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ETHANOL SUSTAINS PHOSPHORYLATED TAU PROTEIN IMMUNOFLUORESCENCE IN THE CULTURED NEONATAL RAT HIPPOCAMPUS: IMPLICATIONS FOR FETAL ALCOHOL SPECTRUM DISORDERS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Arts and Science
at the University of Kentucky

By

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Lexington, Kentucky

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2020

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ABSTRACT OF THESIS

ETHANOL SUSTAINS PHOSPHORYLATED TAU PROTEIN IMMUNOFLUORESCENCE IN THE CULTURED NEONATAL RAT HIPPOCAMPUS: IMPLICATIONS FOR FETAL ALCOHOL SPECTRUM DISORDERS

Fetal Alcohol Spectrum Disorders (FASDs) are comprised of developmental, behavioral, and cognitive abnormalities caused by prenatal alcohol exposure, affecting an estimated 2%-5% of children and costing up to $4 billion annually in the United States alone. Although some behavioral therapies can help, the biochemical mechanisms that underpin FASDs need further elucidation for development of more efficacious therapeutics. The tau protein modulates cytoskeletal structure in neurons, and thereby plays an integral role in proper development and function of the central nervous system, but its function is altered by its phosphorylation state, such that increased phosphorylation reduces tau protein function. The tau protein is highly phosphorylated at birth but becomes dephosphorylated during structural maturation. Aberrant tau hyperphosphorylation is cardinal of neurodegenerative diseases, but less is known about its role in neurodevelopmental disorders, like FASD. In the neonatal rat model of organotypic hippocampal slice culture, tau phosphorylation decremented in all measured regions of the control hippocampus across 24 days in vitro, but tau hyperphosphorylation was sustained in all regions except the CA3 in hippocampal slices that were exposed to alcohol. Future research should investigate the functional, behavioral, and cognitive relevance of hyperphosphorylated tau protein with regard to FASDs to identify potential therapeutics.

KEYWORDS: Tauopathy, Fetal Alcohol Spectrum Disorders, Organotypic Hippocampal Slice Culture

Caleb Seth Bailey

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10/28/2020

Date

ETHANOL SUSTAINS PHOSPHORYLATED TAU PROTEIN IMMUNOFLUORESCENCE IN THE CULTURED NEONATAL RAT HIPPOCAMPUS: IMPLICATIONS FOR FETAL ALOCHOL SPECTRUM DISORDERS

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DEDICATION

Joy Briggs, Daisy, and John Prine

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CHAPTER 1. INTRODUCTION

1.1 Fetal Alcohol Spectrum Disorders

Fetal Alcohol Spectrum Disorders (FASD) is a non-diagnostic umbrella term which includes patients who are diagnosed with partial or full Fetal Alcohol Syndrome (pFAS; FAS), alcohol-related neurodevelopmental disorder (ARND), alcohol-related birth defects (ARBD), and neurobehavioral disorder associated with prenatal alcohol exposure (ND-PAE). These conditions are diagnostically spectral, with FAS patients presenting a greater magnitude of physical (lower birth weight, thin vermilion, smooth philtrum, flat midface, shortened palpebral fissure) and neurodevelopmental (microcephaly, cerebellar hypoplasia) abnormalities compared to other FASDs (Bertrand et al., 2004; Ferreira & Cruz, 2017; Jones & Smith, 1973; Kully-Martens, Denys, Treit, Tamana, & Rasmussen, 2019). Though FASDs are the most preventable causes of mental retardation, an estimated 2-5% of children are affected by FASDs and disburse up to $4 billion in annual costs in the United States alone (Lupton, Burd, & Harwood, 2004; May et al., 2009). Recently, up to 11.5% of women reported drinking during the time of pregnancy, and 3.9% reported binge drinking within the last 30 days of the pregnancy (Denny, Acero, Naimi, & Kim, 2019). In some domains, binge alcohol consumption during pregnancy induces more severe deficits compared to moderate, chronic alcohol consumption (Bonthius, Goodlett, & West, 1988; Sood et al., 2001) though no amount of alcohol consumption is safe at any stage of pregnancy (Williams & Smith, 2015).

Alcohol exposure at different fetal stages has varying effects on the developing central nervous system (CNS); earlier fetal alcohol exposure during the embryonic periods results in the cardinal craniofacial abnormalities in the human along with forebrain and brain stem developmental perturbations in macaques and Sprague-Dawley rats, respectively (Ernhart et al., 1987; Miller, 2007; Mooney & Miller, 2007; Sulik, 2005). Data from both rodent and human studies show that alcohol exposure prior to and briefly after birth has a greater magnitude of influence on neural and glial migration and viability, resulting in hippocampal, cortical, and corpus colossal defects leading to diminished overall brain volume and white matter insulation compared to unexposed groups (Gressens, Lammens, Picard, & Evrard, 1992; Miller, 1995; Riley et al., 2005; Rubert, Miñana, Pascual, & Guerri, 2006; Sowell et al., 2001). These deficits result in several behavioral and cognitive abnormalities in humans which practitioners primarily rely on for diagnosis (Mattson, Crocker, & Nguyen, 2011). Notable deficits observed in FASDs include, but are not limited to, diminished intellectual capacity, attention, executive and adaptive functioning, and learning and memory.

1.1.1 Intelligence

In general, individuals with FASDs have a restricted intellectual capacity with a broad IQ range between 20-120 (Mattson & Riley 1998; Streissguth, Barr, Kogan, & Bookstein, 1996); however, even those most severely affected by FASDs usually have IQs greater than 70 (i.e. do not meet criteria for intellectual disability; Mattson et al., 2011). Chasnoff et al. (2010) showed that there are some intellectual distinctions between FAS, pFAS, and ARND with regard to IQ and language-based memory where, predictably, individuals with FAS scored significantly worse than those with pFAS and

ARND; notably, there were no significant differences in any neurocognitive assessment between individuals with pFAS and ARND. Intelligence research on individuals with lower exposure to prenatal alcohol (pFAS and ARND) is lacking and many outcomes are inconclusive in part due to poor reporting of the timing and amount of alcohol exposure (Henderson, Gray, & Brocklehurst, 2007). Importantly, children with FASDs consistently perform worse in academic domains compared to non-FASDs children even after controlling for IQ, suggesting that general intelligence may only be one component of a milieu of neurocognitive factors that predict suboptimal academic performance (Glass et al., 2017; Goldschmidt et al., 1996).

1.1.2 Attention

Individuals with FASDs also show attention deficits and hyperactivity (Lee, Mattson, & Riley, 2007). Some of the most common attentional deficits seen in FASDs include efficiency of information processing, working memory, impulsivity, and reaction time – symptoms which overlap with attention deficit hyperactive disorder (ADHD; Jacobson, Jacobson, & Sokol, 1994; Kodituwakku, 2009; Rasmussen, 2005; Streissguth, 2007). In fact, the cognitive and behavioral abnormalities between FASDs and ADHD are strikingly similar, though individuals with FASDs generally have lower IQs compared to individuals with ADHD (Bhatara, Loudenberg, & Ellis, 2006; Vaurio, Riley, Mattson, 2008; for a review, see Mattson, Bernes, & Doyle, 2019). FASDs remain the highest risk factor for developing ADHD in children, with prevalence of ADHD in individuals with FASDs reaching up to 94% (Bhatara et al., 2006; Fryer, McGee, Matt, Riley, & Mattson, 2007). This prevalence reflects the similar behavioral and cognitive presentations between FASDs and ADHD, but it should be noted that the diagnostic

distinctions may be muddied. An estimated >90% cases of FASDs are mis- or undiagnosed (Chasnoff, Wells, & King, 2015), which is likely due to the stigmatization and underreporting of maternal alcohol use (Corrigan et al., 2017; Morleo, 2011).

1.1.3 Executive Functioning

Children with FASDs present with poor executive functioning. Executive functioning encompasses some components of attention and appropriate physical activity levels, but additionally incorporates competency in problem solving, concept formation, set-shifting, and planning to achieve goal-directed behaviors (Anderson, 2002; Welsh & Pennington, 1988). Children with FASDs show significant deficits in the ability to generate and appropriately categorize abstract ideas when prompted; additionally, it has been shown that executive functioning may be impaired in individuals with FASDs due to the compounding difficulties with working memory and selective attention (Aragón, 2008; McGee, Schonfeld, Roebuck-Spencer, Riley, & Mattson, 2008; Rasmussen, 2005). Subsequently, these executive functioning deficits impede on or entirely disrupt conceptualization, reasoning, and planning skills (Kodituwakku, Handmaker, Cutler, Weathersby, & Handmaker, 1995; McGee et al., 2008), which ultimately influence daily life and impair adaptive functioning. Taken together, this creates a familial, economic, and societal burden for those affected by FASDs (Greenmyer, Klug, Kambeitz, Popova, & Burd, 2018).

1.1.4 Adaptive Functioning

Diagnostic features of adaptive functioning operate within the three realms of communication, socialization, and daily living skills and are commonly evaluated using

the Vineland-3 assessment for individuals with intellectual disabilities (Sparrow, Cicchetti, & Saulnier, 2016). Cumulatively, these components promote personal and social independence – traits which are substandard in adult individuals with FASDs; these deficits are particularly exacerbated in conjunction with sensory processing deficits which is a common feature in those with FASDs (Åse et al., 2012; Jirikowic, Olson, & Kartin, 2008; Carr, Agnihotri, & Keightley, 2010). The affective impact of poor adaptive functioning in social outcomes in the FASDs population poses a significant risk of developing psychological disorders (Famy, Streissguth, & Unis, 1998; Fryer et al., 2007; O'Connor & Paley, 2009; for a review, see Weyrauch, Schwartz, Hart, Klug, & Burd, 2017). Further, individuals prenatally exposed to alcohol are more likely to present with conduct disorders, substance use disorders, and legal trouble (Alati et al., 2008; Larkby, Goldschmidt, Hanusa, & Day, 2011; Streissguth et al., 2004).

1.1.5 Learning and Memory

Children with FASDs have impaired learning and memory skills regardless of IQ (Lewis et al., 2015; Mattson et al., 2011). Several of the deficits mentioned herein contribute to poor learning and memory outcomes in humans with FASDs, including attention, information processing, cognitive set shifting, working memory, and executive functioning (Aragón, 2008; Lee et al., 2007; Rasmussen, 2005). Poor memory consolidation, slower information processing and inefficient working memory are likely manifestations of the deleterious effects of alcohol on the developing hippocampus; in fact, magnetic resonance imaging (MRI) has revealed that truncated hippocampal development in FASDs is associated with impaired recall as evidenced by reduced hippocampal volume compared to age-matched controls (Willoughby, Sheard, Nash, &

Rovet, 2008). In pre- and neonatal alcohol-exposure rodent models of FASDs, alcohol exposure induces visuospatial learning and recall deficits, which is shown by a marked impairment to reach the escape platform in the Morris Water Maze Task compared to rodents who were not exposed to alcohol (Berman & Hannigan, 2000; Goodlett & Johnson, 1997; Johnson & Goodlett, 2002). Wagner, Zhou, and Goodlett (2014) showed that learning deficits resulting from rodent neonatal binge alcohol exposure (human 3rd trimester equivalent) can persist into adolescence and adulthood. Due to its plastic role in learning and memory and susceptibility to alcohol insult, the hippocampus is an attractive candidate for investigation and potential mitigation of the developmental deficits observed in FASDs.

1.2 Convergent Mechanisms of Pathology in the Hippocampus

The functional role of the hippocampus is primarily involved in the synthesis and retrieval of episodic and contextual memories (Moscovitch, Nadel, Winocur, Gilboa, & Rosenbaum, 2005). Although the role of the hippocampus in long-term memory retrieval is debated, there is evidence to suggest that the hippocampus is necessary for long-term contextual and autobiographical retrieval in rodents and humans, respectively (Rekkas & Constable, 2005; Steinvorth, Levine, & Corkin, 2005; Sutherland, O'Brien, & Lehmann, 2008). The synthesis of new memories is perhaps the most important role of the hippocampus and is certainly the most debilitating deficit in diseased and damaged states of the hippocampus. Memory-related pathologies such as age-related dementia and Alzheimer's Disease (AD) target the hippocampus and the entorhinal cortex, though there

are distinct pathologies for each with respect to subregional hippocampal damage within the perforant pathway (Small, Schobel, Buxton, Witter, & Barnes, 2011).

1.2.1 Neural Cytography

The trisynaptic perforant pathway is comprised of the entorhinal cortex, dentate gyrus (DG), Cornu Ammonis 3 (CA3) subregion, Cornu Ammonis 2 (CA2) subregion, Cornu Ammonis (CA1) subregion, and the subiculum (Cembrowski & Spruston, 2019). The most explored regions of the hippocampus are the DG, CA3, and CA1 while greater contention exists over and less is known about the CA2 subregion (Small et al., 2011). Cell types that pervade the trisynaptic perforant pathway include granule cell layers of the DG which project mossy fiber axons toward interneurons and pyramidal neurons in the CA3; pyramidal neurons project Shaffer collateral axons throughout the pyramidal neurons of the CA3 and CA2 toward pyramidal neurons in the CA1 (Andersen, Bliss, & Skrede, 1971; Small et al., 2011). The heterogeneity of the pyramidal neurons in the Cornu Ammonis (CA) regions remains under investigation, but current research suggests that their functional differences may rely on the length of their distal dendrites which would modify propensities to fire signals (Cembrowski & Spruston, 2019; Ding et al., 2020).

1.2.2 Amino Acid Neurotransmission

The trisynaptic perforant pathway implements GABAergic neurotransmission via laced interneurons and primarily glutamatergic neurotransmission in the granule and pyramidal cells, though it has been reported that there is a GABA presence in the granule cells and mossy fiber axons (Acsády, Kamondi, Sík, Freund, & Buzsáki, 1998; Münster-

Wandowski, Gómez-Lira, & Gutiérrez, 2013; Sloviter, 2003). Constitutive activity in the trisynaptic pathway involves excitatory input from the entorhinal cortex into the granule cells of the DG which release glutamate onto pyramidal cells and interneurons within the CA3 and CA2 regions; this results in at least two cellular responses: 1) pyramidal neurons in these regions propagate excitatory information into subiculum pyramidal cells along the Shaffer collaterals through the pyramidal cells of the CA1; 2) interneurons in the CA3, CA2 and CA1 regions regulate and tune pyramidal neuron activity by lateral inhibition (Banke & McBain, 2006; O'Mara, 2005; Sloviter, 2003). It is through this glutamatergic N-methyl-D-aspartate (NMDA) mechanism that neural plasticity and long-term potentiation occur in the hippocampus (Collingridge, Kehl, & McLennan, 1983; Rebola, Lujan, Cunha, & Mulle, 2008). The interaction of this amino acid neurotransmission is fragile and can contribute to mental illness.

1.2.3 Hippocampal Pathology

In certain pathogenic states, amino acid neurotransmission is dysregulated. Namely, individuals suffering from AD and epilepsy experience heightened hippocampal glutamate activity (Barker & White, 2015; Wang & Reddy, 2017), and individuals with Schizophrenia have a reduced number of hippocampal GABAergic interneurons resulting in unregulated excitation (Konradi et al., 2011). Unbridled glutamate activity can induce calcium-mediated mitochondrial dysfunction and excitotoxic neuronal death (Ankarcrona et al., 1995; Berliocchi, Bano, & Nicotera, 2005). In addition, one hallmark of excessive glutamate activity in the hippocampus is the presence of collateral axonal sprouting and reorganization of axons in hyperexcitable pyramidal neurons which is closely associated with the effects of temporal lobe epilepsy following a sustained seizure (Sutula, Cascino,

Cavazos, Parada, & Ramirez, 1989). The clinical overlap between alcohol-related pathologies and epileptic instances should be noted: the prevalence of epilepsy and reported history of seizures reaches up to 18%-21% in the FASDs population, which is a higher prevalence compared to the general population (Bell et al., 2010; Nicita et al., 2014). This suggests that there may be a common mechanism of hippocampal modification between alcohol-exposure and development of epileptic profiles.

1.2.4 Convergent Mechanisms

Rodent research on temporal lobe epilepsy may provide an important link between glutamate activity, hippocampal axonal reorganization, and subsequent cognitive deficits with relationship to prenatal alcohol exposure. One of the most common outcomes for successful induction of temporal lobe epilepsy in rodent models is hyperexcitability of glutamatergic neurons in the hippocampus such that firing threshold is lower and more sensitive to glutamate (Blümcke et al., 2000; Fritsch, Stott, Joelle Donofrio, & Rogawski, 2010). This glutamate-driven hippocampal hyperexcitability can induce neuronal cell death and axonal reorganization, an effect which is replicated by alcohol exposure and subsequent withdrawal (Ankarcrona et al., 1995; Lindsley, 2006; Sutula et al., 1989; Whittington, Lambert, & Little, 1995). In fact, upon withdrawal from heavy, chronic alcohol exposure, both humans and rodents routinely present with seizures that are perpetuated by robust, imbalanced glutamate activity (Rogawski, 2005).

1.2.5 Alcohol Action

Upon initial exposure, alcohol induces an inhibitory effect on cells by GABA agonism and glutamatergic antagonism; however, upon withdrawal, alcohol induces a

compensatory glutamatergic action, surmounting the inhibitory effects of GABA (Whittington et al., 1995). Importantly, the effects of maternal drinking patterns of exposure and withdrawal are recapitulated in the developing CNS of the fetus (Thomas & Riley, 1998). The differential effects of initial alcohol exposure and subsequent withdrawal on axonal and microtubule organization in the developing rodent brain remains under investigation. It has been shown that initial alcohol exposure suppresses axonal growth and guidance cues, but subsequently exaggerates aberrant axonal sprouting and reorganization in the hippocampus that lasts into adulthood; the nature of this response may depend on the alcohol dose and the period of CNS development in which the alcohol exposure occurs (Hodges & Black, 1981; Lindsley, Kerlin, & Rising, 2003; Sakata-Haga et al., 2003). The hippocampal region most susceptible to alcohol-induced microtubule disorganization appears to be the in axonal bend of the intersection between the CA3 and CA1; this region is imperative for evolution and maintenance of associative memory and may be linked to cognitive deficits seen in rodent models of FASDs (Gruart, Muñoz, & Delgado-García, 2006; Sakata-Haga et al., 2003).

1.2.6 Microtubule Involvement

One culprit that may be involved in the poor developmental outcomes associated with alcohol-induced axonal disorganization could be the regulating effects of microtubule-associated proteins (MAPs) which are sensitive to alcohol exposure and have implications in diseased states (Ahluwalia, Ahmad, Adeyiga, Wesley, & Rajguru, 2000; Evrard et al., 2006; Smith, Butler, & Prendergast, 2013). The sprouting of immature axons incorporates MAPs that directly aid in axonal structural support and indirectly assist neural migration; one structural protein that is sensitive to alcohol and

necessary for appropriate maturation and maintenance of axonal structural support is the tau protein (Gendron, McCartney, Causevic, Ko, & Yen, 2008). Though the tau protein is generally investigated with relationship to neurodegenerative diseases such as AD, Parkinson's, and dementia, its role in the structural support and susceptibility to alcohol-modulation in the developing nervous system must be considered as a potential element in the neurodevelopmental profile of FASDs.

1.3 Tau Protein Function: Modifications and Pathologies

Tau proteins are highly involved in the structural integrity of axonal processes in the central nervous system; the role of tau is integral in several necessary cellular activities including tubulin polymerization, synaptogenesis, neural migration, axonal transport, and sustained support of cytoskeletal function (Cleveland, Hwo, & Kirschner, 1977; Dayanandan et al., 1999; Mandelkow, Stamer, Vogel, Thies, & Mandelkow, 2003; Wang & Liu, 2008). The regulation and function of the tau protein is dynamic and can be altered at the level of genetic expression, alternative splicing, and posttranslational modifications in response to cellular insult.

The tau protein is encoded on human chromosome 17q21 and produces six different isoforms from 16 different exons (Neve, Harris, Kosik, Kurnit, & Donlon, 1986). Tau is in the family of microtubule associated proteins (MAPs) responsible for tubulin assembly and structural support in mature cells. Alterations to the tau gene differentially result in diminished tau activity, including poor microtubule binding, an effect which can result in neurodevelopmental delay and/or degeneration (Bunker, Kamath, Wilson, Jordan, & Feinstein, 2006; Hong et al., 1998; Shaw-Smith et al., 2006).

While not all alterations to the tau gene are associated with any specific pathology, the alternative splicing of tau exon 10 produces isoforms that include either 3 (3R) or 4 (4R) microtubule-binding site repeats with functional and pathogenic impacts (Panda, Samuel, Massie, Feinstein, & Wilson, 2003). Functionally, 4R tau isoforms have triple microtubule binding capacity than do 3R tau isoforms (D'Souza & Schellenberg, 2005). In the non-pathogenic human adult brain, the ratio of 3R to 4R tau isoforms is 1:1 and alterations to this ratio are observed in neurodegenerative diseases such as Parkinson's and Down's syndrome (Heutink, 2000; Yoshida, 2006). Importantly, fetal tau isoforms in both rodents and humans express only 3 microtubule binding repeats suggesting a temporal regulation of tau function (Bunker, Wilson, Jordan, & Feinstein, 2004). There is evidence that tau expression and function is genetically regulated, but posttranslational modifications substantially interfere with constitutive tau activity and can promote pathogenic susceptibility. The tau protein is posttranslationally modified primarily by ubiquitination, glycosylation, and phosphorylation.

1.3.1 Ubiquitination

Ubiquitination of the tau protein results in proteolytic degradation and clearance of the protein. Ubiquitination is site specific on the tau protein with functionally distinct proteolytic activity; in the pathogenic state, tau proteins are ubiquitinated by at least three linkages: Lysine 6, Lysine 11, and Lysine 48 (Wang & Liu, 2008). These linkages have different outcomes for the tau proteins wherein Lysine 48 is necessary for proteolytic recruitment, but Lysine 6 appears to be protective against proteasome recruitment (Cripps et al., 2006). Importantly, ubiquitination is critical for degradation of misfolded proteins that aggregate and jeopardize cell viability if left unmodified. For instance, proteolytic

stress triggers heat shock protein-70 (Hsp70) which interacts with C-terminus of Hsc70-interacting protein (CHIP) ubiquitin ligase, or collectively the E3 ligase; this interaction is effective for clearance of misfolded tau proteins seen in tauopathic brains (Connell et al., 2001). Moreover, the Hsp70-CHIP interaction is being considered as a diagnostic and therapeutic tool for tauopathies because of its elevated activity in tauopathies such as AD (Kumar, Jha, Jha, Ramani, & Ambasta, 2015). In fact, Hsp70-CHIP activity appears to coincide with hyperphosphorylation of the tau protein, a cardinal correlate of tauopathies (Shimura, Schwartz, Gygi, & Kosik, 2004).

1.3.2 Glycosylation

Glycosylation is a modification involving the attachment of a carbohydrate group to an amine side chain (N-linked) or a hydroxyl group (O-linked) of a protein which alters its structure and function. Glycosylation and deglycosylation are site-specific on the tau protein and have functional differences dependent on glycosylation site (Gong, Liu, Grundke-Iqbal, & Iqbal, 2005). Specifically, O-linked monosaccharide β-N-acetylglucosamine glycosylation, or O-GlcNAcylation, occurs when the monosaccharide molecule is attached to the hydroxyl group of the serine or threonine residues of the tau protein. This glycosylation can result in subsequent downstream tau phosphorylation that may induce aberrant tau protein aggregation and/or neuronal membrane permeability, subcellular hallmarks of neurodegenerative diseases such as dementia and AD (Arnold et al., 1996; Lui et al., 2002; Takahashi et al., 1999; Torres & Hart, 1984; Wang, Grundke-Iqbal, & Iqbal, 1996; Yuzwa & Vocadlo, 2014). Thus, glycosylation may be involved in the initiation and maintenance of the cytosolic and structural abnormalities resulting from tau hyperphosphorylation.

1.3.3 Hyperphosphorylation

Hyperphosphorylation is the result of overactive kinase activity wherein phosphate molecules are attached at various residue sites of proteins which can impede on protein function. Hyperphosphorylation of the tau protein is one hallmark of neurodegenerative pathologies, though the phosphorylation state of the tau protein is regulated throughout CNS development in a non-pathogenetic manner (Iqbal, Liu, Gong, & Grundke-Iqbal, 2010). The most clinically investigated tauopathy is AD, which involves hippocampal and entorhinal cortical white-matter degeneration, unregulated tau phosphorylation, development of amyloid-β plaques, and neuronal death, all of which contribute to progressive loss of memory and cognitive function (for a review, see Orr, Sullivan, & Frost, 2017). The mechanisms of phosphorylation rely on kinase activity at one or several phosphorylation sites on the protein structure. For example, glycogen synthase kinase-3β (GSK-3β) phosphorylation of the tau protein upstream from (Threonine 231) or directly on the microtubule-binding domain (Serine 262) disrupts the optimal binding capacity and structural function of the tau protein in diseased states (Gong et al., 2005). Tau interacts with several kinases, but GSK-3β is an attractive candidate due to its developmental regulation and sensitivity to alcohol.

GSK-3β activity is implicated in the developing fetus where its role is dynamic and highly involved in the regulation of cellular glucose metabolism and subsequent neuronal maturation (Jope & Johnson, 2004). As a kinase, GSK-3β is a protein structure with its own amino acid residues that can be ubiquitinated, glycosylated, and phosphorylated which dictate its activity. When GSK-3β is dephosphorylated at its Serine 9 site, it becomes active and such action is elevated following alcohol exposure

(Chen et al., 2009). It has been reported that unregulated activity of GSK-3β may be related to alcohol-driven compensatory Ca2+ activity (Hartigan & Johnson, 1999). GSK-3β overactivity can result in deleterious fetal effects regarding neurite outgrowth, axon extension, and microtubule function in the developing CNS which are directly related to the phosphorylation state of the tau protein; GSK-3β overactivity increases tau phosphorylation at several amino acid residues and impairs the protein function (Dill, Wang, Zhou, & Li, 2008; Xu et al., 2004).

1.4 Purpose

Taken together, the observations outlined in the sections above would suggest: (1) fetal alcohol exposure aberrantly affects microtubule organization in the hippocampus; (2) this effect is likely modified by alcohol-induced Ca2+ activity which affects fetal axonal arrangement and increases GSK-3β activity; (3) resulting in sustained tau phosphorylation within fetal hippocampal axons. We hypothesize that phosphorylated tau protein immunofluorescence will decrement in control slices; however, we predict that this immunofluorescence will not decrement in slices that are exposed to alcohol. The role of alcohol exposure on tau protein phosphorylation in the developing hippocampus is explored below.

CHAPTER 2. METHODS

2.1 Organotypic Hippocampal Slice Culture

Ten-day-old male and female Sprague Dawley pups (Harlan Laboratories; Indianapolis, IN) were humanely euthanized and full brains were aseptically removed and placed into chilled dissecting media solution containing: Minimum Essential Media (MEM; Invitrogen, Carlsbad, CA), 4-(*2-Hydroxyethyl*)-1-piperazineethanesulfonic acid (HEPES; Sigma, St. Louis, MO), streptomycin/penicillin (Invitrogen), and Amphotericin B solution (Sigma). Brains were then midsagittally transected, and hippocampi were removed. Any excess tissue that was attached to the hippocampi following harvest was removed using a No. 15 scalpel. These hippocampi were then flatly positioned upon a plastic platform and sectioned into 200µm slices using the McIllwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Slices which retained the CA1, CA3, Genu, and Dentate Gyrus (DG) were then selected to be plated upon Millicell biopore membranes (0.4µm, 30mm diameter; MilliporeSigma, Burlington, Massachusetts); each membrane supported 3 to 4 hippocampal slices to prevent tissue overlap. Membranes were then inserted into 6-well plates (5mL, 9.5cm^2 area; VWR, Radnor, PA) containing approximately 1.5mL of culturing media for humidity-controlled incubation at 37 °C, 5% CO_2, and 95% air. Any excess culturing media that pooled atop the membrane or around any of the hippocampal slices was removed using a micropipette to improve slice adherence and ensure oxygen exposure. Slices were permitted 6 days of uninterrupted incubation to heal from removal and securely anchor into the membrane prior to any manipulations.

2.2 Experiment 1: Temporal Assessment of Tau Protein Phosphorylation in the Hippocampus

At 6 days in vitro (DIV), slices were either transferred into fresh culture media for later assessment or underwent the IHC procedure for assessment of tau phosphorylation at 6DIV. The IHC procedure recurred incrementally at 12DIV, 18DIV, and 24DIV to track the trajectory of tau phosphorylation states across the experimental timeline (Figure 2.2.1). Regions of interest in Experiment 1 included the CA1, CA3, and DG of the hippocampus (Figure 2.2.2).

Figure 2.2.1 Experimental Timeline. Schematic of timepoints measuring tau phosphorylation across 6DIV, 12DIV, 18DIV, and 24DIV.

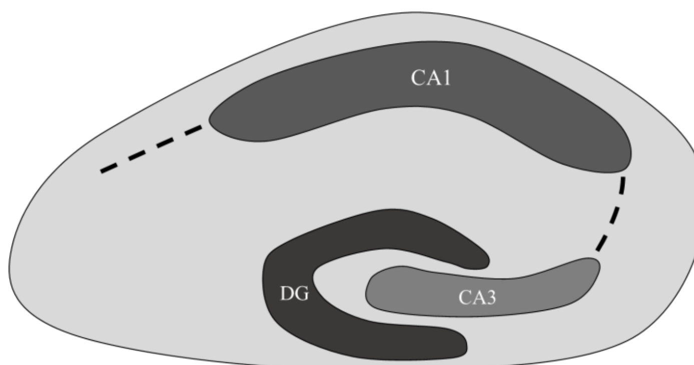

Figure 2.2.2 Regions of Interest for Experiment 1. Schematic of areas of interest in the temporal decrement of tau protein phosphorylation.

2.3 Experiment 2 Effect of Initial Alcohol Exposure on Temporal Decrement of Tau Protein Phosphorylation in the Hippocampus

Experiment 2 assessed tau phosphorylation in the CA1, CA3, DG, and genu of the hippocampus at four timepoints: 6DIV, 12DIV, 18DIV, 24DIV. At each timepoint following 6DIV, there was an EtOH-exposed condition and a control condition. Control slices received plain culture media at all timepoints. Experimental slices received five days of 50mM EtOH exposure in culture media from 6DIV to 11DIV, and then received plain culture media for the remainder of the experimental timeline; this was meant to mimic a human third-trimester equivalent maternal alcohol binge of approximately 0.23BAC.

Plates housing control slices were placed into a Tupperware container with 50mL of distilled water and placed into a Ziploc bag, filled with 5% CO_2 and 95% air, and then sealed and placed into the incubator. Ethanol-exposed slices which received 50mM ethanol media between 6-11DIV were similarly placed in a Tupperware with 50mM ethanol water, placed into a Ziploc bag filled with 5% CO_2 and 95% air, and then sealed and placed into the incubator. Atmospheric treatment was meant to prevent alcohol evaporation during the five-day exposure. Regions of interest in Experiment 2 included the CA1, CA3, DG, and genu of the hippocampus (Figure 2.3.1). Because the modifications of interest for this experiment involve the effect of ethanol on tau protein phosphorylation associated with immature microtubules within mossy fibers and Shaffer collaterals between the CA3 and CA1, we included the additional measure of the hippocampal genu to comprehensively address changes in tau protein phosphorylation in and between these regions following alcohol exposure. Because this area is an ambiguous

and contested region, traditional cytographic regional distinctions did not suffice for exhaustive exploration of tau phosphorylation.

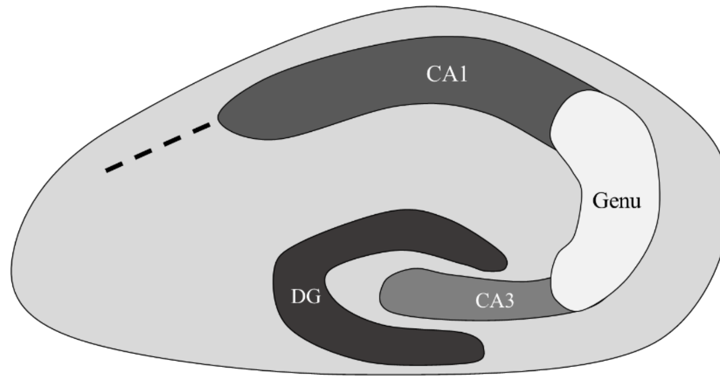

Figure 2.3.1 Regions of Interest for Experiment 2. Schematic of areas of interest in the temporal decrement of tau protein phosphorylation following an initial 5-day alcohol exposure.

2.4 Immunohistochemistry Procedure

For analysis of tau phosphorylation using immunohistochemistry (IHC), all slices were first fixed in 10% formalin. Then the slices were washed twice in 1x PBS solution and placed into an IHC buffer solution containing: 1x PBS, Triton detergent, and Bovine Serum Albumin for 30 minutes. After, slices were again washed twice in 1x PBS solution. The primary antibody used in these experiments was Phospho-Tau (Thr231) Monoclonal Antibody (AT180) (Thermo Fisher, Waltham, MA). The antibody was diluted 1:40 in IHC buffer solution and administered onto slices for 24 hours at 4°C; the slices were then washed twice in 1x PBS. The secondary antibody used in these experiments was Goat anti-Mouse IgG (H+L) Secondary Antibody, TRITC (Thermo Fisher). The secondary antibody was diluted 1:200 in IHC buffer solution and administered onto slices for 24 hours at 4°C; the slices were then washed twice in 1x PBS prior to microscopic fluorescent imaging at 570 nm. Fluorescent intensity of all imaged

slices was measured using optical densitometry where greater fluorescent intensity indicated greater concentration of threonine 231 phosphorylation on tau proteins.

2.5 Statistical Analysis

Statistical analyses were conducted to determine the effect of age and alcohol exposure on the fluorescence of phosphorylated tau proteins in the hippocampus. Multilevel model regression analyses were conducted in SAS using the DG as the reference region for both Experiments 1 and 2 unless otherwise noted. The variables that were included in the analyses were the effect of sex (male or female), EtOH exposure, age, and an interaction between EtOH and age. Model assumptions were not violated. Significance level was set at $p \leq 0.05$, but marginal significance was reported if $p \leq 0.1$. Figures from Experiment 2 have been linearly transformed such that values are depicted as %6DIV.

CHAPTER 3. RESULTS

3.1 Experiment 1.

Results of multilevel models are presented in Table 3.1.1. The initial unconditional means model (Model A) indicated an ICC of 0.38. Next, hippocampal region (CA1, CA3) was added to the model, which significantly improved model fit, Δ deviance = 83.9, $p < 0.001$ (Model B). Adding male to the model did not significantly improve model fit, Δ deviance = 1.4, n.s., and was therefore removed (Model C). However, adding age to the model did significantly improve model fit, Δ deviance = 42.1, $p < 0.001$ (Model D). Evaluation of model coefficients indicated that age was a significant predictor of the Level 1 intercept but not the effects of CA1 or CA3. Therefore, an additional model was fit removing these interaction terms from the model (Model E). This model fit the data equally well as Model D, Δ deviance = 3.0, n.s., and is therefore the preferred model due to parsimony.

In Model E, intensity is estimated to be 24.83, $p < 0.001$, for the DG region at 6DIV. Intensity is 4.43, $p < 0.001$, lower for each subsequent age group (12DIV, 18DIV, and 24DIV). These age group differences are the same for each region (Figure 3.1.1). However, overall intensity is 7.81, $p < 0.001$, higher in the CA1 region compared to the DG region. Intensity was equivalent for CA3 and DG regions.

Table 3.1.1 Results from the multilevel model analysis for Experiment 1.

Effect	Model A	Model B	Model C	Model D	Model E
Intercept					
Intercept	20.89***	18.23***	18.73***	24.28***	24.83***
Male			-1.20		
Age				-4.07***	-4.43***
Residual Var	18.88**	25.51***	24.96***	7.30**	7.17**
CA1					
Intercept		7.81***	8.22***	9.49***	7.81***
Male			-1.00		
Age				-1.13	
CA3					
Intercept		0.18	0.19	0.13	0.18
Male			-0.03		
Age				0.03	
Level 1 Res Var	31.04***	11.16***	11.09***	10.76***	11.16***
Deviance	814.2	730.3	728.9	686.8	689.8
Delta Deviance		83.9***	1.4	42.1***	3

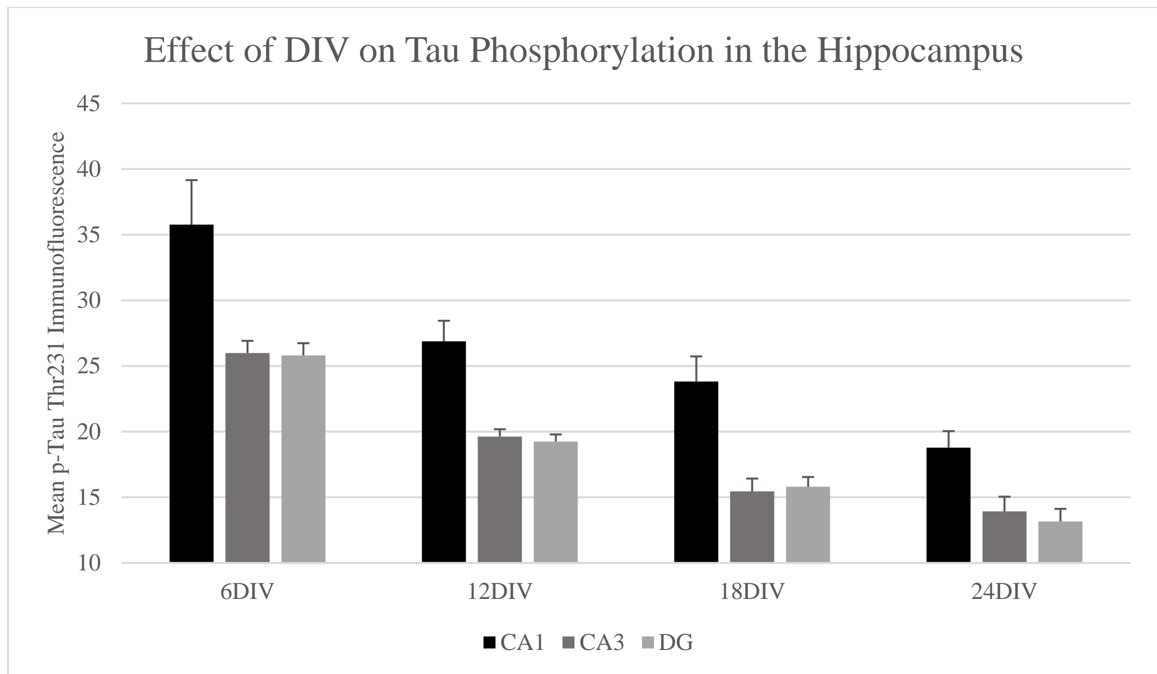

Figure 3.1.1 Average effect of days *in vitro* on p-Tau Thr231 immunofluorescence across 6, 12, 18, and 24DIV.

3.2 Experiment 2

Results of multilevel models are presented in Table 3.2.1. The initial unconditional means model (Model A) indicated an ICC of 0.55. Next, hippocampal region (CA1, CA3, and Genu) was added to the model, which significantly improved model fit, Δ deviance = 219.00, $p < 0.001$ (Model B). Adding male to the model did not significantly improve model fit, Δ deviance = 6.1, n.s., and was therefore removed (Model C). However, adding age to the model did significantly improve model fit, Δ deviance = 22.6, $p < 0.001$ (Model D), as did adding EtOH, Δ deviance = 20.2, $p < 0.001$ (Model E), and the interaction between Age and EtOH, Δ deviance = 15.9, $p < 0.01$ (Model F). Therefore, Model F was the preferred model.

Model F (Table 3.2.1) indicated the mean intensity for control samples at 6DIV in the DG region was 21.11, $p < 0.001$. Intensity is 1.76, $p < 0.001$, lower for each subsequent age group (12DIV, 18DIV, and 24DIV) for control samples in the DG region. Intensity is 5.44, $p < 0.001$, higher in the CA1 region compared to the DG region in control samples at 6DIV. There is a significant difference in intensity between EtOH samples in the Genu region compared to the DG region at 6DIV but not between control samples, 2.44, $p < 0.05$.

Model F also indicated a significant EtOH by Age interaction due to the significant improvement in model fit. However, none of these interaction terms were significant when using DG as the reference region. Further probing of the interaction identified significant terms only when CA1 was the reference region. Specifically, the EtOH by Age interaction significantly predicted the difference between CA1 and CA3 on

intensity, -2.21, $p < 0.001$ and marginally predicted the difference between CA1 and genu on intensity, -1.20, $p = 0.06$ and the difference between CA1 and DG on intensity, -1.23, $p = 0.06$.

Table 3.2.1 Results from the multilevel model analysis for Experiment 2.

Effect	**Model A**	**Model B**	**Model C**	**Model D**	**Model E**	**Model F**
Intercept						
Intercept	20.23***	18.35***	18.71***	20.38***	20.24***	21.11***
Male			-0.67			
Age				-1.24***	-1.24***	-1.76***
EtOH					0.26	-1.71
EtOH*x*Age						1.20
Residual Var	24.20***	25.50***	25.50***	23.40***	23.25***	22.84***
CA1						
Intercept		5.11***	4.91***	4.79***	4.55***	5.44***
Male			0.38			
Age				0.20	0.20	-0.33
EtOH					0.45	-1.56
EtOH*x*Age						1.23
CA3						
Intercept		0.59	0.34	1.29*	1.32	0.61
Male			0.46			
Age				-0.43	-0.43	-0.00
EtOH					-0.05	1.55
EtOH*x*Age						-0.98
Genu						
Intercept		1.81***	1.45**	2.24***	0.94	0.96
Male			0.66			
Age				-0.26	-0.24	-0.25
EtOH					2.49***	2.44*
EtOH*x*Age						0.03
Level 1 Res Var	20.03***	14.81***	14.81***	14.73***	14.37***	14.14***
Deviance	6075.1	5856.1	5850.0	5833.5	5813.3	5797.4
Delta Deviance		219***	6.1	22.6***	20.2***	15.9**

3.2.1 Differences in intensity across age groups based on region and EtOH exposure

Additional probing of the EtOH by Age interaction terms revealed that there was a significant effect of age for control samples in each of the four regions: -1.76, $p < 0.001$ for DG (Figure 3.2.1.1); -1.76, $p < 0.001$ for CA3 (Figure 3.2.1.2); -2.09, $p < 0.001$ for CA1 (Figure 3.2.1.3); -2.01, $p < 0.001$ for genu (Figure 3.2.1.4). However, there was only a significant effect of age for EtOH samples in the CA3 region, -1.53, $p < 0.01$ (Figure 3.2.1.2). There was no significant effect of age for the other three regions: -.56 for DG (Figure 3.2.1.1); .34 for CA1 (Figure 3.2.1.3); -.78 for genu (Figure 3.2.1.4). In the CA1 region, the effect of age was significantly stronger for control samples than EtOH samples, 2.43, $p < 0.001$. In the genu region, the effect of age was marginally stronger for control samples than EtOH samples, 1.24, $p = 0.09$. The figures for the regional means for these data have been linearly transformed by the 6DIV average for their respective condition, such that condition is represented as %6DIV.

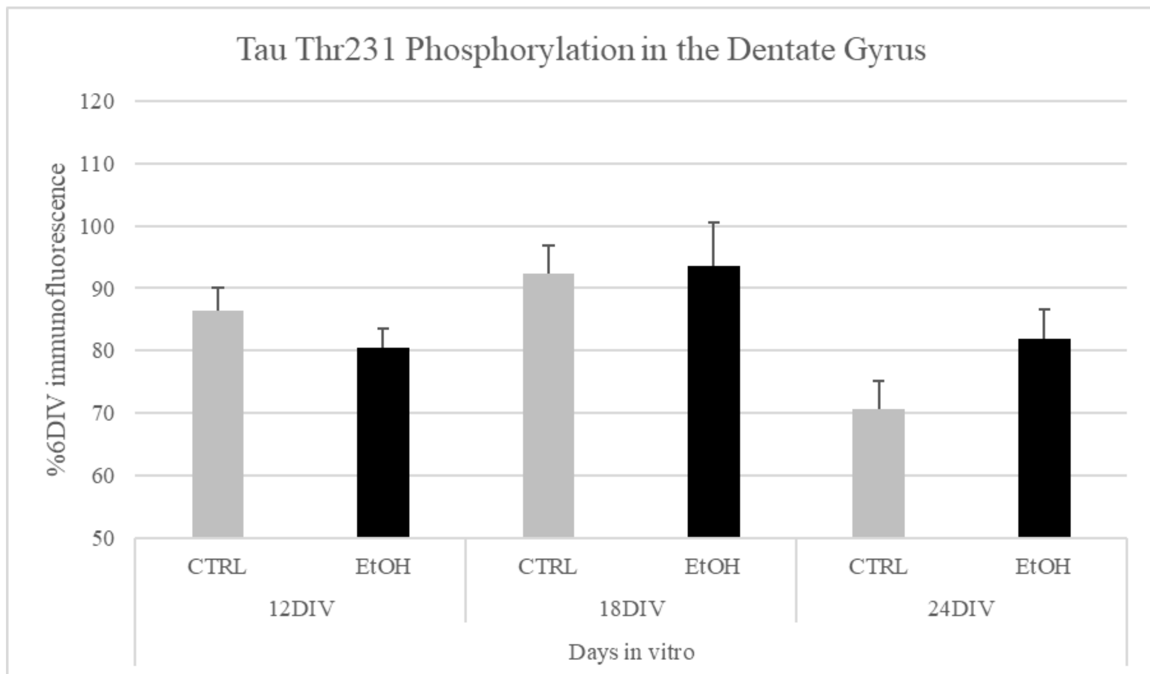

Figure 3.2.1.1 Effect alcohol and days *in vitro* on p-Tau Thr231 immunofluorescence across 12, 18, and 24DIV in the dentate gyrus, shown as %6DIV. Tau phosphorylation significantly declined between 6DIV and 24DIV for control samples, $B = -1.76$, $p < 0.001$. Tau phosphorylation did not significantly change between 6DIV and 24DIV for samples exposed to alcohol, $B = -0.56$, n.s.

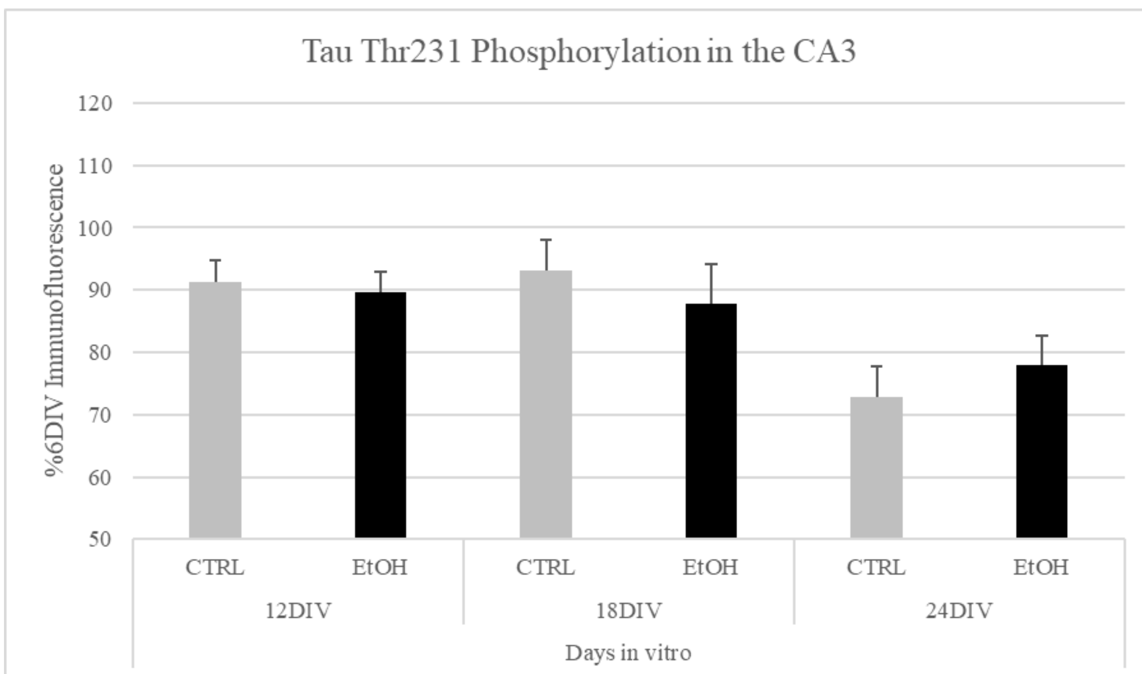

Figure 3.2.1.2 Effect alcohol and days *in vitro* on p-Tau Thr231 immunofluorescence across 12, 18, and 24DIV in the CA3, shown as %6DIV. Tau phosphorylation significantly declined between 6DIV and 24DIV for both control samples, $B = -1.76$, $p < 0.001$ and samples exposed to alcohol, $B = -1.53$, $p < 0.01$.

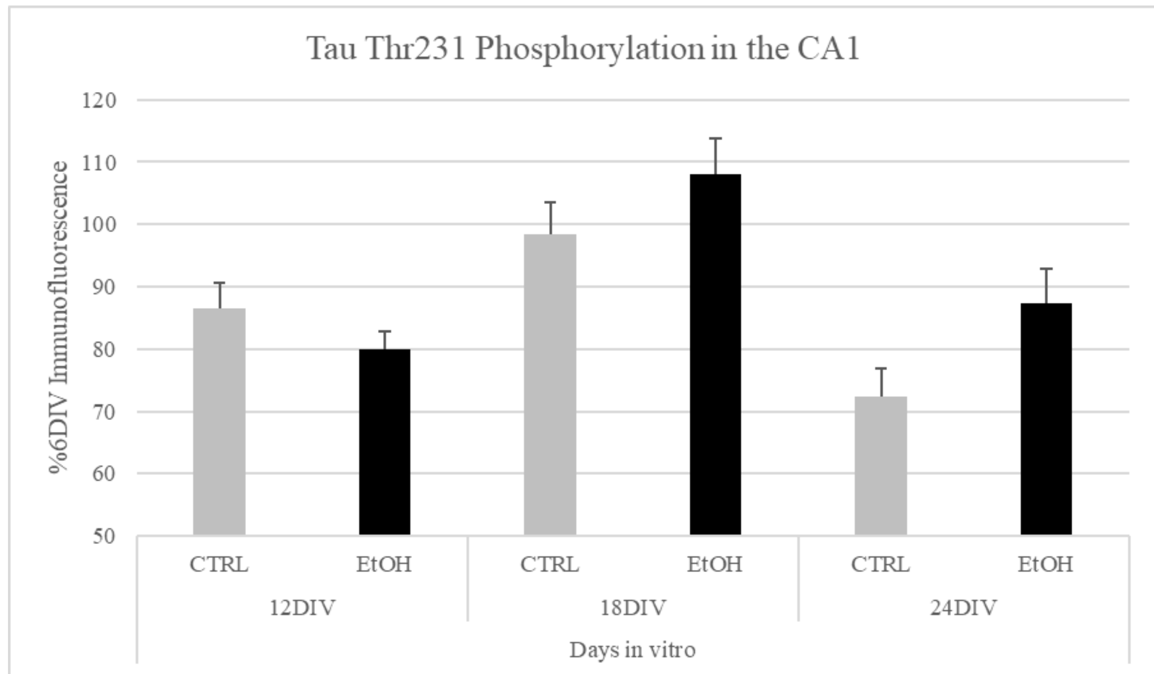

Figure 3.2.1.3 Effect alcohol and days *in vitro* on p-Tau Thr231 immunofluorescence across 12, 18, and 24DIV in the CA1, shown as %6DIV. Tau phosphorylation significantly declined between 6DIV and 24DIV for control samples, $B = -2.09$, $p < 0.001$. Tau phosphorylation did not significantly change between 6DIV and 24DIV for samples exposed to alcohol, $B = 0.34$, n.s.

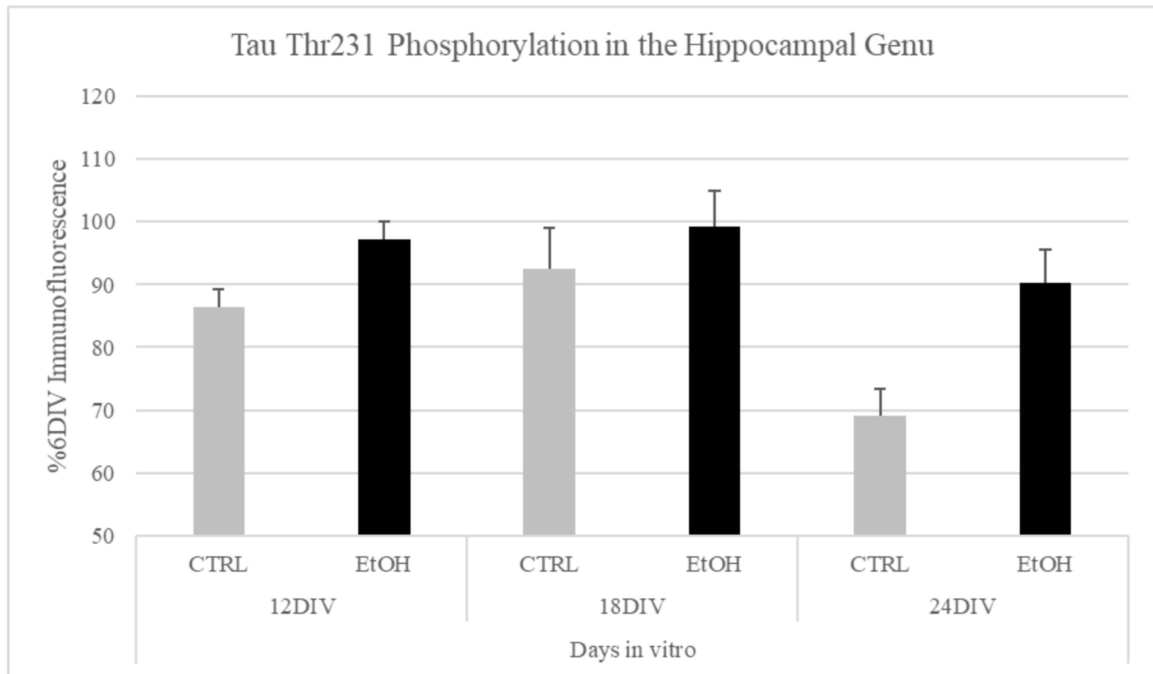

Figure 3.2.1.4 Effect of alcohol and days *in vitro* on p-Tau Thr231 immunofluorescence across 12, 18, and 24DIV in the hippocampal genu, shown as %6DIV. Tau phosphorylation significantly declined between 6DIV and 24DIV for control samples, $B = -2.01$, $p < 0.001$. Tau phosphorylation did not significantly change between 6DIV and 24DIV for samples exposed to alcohol, $B = -0.78$, n.s.

CHAPTER 4. DISCUSSION

4.1 Implications

Because the role of phosphorylated tau protein in the developing nervous system has received little to no experimental or clinical attention, we intended to explore its relationship with alcohol to draw implications related to FASDs. Above we have shown that, under control conditions across 24 days *in vitro* (DIV), the fluorescence of phosphorylated tau protein decrements in all regions of the rat hippocampus studied. However, this effect is widely absent in tissue that encountered alcohol.

4.1.1 Experiment 1

In Experiment 1, we established the decremental nature of phosphorylated tau fluorescence in the CA1, CA3, and DG over brief *in vitro* neonatal hippocampal development. Because tau phosphorylation is elevated and associated with axonal disorganization before and at birth, we anticipated that phosphorylated tau protein fluorescence would be the brightest in the hippocampus when imaged closer to birth (Brion, Smith, Couck, Gallo, & Anderto, 1993); the reduction of tau phosphorylation from 6DIV to 24DIV, therefore, was unsurprising considering the control condition permitted an unmodified environment for culturing healthy tissue.

The reduction in phosphorylated tau protein fluorescence could be a result of at least two temporally regulated and functionally related processes that converge within the insulin pathway: 1) diminished kinase activity of the GSK-3β protein, and/or 2) increased phosphatase activity of protein phosphatase 2A (PP2A). As previously discussed, GSK-

3β phosphorylates the tau protein at several amino acid residues, and GSK-3β is directly implicated in the hyperphosphorylated state of the tau protein in damaged or diseased hippocampi (Gong et al., 2005). Because GSK-3β is dynamically regulated during the development and maturation of cells, GSK-3β activity may be associated with the phosphorylation state of the tau protein across the duration of tissue culturing, such that, as the tissue matured *in vitro*, GSK-3β hyperactivity became relatively stable under control conditions which concurrently reduced tau protein phosphorylation (Jope & Johnson, 2004).

Just as kinase activity is involved with phosphorylation of proteins, phosphatase activity is involved with dephosphorylation of proteins. The most abundantly known phosphatase in the CNS is the PP2A phosphatase which is responsible for dephosphorylating 70%-90% of phosphorylated proteins, including the tau protein (Liu, Grundke-Iqbal, Iqbal, & Gong, 2005; Eichhorn, Creyghton, & Bernards, 2009). In fact, the phosphorylation state of serine/threonine residues on the tau protein is chiefly regulated by GSK-3β phosphorylation and PP2A dephosphorylation, where PP2A dephosphorylation is necessary for cellular morphogenesis and maturation (Avila, 2008; Olsen et al., 2006). It is likely the inverse relationship between GSK-3β and PP2A that resulted in the decrement of tau phosphorylation under control conditions in our tissue, though metrics directly assessing the roles of PP2A and GSK-3β were not included in these experiments.

4.1.2 Experiment 2

In Experiment 2, we established the decremental nature of phosphorylated tau protein in the CA1, CA3, DG, and Genu over brief *in vitro* neonatal hippocampal culture

in the control condition; however, when tissue encountered alcohol, subsequent culturing indicated that fluorescence of tau phosphorylation does not significantly change from 6DIV to 24DIV in the CA1, DG, or Genu. Our hypothesis regarding the temporally sustained nature of tau phosphorylation in the alcohol-treated slices was supported by these findings, except in the CA3 region. This is not unsurprising considering the heterogeneity of glutamatergic NMDA receptor densities on pyramidal cell bodies within the hippocampal subregions; autoradiographic experiments measuring [^{125}I]MK-801 binding show that the CA1 region has the highest density of NMDARs compared to the CA3 and DG regions (Butler et al., 2010). Because the hypothesized effect of alcohol exposure and subsequent withdrawal is ultimately Ca^{2+}-driven with relationship to GSK-3β, higher densities of NMDARs would indicate a greater susceptibility to Ca^{2+}-related kinase activity (Dill, Wang, Zhou, & Li, 2008; Hartigan & Johnson, 1999; Xu et al., 2004); this NMDAR/Ca^{2+} rationale, however, does not explain why tau phosphorylation in the DG was sensitive to alcohol, but not the CA3, considering no significant differences in NMDAR densities between the regions exist. Further investigation should consider differential expression of GSK-3β by region. The heterogeneity of tau phosphorylation between the CA3 and other hippocampal regions could illuminate differences between mossy fiber and Shaffer collateral axonal development and how constitutive tau phosphorylation and function is modified by alcohol exposure.

One possible explanation for the sustained p-tau florescence following alcohol withdrawal is that the inverse relationship between PP2A and GSK-3β may have been disrupted by alcohol exposure such that competition for phosphate homeostasis was amplified and preferential for GSK-3β in the presence of cellular insult. The association

between alcohol exposure, elevated Ca^{2+}, and GSK-3β hyperactivity supports this hypothesis (Chen et al., 2009; Hartigan & Johnson, 1999). As previously mentioned, GSK-3β activity is primarily dictated by a cascade of events originating at the insulin receptor, a receptor which becomes less sensitive to insulin following alcohol exposure, promoting GSK-3β (Seiler, Henderson, & Rubin, 2000). This element of GSK-3β tau hyperphosphorylation resulting from insulin insensitivity is well-established; however, the role of PP2A and insulin signaling in relationship to tau hyperphosphorylation remains under investigation (for a review, see Mandavia & Sowers, 2012).

4.2 Limitations

Notably, the fluorescence of tau phosphorylation is sustained following alcohol exposure – whether that is the sustained phosphorylation of initially-expressed tau proteins or detection of newly-expressed phosphorylated tau proteins remains unclear. There is evidence that alcohol increases total tau protein concentrations (Hoffman et al., 2019); therefore, it is possible that alcohol simply increased the expression of tau proteins phosphorylated at threonine 231 (Thr231) rather than sustaining the initial tau phosphorylation state. It may be erroneous to assume that tau phosphorylation is saturated and immunofluorescent intensity is highest at birth, despite what we have shown here. The functional difference between newly expressed Thr231 phosphorylated tau proteins and sustained Thr231 phosphorylation on initially expressed tau proteins would be difficult to discriminate in our model. Total tau immunofluorescence may be an avenue to consider.

The organotypic hippocampal slice culture (OHSC) model is integral for investigating histological changes in developmental pathologies of neural tissues that are otherwise inaccessible in humans. OHSC minimizes animal resources with an unmatched degree of experimental control which is necessary for delineation of biochemical pathways and subsequent potential therapeutic targets. However, the benefits of the OHSC model are also its limitations: the tissue is divorced from the rest of the CNS and peripheral nervous system (PNS) which are elements, in conjunction with learning and behavior, that would undoubtedly influence CNS development with regard to alcohol exposure.

We are interpreting the decrement of phosphorylated tau protein fluorescence as a crude indication of axonal organization and maturation, though no complementary confirmation was utilized in these experiments. Using myelin basic protein staining may be an avenue by which such a confirmation could be made, where an inverse interaction would better indicate axonal maturation and could provide insight into the relationship between tau phosphorylation and neonatal myelination.

Finally, while our findings demonstrate that tau protein phosphorylation remains elevated in all regions of the hippocampus except the CA3 following alcohol exposure, these findings do not indicate that sustained tau phosphorylation confers any behavioral or cognitive abnormalities observed in rodent models of or human patients with FASDs. Taken together, behavioral and cognitive translational work must be conducted to legitimize FASDs as a tauopathy in order to proceed with any meaningful therapeutic pursuits.

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