Spring 3-14-2024

Neuroplasticity in the Dentate Gyrus: A multimethod investigation on hippocampal LTP following prenatal alcohol exposure

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NEUROPLASTICITY IN THE DENTATE GYRUS: A MULTIMETHOD INVESTIGATION ON HIPPOCAMPAL LTP FOLLOWING MODERATE PRENATAL ALCOHOL EXPOSURE.

By

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DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Psychology

The University of New Mexico
Albuquerque, New Mexico
May 2024

ACKNOWLEDGEMENTS

To:

God, Almighty,

For the everlasting mercy, yesterday, today, and forever!

My beloved parents,

For being my pillars and my strength. For all the love, encouragement, support, guidance, advice, and prayers. I love you!

My sister,

For being my better half.

My best friend,

For all the little things, the big things and everything in between!

All my family and friends,

For being my endless support system. Nobody can thrive alone, so I'm blessed to have you all with me, helping me keep my chin up and move forward.

The *"Latte ladies",*

[to honor my own research and inspired by the Hebbian postulate] – "friends who stick together, graduate together."

My academic mentor,

For taking care of Meeko, the cat!

Additionally, I would like to express my sincere gratitude for accepting me as a student and guiding me throughout these 4 years and 7 months of my Ph.D. journey. Thank you for your teachings, guidance, support, and, more importantly, for standing up for me and believing that I could do it!

My dissertation committee members,

For your availability, expertise, and encouragement.

The Psychology department staff,

For your help and services to make everything work.

"In everything give thanks: for this is the will of God in Christ Jesus" – 1 Th 5:18

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ABSTRACT

Studies have demonstrated alcohol-related deficits in LTP associated with histaminergic and glutamatergic impairments. The histaminergic H3R inverse agonist ABT-239 has shown promise in reversing these deficits. The present study focuses on another H3R inverse agonist, SAR-152594, and its impact on LTP deficits following moderate PAE. The findings reveal that systemic administration of 1 mg/kg of SAR-152594 reverses deficits in potentiation fEPSPs in adult male rats exposed to moderate PAE. Time-frequency analyses of evoked responses indicate PAE-related reductions in power during the fEPSP and increased power during later components of evoked responses, both reversed by SAR-152594. These results provide further evidence that H3R inverse agonism is a potential therapeutic strategy to address deficits in synaptic plasticity associated with PAE. This study also investigated extracellular glutamate levels with a glutamate biosensor in vivo. Following HFS stimulation, the results show differences in fractional change in amplitude of the evoked responses in PAE animals when compared with controls. These results support the alcohol-related deficits in LTP and support the hypothesis that there is a disruption in glutamate release from the presynaptic terminal.

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Introduction

Learning and Memory

It is well-established that prenatal alcohol exposure (PAE) may result in a series of life-lasting developmental deficits in affected individuals. These deficits can manifest as morphological and neurobiological alterations, which affect several domains. One of the domains altered by PAE is learning and memory processes. Those deficits vary from individual to individual and are dependent on the time, length, and quantity of alcohol exposure (Calhoun & Warren, 2007; Flak et al., 2014; Fontaine et al., 2016; Hamilton DA et al., 2014; Khoury et al., 2018). Together, those alterations comprise what is known as Fetal Alcohol Spectrum Disorders (FASDs).

Learning and memory are fundamental cognitive processes that involve acquiring, storing, and retrieving information. These processes have been studied for decades. The modern era of memory research is marked by the remarkable findings of the acquired amnesia of patient H.M. following the surgical removal of parts of the medial temporal lobe (MTL) – which included the hippocampal formation (Clark & Squire, 2010; Scoville & Milner, 1957; Squire, 1992). The hippocampus, perhaps the most extensively studied structure within the MTL, is thought to be responsible for the formation and retrieval of episodic and spatial memories (Bonthius et al., 2001; Burgess et al., 2002; Eichenbaum & Cohen, 2014; Lynch, 2004). Most of what is known about the neurobiology of learning and memory processes came from lesion studies in human and non-human animals that contributed to the understanding of the role of hippocampal formation in the memory consolidation (Aggleton

& Brown, 1999; Eichenbaum, 2000; Malouin et al., 2003; Scoville & Milner, 1957; Squire & Zola-Morgan, 1991; Sutherland et al., 2001).

Prenatal Alcohol Exposure – Clinical Overview

The first clinical article describing alterations in the offspring of chronic prenatal alcohol exposure – *termed Fetal Alcohol Syndrome (FAS)* – and providing diagnostic criteria was published in 1973 (Calhoun & Warren, 2007; Jones & Smith, 1973). FAS is characterized by facial abnormalities, growth deficits, and cognitive impairments (Lim et al., 2022; Sampson et al., 1997). However, to date, the outcomes associated with PAE remain poorly understood as not all prenatally exposed individuals will meet the criteria for FAS. There are many different factors that can contribute to the diverse outcomes of prenatal alcohol exposure. A proper assessment and diagnosis are still challenging (Bakhireva et al., 2017; Salem et al., 2020; Sampson et al., 1997; Subramoney et al., 2018) for several factors including a lack of consensus on diagnostic criteria, awareness of FASD, appropriate screening, and known biomarkers for alcohol (Coles, 2011; Lim et al., 2022). Those factors make it difficult to have a proper diagnosis and/or anticipate possible cognitive impairments an individual may experience.

The damage associated with moderate PAE may not result in morphological alterations like FAS. Prenatal alcohol exposure can have mild lasting effects on a child's cognitive development, leading to a range of problems such as learning difficulties, attention deficits, memory impairments, and hyperactivity (Kodituwakku, 2009; Mattson et al., 2019) that may affect the exposed individual's academic and social functioning.

Neuropsychological profile

In the absence of classic morphological alterations, there has been a great effort among clinical researchers attempting to delineate a neurocognitive profile of the PAE (Kodituwakku, 2009; Lange et al., 2019; McLachlan et al., 2023). FASD shares several cognitive and behavioral characteristics with other neurodevelopmental disorders such as attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), ODD (Oppositional Defiant Disorder) (Coles, 2011; Lange et al., 2019; Malisza et al., 2012), sleep disturbances (Mughal et al., 2020), mood disorders, and schizophrenia (Maya-Enero et al., 2021). In addition, these disorders are common comorbidities of FASD and have a higher prevalence in individuals with FASD compared to non-FASD individuals (Fig. 1). These disorders also have overlapping diagnostic criteria leading to misdiagnosis. The stigmatization common with FASD is another factor that may lead children with FASD to receive multiple diagnoses of other externalizing disorders before receiving an appropriate assessment (Lange et al., 2019).

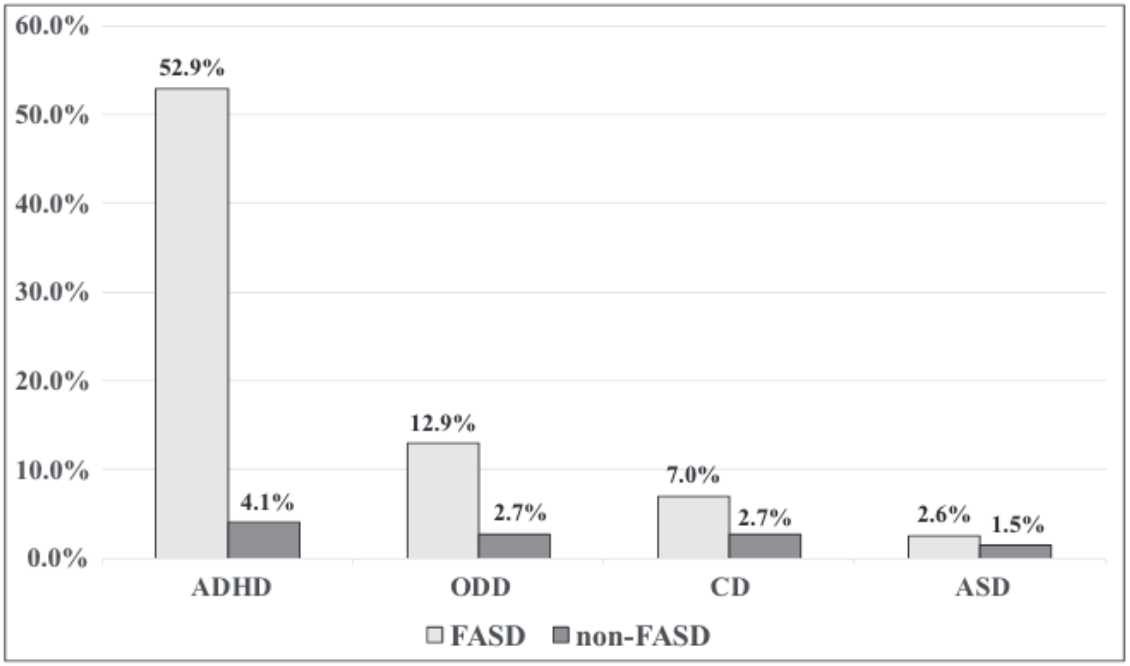

Figure 1. The prevalence of ADHD, ASD, CD, and ODD among children with FASD and without FASD (i.e., the general population of the USA). ADHD, Attention-Deficit Hyperactivity Disorder; ASD, Autism Spectrum Disorders; CD, Conduct Disorder; ODD, Oppositional Defiant Disorder; FASD, Fetal Alcohol Spectrum Disorder. Figure retrieved from Lange et al., 2019.

The lack of morphological alterations, biomarkers for alcohol exposure, and a unique neurobehavioral profile of FASD might result in individuals with FASD not being properly diagnosed or being misdiagnosed. This delay in identification may prevent timely intervention, particularly during the crucial early neurodevelopmental years (Lange et al., 2019; Mattson et al., 2019; Maya-Enero et al., 2021; Subramoney et al., 2018). Coles et al., (2011) state that appropriate methods that provide a more detailed examination of cognitive and behavioral characteristics allow for differentiation between FASD and ADHD. In their investigation of PAE children and children with diagnosed with ADHD - with similar social economic status (SES) and from the same minority population group – the results showed that PAE children had fewer behavior problems when compared to the ADHD non-PAE group, higher deficits in information encoding, and attention switches. Children in the ADHD non-

PAE group presented more externalizing disorders (Coles, 2011). Their results suggest that a better understanding of the cognitive deficits and the associated behavioral outcomes may allow for better discrimination among disorders – particularly FASD and ADHD.

Diagnosing and treating FASD presents a unique challenge when compared to other neurodevelopmental disorders, such as ASD or ADHD, due to the high proportion of individuals with FASD who are adopted and may not receive a diagnosis until later in life. In contrast, individuals with ASD or ADHD often live with their biological families, making it easier to gather accurate health history information and obtain an early diagnosis. This delay in the diagnosis of FASD can hinder the timely provision of appropriate interventions, highlighting the need for increased awareness and access to diagnostic services for individuals with prenatal alcohol exposure. Living in foster care or being placed in an adoptive family can be a major contributor to additional insults (e.g., trauma, stress, and neglect) that can confound with possible outcomes of PAE (Maya-Enero et al., 2021). Therefore, researchers should be cautious when interpreting possible assessment results by taking family history into consideration (Carpita et al., 2022).

Currently, there is no known unique neurodevelopmental profile associated with PAE. Throughout the years, tremendous progress has been made in elucidating the possible neurodevelopmental outcomes that may arise as a consequence of prenatal alcohol exposure. While the currently available assessment methods are crucial in identifying the effects of prenatal alcohol exposure, the lack of consistency in the information collected and reported across institutions poses a significant challenge in interpreting the results, especially in cases of lower levels of exposure. Future clinical research should focus on increasing

assessment rigor across institutions to minimize the heterogeneity in the results of the studies (Bandoli, 2023; Flak et al., 2014; Pyman et al., 2021; Subramoney et al., 2018), to increase the understanding of the outcomes associated with PAE. Additionally, animal research suggests that the severity of the cognitive impairments may be affected by the timing of exposure (Mattson et al., 2019), therefore further investigation into the timing of exposure is needed which has been rarely addressed in human research.

Hippocampal development and alcohol insults

During development in utero, the hippocampus – similarly to other brain regions – is susceptible to a variety of insults such as toxins, nutritional deficiencies, and others. Prenatal alcohol exposure can result in life-lasting damage to the offspring. The hippocampus – and other brain regions – is vulnerable to the teratogenic effects of alcohol during prenatal development as alcohol freely crosses the placenta barrier and the enzymatic activity in the developing fetus is low – compared to adults – and cannot metabolize the alcohol properly (Fontaine et al., 2016; Lange et al., 2019).

Anatomy of the hippocampal formation and developmental neurogenesis

The hippocampal formation is part of the MTL and includes the hippocampus, entorhinal cortex, and subicular complex. The hippocampus proper is divided into subregions – the dentate gyrus (DG), CA1-CA3 fields, and the subiculum – with the corresponding neurons organized in layers. The DG is located at the posterior portion of the hippocampus and is characterized by a densely packed layer of granule cells. The CA1-CA3 fields are in the middle portion of the hippocampal formation and are characterized by pyramidal cells. The primary input into the hippocampus comes from the entorhinal cortex (EC) which projects onto the DG via the perforant pathway. The DG sends output to the CA3 via mossy fibers, and the CA3 region sends output to CA1 via the Schaffer collaterals. These projections are part of the trisynaptic circuit (Fontaine et al., 2016; Madison et al., 1991) (Fig. 2).

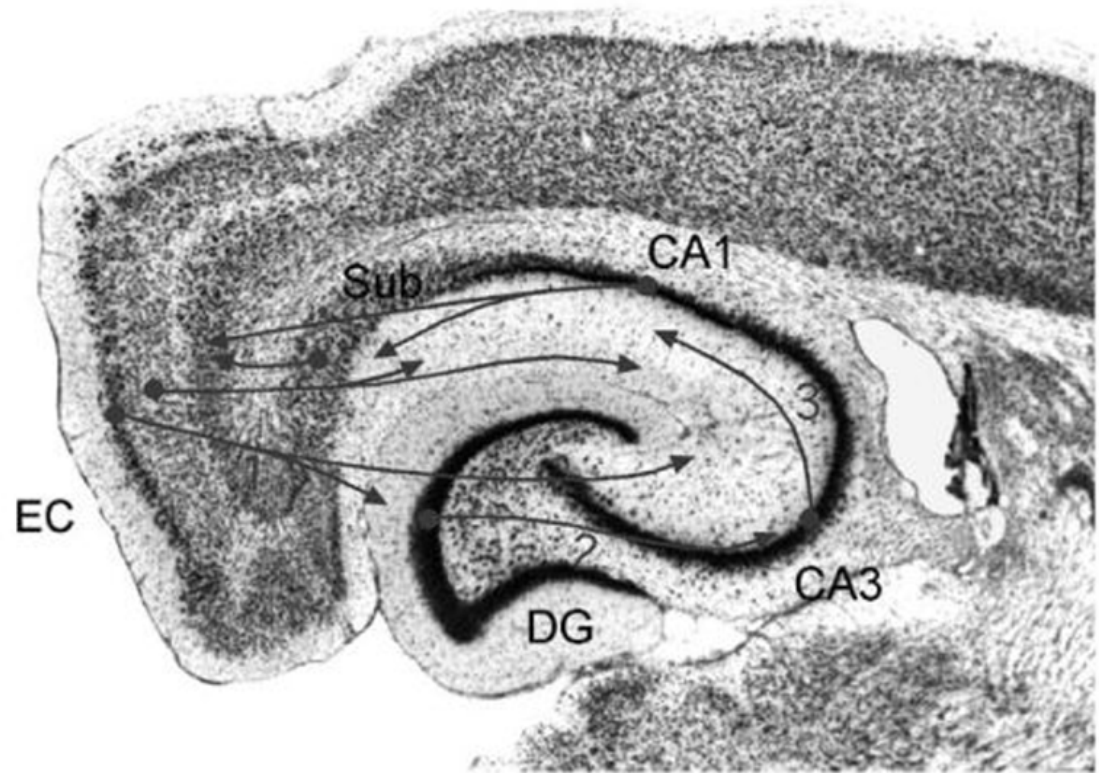

Figure 2. The image shows the projections from the EC onto the hippocampus illustrating the trisynaptic and monosynaptic pathways. [EC → DG → CA3 → CA1]. (EC = entorhinal cortex, Sub= Subiculum, DG = Dentate Gyrus, CA1/CA3 = Cornus Amonnis. Retrieved from Amaral et al., 2007.

The hippocampal area undergoes mid to late neurogenesis during brain development (Stockman et al., 2022) and continues to develop from birth throughout adulthood (Fig. 3). DG granule cells arise on G20 and continue to develop postnatally, only around 15% of the cells are formed prenatally (Bayer, 1980a, 1980b). The remaining cells go through quick development and maturation during the first week postnatally, however, DG neurogenesis persists throughout the lifespan (Christie & Cameron, 2006), though, in adults the number of granule cells does not vary (Amaral et al., 2007). The fibers from the entorhinal cortex arise around G17 in the CA fields and G18 in the DG (Supèr & Soriano, 1994). Animal models have

demonstrated altered hippocampal neurogenesis following PAE (Berman & Hannigan, 2000; Mattson et al., 2019).

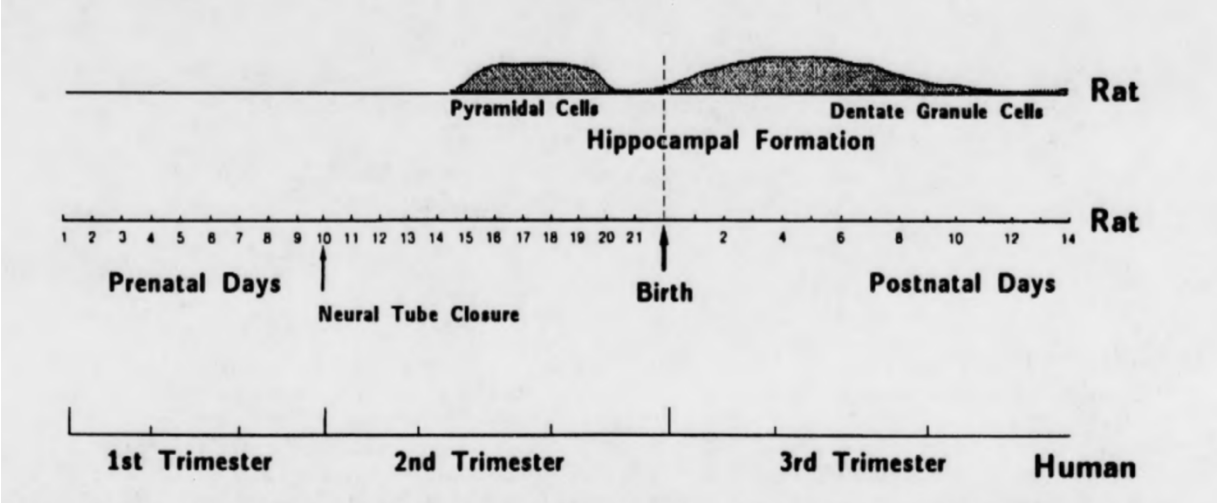

Figure 3. Timeline of the hippocampal formation development in rats (days) and equivalent in humans (weeks). The top line illustrates the pyramidal and dentate granule cells' neurogenesis. Diagram directly retrieved from Savage et al., 1992.

Aims

Given the above-mentioned - and briefly described - challenges associated with prenatal alcohol exposure in clinical population this work – using an animal model of moderate PAE - aim to understand 1) how moderate alcohol exposure impacts cognition – specifically long-term potentiation - and the neurobiological processes supporting learning and memory processes that may be impacted by moderate PAE; 2) if – and how - procognitive pharmacological agents can potentially revert PAE-related deficits; 3) potential new investigative and analytical techniques for assess PAE-related deficits.

Aim 1 - The essential role of synaptic plasticity in long-term memory is well-established, yet the precise mechanisms underlying long-term potentiation (LTP) remain elusive. Prenatal alcohol exposure (PAE) leading to considerable morphological and cognitive impairments raises questions about the impact of moderate PAE on LTP. Additionally, exploring the potential of inverse agonist drugs, based on previous procognitive reports, to reverse deficits associated with LTP presents an intriguing avenue for investigation.

H3Rs are responsible for mediating glutamate release. Research in this field suggests that there is a disruption in a presynaptic component of LTP involved in the glutamate release (Varaschin et al., 2010, 2014, 2018) mediated by H3 receptors (H_3R). In the current study, we aim to investigate the effects of a new inverse agonist agent (SAR152954) on LTP. We hypothesize that if there is a diminished glutamate release level that is related to heightened H3R activity (which inhibits the release of glutamate), the compound would reverse the deficits in neurotransmitter release and possibly reverse those deficits.

Aim 2 - At the synaptic level, the neurotransmitter glutamate is known to play a crucial role in supporting the occurrence of long-term potentiation (LTP). Could disruptions in

presynaptic mechanisms due to prenatal alcohol exposure (PAE) lead to altered glutamate release, potentially resulting in impaired long-term potentiation (LTP) at the synaptic level? To address this issue in vivo electrophysiology will be performed with the implant of a glutamate biosensor in the dentate gyrus to assess the neurotransmitter release level following HFS. Glutamate is crucial for the occurrence of LTP, given that there is a deficit in potentiation following moderate PAE, we hypothesize that the difference may be driven by lower levels of glutamate release into the synaptic cleft.

Aim 3 - Time-frequency analysis is a robust and valuable technique that enables researchers to delve into the intricate temporal changes of neural oscillations. LTP involves a sequence of events following HFS. Immediately after HFS, there is what is defined as post-tetanic potentiation and the feedback circuit. Time-frequency analysis can provide insight into the mechanisms associated with the induction and maintenance of LTP. The approach also provides information about the entire duration of the response rather than being limited to specific points in time. This allows for analysis of some aspects of the response that do not lend themselves well to traditional amplitude-based measures, such as the aspects of the evoked response that follow the second peak (following the population spike [ps]) which are associated with feedback within the dentate gyrus. Though there is a downside to the time-frequency - the complexity of the analysis and interpretation compared to traditional amplitude-based metrics-; time-frequency analyses may provide for more sensitive measures of group differences at or around time points associated with traditional targets of analysis. Following moderate PAE, the analysis of the fEPSP slope reveals deficits in potentiation. Furthermore, we will investigate the remaining segments of the evoked

responses to determine if there are any differences in the later parts that may contribute to a disturbance in the balance between excitation and inhibition following PAE.

The above-mentioned aims do not correspond to each of the following chapters – e.g., time-frequency analyses are discussed in Chapter 3 sections 1 and 2 – and may be discussed in more than one session. Additionally – but not less important – the main goal of this work was not only an attempt to contribute to the field of molecular neuroscience, but to offer an opportunity to – with the results – contribute to the clinical population. When I started working on this project, my ultimate goal was to help others by raising awareness of the issues associated with prenatal alcohol exposure and how those impact the neural mechanisms underlying learning and memory processes.

Chapter 1 – Review of hippocampal LTP and prenatal alcohol exposure

Note: At the moment of the presentation of this work, this chapter has been already published and is reproduced here without alterations. The references are included in the reference session of this document.
DOI: 10.3389/fnmol.2023.1326089

Unraveling the complex relationship between prenatal alcohol exposure, hippocampal LTP, and learning and memory

Monica Goncalves-Garcia and Derek Alexander Hamilton

Abstract: Prenatal alcohol exposure (PAE) has been extensively studied for its profound impact on neurodevelopment, synaptic plasticity, and cognitive outcomes. While PAE, particularly at moderate levels, has long-lasting cognitive implications for the exposed individuals, there remains a substantial gap in our understanding of the precise mechanisms underlying these deficits. This review provides a framework for comprehending the neurobiological basis of learning and memory processes that are negatively impacted by PAE. Sex differences, diverse PAE protocols, and the timing of exposure are explored as potential variables influencing the diverse outcomes of PAE on long-term potentiation (LTP). Additionally, potential interventions, both pharmacological and nonpharmacological, are reviewed, offering promising avenues for mitigating the detrimental effects of PAE on cognitive processes. While significant progress has been made, further research is required to enhance our understanding of how prenatal alcohol exposure affects neural plasticity and cognitive functions and to develop effective therapeutic interventions for those impacted. Ultimately, this work aims to advance the comprehension of the consequences of PAE on the brain and cognitive functions.

1. Introduction

Deficits following prenatal alcohol exposure (PAE) have been extensively investigated for decades. It is widely accepted that binge drinking results in a series of deficits, including morphological and cognitive alterations. However, less is known about impairments associated with moderate PAE. Over the years, significant progress has been made in understanding the neurobiological mechanisms underlying and contributing to the expressions of behaviors that are impaired following PAE. Given the similarities across the

mammalian brain (Gil-Mohapel et al., 2010; Patten et al., 2014), rat models of PAE are essential and valid for elucidating the effects of PAE on the mechanisms involved in brain the plasticity (Fontaine et al., 2016). Furthermore, this research may contribute valuable insights for the development of interventions for the clinical population.

The overarching goal of this review is to offer a comprehensive exploration of the current research on the impacts of PAE, with a particular focus on moderate levels of exposure, when data is available. This review aims to delve into the neurobiological basis of learning and memory processes affected by PAE. The objective is to establish a coherent understanding across different domains. It is evident that even low levels of prenatal alcohol exposure can lead to enduring effects on exposed individuals. While it is widely recognized that the hippocampal formation is particularly sensitive to developmental alcohol exposure, there are significant gaps remaining in understanding impairments of neurobiological mechanisms and subsequent cognitive manifestations. This review seeks to provide a foundational framework for further investigation, with the goal of comprehending the underlying mechanisms behind the negative impacts of PAE on learning and memory, enabling early diagnosis and therapeutic interventions for affected individuals.

1.2. Neural Basis of Learning and Memory

The ability of the brain to adapt to experience and the mechanisms involved in the strengthening - or weakening - of neural connections has been investigated for over 100 years (Bliss & Collingridge, 1993; Nicoll, 2017). Synaptic plasticity can be defined as an activity-dependent alteration of the strength of synapses. The long-lasting forms of synaptic plasticity include long-term potentiation (LTP) – persistent strengthening of synapses - and long-term

depression (LTD, *for review see* (Fontaine et al., 2016) – persisting decreasing in the strength of synapses. In the early 70s, there was a breakthrough in understanding the neural mechanisms underlying synaptic plasticity with the discovery of LTP in the granule cells of the dentate gyrus following high-frequency stimulation (HFS) to the rabbit perforant pathway (Bliss & Lømo, 1973). Following the publication of that study, significant progress has been made in the advancement of the understanding of the cellular and molecular mechanisms underlying synaptic plasticity. The phenomenon of LTP has been identified at synapses across the brain (Lynch, 2004), however, it is mostly studied in the hippocampus as the area plays a major role in spatial navigation, learning, and memory (Bonthius et al., 2001). Investigations of LTP have been extensively conducted at the hippocampal formation as the discoveries associated with LTP provide evidence for the cellular basis of learning and memory (Bashir et al., 1993; Larson & Lynch, 1986; Lynch, 2004; McNaughton et al., 1986; Nicoll, 2017; Sutherland et al., 1998).

1.3. Properties of LTP

LTP requires simultaneous depolarization of the pre-and post-synaptic terminals. The basic properties of LTP are cooperativity (co-activation of multiple excitatory synapses simultaneously or in close temporal proximity), associativity (weak stimulation of a single pathway can induce LTP if strong stimulation of another pathway is delivered simultaneously), and input specificity (occurrence of LTP at a single synapse without spreading to others). Together these properties ensure the accuracy of memory storage and maintenance (Abraham et al., 2019).

Briefly, the mechanisms underlying LTP are mediated by the release of the excitatory neurotransmitter glutamate – which plays a crucial role in the mammalian brain by facilitating most excitatory transmission and by its involvement in cognition, learning, and memory – and the activation of the glutamate receptors NMDA (Collingridge et al., 1983) and AMPA in the postsynaptic cell (Bliss et al., 2018; Bordi, 1996). During low-frequency stimulation, glutamate from the presynaptic terminal binds to NMDAR and AMPAR channels in the postsynaptic terminal (Fig. 4). The AMPAR channel is open and allows for the flow of Na+ and K+. The NMDAR channel is blocked by Mg2+ which is removed following HFS allowing, then, for Ca^{2+} to flow through the channel (Nicoll, 2017). However, NMDAR activation is not necessary for the induction of all forms of LTP (Bashir et al., 1993; Lynch, 2004) in the CA1 region. Unlike NMDA receptor-dependent LTP, mossy fiber LTP can occur independently of NMDA receptors. Notably, inducing LTP in CA1 without NMDA receptors is possible, but generally, Schaffer-commissural LTP requires NMDAR (Lynch, 2004). Ongoing research explores mossy fiber LTP's mechanisms, enduring changes, and diverse roles across brain regions (Malenka & Bear, 2004; Nicoll & Schmitz, 2005).

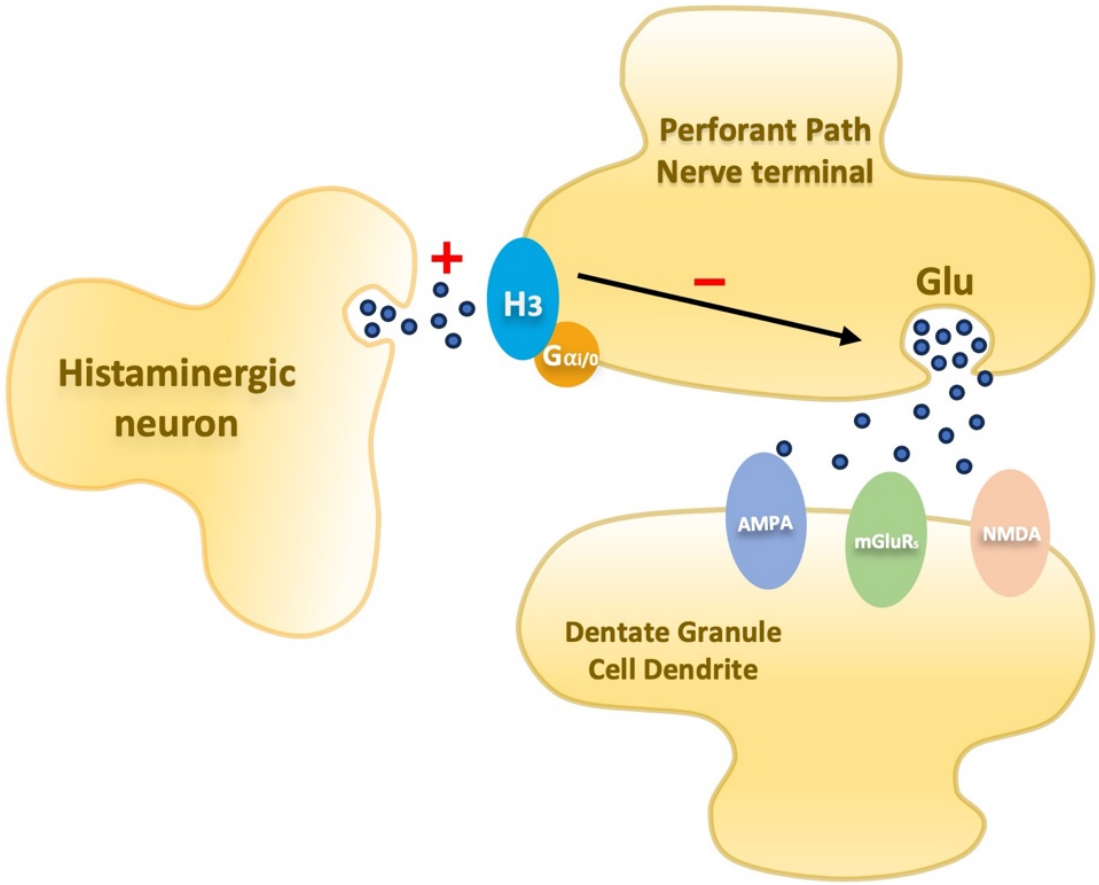

Figure 4. Schematic diagram illustrating an entorhinal perforant path nerve terminal and postsynaptic dentate granule cell dendrite. Presynaptic histamine H3 receptors mediate the inhibition of glutamate release. In PAE animals the inhibitory mechanism is elevated.

In addition, other features of synaptic transmission are NMDA-receptor independent, such as paired-pulse facilitation (PPF) - an enhanced response to two closely spaced stimuli; provides insights into short-term synaptic plasticity and the interplay between presynaptic and postsynaptic mechanisms in neurotransmission studies - and post-tetanic potentiation (PTP) - a short-term enhancement of synaptic transmission observed after a brief period of high-frequency stimulation, reflecting the heightened release of neurotransmitters at the synapse (Bliss & Collingridge, 1993). To investigate the involvement of the neurotransmitter glutamate in the induction of LTP, studies investigated hippocampal LTP in mice lacking $mGlu_1$ – a glutamate receptor subtype. The results suggest a reduction in LTP compared with control

animals which supports the implication of glutamate receptors in the LTP induction (Bashir et al., 1993; Bordi, 1996). NMDAR is referred to as a 'coincidence detector' because of the necessity to activate both presynaptic glutamate release and postsynaptic depolarization (Citri & Malenka, 2008). LTP is comprised of early (e-LTP) and late (l-LTP) phases. The former is independent of protein synthesis and lasts for about 1h, while the latter requires de novo protein synthesis for the maintenance of the LTP. Briefly, e-LTP is frequently understood as being the outcome of a single HFS episode and of shorter duration (~1h). During the induction phase, increased synaptic input triggers the activation of various signaling pathways. Calcium influx into the postsynaptic neuron activates Ca2+/calmodulin-dependent protein kinase II (CaMKII) and other calcium-dependent kinases. These kinases, in turn, phosphorylate AMPA receptors, facilitating their trafficking to the postsynaptic membrane. Additionally, protein kinase C (PKC) is activated, contributing to the modulation of synaptic efficacy. These phosphorylation events play a crucial role in the expression of LTP, where the strengthened synaptic connections are maintained (Citri & Malenka, 2008; Fontaine et al., 2016; Herring & Nicoll, 2016; Tao et al., 2021). The early phase sets the stage for the subsequent molecular and structural changes that underlie the long-lasting modifications associated with LTP, ultimately contributing to the cellular basis of learning and memory. l-LTP requires multiple episodes of HFS and is dependent upon the activation of protein kinase A (PKA) and CaMKII, which develops over time and could last for several hours. Gene transcription and protein synthesis (Malenka & Bear, 2004) contribute to the synthesis of new proteins necessary for maintaining the strengthening of the synapses. PKMzeta has been linked to memory (Pastalkova et al., 2006) and maintenance of LTP through trafficking and expression of

AMPARs (Bingor et al., 2020; Yao et al., 2008) and NMDARs (Shema et al., 2007; Yao et al., 2008). Other potential processes that contribute to induction and maintenance of LTP include retrograde signaling of Nitric Oxide (NO) (Bon et al., 1992; Mizutani et al., 1993), extra synaptic AMPARs and spillover of glutamate (Kullmann & Asztely, 1998), and modified sensitivity of metabotropic glutamate receptors (Aronica et al., 1991). The reader is referred to the reviews of Hayashi (2022) and Herring and Nicoll (2016) for broader treatment of LTP mechanisms.

l-LTP requires multiple episodes of HFS and is dependent upon the activation of protein kinase A (PKA) and CaMKII, which develops over time and could last for several hours. Gene transcription and protein synthesis (Malenka & Bear, 2004) contribute to the synthesis of new proteins necessary for maintaining the strengthening of the synapses. Some studies of in vivo LTP showed evidence for the induction of LTP that lasted for several hours following a single instance of HFS (McNaughton et al., 1986; also see Park et al., 2014 for additional details on different forms of LTP that are dependent on the timing of the stimuli and are pharmacologically distinct).

Given that multiple actions of distinct nature and location must take place for potentiation to occur, identification of those poses a challenge when attempting to understand possible alterations following developmental insults. The first conclusions about LTP were that it was purely a postsynaptic mechanism. However, with the emergence of more research in the matter of nature and loci of expression, the conclusions gravitated between pre- or post-, to a mix of both, to both pre-and postsynaptic mechanisms (Bliss & Collingridge, 1993) that characterize different types of LTP. Presynaptic mechanisms like H3 receptors and

mGluRs (metabotropic glutamate receptors) participate in regulating neurotransmitter release and represent promising targets for manipulation and interventions aimed at modulating long-term potentiation. Additionally, PAE can lead to different long-lasting alterations in synaptic plasticity mechanisms that includes reduced neurogenesis and cell loss (Bird et al., 2018; Choi et al., 2005; Gil-Mohapel et al., 2010; Y. Yang et al., 2017), neuronal morphology and spine architecture (Berman & Hannigan, 2000; D. A. Hamilton et al., 2010; Mira et al., 2020), and NMDAR level alterations (Bird et al., 2020; Hughes et al., 1998; Plaza-Briceño et al., 2020; Samudio-Ruiz et al., 2010). These are some of the mechanisms that could be altered by PAE and have a negative impact on synaptic plasticity. Understanding the deficits and mechanisms of interactions are key to develop potential interventions that could mitigate those long-lasting impairments.

1.4. LTP and Prenatal Alcohol Exposure

Animal models of PAE using in vitro or in vivo electrophysiology to investigate LTP in the hippocampal formation suggest that there are fundamental alterations in the neural mechanisms when compared to non-exposed animals. Even though lower levels of PAE do not result in physical deficiencies, studies on the neurobiology of learning and memory processes have identified cognitive impairments following moderate PAE (Sutherland et al., 1998). Significant decreases in LTP have been observed in the dentate granule cells following high-frequency stimulation (HFS – trains, or tetanus, of stimulation, or theta burst stimulation [TBS]) in the perforant pathway (Brady et al., 2013; Harvey et al., 2020; Sutherland et al., 1998; Varaschin et al., 2010). While CA1 LTP has been extensively studied, the dentate gyrus (DG) is of particular interest as the primary region within the hippocampal formation to

receive input from the entorhinal cortex via the Perforant pathway (PP). Furthermore, studies on DG LTP have consistently reported alterations following prenatal alcohol exposure (Brady et al., 2013; Sutherland et al., 1998; Titterness & Christie, 2012; Varaschin et al., 2010).

One of the first studies reporting electrophysiological impairment following PAE used paired-pulse facilitation in hippocampal slices, specifically in the CA1 region, with interpulse intervals varying from 5 to 400 μs (Hablitz, 1986). The results showed diminished paired-pulse response inhibition at shorter inter-pulse intervals [5-100μs] – which involves activation of recurrent inhibitory pathways – in the PAE group compared to controls, but similar potentiation at longer intervals [200-400μs] (Hablitz, 1986). Similar results were reported in a replication study expanding to two levels of ethanol exposure. One group of animals received a high dose of ethanol (35% ethanol-derived calories [EDC]), while the other received a low dose (17.5% EDC) group. The results were similar to the first study but only in the 35% EDC group at lower PP intervals. The 35% EDC group also showed little evidence of LTP compared to the other diet conditions (Tan et al., 1990). In contrast, another study, using a different method of alcohol administration (intragastric gavage – GD8-21 - 0, 4, or 6 g/kg/day) and brain slices from two age groups (PN25-32 and PN63-77), reported no difference in input/output profiles or paired-pulse responses at any group. There was a significant reduction in the amplitude of the maximal evoked population spike (PS) in the higher dose in the younger group compared to the other two groups (Berman & Hannigan, 2000; Krahl et al., 1999). This result is similar to the results reported by Sutherland et al. (1997) using a 5% ethanol liquid diet, investigating in vivo DG LTP in 5-month-old rats. In terms of timing, a study showed that the effects of developmental alcohol exposure yield different

outcomes in DG LTP. PAE during GD10-21 (2nd trimester-equivalent) only resulted in less potentiation while exposure during GD1-9 and PN1-9 (1st and 3rd trimester-equivalent) did not have a significant effect (Helfer et al., 2012). While these results support timing as a factor, there is still a need for clarification on the teratogenic effects of alcohol. During the 2nd trimester equivalent, the DG granule cells and interneurons undergo neurogenesis. Exposure during the early stages of development may alter cell migration, neuron and glial proliferation, and the formation of neural networks. In addition to the timing of exposure, the pattern/ route of ethanol administration, as well as age during the assessment, are important factors to consider when interpreting the results from different studies of similar investigations as those may lead to diverse outcomes (table 1).

Table 1. LTP protocols on the DG from different studies shows the variability of the alcohol exposure paradigm, stimulation protocol, and stimulation outcomes. Also, this table shows the reduced number of studies on LTP on the DG following throughout the years.

Alcohol Exposure Paradigm/BAC	**Stimulation Paradigm**	**Effects**	**References**
Liquid Diet; GD 1–22; 5% v/v; BAC 83.2 mg/dL	Pulse frequency: 30s Induction: 10 × 400 Hz	LTP M↓	(Sutherland et al., 1997)
Liquid Diet; GD 1–22; 6.61% v/v; BAC 184 mg/dL	Pulse frequency: 15s Induction: 5 × (10 × 5 Pulses, 100 Hz)	LTP M↓	(Christie et al., 2005)
Liquid Diet; GD 1–22; 5% v/v; BAC 84 mg/dL	Induction: 3 × 400 Hz, 25ms or 10 × 400 Hz, 25ms	LTP M↓ with 3 × 400 Hz protocol	(Varaschin et al., 2010)
Liquid diet; GD11–21; 6.6% v/v; BAC 142 mg/dL	Pulse frequency: 30s Induction: HFS 4 x 50 pulses, 100 Hz or TBS 5 x (4 x 4 Pulses, 100 Hz)	LTP M↓	(Helfer et al., 2012)
Drinking Water; GD 1–22; 5% v/v; BAC 84 mg/dL	Induction: 4 × (10 × 5 Pulses, 400 Hz)	LTP M↓; F−	(Patten, Brocardo, et al., 2013)
Liquid Diet; GD 1–22; 6.61% v/v; BAC 101.5 mg/dL	Induction: 4 × (10 × 5 Pulses, 400 Hz)	LTP M↓; F−	(Patten, Sickmann, et al., 2013)
Liquid Diet; GD 1–22; 6.6% v/v; BAC 146.32 mg/dL	Induction: 4 × (10 × 5 pulses, 400 Hz)	LTP M↓; F−	(Sickmann et al., 2014)
Drinking Water; GD 1–22; 5% v/v; BAC 84 mg/dL	Pulse Frequency: 30s Induction: 3×400 Hz	LTP M↓	Varaschin et al., 2014
Liquid diet; GD1–22; 6.6%v/v; BAC 80–180 mg/dL	Pulse frequency: 30s Induction: 4 trains x 50 pulses, 100 Hz	LTP M↓; F↓	(Fontaine et al., 2019)
Alcohol solution; GD1–PD7; 10% v/v; BAC: 62 mg/dL	Pulse frequency: 30s Induction: HFS 8 x (3 x 8 pulses, 200 Hz)	LTP M↓	(Plaza-Briceño et al., 2020)
Liquid diet; GD1–22; 6.6% v/v; BAC 80–180 mg/dL	Pulse frequency: 30s Induction: HFS 4 x 50 pulses, 100 Hz	LTP M↓; F−	(Grafe et al., 2022a)

1.5. Histaminergic and glutamatergic transmissions

Over the years, since the discovery of LTP, significant progress has been made in understanding the components of the complex neurobiological network supporting synaptic plasticity. As a result, a better understanding of what may be altered by prenatal insults has emerged offering potential avenues for interventions. Research in this field suggests that there is a disruption in a presynaptic component of LTP involved in the glutamate release (Varaschin et al., 2010, 2014, 2018) mediated by H3 receptors (H_3R). These receptors are autoreceptors – modulating histamine release – that also acts as heteroreceptors modulating release of other neurotransmitters, including glutamate. Passani et al. (2004), reviewed the role of the histamine H_3R as a possible target for pharmacological interventions to enhance cognition and treat possible disorders associated with sleep, stress, and anxiety. The researchers highlight the possible association of the histaminergic system in cognitive processes and describe results from studies using H_3R antagonists and inverse agonists that improved cognitive performances in cognitively impaired animals. Together, these suggest that neurotransmitters modulated by H_3R are involved in cognition and that H_3R antagonists or inverse agonists could potentially reverse cognitive deficits (Passani et al., 2004). In the dentate gyrus, H_3Rs are located on the perforant pathway and have been demonstrated to inhibit glutamate release via histamine-mediated depression in calcium influx during the presynaptic action potential (Brown & Haas, 1999) (fig. 4).

Glutamatergic neurotransmission is part of the presynaptic components of LTP and may be involved in activity-dependent potentiation deficits following PAE (Savage et al., 1998, 2010; Varaschin et al., 2014). Studies have investigated glutamate receptor release by

utilizing a glutamate reuptake inhibitor that allowed for the measurement of the electrically evoked release of [^{3}H]-D-ASP in brain slice preparations (Savage et al., 1998, 2001). Results suggest activity-dependent potentiation of D-ASP release following moderate PAE suggesting impairment in complex activity-dependent modifications in the neurotransmission (Savage et al., 1998). Even though the presynaptic neurochemical basis for this impairment remains unclear, studies established that one of the neurochemical mechanisms underlying deficits associated with PAE is a reduction in glutamate receptor-mediated potentiation of glutamate release at the synapses of the dentate granule cells (Galindo et al., 2004; Varaschin et al., 2010).

1.6. Sex differences

Male animals are more commonly used in studies investigating synaptic plasticity, with fewer studies exploring both male and female animals or exclusively female animals (An & Zhang, 2015; Sickmann et al., 2014; Titterness & Christie, 2012). These studies focused on synaptic plasticity in adolescent rats (~PND30-35) and reported bidirectional findings. PAE animals showed reduced LTP after HFS relative to control and pair-fed groups, while females exhibited enhanced LTP compared to the other groups. Titterness & Christie (2012) suggested that sex differences could be due to sex-specific alterations to NMDAR-dependent DG LTP. Subsequent work from the same group expanded on the investigation in NMDAR function and expression, but did not support sex differences. Additionally, they did not find sexually dimorphic effects on DG LTP in adulthood (PND 55-70) (Sickmann et al., 2014). Another study, including only female rats, reported enhanced LTP compared to the control groups (An &

Zhang, 2015). The first two groups mentioned, used a liquid diet (35% EDC) and Sprague-Dawley rats, while the last used oral gavage in Wistar rats.

The limited number of studies investigating sexual dimorphism in LTP does not provide sufficient information to 1) accept that sex differences are significant; 2) conclude that the differences reported in adolescence, but not in adulthood, have an impact on plasticity and/or behavioral expression later in life; 3) understand the source of the differences; or 4) elucidate how sex differences may or may not be related to strain, route of ethanol administration, and – timing. Further investigations should focus on expanding on current plasticity models to include both sexes while decreasing possible confounding factors related to diet, route/timing of administration, and animal strain.

Behavioral tasks investigations show that males are more impaired than females in the probe trials of the Morris Water Task (MWT), but both are impaired in the acquisition phases of the test. While other studies report the opposite effect (Berman & Hannigan, 2000). In open-field tests, PAE male rodents are typically more hyperactive than females (Osterlund Oltmanns et al., 2022), show more perseveration errors during reversal learning in the MWT (Rodriguez et al., 2016), and exhibit spatial information retention impairment (Rodriguez et al., 2016). Studies PAE consistently show parallels between animal and human data. Hamilton et al. (2003) found that PAE children, like rodents, had greater distances in a virtual Morris Water Task. Woods et al. (2018) using a similar setup with fMRI, observed longer latencies in PAE boys but no differences in girls. Dodge et al. (2019) found deficits in place learning for syndromal boys and girls, and non-syndromal girls, but not in low-to-moderate PAE. All

studies note differences in place learning, aligning with findings in animal models (Dodge et al., 2020; Goodlett & Johnson, 1997).

These differences can also be related to sexual dimorphisms in neural development. A study by Hamilton et al (2011), shows greater PAE effects on dendritic morphology, structural plasticity, and IEG expression in males than in females. Sexual dimorphisms have been reported in hippocampal neurotransmissions and LTP (Osterlund Oltmanns et al., 2022; Sickmann et al., 2014). Although there has been numerous evidence on sexual dimorphisms and how PAE may affect males and females differently, a thorough discussion on sexual dimorphisms is beyond the intended scope of this review. It is important, however, to stress that possible sex differences must be taken into consideration as investigation on just one or the other sex may or may not reveal subtle differences between sexes.

A recent study by Stockman et al., (2022) investigated neurogenesis in the neonatal rat hippocampus and found evidence for a sexually dimorphic epigenetically based regulation of neurogenesis – specifically in the DG. Their results suggest that there is a developmental sex difference in DG cell genesis. It suggests that there is a modulatory DNA difference with elevated methylation in the males and elevated histone acetylation in females – which suppresses neurogenesis. They also state that in adulthood the overall size of male and female hippocampus does not differ. The early developmental differences – as female neurons mature earlier than male neurons – evidenced in enhanced learning and LTP in females compared to males, reverse with the achievement of reproductivity maturity (Le et al., 2022; Stockman et al., 2022). A potential explanation for sex differences in LTP and behavioral task, has been linked to possible differences in the composition of GABAa

receptors before and after puberty in females. Further investigators in the field should take this information into account when interpreting the results of possible sex differences, particularly if those hippocampal-dependent tests are performed before or after adolescence. Considering the new discoveries about neurogenesis and sex differences, researchers should be mindful of the timing of exposure/ assessment to have a better interpretation of results showing sex differences. Those changes could be associated with the onset of the estrous cycle. Alternatively, late maturation of interneurons and related connections, could be involved in the sex differences in hippocampal LTP before and after puberty (Le et al., 2022). Additionally, recognizing fundamental sex differences in brain development is crucial for understanding how and when these distinctions manifest. This knowledge is essential for informing the development and direction of potential interventions.

1.7. Non-pharmacological Interventions

A growing body of research focuses on potential therapies following PAE and prevention of the possible outcomes by nutraceutical interventions. Choline supplementation has been of interest because it is also involved in the formation of the neurotransmitter acetylcholine. Research has shown that choline supplementation during pregnancy has positive benefits on cognitive scores which could be a potential prenatal intervention to minimize or prevent Fetal Alcohol Spectrum Disorders (FASDs). Because individuals do not normally get diagnosed until later, there are questions about the benefits of postnatal supplementation. Animal models of PAE investigated the benefits of postnatal choline supplementation and the results showed improvement in the MWT (Grafe et al.,

2022a). In the pediatric clinical population, the results of postnatal supplementation have been unclear as the results seem to differ according to the age group (Grafe et al., 2022a; Wozniak et al., 2020). The data on choline supplementation is still scarce. There is a general agreement that choline supplementation is possibly acting on altering hippocampal cholinergic functioning. However, choline can also influence methylation patterns and is linked with other signaling pathways, which are known to be altered by alcohol exposure (Monk et al., 2012). More investigation is needed to 1) understand the mechanisms of action of choline supplementation, 2) determine the benefits of perinatal and postnatal supplementation, 3) assess the long-lasting benefits of choline, and 4) define the ideal window of possible interventions.

Other possible interventions include - but are not restricted to - omega-3 fatty acid supplementation (Patten, Sickmann, et al., 2013), vitamin E, betaine, folic acid, methionine, zinc , voluntary exercises (Christie et al., 2005), enriched environment (animal model) (Gursky & Klintsova, 2017; G. F. Hamilton et al., 2014; Kajimoto et al., 2016) to diminish alcohol-induced changes to the hippocampus. In addition, studies in epigenetics – alterations in gene expression that can be influenced by environmental factors – suggest that alterations in basic cellular processes following PAE may be related to long-lasting effects that include dendritic development and synaptic plasticity, as suggested by reports of reduced hippocampal cell numbers in FASD models (Stockman et al., 2022; Varadinova & Boyadjieva, 2015). These modifications in gene expression alterations include DNA methylation, histone modification, and non-coding RNA regulation, and can disrupt the development of the nervous systems leading to long-lasting impairments (Ehrhart et al., 2019; Varadinova & Boyadjieva, 2015).

These may lead to disruption in the induction and maintenance of LTP, for example. More research is needed to understand the effects of alcohol on gene expression alterations. These are promising outcomes, but more research is needed to understand the possible benefits of the mechanistic bases of those effects.

1.8. Pharmacological manipulations

Pharmacological manipulations with agents that have known receptor interactions can provide insights into receptor-level mechanisms and identify potential pharmacotherapeutic interventions. “Procognitive agents” have been shown to facilitate learning and memory (Sadek et al., 2016) and used to examine potential developmental alterations in histaminergic and glutamatergic neurotransmissions following PAE. Studies have specifically investigated the effects of the histamine H_3R antagonist ABT-239 on both LTP and spatial navigation tasks in PAE animals (Savage et al., 2010; Varaschin et al., 2010). Assessment of the effects of the ABT-239 agent in moderate PAE animals in the MWT showed that the escape latency differences (PAE > saline controls) were reversed. The agent was injected 30 min prior to the training on day 1 and PAE animals treated with ABT-239 had performances similar to the saline-treated control animals (Savage et al., 2010). The use of ABT-239 prior to in vivo electrophysiology recordings demonstrated that the agent improved DG LTP in PAE animals to levels similar to those of saline-treated control animals (Varaschin et al., 2010).

The researchers speculated that ABT-239 facilitated glutamate release. However, they added that there is also a possibility that the inhibition of H_3R on cholinergic nerve terminals facilitates acetylcholine release which could facilitate glutamate release. Also, the ABT-239

agent could be acting on the inhibition of H_3 autoreceptors that promote histamine release and could facilitate the excitation of glutamatergic neurons via H_1 and H_2 receptors (Varaschin et al., 2010). None of those studies found any effect of the agent on control animals.

A recent study using immunohistochemistry, biochemical, and radiohistochemical approaches investigated histamine H_2R density and H_2 receptor-effector coupling in several nerve terminal regions of moderate PAE rats. The results found no significant PAE-related differences in the density of H_2R binding (Davies et al., 2019) in contrast to alterations in the H_3Rs (Varaschin et al., 2018).

Together these data provide evidence for presynaptic alteration following PAE, specifically at moderate levels – which reinforces the fact that there is no known safe amount of alcohol to be taken during pregnancy. The identified presynaptic alterations in the context of moderate PAE highlight the significance of glutamate modulation, possibly through histamine receptor interactions. At this level of PAE investigations, there seems not to report on sex differences investigations.

Although there are not enough studies investigating the effects of procognitive agents on DG LTP in PAE, this discovery not only emphasizes the central role of glutamate in synaptic plasticity but also opens the door to the development of novel pharmacological interventions aimed at ameliorating the effects of PAE on cognitive function, which could have broader implications for individuals affected by FASD. Other research groups have investigated different pharmacological agents to enhance PAE-related cognitive deficits. Slice physiology studies have demonstrated reversal of cognitive deficits via positive modulation the AMPAR following administration of Aniracetam (allosteric modulator of AMPAR and Piracetam

analogon) (Vaglenova et al., 2008; Wijayawardhane et al., 2008). Administration of Piracetam have demonstrated alleviation of PAE-related deficits in CA1-LTP, showed improvement in hippocampal cell viability and reduction of PAE-induced cell apoptosis (Y. Yang et al., 2017)

1.9. Summary, Conclusion, and Future Considerations

This review highlights the extent to which a wide range of research has contributed to understanding some mechanisms and impairments associated with prenatal alcohol exposure. Various studies have consistently demonstrated alterations in synaptic plasticity following moderate levels of prenatal alcohol exposure, highlighting the need for ongoing investigations. Our understanding of the underlying neurobiological mechanisms and their impact on learning and memory processes has significantly improved. Animal models have demonstrated altered hippocampal neurogenesis following PAE ((Berman & Hannigan, 2000; Mattson et al., 2019)). Human studies on trajectories of brain development following PAE, have extensively investigated neuroanatomical differences in PAE and typically developing individuals. However, few studies have assessed brain development over time, which leaves a gap in understanding potential alterations in patterns of development following PAE (for review in humans and neuroimaging techniques, see Moore & Xia, 2022). Taken together, this information is important to provide evidence of the relationship between time of exposure, neural development, and behavior outcomes (Lange et al., 2019). However, many questions remain, particularly regarding sex differences, and more research is needed to determine how the specific timing and dose of exposure affect the mechanisms supporting LTP.

While it is well-accepted that the hippocampal formation is sensitive to any amount of developmental alcohol exposure, there is still a gap in understanding those deficits and the related behavioral and cognitive manifestations. Over the past few decades, technological advancement, better assessment tests, and multi-level investigations have provided great knowledge on the detrimental effects of PAE. Studies on LTP provide a well-established model system for evaluating prospective treatments and identifying potential neural bases of learning and memory deficits observed in PAE. Unfortunately, there have been relatively few studies in LTP conducted for a small number of laboratories in the past few decades and the mechanisms remain to be understood. The development of treatments clinically depends on more research in the area, which is needed to amplify our basic understanding of how PAE affects synaptic plasticity.

Promisingly, this review has shed light on potential interventions, encompassing both pharmacological and non-pharmacological approaches, offering potential information in mitigating the enduring impacts of PAE on cognitive functions.

However, it is imperative to emphasize that this review stresses the need for further research, in-depth investigations, and a better comprehension of how prenatal alcohol exposure negatively impacts synaptic plasticity and cognitive functions. The ultimate aim persists in advancing our understanding of the intricate consequences of PAE on the developing brain and cognitive processes, while also paving the way for the development of effective therapeutic interventions that can enhance the lives of those burdened by prenatal alcohol exposure.

Chapter 2 - Glutamate – biosensor

2.1. Introduction

Glutamate, a neurotransmitter in the central nervous system (CNS), plays a crucial role in various neurophysiological and neuropathological processes. As the most prevalent and primary excitatory signaling molecule, glutamate facilitates communication among neurons, contributing to cognitive processes including those related to learning and memory (Gonçalves-Ribeiro et al., 2019). Glutamate engages in diverse interactions with neurotransmitter systems such as dopaminergic, serotonergic, cholinergic, and histaminergic systems - which plays a significant role in modulating glutamate release and synaptic transmission (Varaschin et al., 2014). The modulation of glutamate release is achieved through a series of mechanisms, including the activation of histamine receptors, particularly, the H3R subtype – located in the perforant path terminals (Brown & Haas, 1999). This receptor subtype engages G-proteins, molecular switches that mediate signal transduction within the cell. When histamine binds to H3 receptors, the associated G-proteins are activated, initiating intracellular signaling pathways (Carthy & Ellender, 2021). The activated G-proteins, in turn, play a pivotal role in the autoinhibition process of histamine release. As autoreceptors, stimulated H3 receptors inhibit further histamine release through the modulation of voltage-gated calcium channels or adenylate cyclase. This G-protein-mediated autoinhibition subsequently leads to a reduction in the inhibition of glutamate release. Dysregulation of glutamate signaling has been linked to various neurological disorders, such as Alzheimer's, Parkinson's, Huntington's disease (HD), and Amyotrophic

lateral sclerosis (ALS) (Gonçalves-Ribeiro et al., 2019; Miladinovic et al., 2015; J.-L. Yang et al., 2011). PAE deficits could be related to alterations in glutamate release.

Studies using animal models of PAE have demonstrated that moderate PAE lead to disruption in presynaptic mechanisms supporting plasticity. Galindo et al. (2004) reported diminished mGluR5 receptor protein levels in dentate gyrus of PAE animals when compared with control animals. The study suggests that the diminished levels of mGluR5 – a metabotropic glutamate receptor - may be implicated in synaptic deficits following PAE. Previously, Sutherland et al. (1998) – investigating glutamatergic neurotransmission -, demonstrated that moderate PAE resulted in LTP deficits.

Real-time monitoring of glutamate release level is important to assess differences following PAE. Because glutamate cannot be detected by voltammetry (Ganesana et al., 2019), we combined in vivo electrophysiology surgical procedures with the addition of an enzyme-based electrochemical biosensor to record potential differences in extracellular glutamate concentration in the DG following HFS.

2.2. Methods

2.2.1. Animals

20 adult male Long-Evans rats long-Evans rats (PAE = 10) 3- to 9-month-old (University of New Mexico Health Sciences Center Animal Resource Facility) were used in the present study. All animals were pair-housed in plastic cages on a 12-hour light-dark cycle with food and water available ad libitum. The experiments reported here were approved by the Institutional Animal Care and Use Committee (IACUC) of the Health Sciences Center and Main Campus of the University of New Mexico.

Breeding and Voluntary Ethanol Consumption During Gestation

The breeding and ethanol exposure procedures are described in detail in Davies et al. (2023). All breeding procedures were conducted in the University of New Mexico HSC Animal Resource Facility (ARF). Two-month-old Long-Evans rat breeders (Envigo Corporation, Indianapolis, IN) were single-housed in plastic cages at 22°C and kept on a reverse 12-hour light/dark schedule (lights on from 2100 to 0900 hours) with Purina Breeder Block rat chow and tap water ad libitum. After at least one week of acclimation to the animal facility, all female rats were provided 0.066% saccharin in tap water for four hours each day from 1000 to 1400 hours. On Days 1 and 2, the saccharin water contained 0% ethanol, on Days 3 and 4, saccharin water contained 2.5% ethanol (v/v). On Day 5 and thereafter, saccharin water contained 5% ethanol (v/v). Daily four-hour ethanol consumption was monitored for at least two weeks and then the mean daily ethanol consumption was determined for each female rat. Following two weeks of daily ethanol consumption, females that drank at levels more than one standard deviation below the mean of the entire group were removed from the

study. The remainder of the females were assigned to either a saccharin control or 5% ethanol drinking group and matched such that the mean pre-pregnancy ethanol consumption by each group was comparable.

Subsequently, females were placed with proven male breeders until pregnant as evidenced by the presence of a vaginal plug. Female rats did not consume ethanol during the breeding procedure. Beginning on Gestational Day 1, rat dams were provided saccharin water containing either 0% or 5% ethanol for four hours a day, beginning at 1000 hours (1 hour following the onset of the dark cycle). The volume of 0% ethanol saccharin water provided to the controls was matched to the mean volume of 5% ethanol in saccharin water consumed by the ethanol-drinking group, which has remained relatively consistent at about 16 mL per four-hour drinking period over multiple breeding rounds. Food was available ad libitum during both the drinking and non-drinking periods. Daily four-hour ethanol consumption was recorded for each dam. Ethanol consumption by the rat dams producing offspring for this study was 2.25 + 0.09 g/kg body weight (Table 2). Ethanol consumption was discontinued at birth, and all litters were weighed and culled to 10 pups. This level of ethanol consumption by another set of rat dams not used in the present study produced serum ethanol concentrations of 46.0 + 3.2 mg/dL (Davies et al., 2023). Maternal weight gain during pregnancy and offspring litter size and birth-weight did not differ based on prenatal treatment group (Table 2). Offspring were weaned at 24 days of age and transferred from the HSC-ARF to the Department of Psychology ARF on Main Campus. To minimize potential litter effects only 1-2 rat offspring per litter were included in the present study.

Table 2. Mean + SEM daily ethanol consumption, maternal weight gain, litter size, and birth weight for SAC and PAE groups.

	Saccharin Control	**5% Ethanol**
Daily Four-Hour Ethanol Consumption[a]	N/A	2.06 ± 0.13
Maternal Weight Gain[b]	113.4 ± 6.6	113.7 ± 9.4
Number of Live Births[c]	12.4 ± 0.5	11.3 ± 0.5
Pup Birthweight[d]	6.69 ± 0.23	6.89 ± 0.27

a - Mean ± SEM grams ethanol consumed/kg body weight/day

- Mean ± SEM grams increase in maternal body weight from GD 1 through GD 21

c - Mean ± SEM grams increase in maternal body weight from GD 1

d - Mean ± SEM number of live births/litter through GD 21

N/A - Not applicable

Anesthesia and surgery:

Urethane (Sigma-Aldrich) solution (1.1 mg/kg; 1ml/kg) was delivered intraperitoneally (i.p.) divided into 2 injections 30 minutes apart. When the interdigital reflex could no longer be detected, animals were placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). At the site of the incision, Lidocaine HCL (2%) was injected subcutaneously for local anesthesia. Vital signs and body temperature were monitored throughout the entire surgical and recording procedures using a PhysioSuite® Systems (Kent Scientific, Torrington, CT).

After exposing the skull, four holes were drilled for the attaching self-tapping screws - with two serving as ground and reference for the recording circuit, and one serving as the return component of the stimulating circuits – and for the biosensor external reference electrode. Two holes were drilled for monopolar stimulating and recording electrodes (Teflon-coated, stainless steel, 114µm diameter, AM Systems, Poulsbo, WA). Following the Paxinos and Watson, 2004, atlas, the recording electrode was implanted into the hilus of the DG (-3.5 mm posterior and 1.8 mm lateral to the bregma), and the stimulating electrode was

implanted into the angular bundle of the perforant path (-8.1 mm posterior and 4.3 mm lateral to bregma). The biosensor electrode was implanted in the same location as the recording electrode (Pinnacle Technology, Inc. USA) and a reference electrode, 100 µm Ag/AgCl wire was inserted on the contralateral side of the brain. Following the glutamate biosensor placement, the animals received an injection of SAR152954 (1.0 mg/kg) diluted in sterile PBS (volume 1mL/100g) or an equivalent volume of PBS and were monitored for 30 minutes before the recording session.

in vivo Electrophysiology protocol:

The electrodes were connected to an isolated pulse stimulator (Model 2100; A-M Systems) and to a differential AC amplifier (Model 1800; A-M Systems). Recording signals were amplified (10000X), band pass-filtered (0.1 Hz to 10 kHz) and transferred to a personal computer via an analog-to-digital converter (Models PCI-6221 and BNC-2090; National Instruments, Austin, TX). The responses were evoked by 400 µA stimulus (0.1ms, 1/10 sec), and the electrodes were lowered into the tissue by 50 – 100 µm increments until optimally placed. A positive-going excitatory postsynaptic potential (EPSP), with a superimposed population spike at a latency of less than 5 mV was indication that the recording electrode was in the hilus of the DG and that enough lateral fibers were being stimulated.

Following the electrodes placement, an input-output curve (50 - 500 µA, each of 6 current intensities repeated 5 times, 1/30 sec) was used to determine the EC40 (intensity equal to 40% of maximum response) of each animal. EC40 current intensities ranged from 200-300µA. Once the EC40 was obtained, the recording electrode was removed, and -

following the same coordinates as the recording electrode, fig. 5 - the glutamate biosensor (fig. 6) was implanted in the hilus of the DG.

Baseline evoked recordings were obtained for 30 min. Synaptic potentiation was induced by 5 trains of high-frequency stimulation (HFS; 400Hz, 25ms duration) with 30s inter-train intervals. Post-HFS evoked recordings were obtained for 60 min. Slope and amplitude of responses for glutamate. Fractional change of the post-HFS fEPSP relative to baseline was calculated for 1 min intervals. Additionally, continuous data were obtained at 1kHz throughout the baseline and post-HFS phases. Continuous data were analyzed with the following formula to calculate fractional changes between the baseline and postHFS phases for each of the 60 timepoints.

$$Fractional\ change\ postHFS = \frac{(postHFS - [mean(baseline)]}{[mean(baseline)]}$$

RM-ANOVAs were perfomed on fractional changes of each timepoint with Diet as between-subject factor.

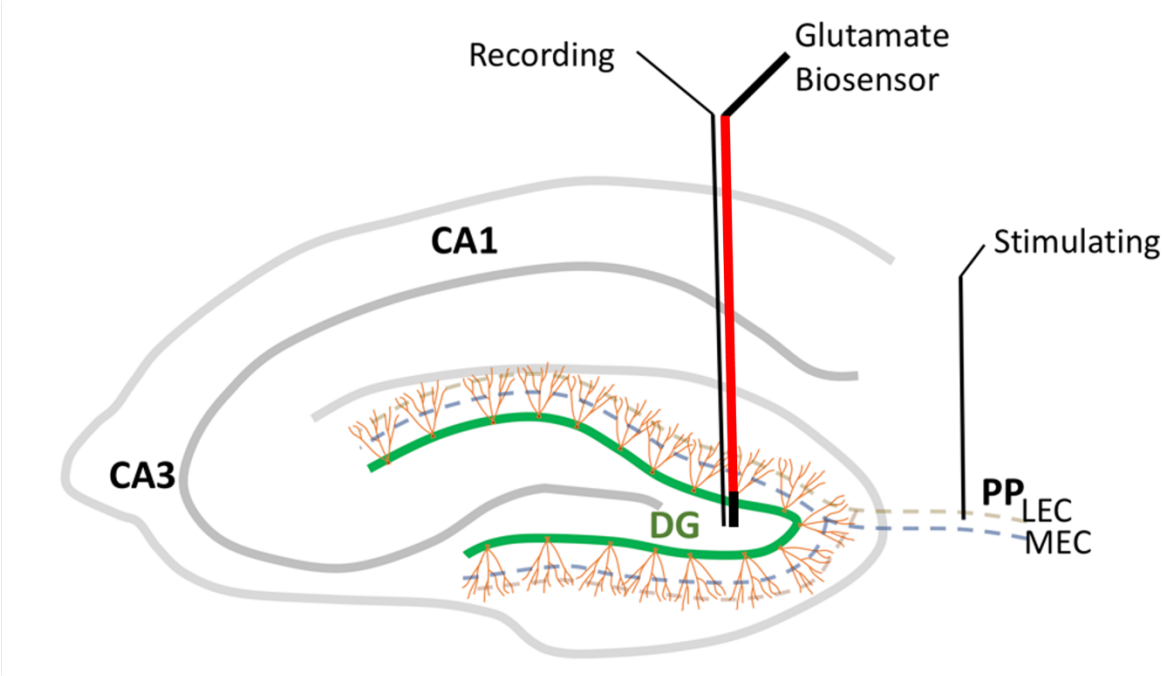

Figure 5. Diagram of the hippocampal formation showing the dentate gyrus and the locations of electrode implantations. After the I/O curve was obtained – for the biosensor recording – the final coordinate of the recording electrode was recorded, the electrode was then removed and replaced by the glutamate biosensor at that same coordinate. The red line represents the active portion of the biosensor and the bottom black represents the epoxy tip (inactive).

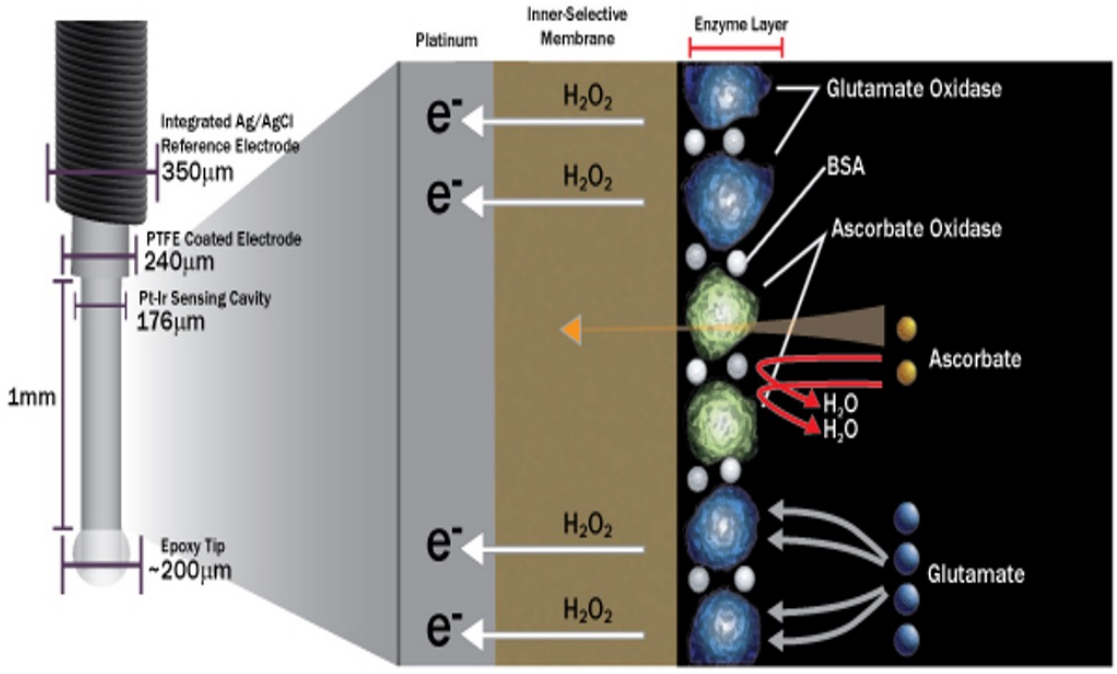

Figure 6. Glutamate biosensor from Pinnacle showing the specifics about measurements and active enzymes.

2.2.2. Statistical analyses

Post-HFS fractional change of values from the continuous data were analyzed with timepoint as a repeated measure (60 values), normalized to for each rat's average baseline, (Fig. 7 shows representative traces of the evoked responses in terms of both amplitude and slope metrics (medians). However, it's worth noting that the delayed return to baseline typically does not accurately reflect responses to PAE). Prenatal Exposure was included as between-subjects factor. The RM-ANOVA analyses were performed between the SAC and PAE groups.

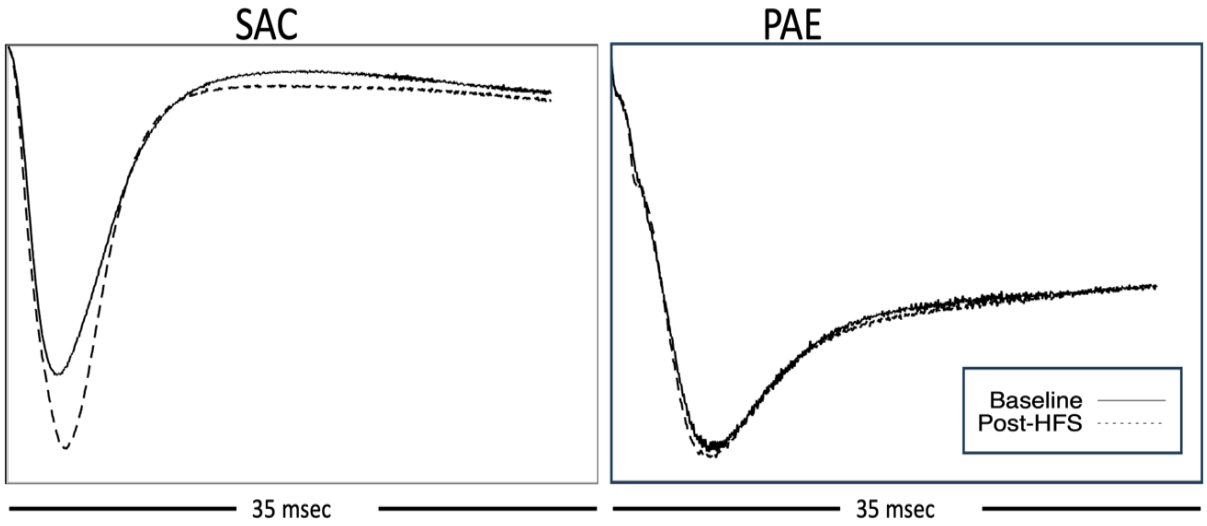

Figure 7.Traces exhibit representative characteristics of the glutamate biosensor evoked responses in terms of both amplitude and slope metrics (medians). However, it's worth noting that the delayed return to baseline typically does not accurately reflect responses to PAE.

2.3. Results

Glutamate amplitude measures

One-way ANOVA revealed significant difference in fractional change amplitude (SAC > PAE), $F(19) = 5.317$, $p = 0.033$, $\eta^2p = .228$, there was no significant difference in the slope ($p = .132$). Fig. 8 shows the bar plots for fractional change in amplitude and slope. The amplitude

for the PAE group decreased after HFS. There was a numeral difference in the slope which was not statistically significant.

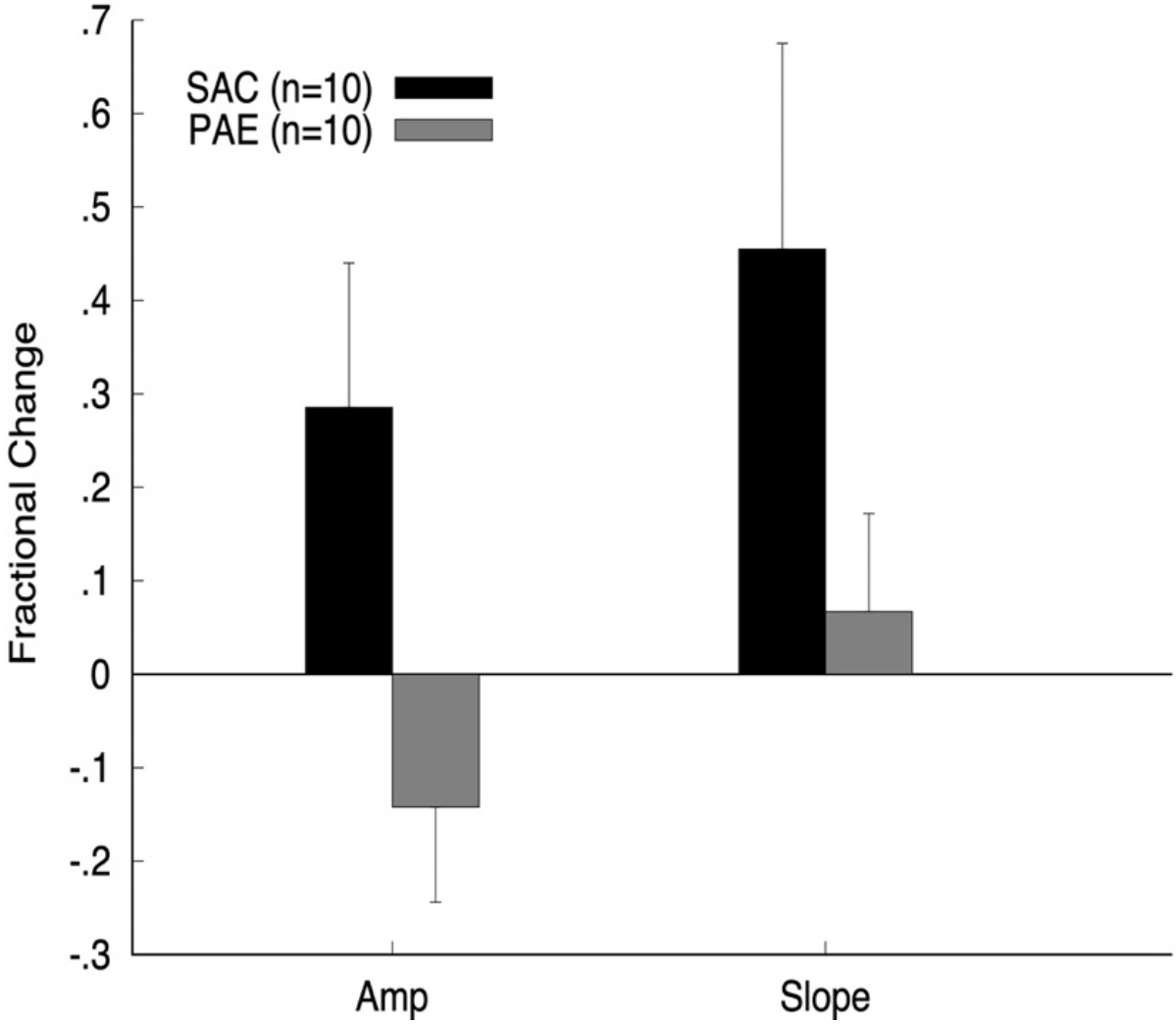

Figure 8. Statistical summary of the impact of prenatal ethanol exposure on glutamate release levels in the dentate gyrus. Data bars are the mean + SEM fractional change in amplitude (p = 0.033) and slope (p = 0.132).

SAC-PAE

Results from the continuous data of the glutamate biosensor, provide evidence for a difference in extracellular glutamate levels following moderate PAE when compared to non-exposed animals. Following HFS, a significant main effect of glutamate level was observed (fig. 9), $F(1, 14) = 4.80$, $p = 0.046$, $\eta^2p = 0.255$, indicating differences across different time

points during the postHFS phase when compared with the baseline phase. The diet was found to be statistically significant, $F(58) = 3.20$, $p < 0.001$, $\eta^2p = 0.186$, providing evidence for the PAE effects on extracellular glutamate levels.

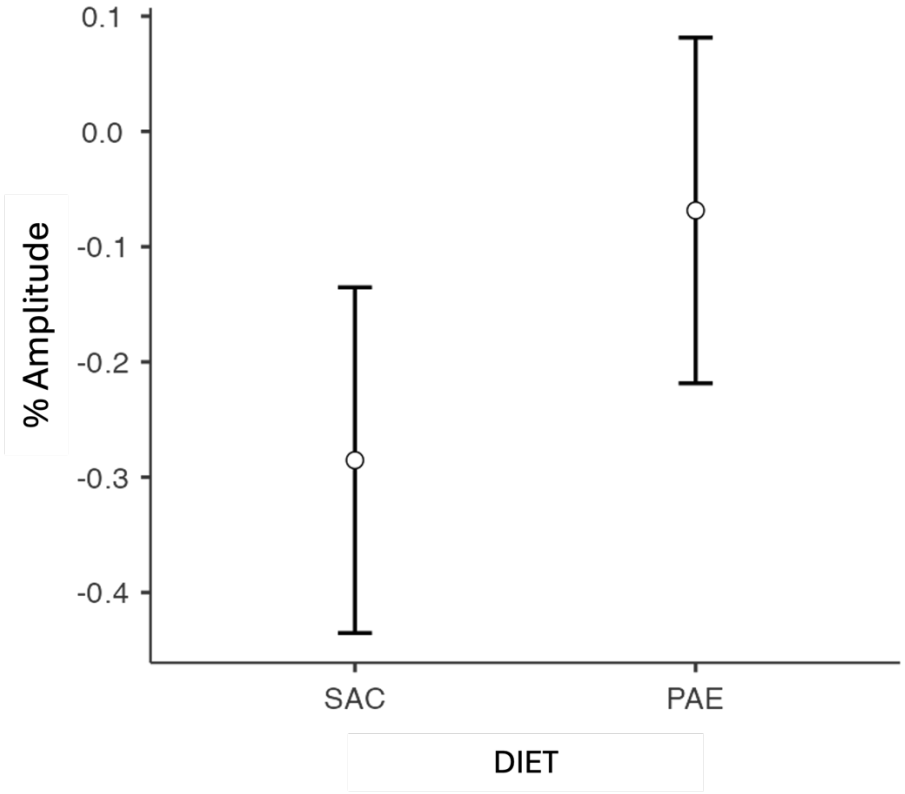

Figure 9. Estimated marginal means for the fractional change in amplitude postHFS for the SAC and PAE groups. RM-ANOVA shows a main effect of diet on glutamate release levels (p = 0.046).

2.4. Discussion and conclusion

To my knowledge, this is the first study to combine in vivo LTP and glutamate biosensor recordings in an animal model of moderate PAE to record in vivo extracellular level of glutamate following HFS. The results show a difference in fractional change in the amplitude of the evoked response following HFS, but not in the slope. Analysis of the continuous data of the biosensor reveals diminished extracellular glutamate level after HFS

in exposed animals when compared with control animals. These results support previous electrophysiological data showing alcohol-related deficits in potentiation as shown and discussed in chapter 3. The combination of analyses of the evoked-responses and the continuous data of the glutamate biosensor adds to the existing literature reporting deficits in the neurocircuit of the hippocampal area. These results provide evidence of some impairment in the glutamatergic system following moderate PAE and support previous literature suggesting an alteration in glutamate release which is consistent with presynaptic disruptions (Varaschin et al., 2014, 2018).

The amplitude-based analysis of fractional change of the continuous data between baseline and postHFS do not measure the absolute value of extracellular glutamate concentration. Future studies aiming to investigate the concentration of glutamate should focus on the absolute values by doing a biosensor calibration prior to recording to have a reference value.

Chapter 3 (section 1) – PAE-related deficits in LTP and pharmacological intervention

Note: At the time of the presentation of this work, this chapter has been submitted for publication (currently under review) and is reproduced here as originally submitted to the journal which may be slightly different from the final published article. References are listed in the reference session of this document.

The histamine H3 receptor inverse agonist SAR-152954 reverses deficits in long-term potentiation associated with moderate prenatal alcohol exposure

Monica Goncalves-Garcia, M.S., Suzy Davies, Ph.D., Daniel D. Savage, Ph.D., Derek Hamilton, Ph.D.

Prenatal alcohol exposure can have persistent effects on learning, memory, and synaptic plasticity. Previous work from our group has demonstrated deficits in long- term potentiation (LTP) of excitatory synapses on dentate gyrus granule cells in adult offspring of rat dams that consumed moderate levels of alcohol during pregnancy. At present, there are no pharmacotherapeutic agents approved for these deficits. Prior work established that systemic administration of the histaminergic H3R inverse agonist ABT-239 reversed deficits in LTP observed following moderate PAE. The present study examines the effect of a separate H3R inverse agonist, SAR-152594, on LTP deficits following moderate PAE. We demonstrate that systemic administration of 1 mg/kg of SAR-152594 reverses deficits in potentiation of field excitatory post-synaptic potentials (fEPSPs) in adult male rats exposed to moderate PAE. Time-frequency analyses of evoked responses revealed PAE-related reductions in power during the fEPSP, and increased power during later components of evoked responses which are associated with feedback circuitry that are typically not assessed with traditional amplitude-based measures. Both effects were reversed by SAR152954. These findings provide further evidence that H3R inverse agonism is a potential therapeutic strategy to address deficits in synaptic plasticity associated with PAE.

3.1. Introduction

Prenatal alcohol exposure (PAE) leads to a well-established condition known as Fetal Alcohol Spectrum Disorder (FASD) – with a prevalence of 1-5% in the United States, which constitutes a public health major concern. Persistent cognitive impairments represent a subset within the broad range of developmental deficits encompassing morphological and neurobiological alterations (Mattson et al., 2019; Popova et al., 2017). The hippocampal formation has been one site of interest in research due to compelling evidence that it is particularly sensitive to the harmful effects of PAE (Brady et al., 2013; Sutherland et al., 1998;

Valenzuela et al., 2012). Our laboratories have also reported spatial (Rodriguez et al., 2016; Savage et al., 2010) and associative learning and memory deficits (Savage et al., 2010) in adult rat offspring whose mothers consumed moderate quantities of ethanol during pregnancy (blood alcohol concentration [BAC] 60-80 mg/dL). Animal models of PAE have demonstrated that PAE-associated learning deficits were more apparent with increasingly challenging versions of behavioral tasks (Hamilton et al., 2014; Harvey et al., 2020; Sanchez et al., 2019; Savage et al., 2010). Additionally, research has revealed that, at the synaptic level, moderate PAE contributes to disruptions of the molecular mechanisms supporting long-term potentiation (LTP)(Brady et al., 2013; Savage et al., 2010; Sickmann et al., 2014; Valenzuela et al., 2012). The phenomenon of LTP - persistent strengthening of synapses - has been identified at synapses across the brain (Lynch, 2004), however, investigations of LTP have been extensively conducted in the hippocampal formation as LTP is thought to be one of the principal processes undergirding the synaptic basis of learning and memory (Bashir et al., 1993; Larson & Lynch, 1986; Lynch, 2004; McNaughton et al., 1986; Nicoll, 2017; Sutherland et al., 1998).

LTP has been extensively investigated in multiple cortical and hippocampal circuits, however, there has been considerable interest in the input from the entorhinal cortex via the perforant pathway (PP) fiber bundle to dentate gyrus (DG) granule cells following PAE (Brady et al., 2013; Sutherland et al., 1998; Titterness & Christie, 2012; Varaschin et al., 2010). Animal models of PAE using in vitro or in vivo electrophysiology to investigate LTP in the hippocampal formation suggest that there are fundamental differences in the neural mechanisms subserving LTP when compared to non-exposed animals. Even though lower levels of PAE do

not result in physical teratology, studies on the neurobiology of learning and memory processes have identified cognitive impairments (Sutherland et al., 1998). Significant decreases in LTP have been observed in the DG cells following high-frequency stimulation (HFS – trains, or tetanus, of stimulation, or theta burst stimulation [TBS]) in the perforant pathway (Brady et al., 2013; Harvey et al., 2020; Sutherland et al., 1998; Varaschin et al., 2010). The precise mechanisms underlying these outcomes are, however, not fully understood.

Neurochemical and physiological studies (Galindo et al., 2004; Perrone-Bizzozero et al., 1998; Savage et al., 2002) have established that moderate PAE disrupts presynaptic mechanisms influencing glutamate release in DG, contributing to synaptic plasticity and learning deficits in PAE rats (Varaschin et al., 2010, 2014, 2018). Type 3 histamine receptors (H3Rs) on nerve terminals inhibit the release of various transmitters including histamine (Arrang et al., 1983), other monoamines (Schlicker et al., 1988, 1989, 1993), acetylcholine (Clapham & Kilpatrick, 1992) and glutamate (Garduño-Torres et al., 2007). H3R inverse agonists interfere with H3R function, enhancing neurotransmitter release and showing procognitive effects in diverse animal models of learning and memory processes (see reviews by Haas et al., 2008; Esbenshade et al., 2008; Brioni et al., 2011; Nikolic et al., 2014). These observations motivated investigation of H3R inverse agonists as a potential pharmacotherapeutic intervention for PAE. A 1.0 mg/kg dose of the H3R inverse agonist ABT-239, improved contextual fear conditioning and spatial memory deficits in PAE rats (Savage et al., 2010). ABT-239 also reversed deficits in DG LTP induced by moderate PAE (Varaschin et al., 2010), while the selective H3R agonist methimepip replicated LTP deficits in control

animals (Varaschin et al., 2014). PAE did not impact H3R density as shown by radiohistochemical studies in female rats using the selective H3R antagonist [3H]-A349821 (Varaschin et al., 2018). However, Davies et al. (2023) have also recently demonstrated increased expression of the rH3A and rH3C mRNA isoforms of H3Rs in the dentate gyrus and entorhinal cortex of male, but not female, PAE rats. Further, radiohistochemical studies using the selective H3R agonist methimepip revealed increased H3R function in hippocampal and cortical regions of PAE rats, as measured by enhanced methimepip-stimulated [35S]-GTPγS binding (Varaschin et al., 2018). Subsequent electrophysiology experiments demonstrated that methimepip heightened inhibition of glutamate release in the DG, particularly in PAE rats (Varaschin et al., 2018). The concentrations of methimepip affecting glutamate release were similar to those enhancing GTP binding in the DG of PAE rats. Overall, these findings imply an elevation of H3R-mediated inhibition of glutamate release in the DG and other cortical regions in PAE offspring. Further this heightened H3R-mediated inhibitory influence can be mitigated by H3R inverse agonists like ABT-239.

Even though ABT-239 showed promising results, it did not advance to clinical trials due to cardiac QT prolongation (Hancock, 2006). Aiming to identify a pharmacological intervention for clinical populations and to further examine the effects of H3R inverse agonism on LTP in PAE rats, the present study investigates another H3R inverse agonist agent – SAR-152954 – with a similar mechanism of action as ABT-239, but with no major safety issues in Phase I clinical trials. SAR-152954 is an orally active non-imidazole H3R inverse agonist that readily crosses the blood-brain barrier with a high affinity for binding to the rat (4.9 nM) and human (2.8 nM) H3 receptor. In rats, the IC50 for SAR-152954 inhibition of ∝-

methylhistamine-stimulated [35S]-GTPγS binding is 17 nM. SAR-152954 has a greater than 10,000-fold selectivity for the H3 receptor relative to H1, H2 and H4 receptors and, at 10 nM, it is inactive against more than 150 receptors, ion channels, kinases and other enzyme targets. Oral SAR-152954 is rapidly absorbed, reaching peak plasma levels at approximately 30 minutes, with a plasma half-life of about 2 hours in rats.

In the present study, we investigated how PAE impacts DG granule cells responsiveness and LTP in vivo. We injected SAR-152954 intraperitoneally (i.p). at the same 1.0 mg/kg dose as ABT-239 in our previous study (Varaschin et al., 2010). We hypothesized that SAR-152954, would not impact baseline DG cells input/output function, but would improve the LTP deficits following moderate PAE. In addition to standard amplitude-based measures, we performed time-frequency analyses to assess the effects of both PAE and SAR-152954. Time-frequency analysis of evoked responses involves converting amplitude information to frequency information for the quantification of changes in event-related power with temporal precision. The motivation to include this approach was based on the possibility that time-frequency analyses could detect PAE-related differences in evoked responses that could be missed by traditional amplitude-based measures, and possibly provide a more sensitive measure of PAE-related or treatment-related effects. As this is a novel approach to analysis of LTP deficits observed following PAE, a description of time-frequency analyses intended to be broadly accessible is provided in section 2.3. These findings could further validate the efficacy of H3R inverse agonists for overcoming synaptic plasticity deficits associated with PAE employing an agent with a promising safety profile based on Phase I clinical trial data.

3.2 Methods

3.2.1. Animals

Thirty-six (18 SAC and 18 PAE) 3- to 9-month-old male Long-Evans rat offspring housed at the University of New Mexico Main Campus Animal Resource Facility were used in the present study. All animals were pair-housed in plastic cages on a 12 h light-dark cycle with food and water available ad libitum. The experiments reported here were approved by the Institutional Animal Care and Use Committees (IACUCs) of the Health Sciences Center and Main Campus of the University of New Mexico.

3.2.2. Breeding Colony and Ethanol Consumption Procedures

The breeding and ethanol consumption procedures were the same as described in Chapter 2.

3.2.3. Anesthesia and surgery

Electrophysiology data collection and drug treatment were performed by an experimenter who was blind to prenatal treatment and drug condition. Urethane (Sigma-Aldrich) solution (1.1 mg/kg; 1 ml/kg) was delivered intraperitoneally divided into 2 injections 30 minutes apart. When the interdigital reflex could no longer be detected, animals were placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Lidocaine HCl (2%) was injected subcutaneously into the scalp for local anesthesia prior to incision. Vital signs and body temperature were monitored throughout the entire surgical and recording procedures using a PhysioSuite® Systems (Kent Scientific, Torrington, CT).

After exposing the skull, three holes were drilled for attaching self-tapping screws with two serving as ground and reference for the recording circuit, and one serving as the

return component of the stimulating circuits. Two holes were drilled for monopolar stimulating and recording electrodes (Teflon-coated, stainless steel, 114 µm diameter, AM Systems, Poulsbo, WA). Following the Paxinos and Watson 2004 atlas, the recording electrode was implanted into the hilus of the dentate gyrus (DG) (-3.5 mm posterior and 1.8 mm lateral to the bregma), and the stimulating electrode was implanted into the angular bundle of the perforant path (-8.1 mm posterior and 4.3 mm lateral to bregma).

3.2.4. *in vivo* Electrophysiology protocol and Time-frequency Analyses

The electrodes were connected to an isolated pulse stimulator (Model 2100; A-M Systems) and to a differential AC amplifier (Model 1800; A-M Systems). Recording signals were amplified (10,000X), band pass-filtered (0.1 Hz to 10 kHz) and transferred to a personal computer via an analog-to-digital converter (Models PCI-6221 and BNC-2090; National Instruments, Austin, TX). The responses were evoked by 400 µA stimulus (0.1 ms, 1/10 sec) and the electrodes were lowered into the tissue by 50 - 100 µm increments until optimally placed. A positive-going excitatory postsynaptic potential (EPSP), with a superimposed population spike at a latency of less than 5 mV were indication that the recording electrode was in the hilus of the DG and that an adequate number of lateral fibers were being stimulated.

Following the electrode placements, the animals received an injection of SAR-152954 (1.0 mg/kg) diluted in sterile PBS (volume 1 mL/100 g) or an equivalent volume of PBS and were monitored for 30 minutes prior to the recording session. The drug or vehicle was prepared for the experimenter by another individual to maintain blinding of drug conditions. For each evoked response, fEPSP slopes were measured for an epoch of 500 µs centered on

the midway point of the fEPSP and population spike amplitudes were quantified at the minimum of the population spike using a custom Matlab script that automated identification and quantification of the fEPSP and population spike. All responses were screened for accuracy. An input-output curve (50 - 500 µA, each of 6 current intensities repeated 5 times, 1/30 sec) was used to determine the EC_{40} (current intensity equal to 40% of maximum response) of each animal. Input-output curves were conducted by an experimenter blind to experimental conditions. EC_{40} selection was based on the population spike amplitude to ensure that evoked responses included population spikes. The EC_{40} current was used for all subsequent phases of the study. EC_{40} intensities ranged from 200-300 µA. Baseline recordings were obtained for 20 min. Synaptic potentiation was induced by 5 trains of high frequency stimulation (HFS; 400Hz, 25 ms duration) with 30 s inter-train intervals, which pilot work revealed produced robust deficits of fEPSP LTP following moderate PAE. Post-HFS recordings were obtained for 60 min. Fractional change of the post-HFS fEPSP relative to baseline was calculated for 1 min intervals.

Time-frequency analyses were conducted to evaluate dynamic changes in the frequency composition of evoked responses. Event-related spectral perturbations (ERSPs) are commonly utilized to analyze changes in the frequency domain associated with a specific event. Amplitude information is converted to frequency information, while the timing related to the critical event is maintained. Whereas event-related potentials (ERPs) in electroencephalography (EEG) or magnetoencephalography (MEG) studies focus on the amplitude and timing of the signal of interest, ERSPs provide information about the timing of spectral power within frequency bands of interest (typically those in the range of 1-60 Hz).

With respect to LTP, the critical event is the electrical stimulus and the principal factors quantified for analysis are amplitude-based measures including the slope of the fEPSP (mV/msec) and amplitude of the population spike.

The principal disadvantage to the time-frequency is the complexity of the analysis and interpretation compared to traditional amplitude-based metrics. There are, however, several advantages of time-frequency analysis. Individual (between-animal) differences in response amplitude exist, and LTP studies typically calculate the fractional change in amplitude from baseline to the post-HFS period. Though the variation may not be great, it can complicate interpretation of differences observed at baseline. The time-frequency approach involves conversion of the response to the frequency domain, removing amplitude as a factor. The time-frequency approach also provides information about the entire duration of the response rather than being limited to specific points in time. This allows for analysis of some aspects of the response that do not lend themselves well to traditional amplitude-based measures, such as the aspects of the evoked response that follow the second peak (following the population spike) which are associated with feedback within the dentate gyrus. Time-frequency analyses may also provide for more sensitive measures of group differences at or around time-points associated with traditional targets of analysis.

Time-frequency analyses were performed in Matlab using a custom script based on the wavelet approach described in Cohen.[1] Stimulus artifacts were removed to avoid

[1] EEGLAB is an open-source Matlab toolbox that includes time-frequency analyses. Because signals in the present study were sampled at higher frequency (20kHz) than EEG studies that EEGLAB was designed for, a custom script was prepared to ensure the accuracy of results. This script and a sample evoked response are included in the supplementary materials.

contamination of the time-frequency data. Fourier transformation (FFT) and wavelet transformation (WT) were performed for each evoked response for a frequency range of 1-10 kHz. To provide context, the typical duration of an fEPSP is ~1.25 msec with associated spectral power of ~700-3000 Hz over the course of the fEPSP. Thus, the spectral power expressed in the time-frequency analyses presented here include substantially higher frequencies than observed in studies of continuous or evoked human EEG studies. The resulting values were expressed as percent signal relative to the period before the stimulus. Fractional change in spectral power was calculated in the same manner as the amplitude measures for evoked responses (i.e., (post-HFS – baseline)/baseline) where a value of 0 indicates no change and a value of 1 corresponds to a 100% increase in signal power. Independent samples t-tests (PAE – SAC) were performed separately in the Vehicle control condition and SAR-152954 condition. The data from the post-HFS period were limited to responses obtained over the final 48 min. (comprised of the last eight 6-min epochs), which was selected to ensure that the evoked responses were stable (asymptotic). Thus, the time frequency analyses do not include responses during the PTP period of the post-HFS period.

3.2.5. Statistical analyses:

For RM-ANOVAs, significant Mauchly's tests for sphericity were followed by Greenhouse-Geisser corrected tests. For LTP, the mean fEPSP slope values were averaged for each minute (2 observations at 1/30sec) and normalized for each rat to the rat's average baseline fEPSP response, providing a fractional change for each post-HFS observation. Post-HFS fractional change values were analyzed at each time point as a repeated measure (60 values). Prenatal Exposure and Drug Treatment were included as between-subjects factors. Follow-up analyses included separate assessments of Prenatal Exposure and Drug Treatment main effects and interactions for each of 10 post-HFS epochs that were 6 min in duration.

For time-frequency analyses, an independent samples t-test were performed in the vehicle condition (PAE – SAC) to evaluate effects of PAE. A separate independent samples t-test was performed to evaluate the effects of SAR-152594 in the PAE group. An alpha of $p < 0.025$ was applied to these tests. If SAR-152594 normalizes responses in PAE rats, then a comparison (PAE-SAC) in the SAR152594 condition should yield null effects.

3.3. Results

Mean ethanol consumption, maternal weight gain, litter size, and birth weight are provided in Table 2.

IO Curve

Mean IO Curves for each combination of Prenatal Exposure and Drug Treatment are shown in Fig. 10. fEPSP slopes increased as a function of current [Greenhouse-Geisser $F(1.109, 35.494) = 129.13$, $p < 0.001$, $\eta_p^2 = .801$]. Although there was a numerical decrease in responses in animals that received treatment with SAR-152954, there were no significant main effects of Drug Treatment or Prenatal Exposure, and no interaction between variables [all ps > 0.49].

LTP. Amplitude Measures

Sample evoked responses from each group at baseline and following high-frequency stimulation are shown in Fig. 11. Mean fractional change in fEPSP values for each combination of Prenatal Exposure and Drug Treatment are shown in Fig. 12. There was a significant Time-point X Prenatal Exposure X Drug Treatment interaction [$F(59, 1888) = 1.70$, $p < 0.001$, $\eta_p^2 = .051$]. The main effects of Prenatal Exposure and Drug Treatment, and the interaction between these factors were not significant [all ps > 0.066]. Follow-up analyses revealed a significant main effect of Prenatal Exposure [SAC > PAE] within the Vehicle Drug Treatment group [$F(1, 16) = 6.30$, $p = 0.023$, $\eta_p^2 = .282$]. The Time-point X Prenatal Exposure interaction within the Vehicle group was not significant [$p = 0.53$]. Within the SAR-152954 group the Prenatal Exposure effect and the Time-point X Prenatal Exposure interaction were not significant [all ps > 0.72].

ANOVAs performed on the 10 six-minute periods following HFS revealed significant Prenatal Exposure effects [SAC > PAE] in the Vehicle condition for periods 3-10 (13-60 minutes post-HFS; all ps < 0.035, see Fig. 12), and no significant Prenatal Exposure effects for periods 1-2 (1-12 minutes post-HFS) which is inclusive of the immediate post-tetanic potentiation period. Within the SAR-152954 group, there were no significant Prenatal Exposure effects for any of the 10 periods (1-60 min; all ps > 0.3). A separate ANOVA on the mean fractional fEPSP during the final 6-min period (55-60 minutes post-HFS; see Fig. 13) revealed a significant Prenatal Exposure X Drug Treatment interaction [F(1, 32) = 5.09, p = 0.031, η_p^2 = 0.137]. The interaction resulted from a significant Prenatal Exposure effect [SAC>PAE] for animals in the Vehicle condition [F(1, 16) = 5.78, p = 0.029, η_p^2 = 0.265] that was not present for animals that received treatment with SAR-152954 (p > 0.43).

LTP. Time-frequency analyses

Figure 14 shows an example of a time-frequency response with the associated evoked response overlaid to illustrate the relationship between principal events of the evoked response (fEPSP, population spike, etc.) and time-frequency data. Mean fractional change in time-frequency responses during the post-HFS period for each condition is illustrated in Figure 15. Similar changes in spectral power are evident in all conditions. Following stimulation (0-5 msec), there was a large increase in spectral power from ~400 Hz - ~3000 Hz during the rising phase of the evoked response, inclusive of the population spike and overall peak in the evoked responses. After the overall peak (see peak 2 in Fig. 14) from 5-10 msec after stimulation, which corresponds to the decrease in amplitude of the evoked response and activity in feedback circuitry, there is an increase in spectral power ~100 Hz - ~1000 Hz.

The period from ~10 msec following stimulation to the end of the response (40 msec) includes an increase in power around 100 Hz, and a decrease in power at higher frequencies.

Differences between PAE and saccharin groups in the vehicle condition are shown in Figure 16. Mean values in Fig. 16A highlight two conspicuous outcomes. There is reduced power in several frequency ranges (~1000 Hz - ~3000 Hz) in PAE rats during the timeframe corresponding to the rising phase of the evoked response, including the population spike, and overall peak, and increased power in lower frequency ranges (200 Hz - 1000 Hz) during the time period from 10 msec-40 msec following stimulation. Fig. 16B shows the same values thresholded for statistical significance of $p < 0.025$. There was reduced spectral power during the fEPSP (~2-4 msec following simulation) from ~1200 Hz - ~3000 Hz in PAE rats compared to SAC rats, which appears as two small clusters prior to the marker indicating peak 1 (referenced in Fig. 14). The average test statistic, p-value, and effect size within these two clusters are $t(16) = -2.80$, $p = 0.014$, $\eta_p^2 = 0.329$, respectively. There was increased spectral power from ~10 msec - 40 msec following stimulation from ~100 Hz – 200 Hz. In PAE rats [average $t(16) = 2.64$, $p = 0.018$, $\eta_p^2 = 0.304$]. Differences between the SAR-152594 and vehicle conditions in the PAE group are shown in Fig. 17. Figs. 17A-B show an opposite pattern to that observed for the PAE-SAC comparison in the vehicle condition shown in Figs. 16A-B. In PAE rats that received SAR-152594, there was increased power ~1000 Hz - ~3000 Hz during the first 5 msec after the stimulus, which corresponds to the rising phase of the evoked response, including the population spike, and overall peak, and decreased power in lower frequency ranges (200 Hz - 1000 Hz) during the same time period. There was decreased power in the 100 Hz - 200 Hz range for the period 10 msec - 40 msec after stimulation. There

were a number of clusters with p-values below the 0.025 threshold, and two elements related to the effects of PAE on time frequency outcomes are emphasized here. While there was no apparent reduced spectral power during the fEPSP, as seen in the previous analysis, there were increases in power at ~3000 Hz - 6000 Hz at the overall peak of the evoked response (peak 2 in Figs. 14 and 17). The average test statistic, p-value, and effect size within this two cluster is t(16) = 3.27, p = 0.006, η_p^2 = 0.400, respectively. There was decreased spectral power from ~10 msec – 40 msec following stimulation from ~50 Hz – 100 Hz [averages: t(16) = -3.00, p = 0.008, η_p^2 = .360].

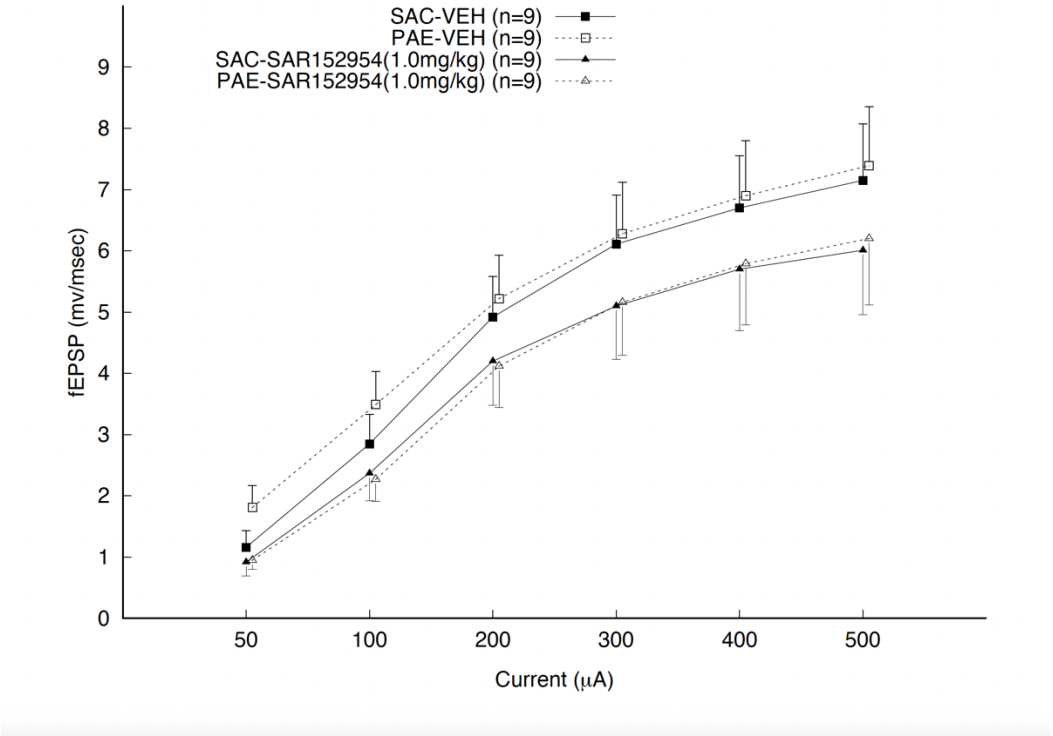

Figure 10. Mean IO Curves for each combination of Prenatal Exposure and Drug Treatment.

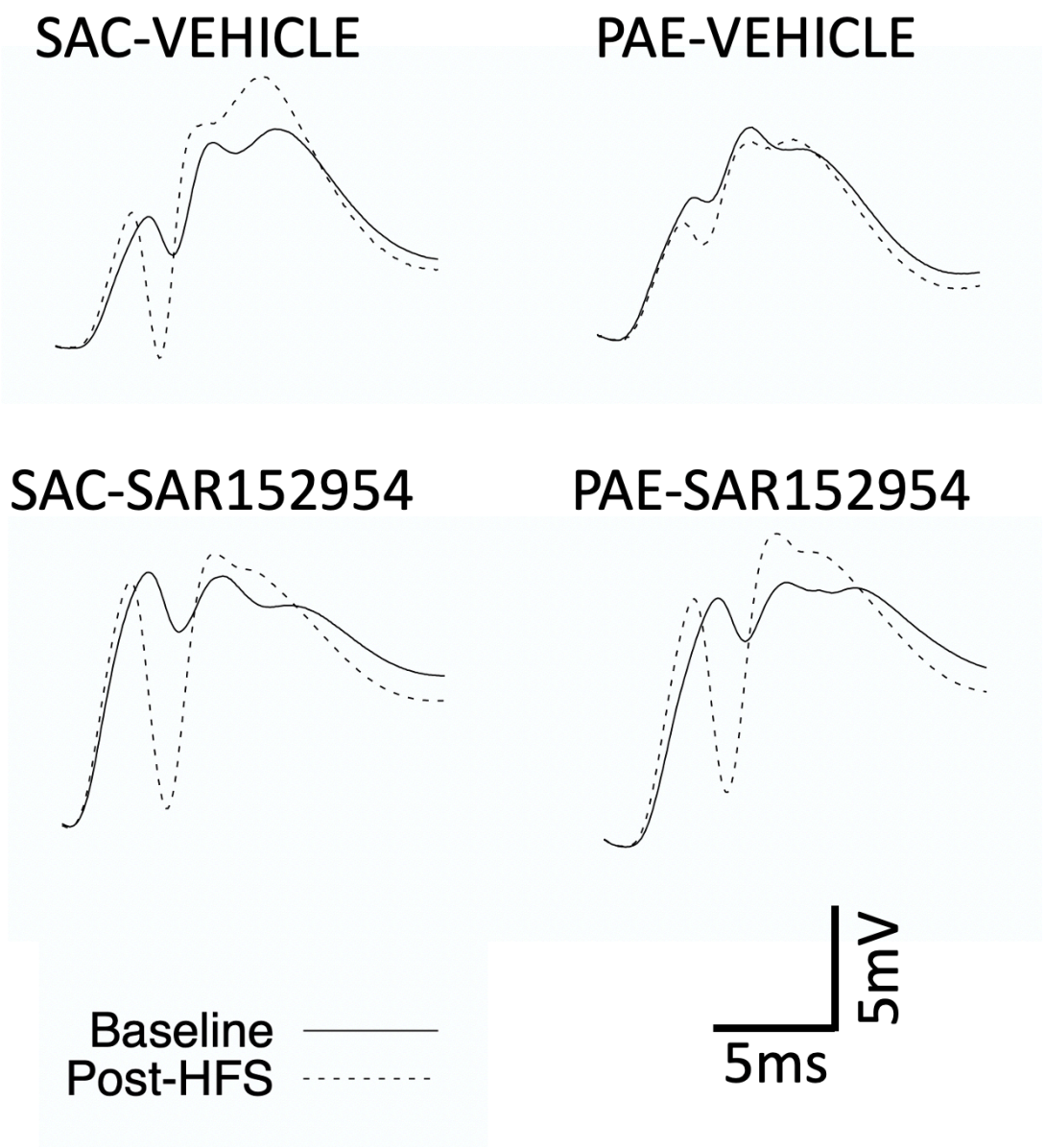

Figure 11. Representative evoked responses during baseline (solid lines) and post-high frequency stimulation (dotted lines) recordings from each group. Sample traces are from an individual animal in each group with median LTP measures during the last 6 minutes of the post-HFS period.

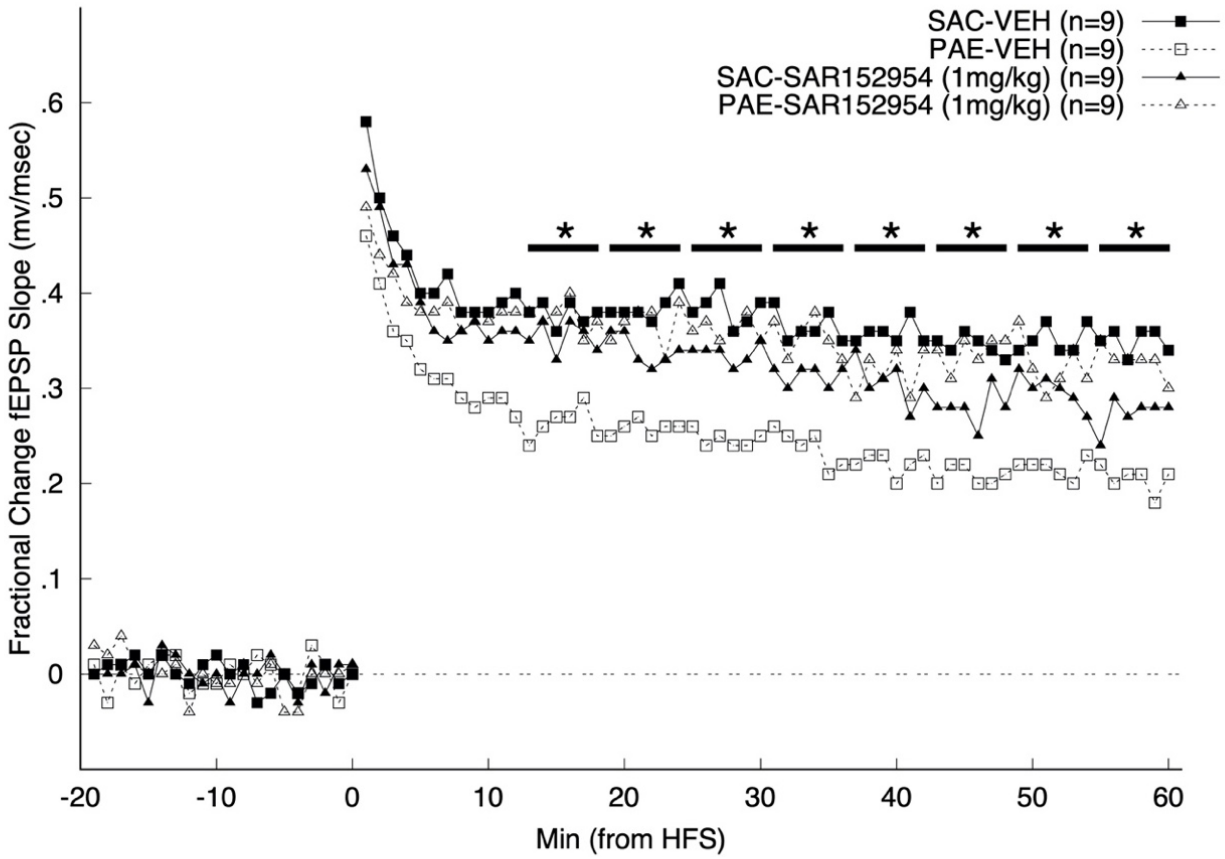

*Figure 12. Mean fractional change in fEPSP for the 20-minute baseline period and 60 minutes after high-frequency stimulation (post-HFS) for each combination of Prenatal Exposure and Drug Treatment * and horizontal bars indicate 6-minute epochs for which there was a significant Prenatal Exposure effect (SAC > PAE) in the Vehicle condition (all p values < 0.035).*

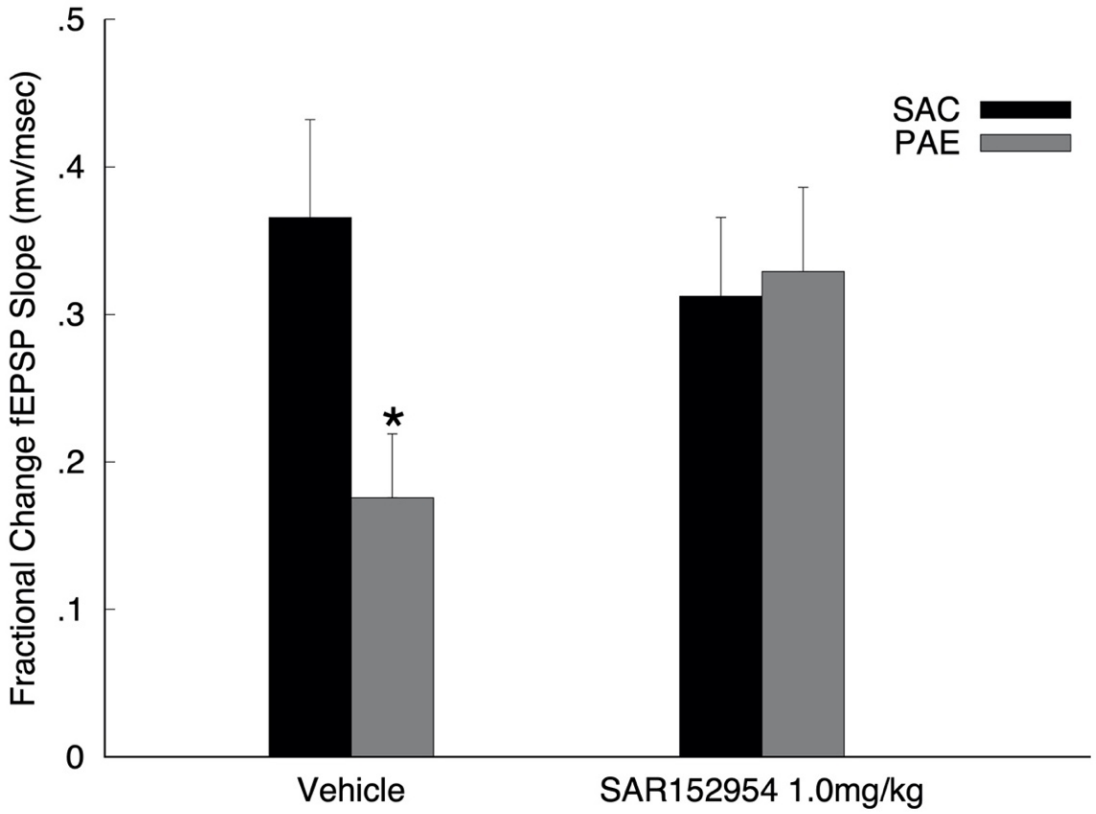

*Figure 13. Mean fractional change in fEPSP for the final 6-minute epoch of the post-HFS period for each combination of Prenatal Exposure and Drug Treatment. * p = 0.029.*

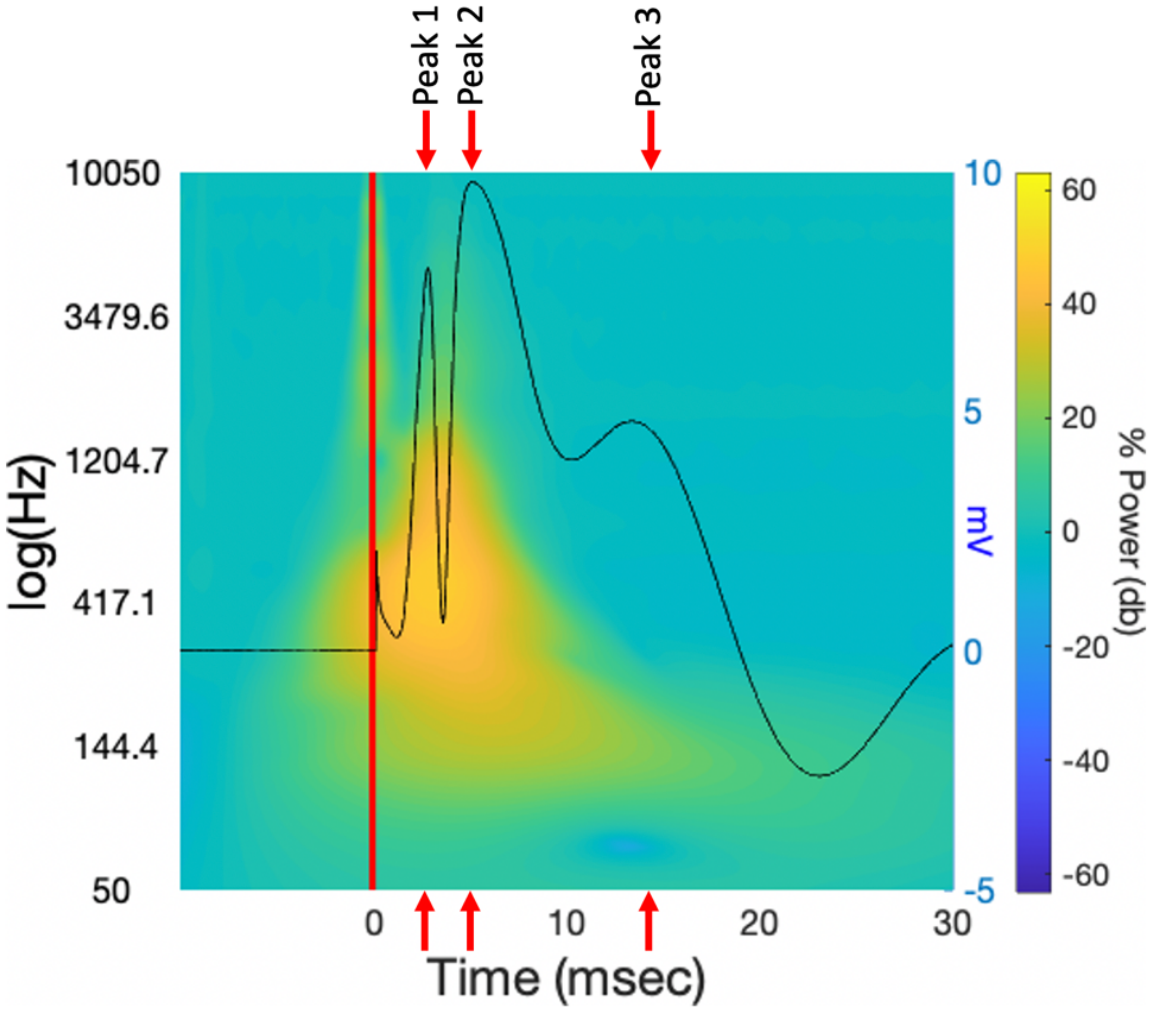

Figure 14. Example average event-related spectral perturbation (ERSP) overlayed by an exemplar evoked response. The y-axis on the left represents the frequency for the ERSP. The y-axis on the right represents the amplitude of the overlayed evoked response (in mV). The x-axis represent time with stimulation occurring at time 0. The colorbar represents the fractional change in signal between the post- and pre-stimulus periods (e.g., 60 represents a 60x increase in signal, -60 represents a 60x decrease). To illustrate the difference between traditional and ERSP measures, consider that the electrical evoked response is analyzed through amplitude-based measures (e.g., fEPSP slope, population spike amplitude), and, therefore, represents a time-amplitude analyses. ERSP analyses involve conversion of amplitude domain signals to frequency domain signals. The time dimension does not differ. A rapid change in amplitude, such as the stimulus artifact at 0 msec (roughly 100-200µsec in duration), will appear as a high-frequency perturbation in the time-frequency domain; see the increase in high-frequency signal at time 0 ranging from ~2500Hz-10KHz. Notes: 1) the sampling frequency was 20KHz, which would allow for signals up to 10KHz to be detected, 2) the relationship of the two y-axis scales in this example figure is arbitrary; the goal is to illustrate the relative timing of frequency-domain signal to the original amplitude signal. Red arrows along the top and bottom of the x-axes are included to mark the timing of prominent events in the evoked responses to aid in the interpretation of time-frequency plots. The first peak is the point in the evoked response and is used to identify the end of the fEPSP for measurement of the fEPSP slope. The second peak marks the highest point of the rising phase of the evoked response. The third peak marks the initial peak in the phase of the evoked response during which the signal amplitude decreases and includes low frequency oscillations related to feedback input.

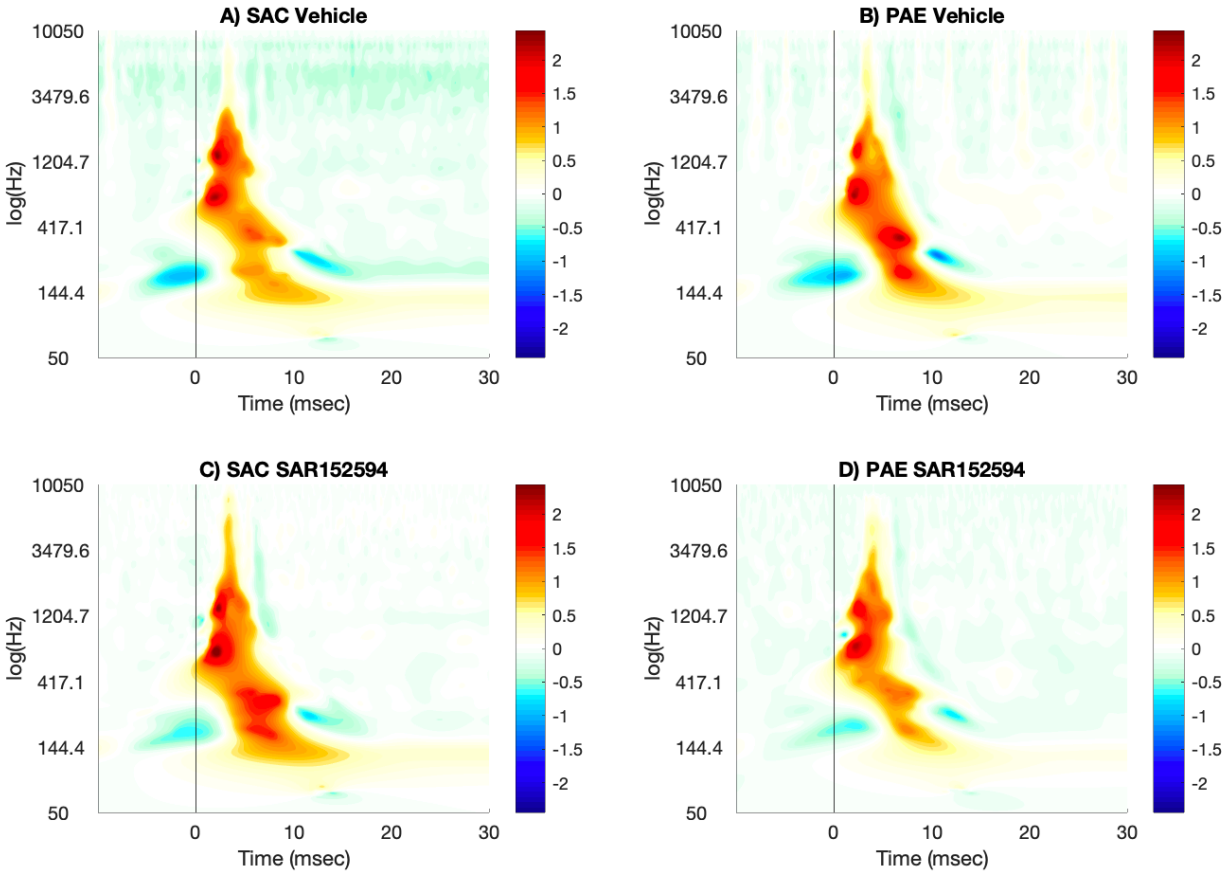

Figure 15. Time-frequency analyses. Plots A (Saccharin Vehicle), B (PAE Vehicle), C (Saccharin SAR152594), and D (PAE SAR152594) show the fractional change in spectral power during the post-HFS period relative to baseline. Warm colors represent an increase in power and cool colors represent a decrease in power.

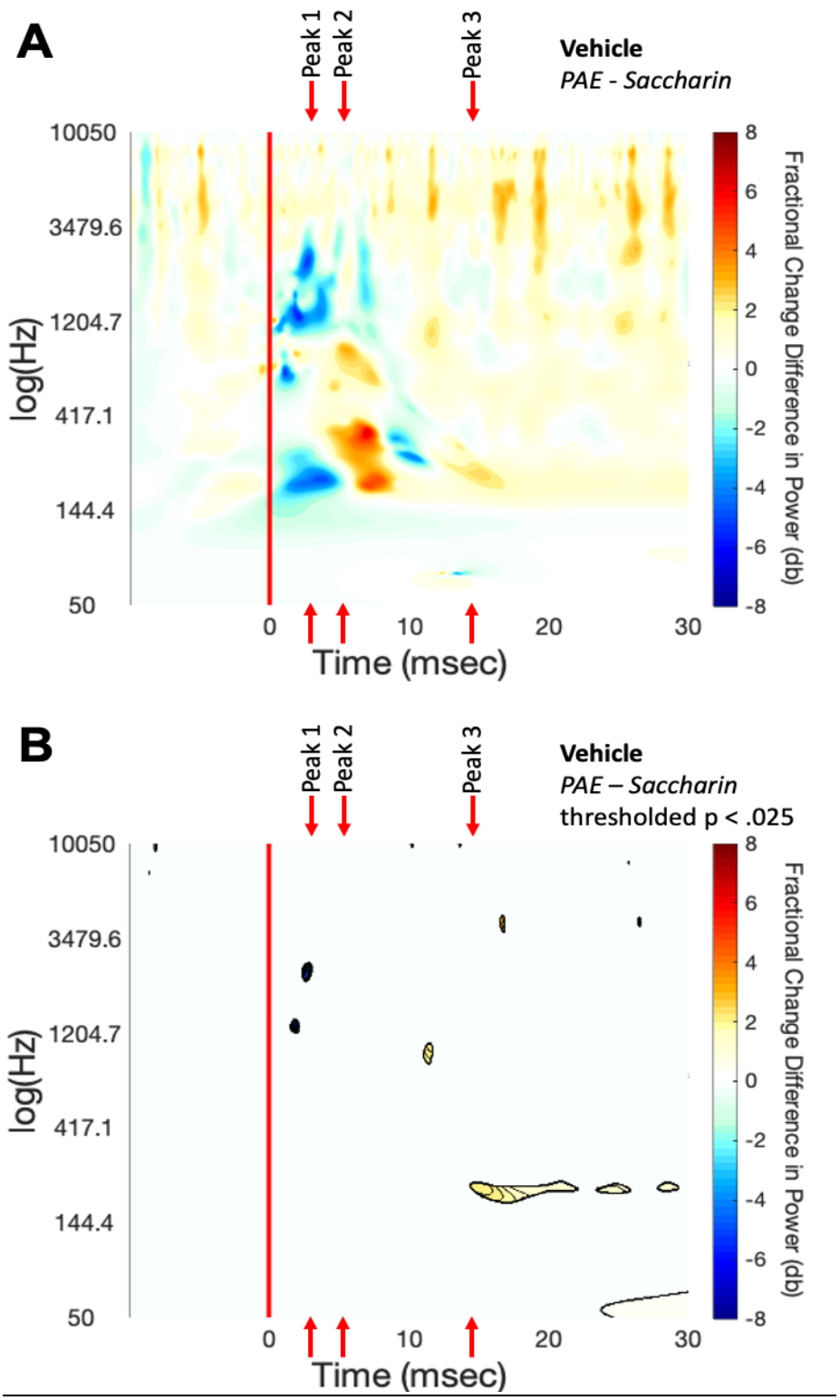

Figure 16. Mean difference (PAE-SAC; Signal in Fig 15B minus signal in Fig. 15A) in fractional change in power in the vehicle condition. Panel A shows the total values unthresholded for statistical significance. Panel B shows the same values thresholded for significance at p < 0.025 outlined (contoured) in black. Red arrows indicate the timing of prominent peaks in the evoked response; refer to Fig. 14 for reference.

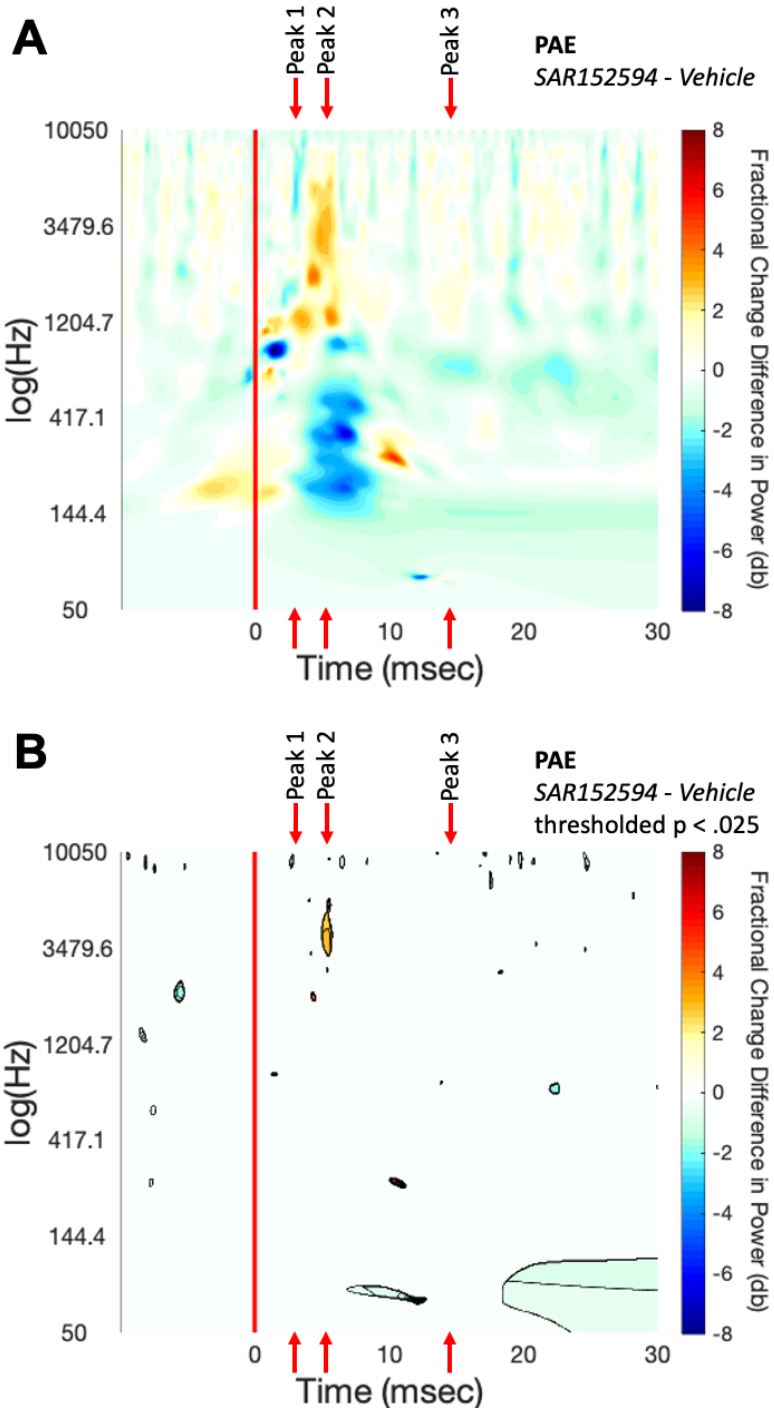

Figure 17. Mean difference (SAR152594 – Vehicle; Signal in Fig 15D minus signal in Fig 15C) in fractional change in power in the PAE group. Panel A shows the total values, unthresholded for statistical significance. Panel B shows the same values thresholded for significance at p<.025 outlined (contoured) in black. Red arrows indicate the timing of prominent peaks in the evoked response; refer to Fig. 14 for reference.

3.4. Discussion

The H3R inverse agonist SAR-152954 reversed LTP-related deficits caused by moderate PAE as measured both by traditional amplitude-based measures and time-frequency analyses. The present findings join our prior reports showing that the H3R inverse agonist ABT-239 improves LTP and learning and memory outcomes following moderate PAE. There were no effects of PAE or SAR-152954 on the population spike or the basic input/output relationships, though there was a numerical decrease in the IO-curve responses with SAR-152954 in both PAE and control animals. These observations suggest that H3R inverse agonism is a potentially fruitful target for treatment of PAE-related deficits in synaptic plasticity, learning, and memory.

Our chosen LTP induction protocol (5 trains of 25 msec, 400 Hz stimulation at EC_{40}) demonstrated reduced fractional change in the fEPSP slope in PAE rats versus saccharin controls. Although no differences were evident in the population spike, this protocol was selected due to its ability, established in pilot work, to consistently reveal robust PAE-related deficits in fEPSP LTP. The selection was based on the expectation that the fEPSP, being most influenced by glutamatergic transmission, aligns with the proposed mechanism of action for H3R inverse agonism. It is important to note that an LTP protocol that included 5 trains of HFS was selected to yield a PAE-related deficit in the fEPSP, as this aspect of the evoked response reflects the rapid effect of presynaptic glutamate release on the post-synaptic target cells. A previous study (Varaschin et al., 2010) revealed significant effects of PAE on the fEPSP and less robust effects on the population spike using three trains of high frequency stimulation (all other factors were the same as described in the present study).

PAE-related impairments in LTP in the vehicle condition replicate prior findings with moderate PAE, and add to the growing body of literature showing that PAE impairs LTP across a broad range of ethanol doses, timing of exposure, and duration of exposure (Helfer et al., 2012; Marquardt & Brigman, 2016; Park et al., 2014; Sickmann et al., 2014; Subramoney et al., 2018; Titterness & Christie, 2012; Varaschin et al., 2010, 2014). A novel aspect of the analytical approach employed here is that application of time-frequency analyses similar to those conducted in human EEG studies revealed notable decreases in spectral power among PAE rats at time points corresponding to the fEPSP. Notably, the effect sizes for these decreases in spectral power were larger than the effect sizes observed for the fEPSP slope measurement suggesting that time frequency measures may be more sensitive to PAE-related effects on potentiation of initial excitatory responses. Furthermore, these analyses revealed increased power among PAE rats during a prolonged period from the peak of the evoked response until the eventual return to baseline, which are typically measured when analyzing evoked responses. These alterations might signify the interplay of excitatory and inhibitory signals within the dentate gyrus feedback circuitry. However, a comprehensive understanding of the precise mechanistic changes underlying these observations exceeds the scope of the present study.

SAR-152954, at a dose of 1.0 mg/kg, appears to have largely reversed PAE-related alterations in potentiation of evoked responses as measured by fEPSP slope as well as with time-frequency analyses. SAR-152594 increased spectral power around the time of the overall peak of the evoked response in PAE rats. However, the observed increase was slightly later than the decreased power observed in PAE rats receiving the vehicle. Further, SAR-

152594 was associated with decreased power in the later components of the evoked response. However, it remains unclear whether this reflects SAR-152954's impact on the initial excitatory response, effects on interneurons, or both. Previous research by Madden et al. (2020) showcased that PAE reduces parvalbumin-containing cells in the dorsal hippocampus, suggesting potential disruptions to interneuron-related functions in PAE offspring. Future investigations should delve deeper into this phenomenon, employing pharmacological interventions that target glutamatergic and GABAergic receptors.

The current findings align with previous observations by Varaschin et al. (2014, 2018) regarding ABT-239 and the H3R agonist methimepip (Sadek et al., 2016). The present findings and prior findings from our laboratories indicate that H3R inverse agonists could be effective at addressing cognitive and behavioral deficits following PAE. However, our studies including the present study, have only utilized a single moderate ethanol exposure protocol. The effectiveness of H3R antagonism and inverse agonism following PAE with higher ethanol exposure levels, durations, and timing of exposure are critical for establishing the potential utility of these pharmacological agents to broadly address behavioral and cognitive consequences of PAE.

As H3Rs inhibit glutamate release, the functional outcome of inverse agonism would be increased glutamate release. We note that other interventions are effective at reversing PAE-related LTP deficits. For example, Grafe et al. (2022) report rescue of PAE-related deficits in LTP by choline supplementation. Acetylcholine increases glutamate release through nAchRs (Ge & Dani, 2005) and activation of nAchRs induces LTP in DG (Matsuyama et al., 2000). Glutamate release might be a final common pathway by which the interventions

rescue PAE-related deficits in LTP. If so, other interventions that impact glutamate release could represent potentially fruitful approaches for addressing a broad range of behavioral, cognitive, and plasticity deficits observed following PAE. We note also that ABT-239 and other H3R inverse agonists display affinity for Sigma-1 receptors (S1Rs) (Riddy et al., 2019; Szczepańska et al., 2021). Because S1Rs play a key role in the modulation of NMDAR activity and plasticity through effects on SK channels (Martina et al., 2007) it is important for future work to address the role of S1Rs in the positive effects of H3R inverse agonism reported here.

There are several additional limitations of the present study to note. The observations from the present study were performed exclusively in male rats. Previous work conducted by Varaschin et al. (2010 and 2014) observed robust LTP deficits in PAE rats that were ameliorated by administering the H3R inverse agonist ABT-239. Varaschin et al. (2018) found no effects of PAE on H3R function in female rats, and Davies et al. (2023) reported greater alterations in H3R isoform expression in males. Collectively, these observations motivated the present focus on male rats in assessing the effectiveness of SAR152954. There is limited research on both sexual dimorphism in LTP and the interaction between LTP and neuropharmacology (Goncalves-Garcia & Hamilton, 2024). Sexual dimorphisms in PAE-related deficits in LTP have been observed by Sickmann et al., (2014), therefore, it will be important to examine potential sex differences in the effectiveness of H3R inverse agonists in future research. The present study also only evaluated LTP and the effects of SAR-152594 in adult rats. While this is a strength for evaluating effectiveness in long-term outcomes, the present study did not evaluate the effectiveness of H3R inverse agonism in younger offspring. Regarding the LTP protocol, we only utilized one induction procedure for LTP and only

evaluated LTP in one pathway. As noted, the induction protocol selected was identified because it yielded robust and reliable PAE-related deficits in LTP. Other induction protocols could yield greater or lesser deficits, for which different doses of H3R inverse agonists could be required to rescue or ameliorate PAE-related deficits. The potential effectiveness of H3R inverse agonism in other pathways was not established. The generality of the present outcomes to other hippocampal or cortical circuits needs to be investigated. Finally, we note that the present study only examined LTP and SAR-152594 effects in an anesthetized preparation. Evaluating PAE effects in awake, freely-moving animals is critical, particularly considering that histaminergic tone should be substantially different in the awake state, compared to sleeping or anesthetized states.

In addition to the future studies described above, we are also motivated to pursue several other scientific questions related to the use of H3R inverse agonists to improve outcomes following PAE. First, the assessment of H3R inverse agonists other than ABT-239 on behavioral, cognitive, learning, and memory outcomes is needed. The present findings suggest that PAE-related learning and memory deficits that are related to alterations in hippocampal LTP could benefit from treatment with SAR-152594. Further, local injection of SAR-152594 into the hippocampal formation could also be critical in confirming the locus of the drug effects reported here for the dentate gyrus.

In summary, the present study replicates prior work illustrating deficits in LTP following moderate PAE in male rats, and prior work showing that H3R inverse agonists are effective at reversing PAE-related deficits in LTP. We further demonstrated that time-frequency analyses provide a novel and more sensitive assessment of PAE effects on LTP.

These analyses also highlight PAE-related alterations in feedback circuity, possibly revealing novel information about PAE effects on excitatory and/or inhibitory feedback signaling in the hippocampus, and potential impacts of PAE on interneuron function that warrant additional investigation. Time-frequency analyses also provide a sensitive assessment of drug effects, and therefore, could represent an important tool for evaluating treatments designed to ameliorate effects of PAE.

Chapter3 (section 2) – Additional analyses of electrophysiological signals associated with feedback circuitry.

3.5. Introduction

Time-frequency analysis is a robust and valuable technique that enables researchers to study how the frequency content of a signal changes over time. It provides a way to examine the varying frequency components present in a signal as it evolves through different time intervals. LTP involves a sequence of events following HFS. Immediately after HFS, there is what is defined as post-tetanic potentiation followed by the feedback circuit. The classic measures of LTP are focused on the early portions of the evoked response. However, the overall waveform of the evoked responses raises the question if there is more information that can be extracted from those later portions, after the second peak (following the population spike [PS]), of the response that could – potentially – be affected by PAE. Time-frequency analysis can provide insight into the mechanisms associated with the induction and maintenance of LTP and the interplay of excitatory and inhibitory signals within the dentate gyrus feedback circuitry. This approach provides information about the entire duration of the response rather than being limited to specific points early in time.

The dentate gyrus consists of granule cells, which are densely packed neurons known for their distinctive morphology and abundant presence. These granule cells receive input from the entorhinal cortex through the perforant pathway and, in turn, project their axons, called mossy fibers, to the CA3 region of the hippocampus. Basket cells, a type of inhibitory interneuron, are strategically positioned in the dentate gyrus and provide local inhibition, regulating the activity of granule cells. The intricate connectivity and interaction among these

components facilitate both feedforward and feedback mechanisms. Feedforward signals move in a linear manner, from the entorhinal cortex to the dentate gyrus, and then to the CA3 region through the mossy fibers. Feedback, on the other hand, involves information returning from the CA3 region back to the dentate gyrus, creating a loop that refines and modulates the processing of information, contributing to the intricate network dynamics underlying memory formation and retrieval (Amaral et al., 2007; Sloviter, 1991). Locally - in the DG area – granule cells (GC), mossy cells (MC), and basket cells (BC) integrate the interneuron circuit that can be divided into the feedback and feedforward inhibition pathways and the mossy cells associational system (fig. 18). The excitatory input received from the entorhinal cortex (EC) EC projects onto the BC cells – resulting in feedforward inhibition – and the GC cells. The BC projects onto the GC cells – feedback inhibition. The MC sends monosynaptic inputs to the GC and disynaptic signals via the BC cells. The specific communication GC-MC-GC is referred to as the associational circuit (Hendrickson et al.,

2015). Any disruption to the system would lead to alteration in the excitatatory-inhibitory balance.

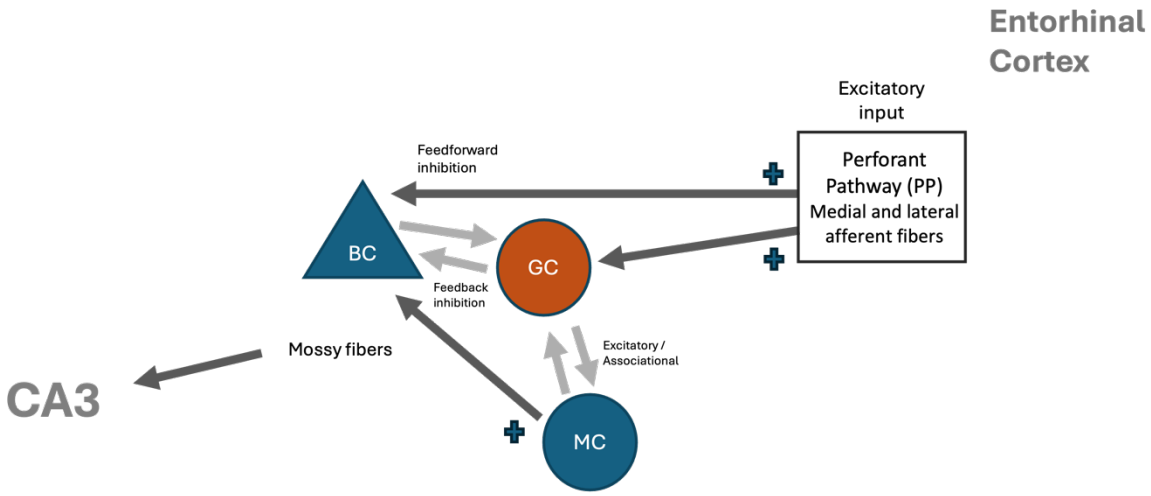

Figure 18. Schematic representation of the Feedback-feedback circuit in the dentate gyrus. The excitatory input from the EC projects onto the BC cells – resulting in feedforward inhibition – and the GC cells. The BC projects onto the GC cells – resulting in feedback inhibition. The MC sends monosynaptic inputs to the GC and disynaptic signals via the BC cells. The specific communication GC-MC-GC is excitatory. BC = basket cells; GD = granule cells; MC = Mossy cells.

This chapter provides additional analysis as follow-up measure to the time-frequency analysis reported in Chapter 3 (section 1).

3.6. Methods

In addition to the standard amplitude-based measures of the fEPSP and the time-frequency analysis described in the methods session of Chapter 2, we analyzed the area under the curve (AUC) between 5 points (3 peaks and 2 troughs) of the evoked-response comparing two phases of the stimulation protocol before and after HFS (baseline and postHFS). Fig. 19 shows a representative waveform of an evoked response with peaks and troughs labeled y1, y2, y3, y4, and y5. There are two AUCs (*y1-y2-y3* and *y3-y4-y5*). An additional analysis included the amplitude difference between y1 and y2 (labeled yp).

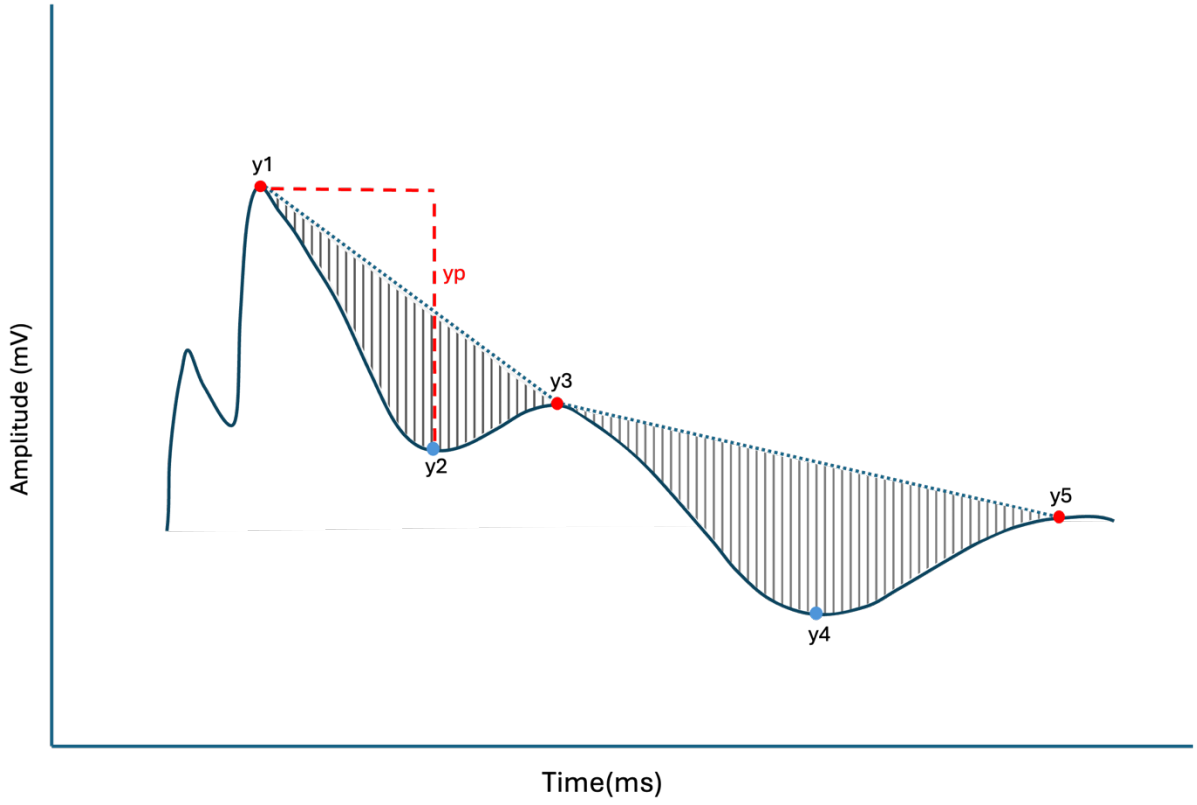

Figure 19. Representative trace of an evoked response with peaks (red dots denoted y1, y3, and y5) and troughs (blue dots, denoted y2 and y4). The AUC was calculated from a peak to the next trough (in the example, y1-y2).

3.6.1 Statistical Analyses

RM-ANOVA was performed for AUC1 (from peak 1 to peak 2) and AUC2 (from peak 2 to peak 3) analyzed in a 2(Diet) x 2(Drug) x 2(Protocol phase) analysis with protocol phase (Baseline-PostHFS) as a within subject factor (table 3). One-way ANOVA was performed for each individual point (peaks and troughs) and amplitude difference (Y2-Y1). The units are mV squared.

3.7. Results

The analysis revealed significant main effects for the AUC1 (PostHFS > Baseline) measure ($F(1, 32) = 73.016$, $p < .001$, $\eta^2p = 0.695$) and AUC2 (PostHFS > Baseline) measure ($F(1, 32) = 30.466$, $p < .001$, $\eta^2p = 0.488$). None of the other measures were statistically significant (ps > 0.69). There was a main effect in the amplitude between peak 1 and trough 1 (PostHFS > Baseline) measure ($F(1, 32) = 76.929$, $p < .001$, $\eta^2p = 0.706$). There were main effects (PostHFS > Baseline) in the individual points as follow: Y1 - ($F(1, 32) = 54.006$, $p < .001$, $\eta^2p = 0.628$); Y2 - ($F(1, 32) = 64.997$, $p < .001$, $\eta^2p = 0.670$); Y3 - ($F(1, 32) = 36.911$, $p < .001$, $\eta^2p = 0.536$); Y4 - ($F(1, 32) = 6.327$, $p = .017$, $\eta^2p = 0.165$); and Y5 - ($F(1, 32) = 23.288$, $p < .001$, $\eta^2p = 0.421$). There were no other main effects or interactions (ps > 0.1).

Table 3. Mean and Standard Deviations for AUC 1 (Y1-Y2-Y3) and AUC 2 (Y2-Y3-Y4), individual amplitude values for all points (Peaks: Y1-Y3-Y4; Troughs: Y2 and Y4), and amplitude Y2-Y1. All measures are for baseline and postHFS.

	SAC		**PAE**	
	Veh	*SAR*	*Veh*	*SAR*
Baseline AUC 1	170.78(64.48)	125.14 (58.79)	157.01 (82.40)	108.76 (44.43)
PostHFS AUC 1	321.76(139.62)	284.67 (141.81)	271.19 (105.93)	196.84 (74.50)
Baseline AUC 2	936.78 (294.95)	859.27 (354.69)	929.20 (288.29)	823.24 (335.67)
PostHFS AUC 2	907.50 (280.26)	814.39 (339.53)	874.65 (273.36)	803.59 (329.52)
Baseline Y1	9.10 (3.02)	8.15 (3.53)	8.87 (2.81)	7.98 (3.10)
PostHFS Y1	10.73 (3.51)	9.86 (4.60)	10.05 (3.49)	8.99 (3.55)
Baseline Y2	4.59 (2.08)	4.30 (2.53)	4.36 (1.85)	4.49 (2.36)
PostHFS Y2	3.76 (1.95)	3.38 (2.11)	3.58 (1.50)	3.97 (2.17)
Baseline Y3	5.15 (2.04)	4.66 (2.49)	4.79 (1.73)	4.74 (2.27)
PostHFS Y3	4.73 (1.97)	4.07 (2.18)	4.26 (1.50)	4.40 (2.12)
Baseline Y4	-2.59 (0.66)	-2.49 (0.90)	-2.78 (0.79)	-2.22 (0.85)
PostHFS Y4	-2.69 (0.65)	-2.57 (0.90)	-2.79 (0.82)	-2.30 (0.88)
Baseline Y5	0.37 (0.23)	0.24 (0.33)	0.24 (0.26)	0.31 (0.18)
PostHFS Y5	0.28 (0.26)	0.14 (0.29)	0.18 (0.26)	0.22 (0.20)
Amplitude Y2-Y1 Baseline	4.51 (1.57)	3.85 (1.67)	4.51 (1.46)	3.49 (1.22)
Amplitude Y2-Y1 PostHFS	6.97 (2.54)	6.48 (3.05)	6.47 (2.28)	5.02 (1.87)

3.8. Discussion and conclusion

Potentiation following HFS is evident in these results which agrees with the classical amplitude measures as reported previously. Analysis of AUCs did not reveal differences between diet conditions and/or the presence of the drug. This additional analysis was an attempt to identify an analytical method that could be more sensitive to capturing potential differences in the synaptic feedback following moderate PAE. It seems that AUC and other amplitude-based measures are sensitive to capturing large differences between the two phases of the protocol (baseline and PostHFS), but not sensitive enough to capture small differences that is evident by other measurements as reported in previous chapters. The motivation to add time-frequency analysis (or, in this case, amplitude measures) was an attempt to analyze the entire response rather than just the early points associated with fEPSP and PS. The time frequency approach appears to provide a more sensitive assessment of alterations related to PAE and could be useful in identifying potential novel alterations that could inform understanding of how PAE affects synaptic plasticity. Even though great progress has been made in understanding what is affect by alcohol, it remains unknown how the dynamic of the system is disrupted by PAE. Considering the intricate cellular system of hippocampal formation – particularly the hippocampus – additional and novel forms of analysis may help enlighten potential ethanol-associated functional disruptions of the molecular system. Considering the tight balance between inhibition and excitation in the normal hippocampus, interference in any part of the structure could disrupt that balance of the entire system supporting learning and memory processes. Ethanol exposure affects various parts of the brain and each structure differently. In the hippocampal formation

GABAergic interneurons represent a minority of neurons but are densely innervated to hundreds of postsynaptic dendrites. These neurons are highly susceptible to alcohol less likely to survive (Bird et al., 2018). Ethanol exposure alters the excitability of neurons that are active or inactive. In the striatum chronic alcohol exposure leads to hyperpolarization in low-threshold spiking interneurons, but induces depolarization in fast-spiking interneurons (Abrahao et al., 2017). Even though the power analysis revealed differences not previously captured by the LTP analyses, it is not clear what may be driving those differences. It is clear, however, that there is a difference in the dynamic of the response of PAE animals when compared to controls. Considering the complexity of the feedback-forward inhibitory system, any disruption during development could impact the balance of the entire circuit. The present analyses provide evidence that traditional amplitude-based measures may not capture impairments in the response that may be associated with the excitatory-inhibitory dynamic system. Beyond the glutamatergic transmission and H3R investigated in the present work, future studies should aim to expand the investigation from specific structures to circuits and functions.

Chapter 4 - Glutamate – biosensor + SAR152954

4.1. Introduction

Following the previously described experiments, an additional experiment was conducted combining the glutamate biosensor with drug administration. This additional experiment only includes PAE + saccharine and PAE + SAR152954 groups because pilot study did not find differences in the PAE-SAR that would justify including a SAC-SAR group, but rather keep only the PAE groups to assess potential differences between the two alcohol-exposed groups. Also, there are ethical implications associated with the use of animals. Therefore, we tried to minimize the numbers of animals needed to collect these data.

The goal of this additional experiment was to assess potential differences in extracellular level of glutamate following SAR-152954 administration. The hypothesis is that if H3R-mediated inhibition is heightened by PAE, there should be less glutamate release in be similar to control animals.

4.2. Methods

4.2.1. Animals

17 adult male Long-Evans rats long-Evans rats (PAE = 10; PAE-SAR = 7) 3- to 9-month-old (University of New Mexico Health Sciences Center Animal Resource Facility) were used in the present study. All animals were pair-housed in plastic cages on a 12-hour light-dark cycle with food and water available ad libitum. The experiments reported here were approved by the Institutional Animal Care and Use Committee (IACUC) of the Health Sciences Center and Main Campus of the University of New Mexico.

Breeding and Voluntary Ethanol Consumption During Gestation

The breeding and exposure procedures are the same as described in the methods session of Chapter 2.

Anesthesia and surgery:

Same as described in chapter 2. Following the glutamate biosensor placement, the animals received an injection of SAR152954 (1.0 mg/kg) diluted in sterile PBS (volume 1mL/100g) or an equivalent volume of PBS and were monitored for 30 minutes before the recording session.

in vivo Electrophysiology protocol:

Same as described in chapter 2.

4.2.2. Statistical analyses

Same as describe in chapter 2 comparing the PAE-saline with the PAE-SAR groups.

4.3. Results

Continuous data

PAE-saline – PAE-SAR

The main effect of glutamate level did not reach statistical significance between the diet conditions (PAE-saline and PAE-SAR) (fig. 20), suggesting no differences in glutamate level over time following HFS when comparing different time points during the postHFS phase and the baseline phase. All ps>0.8.

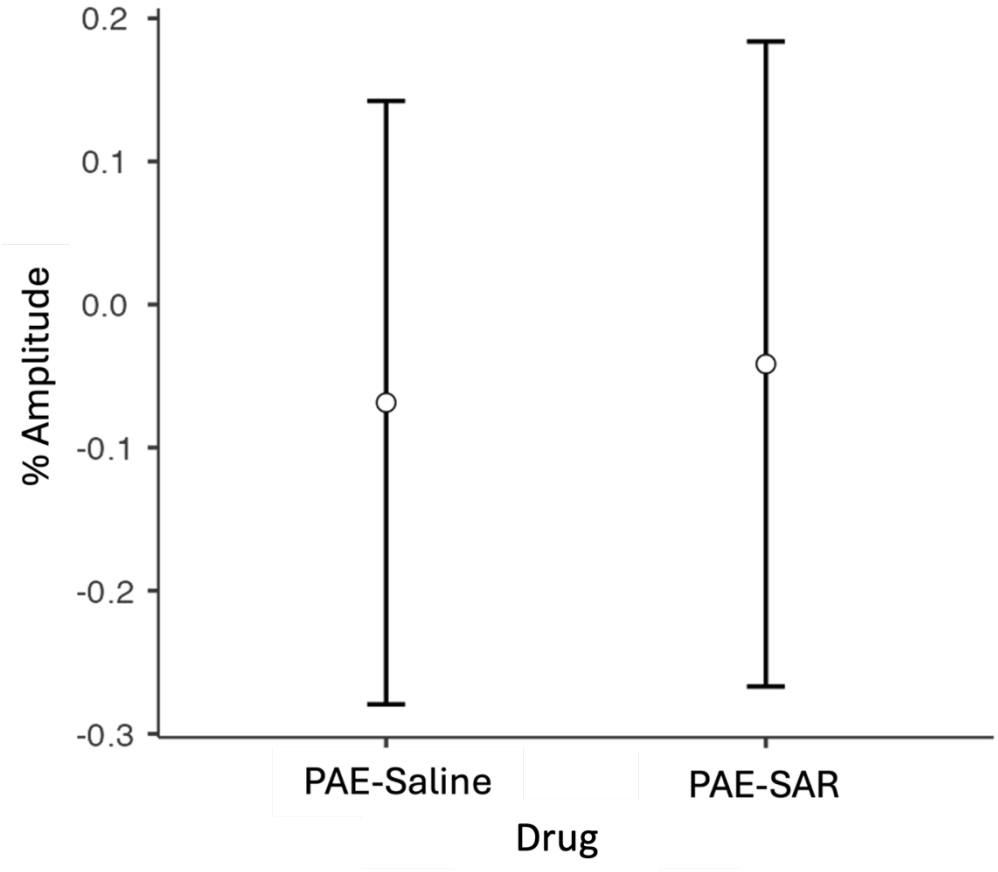

Figure 20. Estimated marginal means for the fractional change in amplitude postHFS for the PAE-saline and PAE-SAR groups. After HFS, there were no statistically significant differences in extracellular glutamate levels following SAR152954 administration when compared with the PAE-saline group (p>0.8).

4.4. Discussion and conclusion

To my knowledge, this is the first study to combine in vivo LTP and glutamate biosensor recordings in an animal model of moderate PAE with the addition of pharmacological intervention. Comparison of extracellular glutamate level of PAE-saline (from chapter 2) and PAE-SAR did not reveal the same differences evident by the electrical responses. Previously, we speculated that the inverse agonist SAR152954 acts on the H3R by ameliorating PAE-related deficits and promoting glutamate release which is involved in plasticity. The reversal of LTP deficits with administration of the drug, provides evidence for the beneficial effects of the drug. However, the glutamate biosensor – that is intended to measure extracellular concentration of glutamate – did not show differences in extracellular glutamate concentration between the PAE-saline and PAE-SAR groups. The amplitude-based analysis of fractional change of the continuous data between baseline and postHFS do not measure the absolute value of extracellular glutamate concentration. Additionally, a potential explanation is related to the analytical performance of the biosensor utilized in this study. The available linear range may have limited sensitivity (Ganesana et al., 2019) to capture the tiny differences in extracellular concentration following drug administration and HFS. Future studies could replicate this study with other types of biosensors that could be more sensitive to detecting differences in extracellular concentrations. Also, the differences that are detectable in the electrical responses may be due to differences in the timing of release. Another potential speculation is that those differences in LTP are time related possibility happening during PTP – when there is an enhancement of neurotransmitter

release. Future studies should focus on pairing the timing of the electrical and enzymatic responses.

Chapter 5 - Conclusion and final considerations

The lack of morphological alterations following moderate PAE may incorrectly lead to the conclusion that small amounts of alcohol are not damaging to exposed individual. It is not until when cognitively challenged – in school years – that those impairments may become evident. Issues with attention and learning, may be linked with conditions such as ADHD – which can be comorbid with PAE. Animal model of prenatal alcohol exposure provides strong evidence for those deficits that can be mild, but life-lasting. Animal models allow for in-depth investigations on the mechanisms supporting cognition. The primary objective of this study was to contribute valuable information to the field of moderate PAE research by providing substantial information on the neurobiological issues that emerge following exposure to alcohol during pregnancy.

The central goal of the present study was to investigate presynaptic mechanisms that could be disrupted by PAE - therefore negatively impacting learning and memory processes –, investigate the potential benefit of pharmacological intervention, and identify measurements that could be more sensitive to capturing smaller changes in the evoked response in LTP. It has been clear that moderate PAE disrupts LTP, however the neural circuits supporting LTP is complex and understanding what may be disrupted by alcohol remains an open question. This study demonstrated that that is PAE-related deficits in potentiation by analyzing the evoked responses with classical and novel measurements. Time-amplitude analyses – converted from time-frequency – revealed spectral power differences that aligns with the fractional changes of the fEPSP, with larger effect sizes, suggesting that power analysis might be a useful and more sensitive tool to analyze LTP. Follow-up analysis of AUC

did not reveal differences that were present in the other analysis, suggesting that AUC may be sensitive to capture larger differences, but not mild. Investigation of the H3R inverse agonist SAR152954, provides a promising target for pharmacological intervention to rescue PAE-related deficits in learning and memory processes.

Overall, the present study demonstrated in various ways that even a very moderate amount of alcohol during the gestational period has a long-term impact on the developing brain. When developing, the entire organism is affected by ethanol exposure in different ways. Each part of the developing brain will be impacted differently; therefore, it is not possible to conclude what, how, and when each brain structure will be impacted. Histaminergic and glutamatergic transmissions are just a subset of the pathways that are affected by alcohol and involved in synaptic plasticity. This study positively demonstrated that those structures are affected, offered alternatives to investigating and analyzing LTP and added to the ongoing work on the pharmacological investigation to help those impacted by the deleterious effects of PAE.

However, much additional research is needed to improve our understanding of PAE-related deficits and LTP. This study did not include females, which is necessary to assess if the results reported here replicate in female and to understand potential sex differences. Additionally, this study investigated a cohort of adult animals, future investigations on adolescent offspring to assess if the deficits manifest in similar way in different age groups. For the biosensor, future research should investigate dual recording combining the biosensor with the electrode for simultaneous recording to assess the relationship of the electrical and enzymatic responses.

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