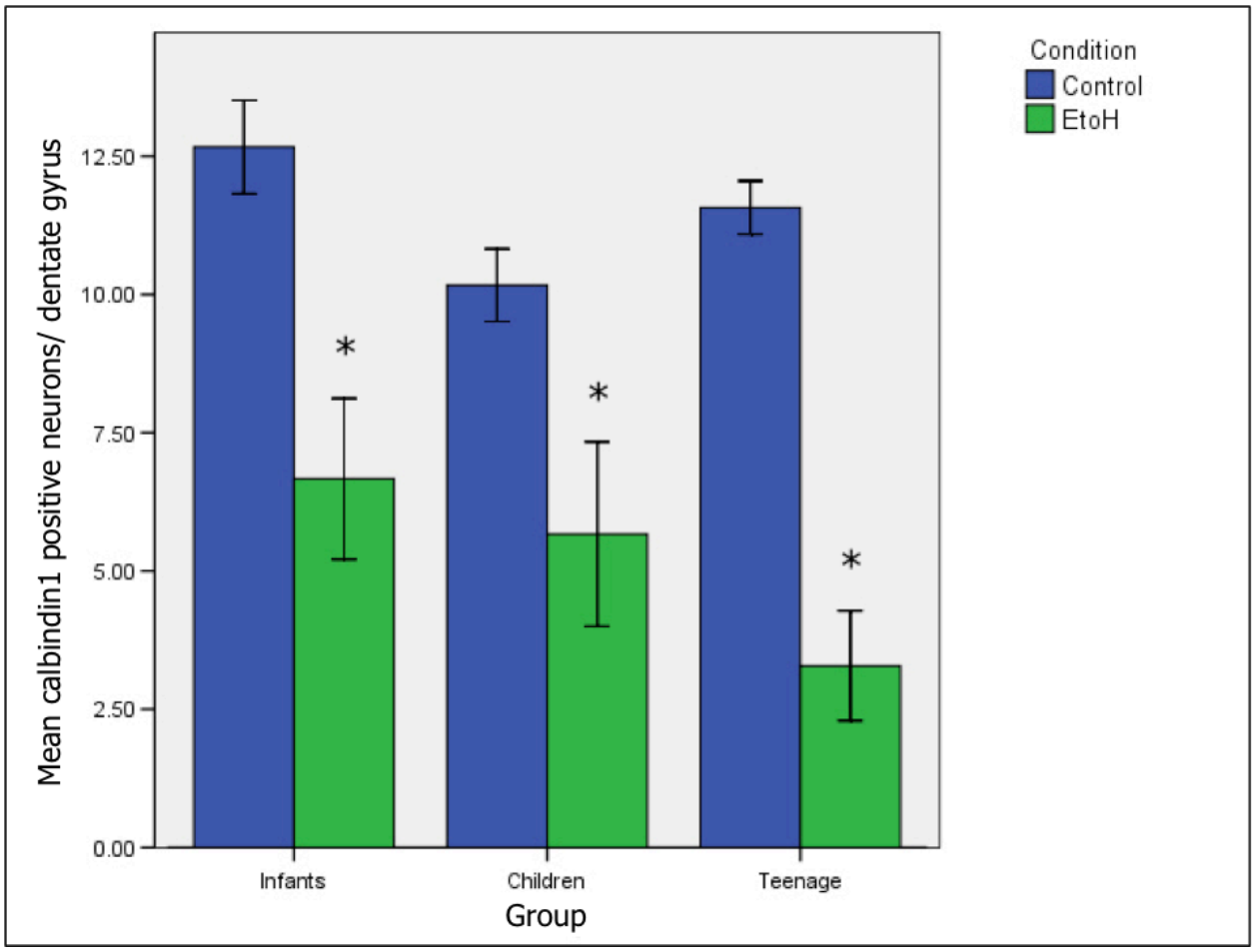

Figure 40: Inhibitory neurons labeled with anti-calbindin1 in dentate gyrus of human infants, children, and teens groups. Within the dentate gyrus, all age groups; infants (n=6) (P value equals 0.0093, t = 4.1079, df = 5), children (n=6) (P value equals 0.0463, t = 2.6349, df = 5) and teens (n=7) (P value equals 0.0004, t = 7.0858, df = 6) showed significant reduction in the mean neurons count expressed to calbindin1 between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

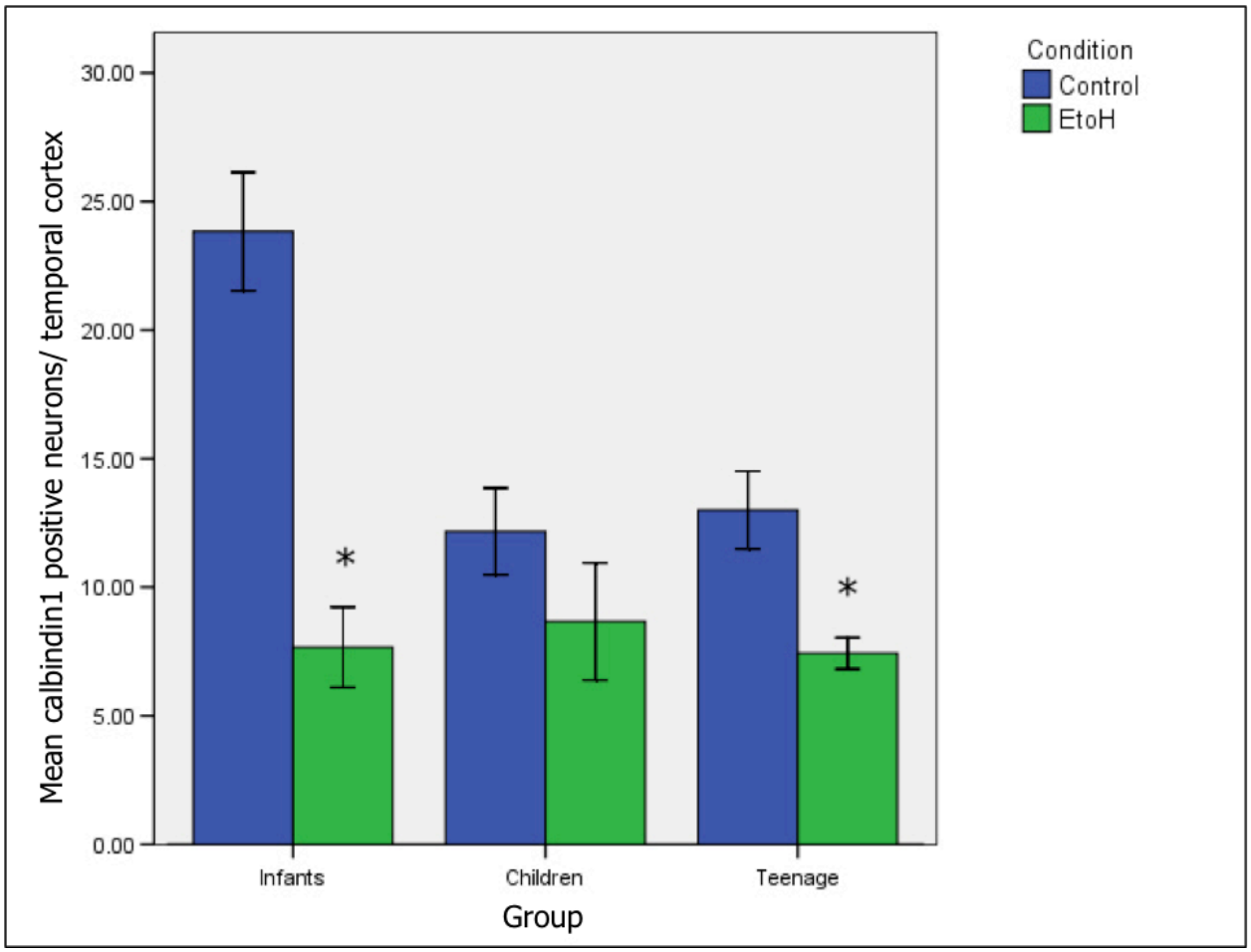

Figure 41: Inhibitory neurons labeled with anti-calbindin1 in temporal cortex of human infants, children, and teens groups. Within the temporal cortex, both infants (n=6) (P value equals 0.0011, t = 6.7748, df = 5) and teens (n=7) (P value equals 0.0020, t = 5.2272, df = 6) showed significant reduction in the mean neurons count expressed to calbindin1 between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

4.3.9 Sex difference in GABA expression in human following ethanol exposure

Both male and female groups revealed significant diminution to parvalbumin (PVALB) following PNEE in CA1 (32.73%, and 26.60%) (figure 42), CA3 (35.51% and 28.44%) (figure 43), and temporal cortex (49.58% and 41.90%) (figure 45). However, only male group in dentate gyrus (45.31%) showed significant reduction in neurons expressed in parvalbumin (figure 44).

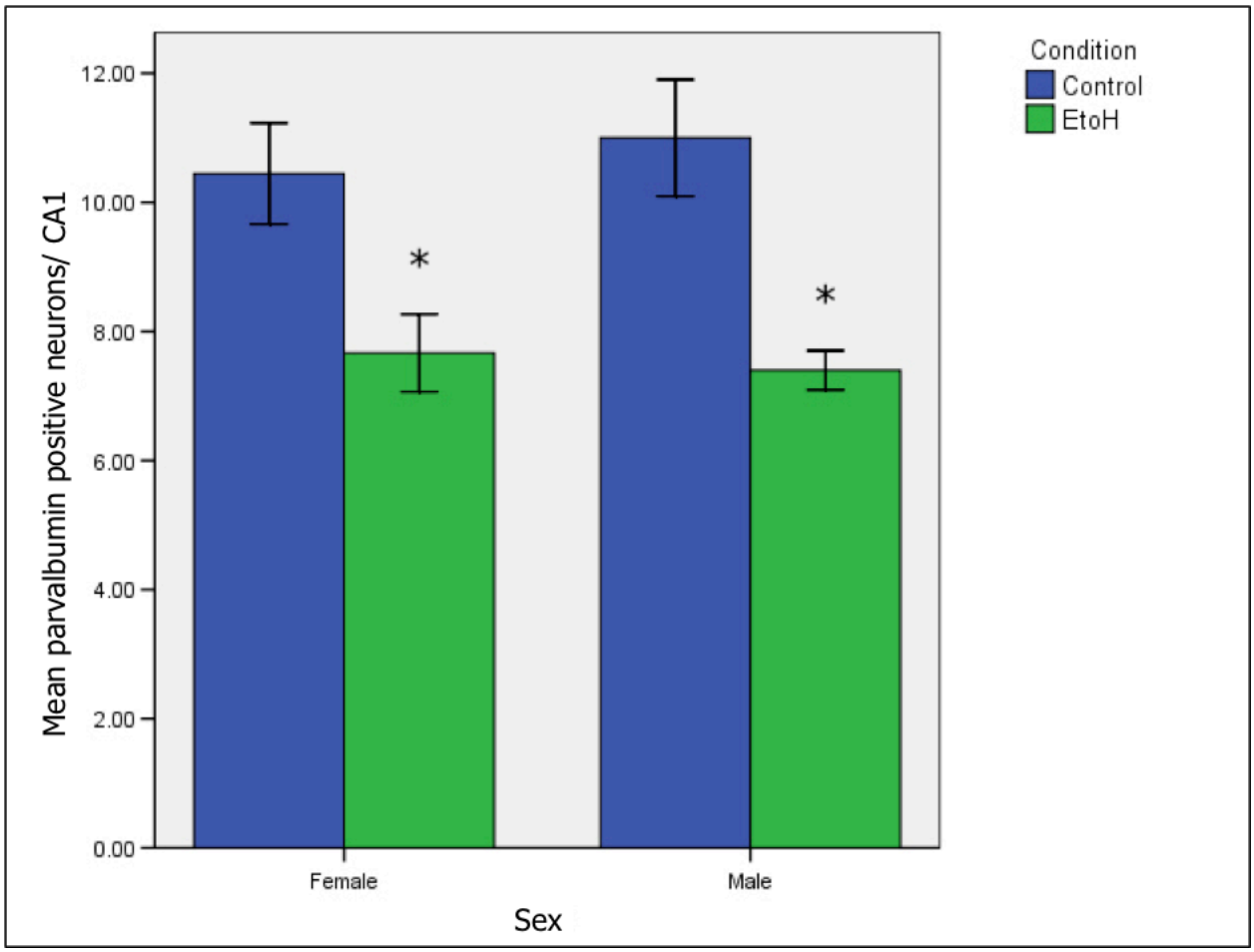

Figure 42: Sex difference in human mean inhibitory neurons labeled with anti-parvalbumin in CA1 region. Within human CA1, both male (n=10) (P value equals 0.0028, t = 4.0704, df = 9) and female (n=9) (P value equals 0.0327, t = 2.5786, df = 8) showed significant reduction in the mean neurons count expressed to parvalbumin between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

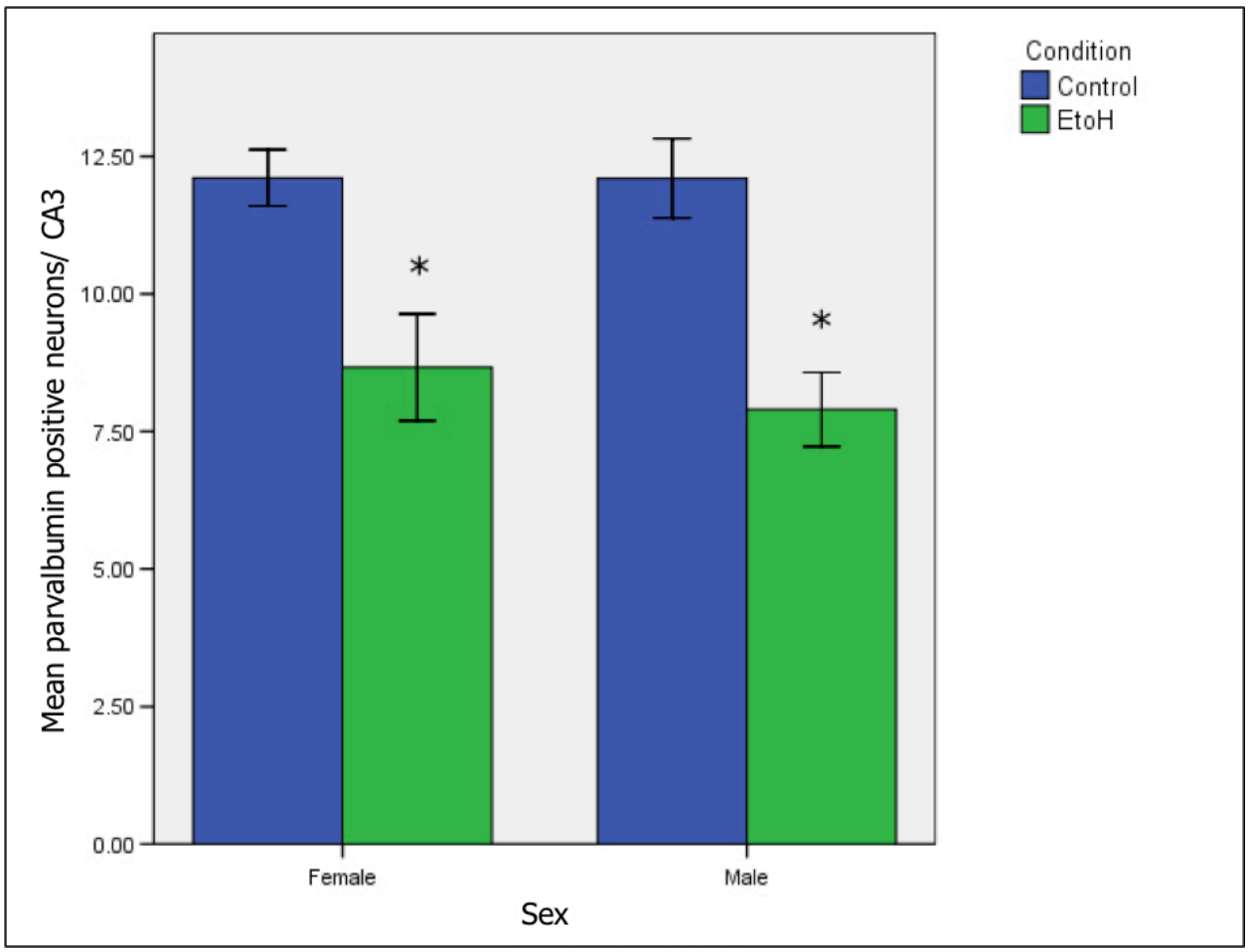

Figure 43: Sex difference in human mean inhibitory neurons labeled with anti-parvalbumin in CA3 region. Within human CA3, both male (n=10) (P value equals 0.0011, t = 4.7088, df = 9) and female (n=9) (P value equals 0.0286, t = 2.6656, df = 8) showed significant reduction in the mean neurons count expressed to parvalbumin between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

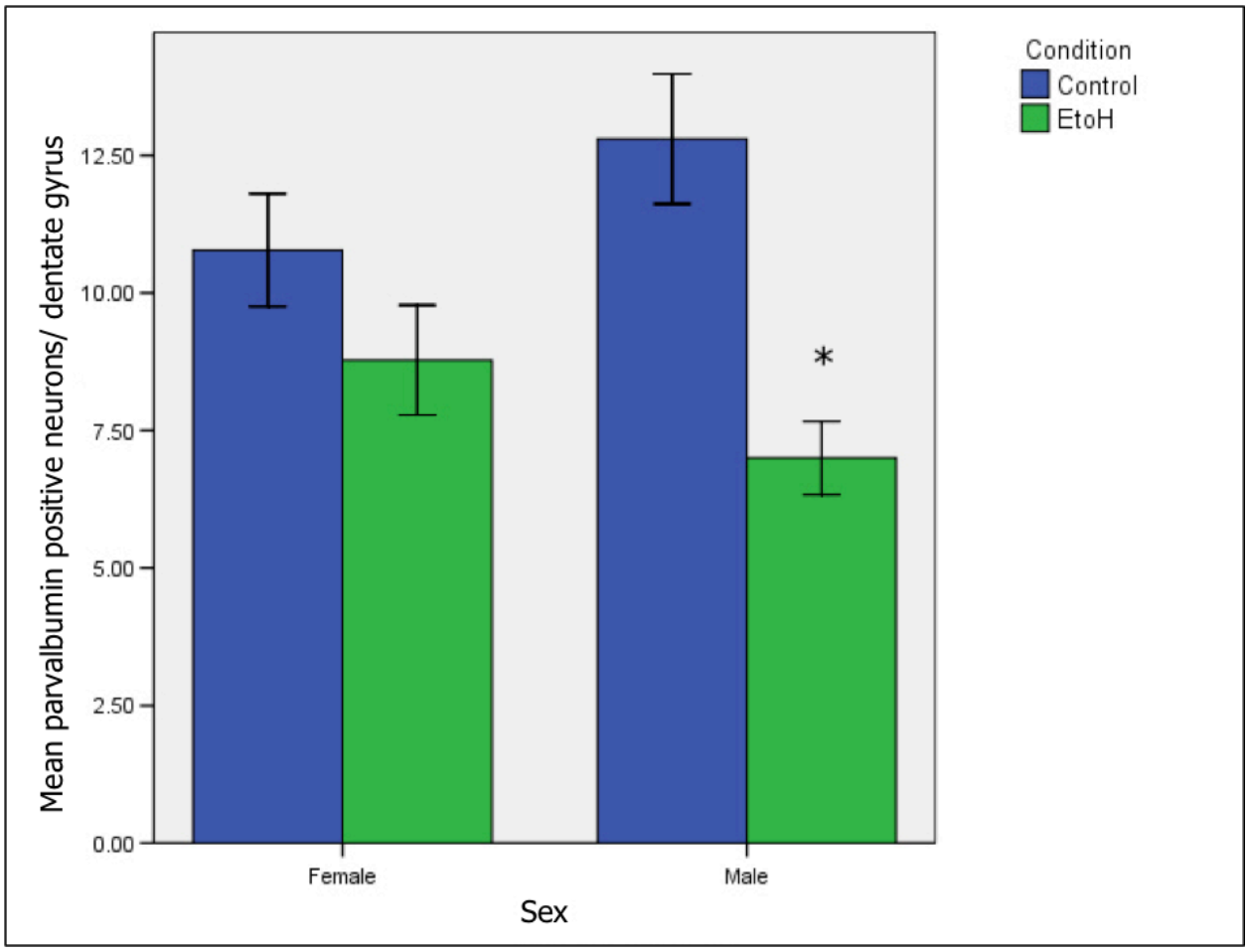

Figure 44: Sex difference in human mean inhibitory neurons labeled with anti-parvalbumin in dentate gyrus. Within human dentate gyrus, male (n=10) (P value equals 0.0006, t = 5.1625, df = 9) showed significant reduction in the mean neurons count expressed to parvalbumin between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

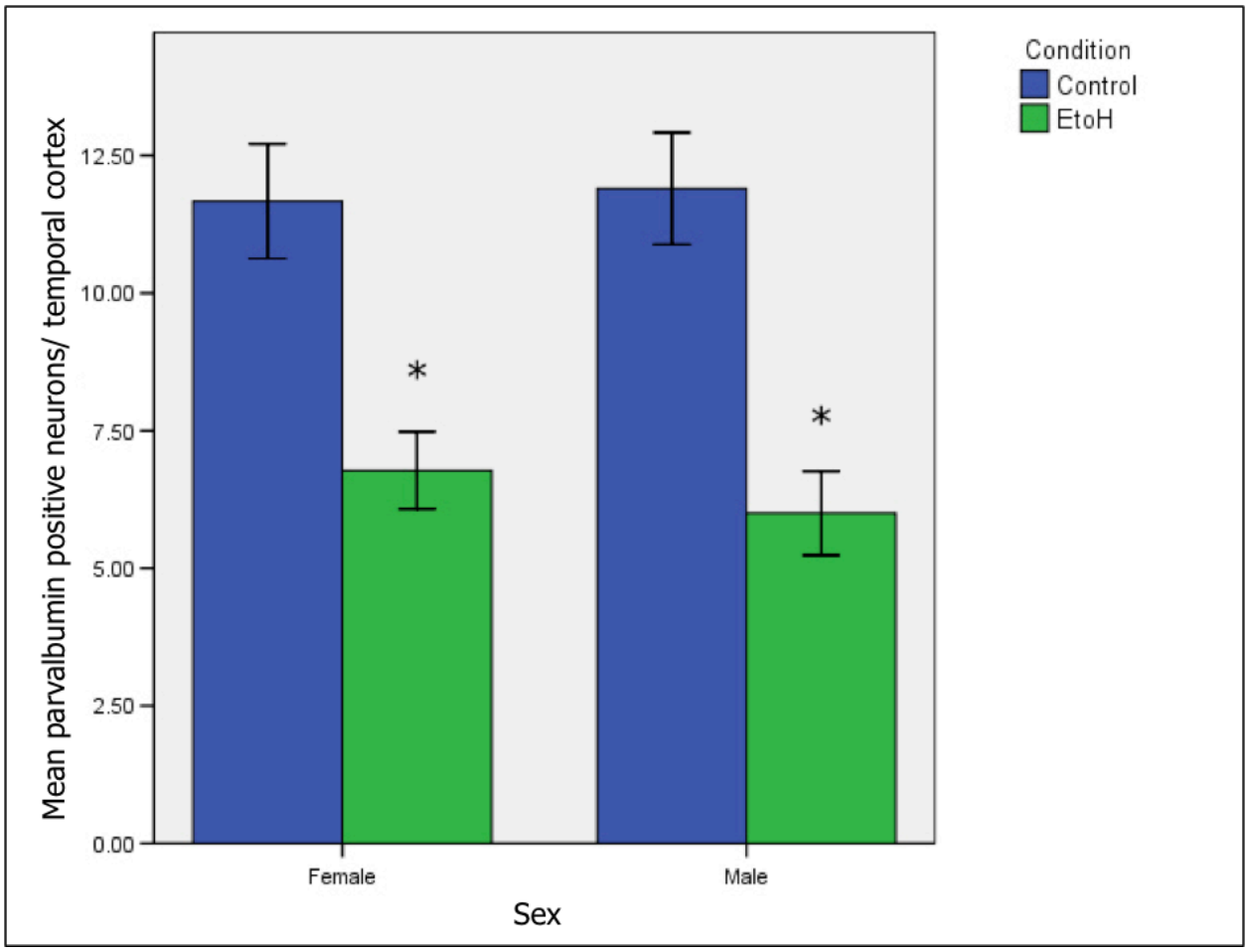

Figure 45: Sex difference in human mean inhibitory neurons labeled with anti-parvalbumin in temporal cortex. Within human temporal cortex, both male (n=10) (P value equals 0.0001, t = 6.9479, df = 9) and female (n=9) (P value equals 0.0050, t = 3.8370, df = 8) showed significant reduction in the mean neurons count expressed to parvalbumin between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

Like parvalbumin, calbindin1 immunoreactivity showed significant reduction following PNEE in both males and females among different tested brain regions; CA3 (57,60% and 56.39%) (figure 47), dentate gyrus (65.52% and 44.12%) (figure 48), and temporal cortex (56.67% and 45.86%) (figure 49) compared with controls. However, CA1 showed a significant reduction in male group only (65.55%) (figure 46),

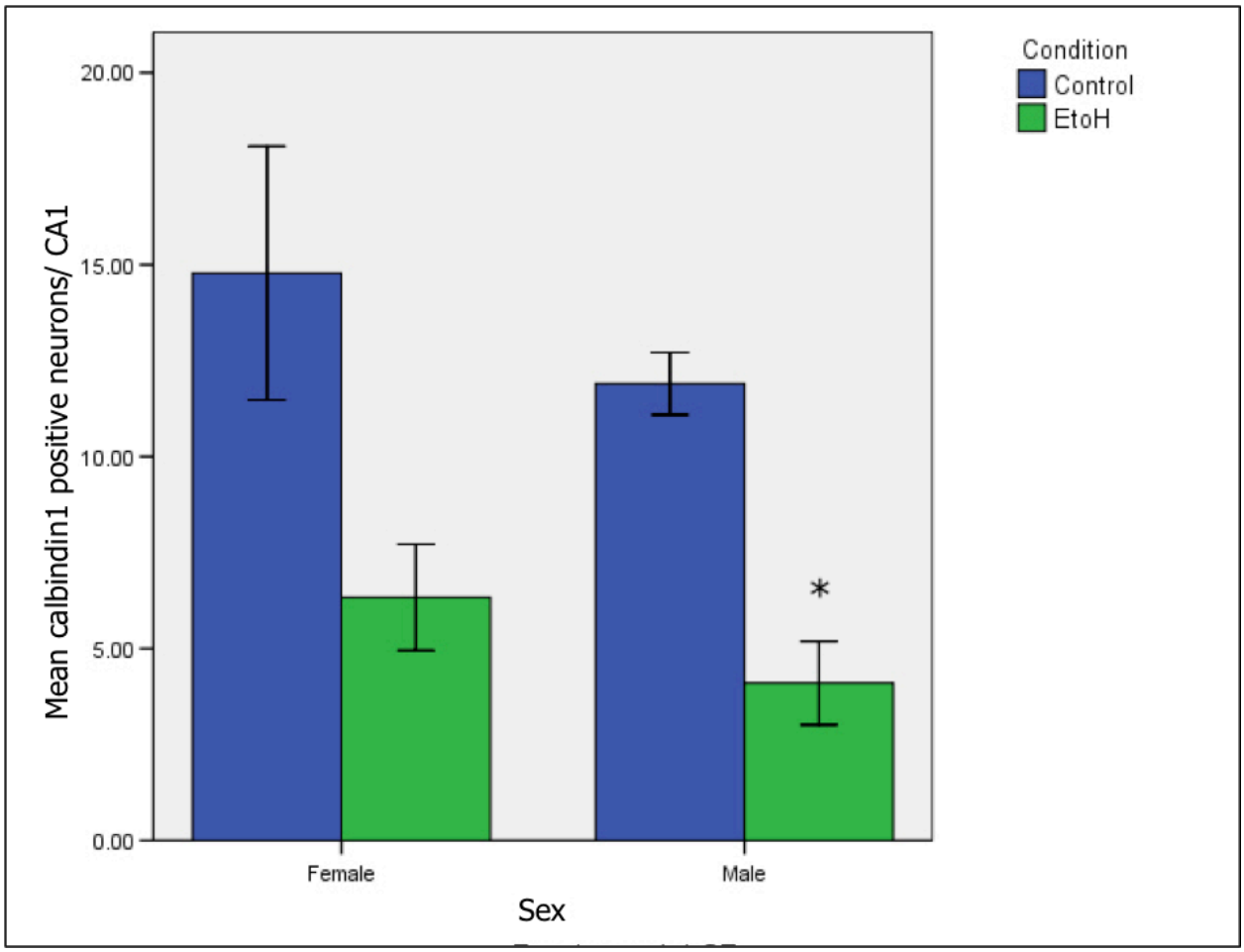

Figure 46: Sex difference in human inhibitory neurons labeled with anti-calbindin1 in CA1 region. Within human CA1, male (n=10) (P value equals 0.0001, t = 8.5105, df = 9) showed significant reduction in the mean neurons count expressed to calbindin1 between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

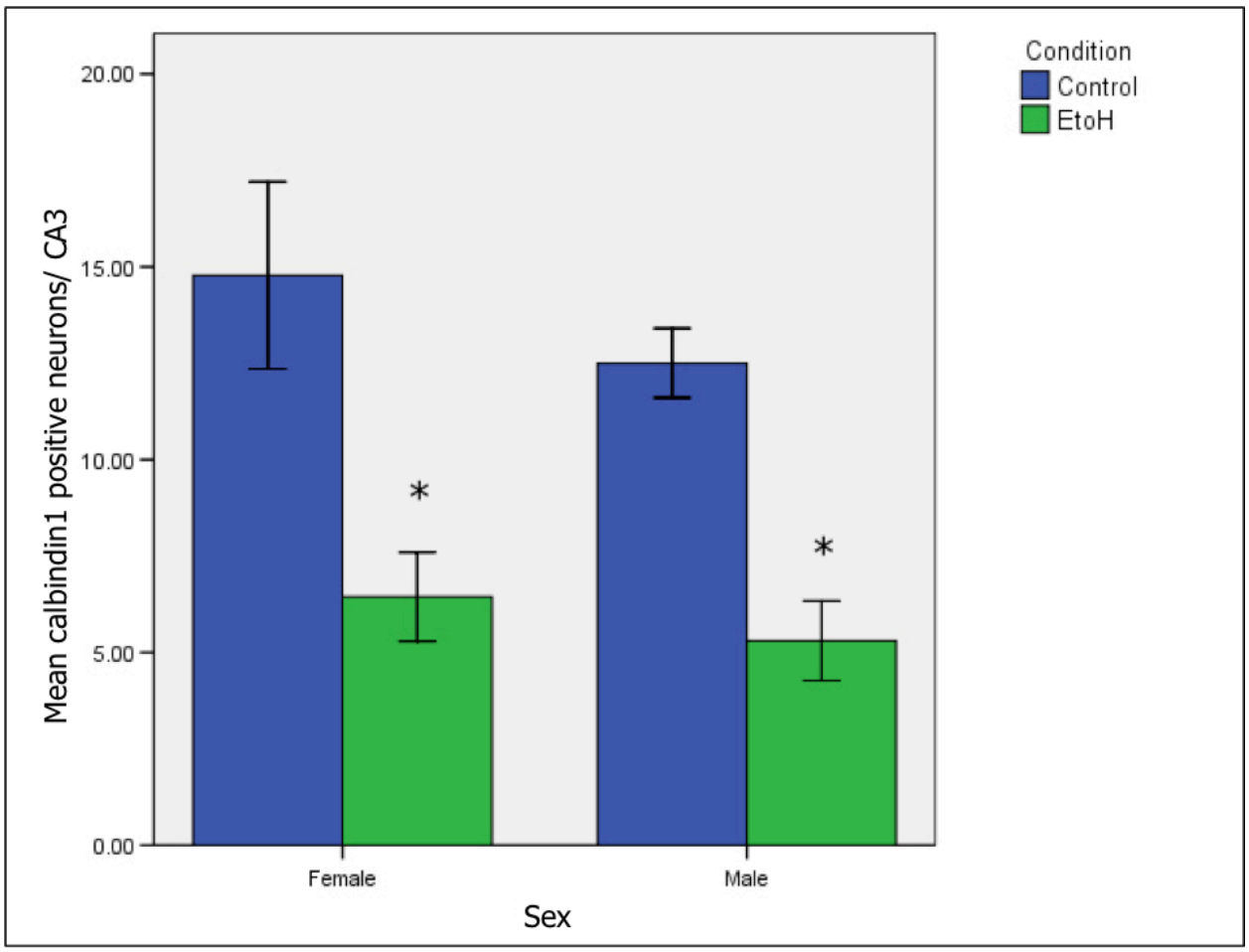

Figure 47: Sex difference in human inhibitory neurons labeled with anti-calbindin1 in CA3 region. Within human CA3, both male (n=10) (P value equals 0.0012, t = 4.6305, df = 9) and female (n=9) (P value equals 0.0232, t = 2.7995, df = 8) showed significant reduction in the mean neurons count expressed to calbindin1 between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

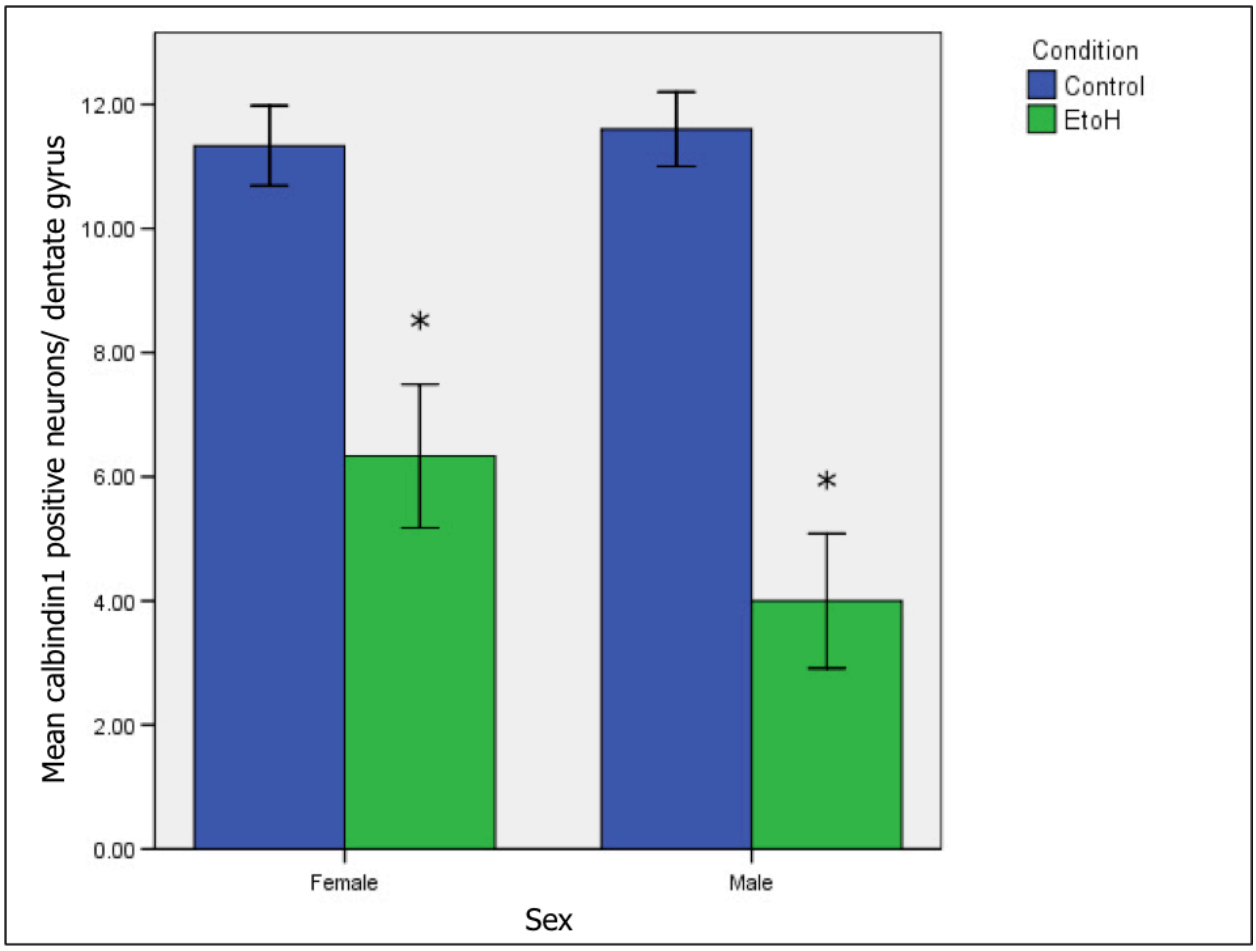

Figure 48: Sex difference in human inhibitory neurons labeled with anti-calbindin1 in dentate gyrus. Within human dentate gyrus, both male (n=10) (P value equals 0.0001, t = 7.1956, df = 9) and female (n=9) (P value equals 0.0052, t = 3.8100, df = 8) showed significant reduction in the mean neurons count expressed to calbindin1 between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

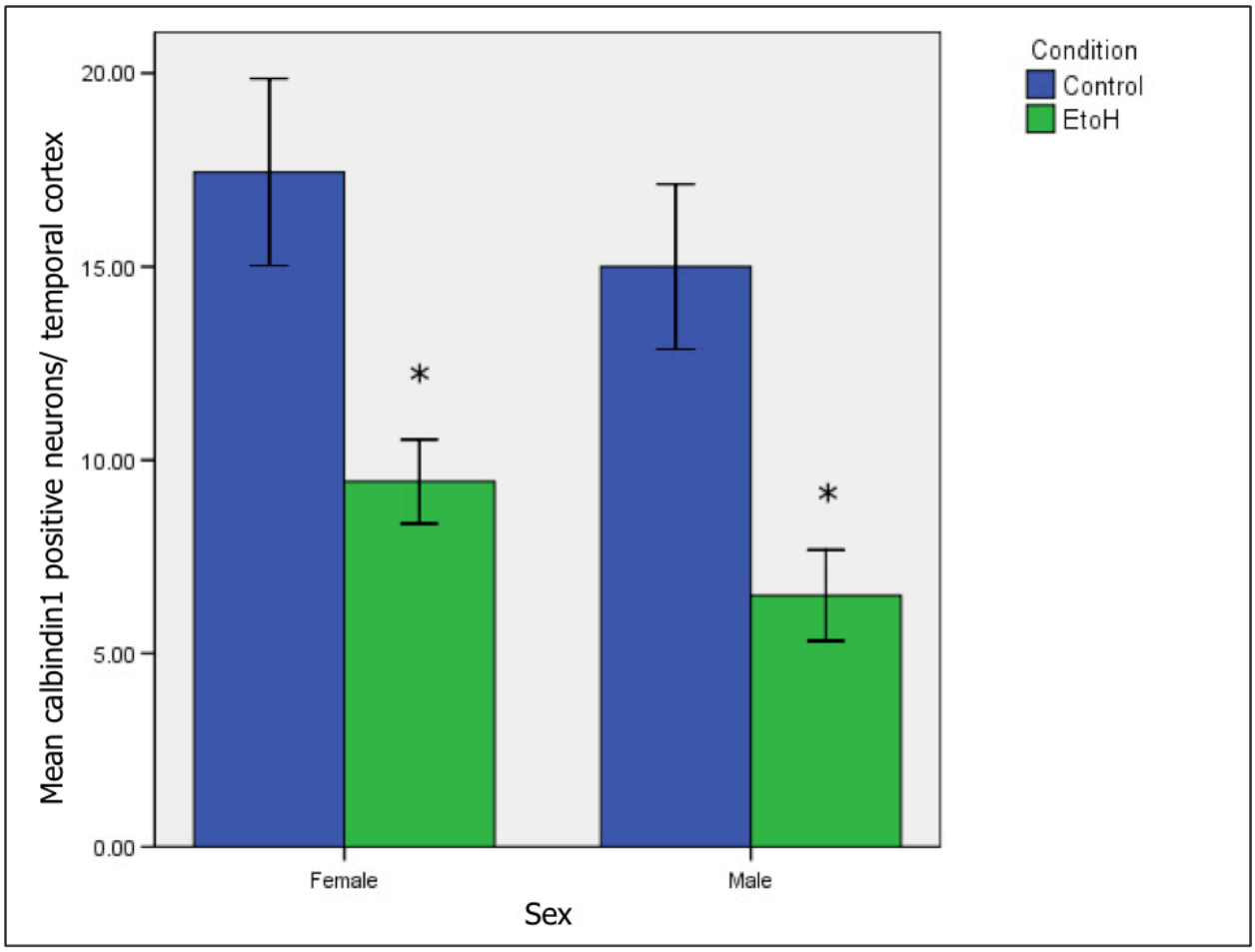

Figure 49: Sex difference in human inhibitory neurons labeled with anti-calbindin1 in temporal cortex. Within human temporal cortex, both male (n=10) (P value equals 0.0044, t = 3.7742, df = 9) and female (n=9) (P value equals 0.0094, t = 3.3941, df = 8) showed significant reduction in the mean neurons count expressed to calbindin1 between controls and ethanol-exposed groups. Error bars = +/- 1 SE.

4.3.10 Glutamatatergic neurons in non-human primate CNS

Glutamatergic neurons in macaque cerebral cortex were best detected (by the author) with rabbit anti-rat EAAT3 (Figure 50). This antibody also reacted with some endothelial cells, however these are easily differentiated from neurons. I found excitatory neurons in the expected laminar distribution in both macaque and human cortices. There is little difference between the localization of Anti-EAAT3 and Anti-EAAC1 in glutamatergic neurons in the human cortex, however only Anti-EAAT3 labels neurons in macaque brains.

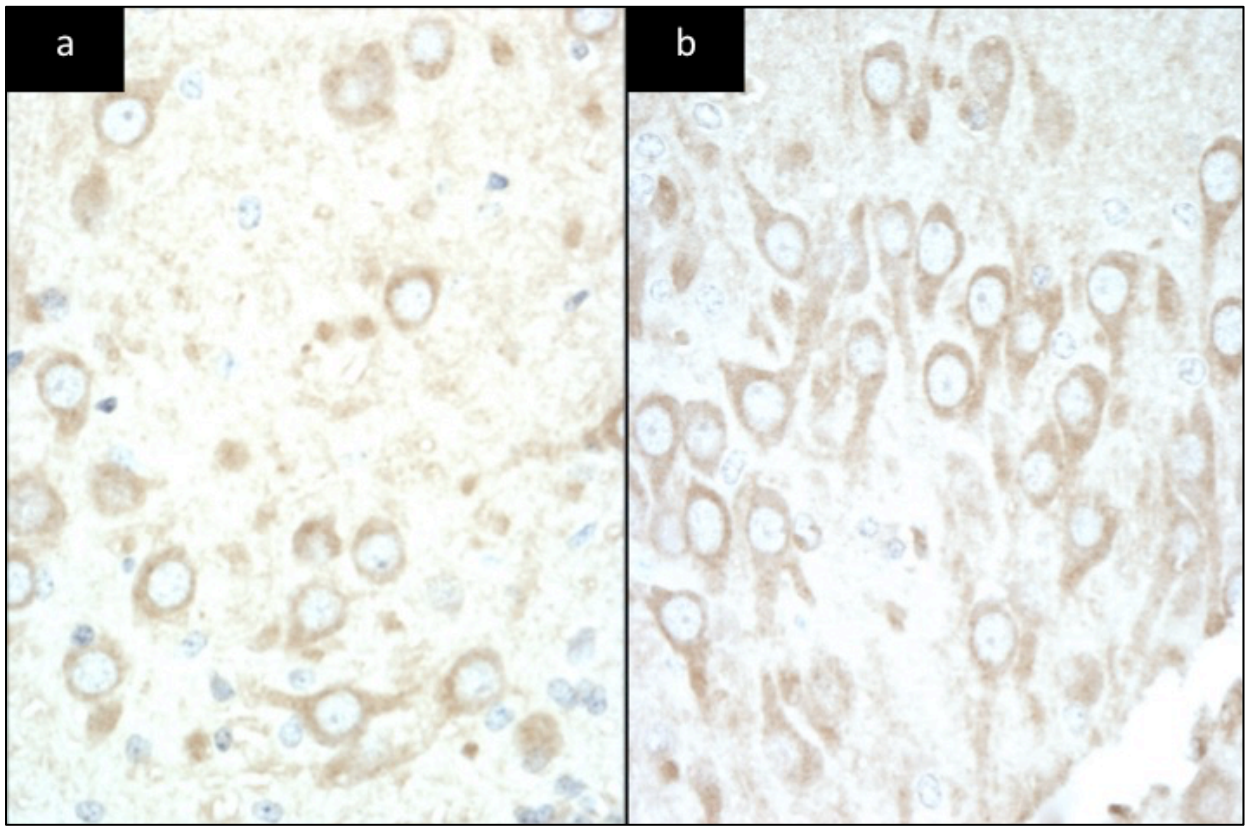

Figure 50: Glutamergic neurons in macaque deep temporal cortical layer. (IHC with 1/800 diluted anti-EAAT3 on paraffin-embedded tissue). a. Control macaque monkey male 6 months age, b. FASD macaque monkey male 6 months age. The control and FASD cases show strongly immunoreactive neurons. 400x magnification.

4.3.11 Non-human primate expresses glutamatatergic transporter following ethanol

I tested neuronal immunoreactivity for EAAT3 in both control and PNEE macaque brain tissues. In the hippocampi and inferior temporal cortices there was no statistically significant difference between the two groups (figure 51).

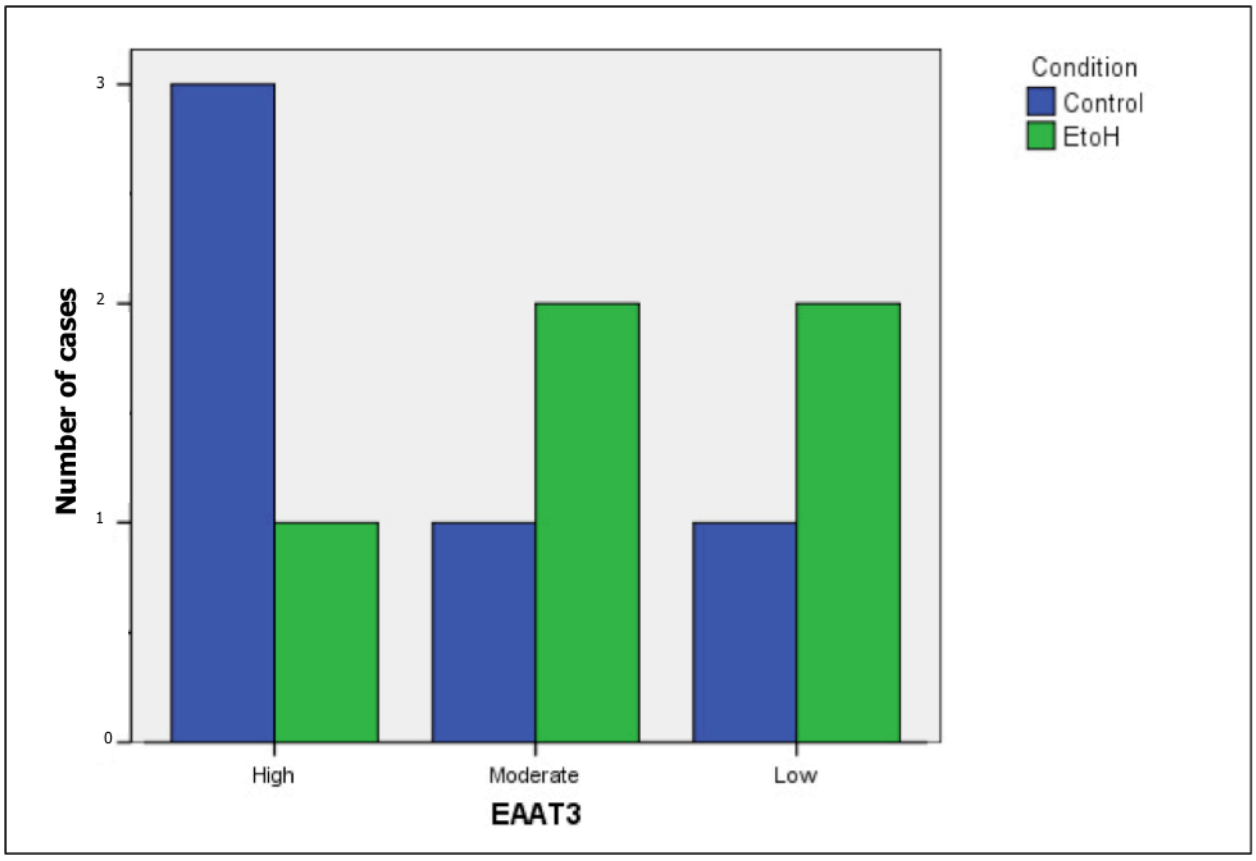

Figure 51: EAAT3 expression in macaque brains. Chi square test have performed on non-numerical variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. (2 cases with no expression are not shown). There was no difference in neuronal immunoreactivity to EAAT3 in macaque brain tissues (n=12) (Chi-square test value=1.667, P= 0.435).

4.3.12 GABA neurons in non-human primate CNS

The anti-calbindin1 (CALB1) antibody demonstrated a limited number of interneurons in layer II of the human cerebral cortex and non-specific binding to myelin in macaque brain tissue. The anti-GAD antibody demonstrated good labeling of inhibitory neurons in the cortex of macaque brains, despite very weak staining of neurons in human cerebral cortex (figure 52).

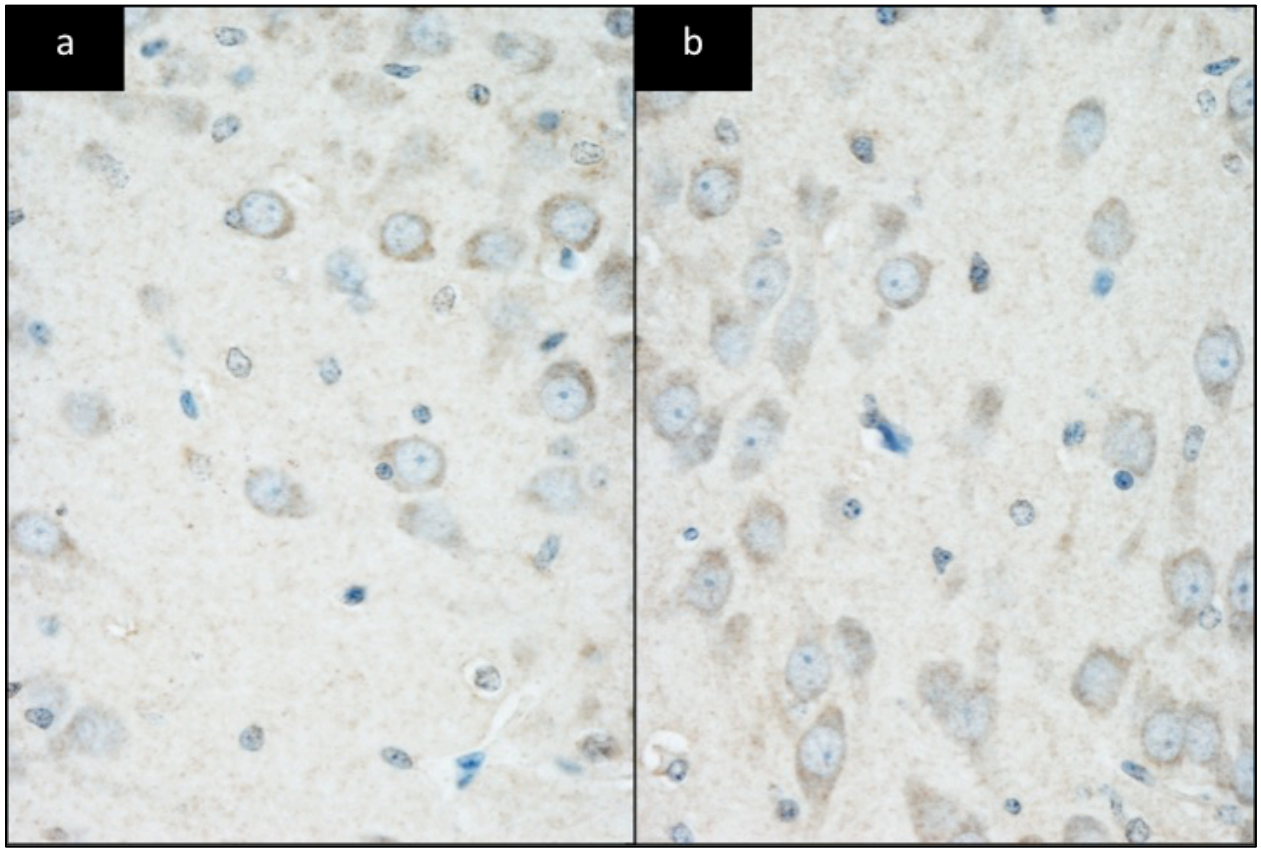

Figure 52: Inhibitory neurons expressed in macaque deep temporal cortical layer. (IHC with 1/200 diluted anti-GAD on paraffin-embedded tissue). a. Control macaque monkey male 6 months age, b. FASD macaque monkey male 6 months age. 400x magnification.

4.3.13 Interneurons in non-human primate following *in utero* alcohol exposure

Although macaque monkey brain tissue showed inhibitory neurons clearly in cortical layer II-III using GAD antibody, PNEE and controls cases did not show significant difference between the two groups. Chi square test have performed on non-numerical variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high (figure 53).

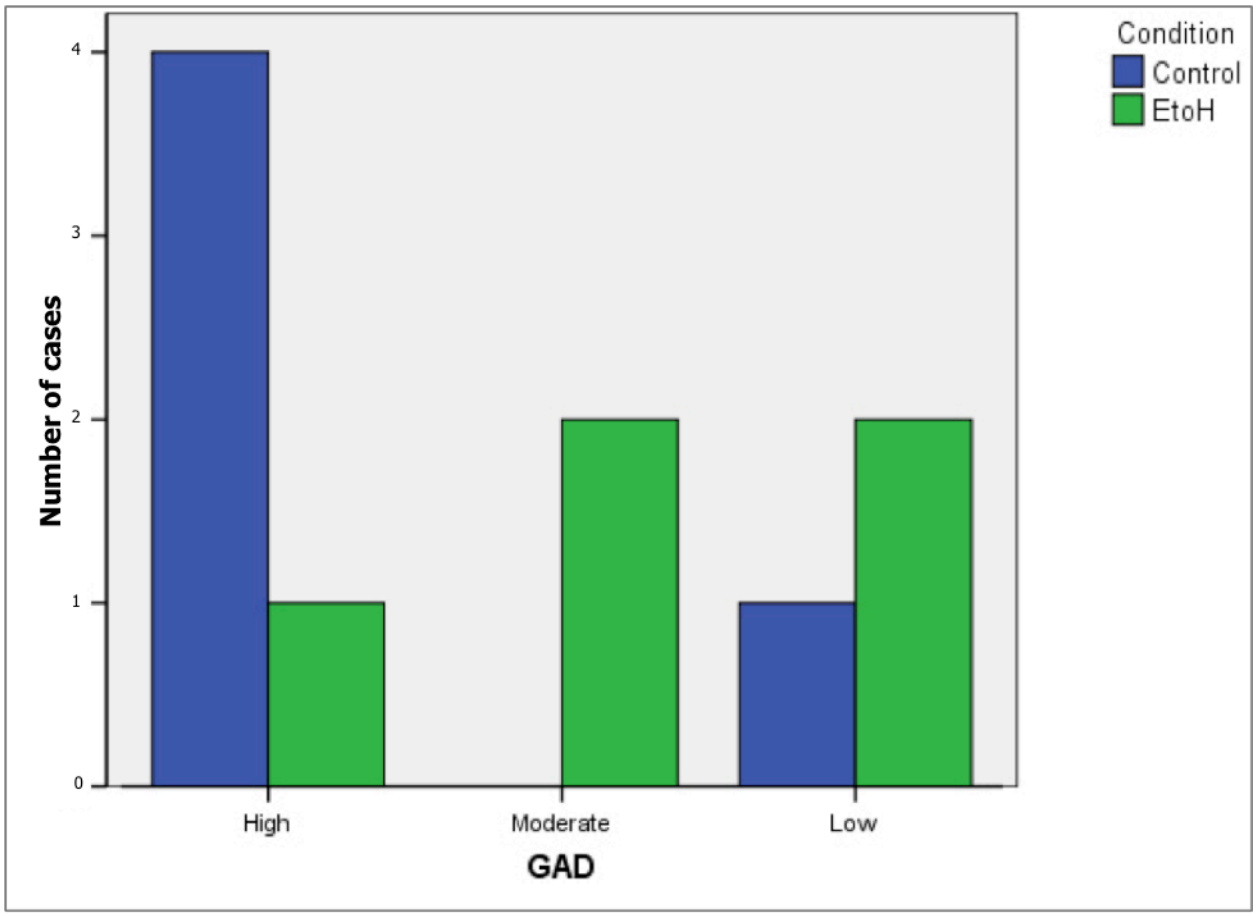

Figure 53: GAD1/GAD67 expression in macaque brains. Chi square test have performed on non-numerical variables according to cell immunoreactivity; 1= not detected, 2= low, 3=moderate, and 4=high. (2 cases with no expression are not shown). There was no difference in neuronal immunoreactivity to GAD1/GAD67 in macaque brain tissues (n=12) (Chi-square test value= 4.133, P= 0.127) (n=12).

Chapter 5. DISCUSSION

5.1 Human neuropathological changes following *in utero* alcohol exposure

Ethanol exposure during pregnancy can cause major negative effects on the developing brain. Although FASD is common in many regions in the world, fewer than 25 cases of human brain autopsies have been described in detail. The reported abnormalities include microencephaly, hydrocephaly, agenesis of corpus callosum, agenesis of anterior commissure, periventricular and leptomeningeal heterotopias, cerebral dysgenesis, and brainstem dysgenesis [21,128,149-153]. However, these findings are documented exclusively severe cases with major abnormalities in the brain and only represent the worst damage in FAS individuals. In fact, FASD has not yet studied thoroughly on human brains.

In the human autopsy cases from Winnipeg Health Sciences Centre, severe neuropathological abnormalities supported the previous studies. However, only a minority of these cases had major abnormalities. Hypoxic changes were commonly found in fetus brain autopsies, which are likely related to the placental insufficiency that led to fetal death.

5.2 Absence of oxidative stress markers in human and monkey FASD

Alcohol is detoxified mainly in liver where byproducts include reactive oxygen species (ROS), including superoxide (O2−•), hydrogen peroxide (H2O2), and hydroxyl radical (OH•) [81]. These products play a vital role in cell signaling and molecule oxidation [154]. ROS products, which oxidize proteins, DNA, and lipids, are normally metabolized inside the cells. Alteration in this balance leads to intracellular destruction followed by apoptotic changes in the cells [81,155].

Numerous studies have been designed to examine these oxidation products using specific antibody markers, e.g., glutamate—cysteine ligase catalytic subunit (GCLC) for protein

expression response to oxidative stress, 8-hydroxydeoxyguanosine (8OHdG) for DNA oxidation [156,157], and malondialdehyde (MDA) [158], acrolein [159,160], and 4-hydroxynonenal (4HNE) for lipid peroxidation [161]. These markers have illustrated that excessive oxidative products are present and might play a mechanistic role in many diseases. Some of these studies correlated the oxidative stress modification markers with cognitive and behavioral deficits in AD and MCI [162,163] (Table 8).

Table 8: Published studies associated oxidative stress markers with neurological disorders

Disorder	Model	Tested materials	Oxidative stress marker	Reference
Alzheimer's (AD)	Human AD autopsy	Brain, CSF, urine	↑8-OHdG	(Nunomura et al., 2006) [164]
		Brain	↑4HNE	(Jomova et al., 2010) [165]
		Frontal cortex	↑3-NT ↑4HNE	(Ansari & Scheff, 2010) [162]
		Hippocampus	↑4HNE	(Perluigi et al., 2009) [166]
Autism	7±2 age autistic children	Cingulate gyrus and cerebellum	↑3-NT	(Sajdel-Sulkowska et al., 2011) [167]
	Adult autistic human	Plasma	↑MDA	(Meguid et al., 2011) [168]
	Autistic human autopsy	Cerebellum	↓GCLC	(Gu et al., 2013) [169]
Parkinson's (PD)	PD human autopsy	Substantia nigra	↑4HNE ↑MDA	(Jomova et al., 2010) [165]
		Blood, urine	↑8-OHdG	(Seet et al., 2010) [170]
Down syndrome (DS)	DS human autopsy	Cortex	↑8-OHdG	(Perluigi & Butterfield, 2012) [171]
Mild cognitive impairment (MCI)	Human autopsy	Brain	↑4HNE	(Reed et al., 2008) [163]

3-nitrotyrosine (3-NT), 8-Hydroxydeoxyguanosine (8OHdG), Malondialdehyde (MDA), 4-Hydroxynonenal (4HNE), Glutamate—cysteine ligase catalytic subunit (GCLC), ↑ increase in level, ↓ decrease in level.

However, I did not detect increased markers of oxidative stress in human fetuses, infants, or children, or in non-human primates prenatally exposed to alcohol. This does not reflect findings in previous rodent FASD studies. There are at least three possible reasons: First, oxidative changes in brain might be a species-specific effect not found in primates. Second, postnatal life circumstances in monkey and human might gradually eliminate markers of *in utero* hypoxic stress. Third, death circumstances in humans cause oxidative changes that might obscure the *in utero* changes; this is supported by the high prevalence of GCLC expression in control and PNEE fetal cases.

Moreover, I cannot ignore technical factors related immunohistochemical detection that might lead to false negative results. First, the blocks of non-human primate tissues were originally embedded in an old type of paraffin, which is difficult to cut into 5μm sections and was therefore re-embedded. Second, the duration of fixation was not reported from imported macaque brain tissue, which could induce false negative results from excessive cross-linking epitopes. Third, oxidative stress markers in human and non-human primate brain tissues could be present but too low to be detected with the antibodies used. Forth, the antigen retrieval method was necessary in positive control specimens, however it can be a factor that produces false negative immunolabeling.

Although the mechanism of oxidative stress damage in FASD individuals has not been established yet, a large number of experiments have been proposed to examine the changes using distinct assays on different animal models. These experimental studies have detected ROS products in brain, antioxidants levels, and molecular damages results from oxidative stress. ELISA, immunohistochemistry, and immunocytochemistry are the main approaches tested for intracellular imbalance of ROS. However, these approaches have both weaknesses and strengths.

Enzyme-Linked Immuno-Sorbant Assay (ELISA), for instance, is very sensitive and inexpensive so it can detect low protein concentration in the tissue, but early immune response might be not detected in ELISA. In addition, this assay is only monoclonal antibody detection so that cross-reactive bindings are arising [172]. In contrast, IHC is less sensitive, and different staining pattern at the same tissue could be misleading in the interpretation. However, it detects monoclonal and polyclonal antibodies and maintains the integrity of cellular components in the tissue.

This metabolic issue has been examined largely in rodent experiments. For example, MDA and 4HNE have been found in rat brain tissue on GD17-18 as well as rat cerebellum, hippocampus, and cerebral cortex on postnatal day (PND) 4-9 when exposed to ethanol *in utero* [87,88]. 8OHdG was present in a high concentration in rat cerebellum following ethanol exposure at GD6-12 [86]. Table 9 is a summary of previous studies on oxidative stress and *in utero* alcohol exposure.

Table 9: Summary of some previous studies on oxidative stress in prenatal alcohol exposure

Model	Time exposure	Time of conducting study	Rout of consumption	Tissue	Effect	Reference
Mice prenatal EtOH	GD17	6h after injection	IP (4g/kg)	Brain	↓8-OHdG	(Miller et al., 2013) [173]
Mice prenatal EtOH	GD8	GD 9	IP (2.9g/kg)	Brain	↑8-OHdG	(Dong et al., 2009) [174]
Rats prenatal EtOH	GD17	NA	Gavage	Brain	↑4HNE	(Ramachandran et al., 2001) [87]
Rats prenatal EtOH	GD 6-21	PND1	Liquid-diet	Cerebellum	↑8-OHdG	(Chu et al., 2007) [86]
Rats prenatal EtOH	GD10, 17	GD18	Gavage 4g/kg	Cortex	↑GCLC	(Narasimhan et al., 2011) [175]

Thiobarbituric acid reactive substances (TBARS), glutathione peroxidase (GPx), glutathione reductase (GR), 8-Hydroxydeoxyguanosine (8OHdG), Malondialdehyde (MDA), 4-Hydroxynonenal (4HNE), and Glutamate—cysteine ligase catalytic subunit (GCLC) treated intraperitoneally (IP).

5.3 Shortcomings and limitations of oxidative stress markers detection

In this study, I selected specific antibodies to glutamate—cysteine ligase catalytic subunit (GCLC), 8-hydroxydeoxyguanosine (8OHdG), malondialdehyde (MDA), and 4-hydroxynonenal (4HNE) for oxidative stress detection. These antibodies were tested in hippocampi and cortices. The major limitation of studying human brain tissues is that the FASD risk factors (quantity, frequency of alcohol, and time of exposure) are seldom reported in detail. Further, I cannot absolutely exclude the possibility that the control samples did not have PNEE. Macaque brain tissues offer some degree of controls in respect of the amount, and frequency of alcohol exposure in addition to specific time of exposure; non-human primates might have species-specific effects.

5.4 Excitatory neurons alterations associated with prenatal alcohol exposure in humans

Excitatory neurons that use the neurotransmitter glutamate are generated during the first and second trimester during early brain development. During this period, modifications in excitatory neuron development have been associated with cognitive deficits in individuals affected with various neurodevelopmental disorders [176-178]. Changes in this neuron population are also associated with a range of neurodegenerative disorders, such as Alzheimer disease [179-181], Huntington disease [182], and motor neuron disease (amyotrophic lateral sclerosis) [183-185] (Table 10).

Table 10: Published studies correlated excitatory neurons with neurological diseases

Disease	Species	Brain region	Excitatory marker	Reference
Schizophrenia	Human	Striatum	↓EAAT-3	(McCullumsmith & Meador-Woodruff, 2002) [186]
Bipolar	Human	Striatum	↓EAAT-3 ↓EAAT-4	(McCullumsmith and Meador-Woodruff, 2002) [186]
Multiple Sclerosis	Human	Brain	↓EAAT-1	(Pitt et al., 2003)
Epilepsy	Rats	Hippocampus	↓GLAST ↑ GLT-1	(Guo et al., 2010) [187]
Huntington	Human	Neostriatum	↓EAAT-2	(Arzberger et al., 1997) [188]
Alzheimer	Human Mice Mice	Cortex Astrocyte Brain	↓EAAT-2 ↓ GLT-1 ↓EAAT-1 ↓EAAT-2	(Masliah et al., 1996) [189] (Dabir et al., 2006) (Masliah et al., 2000)
Parkinson	Human	Substantia nigra	↑EAAT-3	(Plaitakis and Shashidharan, 2000) [190]
ALS	Human	Brain	↓ GLT-1	(Rothstein et al., 1995) [191]

Multiple sclerosis (MS), Amyotrophic lateral sclerosis (ALS), Glutamate/aspartate transporter (GLAST-1), Glutamate Transporter (GLT-1), Excitatory Amino Acid Transporters-1 (EAAT-1)

Excitatory neurotransmitter modification has been documented in animal models following prenatal alcohol exposure (Table 11). I found a 12.2 % and 17.26% reduction in the density of glutamatergic neurons in alcohol-exposed brains (all ages / sexes) in dentate gyrus and temporal cortex, respectively.

5.5 EAAC1 is highly expressed in neurons and dendrites

Glutamate/aspartate transporter (GLAST-1) [192], glutamate transporter (GLT-1) [193], excitatory amino acid carrier-1 (EAAC-1) [194], and excitatory amino acid transporters (EAAT-4) [195] are four membrane transporter proteins. In the brain, these transporters protect the cells from excitotoxicity by decreasing the high glutamatatergic level in the excitatory synapse during resting state [196]. EAAC-1 was chosen as a marker of glutamatergic neurons in my human autopsy investigation, whereas EAAT-3 has been chosen to detect excitatory neurons in macaque monkey brain autopsies. It is not clear why the slightly different proteins were not detected equally in human and macaque brains. EAAC1 and EAAT-3 are both Na+-dependent L-glutamate/D, L-aspartate membrane transport proteins [197].

Previous studies have localized EAAC-1 in neurons and non-neuronal cells in heart, kidney, and muscle. Particularly EAAC-1 transporter proteins are expressed in CNS mainly in the hippocampus regions (stratum radiatum, CA1) of young rats, cerebral cortex layer II-IV, and cerebellum of mice brain [198-200]. In the cerebellum, EAAC-1 was found mainly in neurons expressed in cerebellar Purkinje cells [197]. Moreover, it was expressed in rat astrocyte process, mice cortical astrocytes, and human apical dendrites [201-203]. A later study co-localized EAAC-1 in basal ganglia, dentate gyrus, and olfactory neurons [204], and EAAT-3 has been localized in human cortical pyramidal neurons [205].

5.6 Sex-based difference in EAAC1 expression of human FASD

Previous studies discovered sex specific changes in GS protein expression following alcohol exposure [206,207]. The mechanistic ability of sex hormones, e.g., estrogen infiltration via blood brain barrier throughout the brain was discovered to be one of the main intentions behind sex-based difference following *in utero* ethanol exposure [207]. Therefore, I anticipated sex differences in the expression of glutamatergic neurons in human infants, children, and teenagers. However, contrary to my expectations, my results showed both sexes respond similarly to the prenatal alcohol exposure, i.e., both males and females have reduced expression of glutamatatergic neurons following ethanol exposure.

5.7 Inhibitory neurons expression reduced following prenatal ethanol exposure

Parvalbumin (PVALB) and calbindin1 (CALB1) were selected in my investigation for the effect of PNEE on inhibitory interneurons on human. Whereas, glutamate decarboxylase isoform-65 (GAD65); the precursor of GABA synthesis [208], was selected to test the changes in non-human primate models prenatally exposed to ethanol because preliminary immunohistological tests of parvalbumin (PVALB) and calbindin1 (CALB1) did not show labeling on macaque brain tissues.

A previous study found a substantially decreased number of striatal dendritic inhibitory neurons detected with parvalbumin (PVALB) in newborn rats exposed to long-term postnatal ethanol at P2-P6 [209]. Moreover, hypoxic postnatal (P3-P11) mice previously exposed to ethanol showed 59% reduction in parvalbumin (PVALB) immunoreactivity in interneurons [210]. Numerous studies reported the effect of ethanol on brain development in respect of inhibitory neuron expression (see Table 11).

Table 11: Changes of excitatory and inhibitory neurotransmitter in alcohol exposure

Neurotrans-mitter	Model	Timing of exposure	Rout of consumption	Tissue	Marker	Reference
Excitatory	Guinea Pigs	GD 2-63	Gavage	Hippocampus	↑Glu	(Iqbal et al. 2006)
	Rats	GD15-PD9	BPHC	Hippocampus	↓GLT1 ↓GLAST	(Castaldoa et al., 2009)
	Rats	PD 8	IH	Hippocampus	↑EAAT1 ↑EAAT3	(Zink et al., 2011)
	Rats	NA	NA	Cortex	↑EAAT-1 ↑EAAT-2	(Zink et al., 2004)
	Rats	GD3-21	liquid diet	Dentate gyrus	↓mGluR5	(Galindo et al. 2004)
	Rats	GD5-20	BPHC	Frontal cortex	↑EAAC1	(Castaldo et al., 2007)
Inhibitory	Mice embryos	GD7-18	Liquid diet	Brain	↓5-HT	(Sari and Zhou, 2004)
	Monkeys	GD3-42	Gavage	Cortex	↓GABA	(Miller 2006)
	Newborn rats	NA	NA	Cortex	↑GAD	(Zink and Spanagel, 2005)
	Rats	1PD	IH	Cortex	−PV ↓CALB1 ↑CALB2	(Granato, 2006)

	Mice	NA	Liquid diet	Hippocampus	↓CALB1 ↑GFAP	(Satriotomo et al., 2000)
	Rats	Term	Gavage	Cerebellum	↓PV ↓CALB1 ↑CALB2	(Wierzba-Bobrowicz et al., 2011)

Buccopharyngeal cannula (BPHC); Glutamate/aspartate transporter (GLAST-1); glutamate Transporter (GLT-1), Postnatal day (PD); Inhalation (IH); Parvalbumin (PVALB); calbindin1 (CALB1); Calretinin (CALB2); Level decreased (↓); Level increased (↑); Level did not change (−); Throughout pregnancy (Term)

In my experiment, both male and female PNEE groups showed reduction in inhibitory neuron immunostained with anti-parvalbumin (PVALB) and anti-calbindin1 (CALB1). My results are consistent with previous studies, which did not find sex-specific difference in GAD65 expression in PNEE mice [211].

5.8 Inhibitory neurons in infant brains are vulnerable to prenatal ethanol exposure

The maturation of brain structures plays an integral part in the concentration of GABA interneuron expression. Several studies explicitly elicited the difference in neuron generations and maturations across distinct age groups [204,212,213]. For instance, immunolabeling of GABA receptor was detected greater in early embryonic age (E11–14) PNEE mice, whereas adult PNEE mice did not show a significant difference in parvalbumin (PVALB) expression [214]. Additionally, the expression of calbindin1 (CALB1) increased with developmental age in human cerebellum (children and adults) [116]. In my experiment, GABA neurons detected with parvalbumin (PVALB) and calbindin1 (CALB1) were reduced in all age groups with PNEE. Infants group immunostained with anti- calbindin1 (CALB1) showed the highest difference in GABA expression following alcohol exposure.

5.9 Absence of neuronal differences in non-human primate brains with PNEE

I had expected that the monkey brains would show differences that could not be shown in the human brains. However, there were none. Perhaps the main reason is the limited (once per week) exposure. Neuron generation and migration occurs during a protracted period of development and it is possible that the alcohol doses used (which did not cause reduced fetal viability) were insufficient to cause obvious damage. Another possibility is that all cell populations (including glial cells and endothelial cells) were reduced equally with neurons and therefore no changes could be detected with the counting method used. Unfortunately brain weights of the monkeys are not available to provide support for this explanation.

5.10 Neuronal changes might explain the neurobehavioral abnormalities in FASD

Earlier studies have used antibodies against calcium binding proteins to discover the association between inhibitory neuron alterations and neuronal destruction in individuals diagnosed with some neurological illnesses, for instance, attention deficit hyperactive disorder (ADHD) (Hoekzema et al., 2013), Alzheimer's disease (AD) [215], and schizophrenia [216]. Moreover, seizures in epileptic patients have been found to be associated with glutamate/GABA neurotransmitter alterations in hippocampus [196].

Although the mechanism of ethanol insult to brain interneurons following prenatal alcohol exposure has not yet been established, previously reported results showed immunohistological decreased in calcium binding protein expression in Purkinje cells of rats cerebellum following PNEE [217]. Neuron loss and motor coordination deficits are interconnected [218,219]. For these reasons, I suggested an association between inhibitory neurons and cognitive deficits in individuals with FASD.

My human brain autopsies supported these previously published small animal model studies (Table 11). I recorded interneuron reduction in hippocampus CA1, CA3, and DG in addition to inferior temporal regions expressed in both parvalbumin (PVALB) and calbindin1 (CALB1) following ethanol exposure.

5.11 The pathogenesis of neuronal reduction following alcohol exposure

Based on neuroimaging studies of the human and animal brains [105], I can consider the changes in the volume and cell numbers in brain regions, e.g., hippocampus, cortex, and basal ganglia etc., as a neurobiological response to PNEE. My investigation affirmed previous reports, showing that excitatory neurons were localized predominantly in temporal cortex layer II-V, CA1, CA3 and dentate gyrus of the hippocampus of controls (not exposed to alcohol *in utero*) and FASD human brain in infants, children, and teenage groups.

Neuron loss ought to be explained in order to understand the mechanism of loss and the resulting brain dysfunction. I offer five possible explanations of neuronal reduction in human brains with PNEE: First, interference with neuronal migration from the ventricular zone to the neocortex [61]. Second, delay in neurogenesis follows alcohol exposure during second or third trimester, which are considered critical periods of neuron generation [220]. Third, decreased neuron production from sub-ventricular zone throughout the pregnancy in response to PNEE [221,222]. Fourth, PNEE-induced apoptosis in developing brain, which in turn could decrease the number of neurons in the brain tissue [90,223]. Fifth, exposure to alcohol might alter or inhibit neuronal differentiation in the brain [224].

Because many neurological diseases have been found to correlate with excitatory neurotransmitter destruction [225,226], I speculate that the reduction in excitatory neurons could correlate with cognitive deficits in individuals diagnosed with FASD. Further human studies should be done to specify neuronal dysfunctions and their associations with intellectual impairments in FASD. Relatively greater loss of the inhibitory neurons following PNEE could explain other features of FASD such as tremor and seizures [227].

5.12 Shortcomings and limitations of neuronal detection

Cerebellum, striatum, and occipital cortex should be tested further in order to investigate the neuronal changes in more brain regions. This might help explain the volumetric changes shown using MR imaging in other studies. In addition, use of more neuroanatomical markers such as Calretinin or NeuN would generalize the idea of neuronal reduction following the insults of alcohol prenatally. Because FASD is associated with addictive behaviors, detection of dopamine receptors might be of interest. Macaque experiments have previously relate the behavioral deficits in FASD with dopamine reduction [137].

Chapter 6. CONCLUSIONS

FASD is one hundred percent preventable. However, because of a variety of societal and individual factors, it continues to occur and therefore understanding the disease is critical. There are several major obstacles to understanding mechanistic changes in the developing brain. Interspecies differences make application of the animal models difficult. With respect to alcohol exposure, primary (time, frequency, quantity of alcohol exposure) and secondary (prenatal nutrition, genetics, education, socioeconomic, and father's drinking) biological and non-biological cofactors influence effects on the brain and likelihood of developing FASD.

Many studies have been constructed to investigate the pathogenicity of alcohol exposure *in utero* in animal models. However, few studies have been done on human autopsies. Therefore, I constructed my study of human and nonhuman primates to validate the highly controlled rodent studies of FASD.

My first hypothesis proposed that persistent markers of oxidative stress would be evident by immunohistochemical detection in human and monkey brains following *in utero* alcohol exposure. This was not supported by my data using four oxidative stress markers—GCLC, MDA, 4HNE, and 8OHdG. However, his does not exclude oxidative stress in the overall hypothesis of PNEE-related damage because there are several possibilities (dose of alcohol in the monkeys; postpartum environments and mortal conditions in the humans) that might prevent detection of oxidative markers in the samples I studied.

My second hypothesis proposed that PNEE could alter the balance in excitatory and inhibitory neurotransmitters in human and nonhuman primates in the hippocampus and temporal lobe. This was supported by my data in human brains. Immunohistochemical detection was performed to detect neuronal modifications in glutamatergic and GABAergic neurons. Like

previous studies, I found substantial reductions in the proteins calbindin1 (CALB1) and parvalbumin (PVALB) in human brains (CA1, CA3, dentate gyrus, and temporal cortex) of infants, children, and teenagers prenatally exposed to ethanol. Notably, human infant brains appeared most vulnerable to a reduction of excitatory and inhibitory neurons. Unlike in the human brain, the excitatory and inhibitory neurons markers in macaque brains did not show a significant difference between the controls and those with PNEE.

6.1 Value and limitations of the study

Human tissue must be studied to validate the many small animal studies. Verification of the hypothesized oxidative and/or neuronal changes will be of extreme value to the literature.

The major limitation of human studies is the inherent lack of control and the uncertainty of the actual alcohol exposure (which was often in combination with other potentially damaging agents, including tobacco or illicit drugs such as marijuana or cocaine). Study of the monkey brains offered some degree of control and actual ethanol exposure, but this is limited by the fact that the monkeys were exposed to alcohol only once weekly *in utero* and then survived for 6 months after birth. With respect to studying the oxidative changes, it is not clear how long the markers persist in the tissues. Considering the relative difficulty of conducting the immunostains on the monkey brain tissues, it seems likely that they were subjected to prolonged formalin fixation before paraffin embedding. Consequently, more intense antigen retrieval was required, which raised the possibility of false negative results.

6.2 Future direction of the study

With respect of neuronal modification following PNEE, more brain regions should be studied. Other neurochemical markers, such as those that detect more restricted populations of

acetylcholinergic or dopaminergic neurons might also be of interest to understand some of the behavioral and cognitive features of FASD.

Moreover, neuropathology and neuronal alterations in human cases should be matched so that the changes can explain the more severe neuropathological malformations. More importantly, the quantity of alcohol exposure and time of exposure would be correlated with specific areas of neuronal reduction.

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