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Investigation of the Effects of Prenatal Drug Exposure on Astrocyte-Mediated Synaptogenic Signaling

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INVESTIGATION OF THE EFFECTS OF PRENATAL DRUG EXPOSURE ON ASTROCYTE-MEDIATED SYNAPTOGENIC SIGNALING

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APPROVAL OF THESIS

We, the faculty supervising the work of Taylor Christian Boggess, affirm that the dissertation, *Investigation of the Effects of Prenatal Drug Exposure on Astrocyte-Mediated Synaptogenic Signaling*, meets the high academic standards for original scholarship and creative work established by the Biomedical Research Program and the Joan C. Edwards School of Medicine. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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ABSTRACT

One of the most significant outcomes of the rise in rates of opioid abuse in the United States has been a dramatic increase in the incidence of neonatal abstinence syndrome (NAS), the clinical diagnosis for the collection of withdrawal-like signs and symptoms commonly observed in the newborns of mothers who abused certain drugs, such as opioids, during pregnancy. While a great deal of research has investigated the short-term symptoms and potential treatments for NAS, including my own studies to identify emerging trends among a local population of NAS patients, there remains a critical need to investigate the long-term effects of prenatal drug exposure on neurological development. A potential route through which drugs of abuse likely interact with the developing nervous system lies in astrocyte-mediated synaptic development, more specifically in signaling involving thrombospondins, a class of astrocyte secreted glycoproteins that act through binding to the neuronal cell surface receptor α2δ-1. α2δ-1 is also the receptor for the antiepileptic drug gabapentin. Co-abuse of opioids and gabapentin during pregnancy is associated with a unique presentation of NAS. I have therefore hypothesized that prenatal exposure to drugs of abuse leads to significant disruptions in astrocyte mediated synaptogenic signaling, resulting in permanent deficits in synapse formation and alterations in behaviors associated with addiction. To test this, I treated pregnant mice transgenic for α2δ-1 with one of four treatments: the opioid drug buprenorphine, gabapentin, a combination of both drugs, or a vehicle control. α2δ-1 wild-type, heterozygous, and knockout littermate pups of both sexes at 21 days of age were used for studies involving immunohistochemistry and confocal fluorescence microscopy to investigate synaptic connectivity within brain regions associated with reward. A second cohort of pups at 70 days of age underwent the same studies after first undergoing behavioral experiments examining anxiety, locomotion, and reward seeking. By completing

these studies, I hoped to contribute to a greater understanding of the effects of prenatal opioid exposure on neurological and behavioral development that would inform parents, healthcare providers, and educators looking to meet the unique developmental and educational needs of children impacted by drug abuse.

CHAPTER 1

INTRODUCTION

The use of derivatives of the opium poppy (*Papaver somniferum*) to treat pain and induce euphoria likely predates the oldest surviving records of human history (Bandyopadhyay, 2019; Brownstein, 1993; Dhawan et al., 1996; Pasternak & Pan, 2013). It is also likely that these derivatives and the synthetic drugs modeled after them have led to misuse and addiction for nearly as long. It is only within the past few decades, however (AHRQ, 2021; Scholl L, 2019; USDHHS, 2015), that rates of opioid abuse have risen to levels high enough to have a significant impact on the wider society and be labeled an epidemic, due predominantly to factors such as evolving philosophies surrounding the treatment of pain, the development of increasingly effective and potent drugs, and changes in the prescribing practices of physicians (Cerdá et al., 2015; DeWeerdt, 2019; Haight et al., 2018; Kolodny et al., 2015; Tsang et al., 2008; USDHHS, 2015). The full consequences of this widespread rise in substance abuse are still in the process of being understood with researchers from a wide variety of backgrounds and specialties approaching different aspects of the problem.

Neonatal Abstinence Syndrome

One direct result of the rise in rates of opioid use disorder (OUD) that is being heavily researched is the dramatic rise in the rates of neonatal abstinence syndrome (NAS) (Desai et al., 2014; Haight et al., 2018; Tolia et al., 2015; Winkelman et al., 2018). NAS is the clinical diagnosis used to describe the withdrawal-related signs and symptoms commonly observed in the newborns of mothers who abused certain drugs, such as opioids, during pregnancy (the condition can also be referred to as neonatal opioid withdrawal syndrome, or NOWS, in cases where opioids are known to be the primary substance abused during the pregnancy). Signs and

symptoms of NAS typically begin within 72 hours after birth and commonly include irritability, tremors, excessive crying, sneezing, excessive yawning, tachycardia, hypertension, poor feeding, diarrhea, and, in some severe cases, seizures (Kocherlakota, 2014; Raffaeli et al., 2017). Use of drugs of abuse, such as opioids, during pregnancy has also been correlated with increased risk of sudden infant death syndrome (SIDS; risk ratio as high as 3.6 for methadone-exposed infants) (Cohen et al., 2015; Kandall et al., 1993) and death within the first two years of life (Ostrea et al., 1997). Numerical scoring scales, such as the Finnegan Neonatal Abstinence Scoring System (Finnegan et al., 1975) are used to evaluate the severity of symptoms and help direct decisions concerning treatment and care (Kocherlakota, 2014). Length of stays in hospital neonatal intensive care units or similar dedicated treatment facilities average 16 days for affected infants (Hudak & Tan, 2012) but can widely vary. Pregnant women with OUD are commonly encouraged to enroll in medication-assisted treatment (MAT), which combines the prescription of less-addictive opioid drugs, typically methadone or buprenorphine, with behavioral counseling and other forms of prenatal support in order to treat their addiction and hopefully improve health outcomes for both the mother and her child. However, emerging evidence asserts that any opioid use during pregnancy, even when administered as part of a medical treatment, can have deleterious effects on the developing fetus and MAT does not eliminate the potential for NAS (Belcheva et al., 1998; Burke & Beckwith, 2017; Grecco et al., 2021; H. Johnson et al., 1984; Kayemba-Kay's & Laclyde, 2003).

NAS represents a significant health concern for affected infants and an emotional toll on the families of those infants. It also represents a significant financial burden (Patrick et al., 2015; S. Patrick et al., 2012). Total hospital costs to treat NAS patients who were covered by Medicaid increased from $65.4 million in 2004 to $462 million in 2014 (Winkelman et al., 2018). These

rising medical costs are further complicated by the fact that, while socio-economic status is not necessarily correlated with substance use, a large proportion of substance use disorder patients come from lower socio-economic backgrounds, a group that also historically has poorer access to quality healthcare in general (Becker & Newsom, 2003; Braveman & Gottlieb, 2014; Hanson & Chen, 2007; M. Kim et al., 2018; M. Patrick et al., 2012).

While OUD and NAS are global issues, the United States has been, and remains, the most severely impacted country (Lancet, 2022). The national rate of NAS in the United States was 6.8 per 1000 neonatal hospitalizations in 2018 compared to 1.5 in 1999 (AHRQ, 2021; Haight et al., 2018). However, rates can vary widely between different states and within the same state. Appalachia and more rural regions in the country have been disproportionately affected by OUD and NAS (J. Brown et al., 2018; Hayes & Brown, 2012; Patrick et al., 2015). In West Virginia for example, where access to treatment facilities for opioid abuse is limited compared to other states (J. Brown et al., 2018), the rate of NAS from October 2016 to December 2017 was 52.6 cases per 1,000 live births (Umer et al., 2019). In 2018, the statewide rate was slightly lower at 49.6 per 1000 live births (7.3× the national average) but was still the highest rate of any state in the country (AHRQ, 2021).

The serious emotional toll for the families of affected children and the significant burdens placed on healthcare providers associated with these high incidence rates of NAS have prompted considerable research efforts into better understanding the mechanisms of NAS and improving clinical care and treatment for NAS affected infants. However, compared to the number of research studies that have investigated mechanisms and treatments, information regarding the long-term effects of prenatal exposure to drugs of abuse on neurological development, in humans or in animal models, is limited. Additional research into this area is necessary in order for

healthcare providers, educators, and parents better meet the specific needs of affected children as they grow and develop.

The Endogenous Opioid System Serves a Key Role in CNS Development

All opioid drugs interact with the human body via binding to one or members of a class of G-protein coupled receptors simply referred to as opioid receptors (McDonald & Lambert, 2005). These receptors are expressed in varying levels throughout the body, including the CNS (Merrer et al., 2009; Peng et al., 2012; Rius et al., 1991; Zhu et al., 1998). Each receptor type binds preferentially with one or more members of a group of peptide hormones known as endogenous opioids (McDonald & Lambert, 2005; Zagon & McLaughlin, 2017). The endogenous opioid β-endorphin appears to bind strongly to all three of the classically known receptors: the μ opioid receptor (MOR), the δ opioid receptor (DOR), and the κ opioid receptor (KOR) (McDonald & Lambert, 2005). The endogenous opioids endomorphin 1 and 2 bind most strongly to the MOR, leu- and met-enkephalin bind most strongly to the DOR, and dynorphins A and B bind most strongly to the KOR (Dhawan et al., 1996; McDonald & Lambert, 2005; Zagon & McLaughlin, 2017). The more recently characterized endogenous opioid nociceptin/orphanin FQ opioid peptide (N/OFQ) binds to the most recently discovered receptor, the N/OFQ opioid receptor (NOR) (Toll et al., 2016). The interactions between endogenous opioids and their receptors are often collectively referred to as the endogenous opioid system (McDonald & Lambert, 2005). This system is involved in the activity and regulation of many different biological processes, including analgesia, respiratory/cardiac function, intestinal transit, mood, and hormone secretion, and is essential for normal development and function (Dhawan et al., 1996; Rius et al., 1991; Tan et al., 2018).

There is reason to believe that endogenous opioids and their receptors are particularly vital to healthy nervous system development. For example, MOR and KORs have been detected on the surfaces of rodent embryonic stem cells and neural progenitor cells with evidence that the binding of endogenous opioids can induce embryonic stem cells to differentiate via the extracellular signal-regulated kinase (ERK)/ mitogen-activated protein (MAP) kinase signaling pathway (E. Kim et al., 2006; Tan et al., 2018). The localization of opioid receptors to different regions of the brain may also have a significant impact on development. Immature mice have been shown to have a higher percentage of MOR expression within known sites of neurogenesis, namely the subventricular zone and striatum, compared to adult mice (Stiene-Martin & Hauser, 1991). In neonatal rats, expression of KORs within known sites of cellular proliferation and neurogenesis, including the ventricular and subventricular zones and the hippocampus, has been shown to be higher compared to adults (Tan et al., 2018). Given the importance of endogenous opioids and their receptors in the developing CNS, an introduction of exogenous opioids could have a lasting, dramatic impact on this process.

Exogenous Opioids Have Multiple Effects Within the CNS

All opioid drugs, whether derived from natural sources or developed synthetically, interact with the human body primarily through the same class of receptors as endogenous opioids. Morphine, the prototypical opioid, and its derivatives (heroin, fentanyl, oxycodone, etc.) are primarily active at MORs but also act as weaker KOR and DOR agonists (McDonald & Lambert, 2005). These drugs are believed to alleviate pain through two separate mechanisms: 1. By binding to opioid receptors on the axon terminals of nociceptive afferent neurons from the peripheral nervous system within the dorsal horn of the spinal cord to depress the release of neurotransmitters, predominantly substance P and glutamate; and 2. By binding to opioid

receptors within the CNS (particularly the midbrain, brainstem, and thalamus) to activate descending inhibitory pain pathways (Doyle & Murphy, 2018; Inturrisi, 2002; Lipp, 1991; Lueptow et al., 2018; Pasternak & Pan, 2013). Methadone is another derivative of morphine that also has a high affinity for the MOR and can also be prescribed for pain relief. However, methadone also has additional properties, such as a delayed onset of action and longer half-life (Ball & Ross, 2012; R. Brown et al., 2004; Chestnut, 2020), that have led to its use as a maintenance treatment for OUD ("Dose-response effects of methadone in the treatment of opioid dependence," 1993; Ling et al., 1976). In contrast, the more recently developed opioid buprenorphine, which is also used as an analgesic and has been increasingly used in the treatment of OUD, is a derivative of the opiate alkaloid thebaine. It has been shown to be a partial agonist at MORs but an antagonist at KORs and DORs (Martin et al., 2001; Welsh & Valadez-Meltzer, 2005), likely contributing to its effectiveness as a maintenance therapy. Intriguingly, however, buprenorphine's metabolite norbuprenorphine is an agonist at the DOR and a partial agonist at MORs and KORs (Huang et al., 2001). Clearly, despite all being classified as opioids, individual drugs within this classification can vary widely in both their receptor binding profiles and their pharmacokinetics, thus determining their applications in medicine and potentials for harm or abuse.

It is widely accepted that the primary mechanism by which opioids induce reward and hold the potential for abuse and addiction is via interactions within the mesolimbic dopamine pathway (Carlezon & Wise, 1996; Di Chiara et al., 2004; S. Johnson & North, 1992; Pierce & Kumaresan, 2006; Wachtel et al., 1989) (**Figure 1A**). The mesolimbic dopamine pathway, sometimes referred to as the dopamine reward pathway, normally functions to encode experiences as novel (Bunzeck & Düzel, 2006; Bunzeck et al., 2007; Kutlu et al., 2021) and

pleasurable in order to reinforce future behaviors in an attempt to seek out similar experiences (Knutson & Cooper, 2005; Lisman & Grace, 2005; Wittmann et al., 2005). This process begins within the midbrain in the ventral tegmental area (VTA), where the cell bodies of dopaminergic neurons extend axonal processes to other regions of the brain. The most prominent of these brain regions are the nucleus accumbens (NAC) within the striatum and the prefrontal cortex (PFC) (Adinoff, 2004; Carr et al., 1999; Han et al., 2017; Lisman & Grace, 2005). Increased phasic release of dopamine within the NAC in response to external stimuli has been shown to reinforce pleasurable behaviors including eating food, engaging in sexual activity, or consuming alcohol and/or drugs of abuse (Lingford-Hughes & Kalk, 2012). Opioids act within the VTA by binding to opioid receptors on the surface of GABAergic interneurons that exert inhibitory control on neighboring dopaminergic neurons (Bouarab et al., 2019; Jalabert et al., 2011; S. Johnson & North, 1992), effectively inhibiting the release of GABA and disinhibiting the release of dopamine.

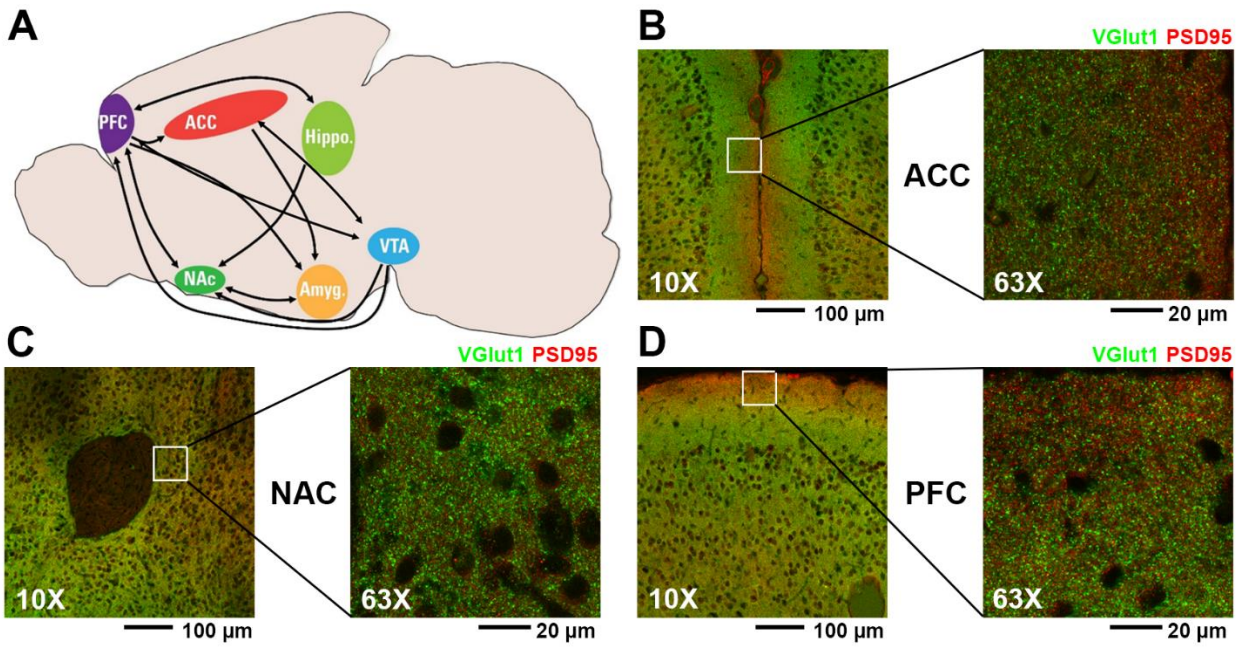

Figure 1: Brain Regions of Interest Within Mesolimbic Dopamine Pathway
(A) Diagram of mouse brain highlighting regions associated with mesolimbic dopamine pathway and the known connections between them (PFC, prefrontal cortex; ACC, anterior cingulate cortex; NAC, nucleus accumbens; VTA, ventral tegmental area; Hippo, hippocampus; Amyg, amygdala). (B–D) Representative fluorescent confocal microscopic images of excitatory synaptic immunohistochemistry staining (presynaptic VGluT1, green; postsynaptic PSD95, red) at 10X and 63X magnification of the (B) ACC, (C) NAC, and (D) PFC.

Interactions between brain regions, both within and outside the mesolimbic dopamine pathway, modulate the activity of this pathway and influence reward motivated behavior. The PFC, the brain region most associated with decision making and impulse control (Holt et al., 2008; Knutson & Cooper, 2005; Pryor & Veselis, 2006), projects to the NAC and modulates its activation in response to dopamine release (Carr et al., 1999; Han et al., 2017; McFarland et al., 2003; Pierce & Kumaresan, 2006; Rebec & Sun, 2005). It has been shown that projections from the medial PFC to the NAC are involved in reinstatement of drug-seeking behavior in response

to stress or drug-associated cues (McFarland et al., 2003; Rebec & Sun, 2005). It has also been shown that the PFC is largely responsible for reward prediction during the performance of behavioral tasks (Knutson & Cooper, 2005). The hippocampus, a brain region crucial to learning and memory (Anand & Dhikav, 2012; Rubin et al., 2014), appears to form a functional loop with the VTA to promote the seeking out of novel experiences and expecting reward when engaging in behaviors associated with pleasurable memories (Lisman & Grace, 2005; Wittmann et al., 2005). The anterior cingulate cortex (ACC) is involved in a variety of functions, including reward anticipation, motivational persistence, and modulation of goal oriented motor activity (Blanchard et al., 2015; Walton et al., 2003), and has been shown to influence motivation via modulation of VTA activity (Elston & Bilkey, 2017; Narita et al., 2010; Y. Yuan et al., 2009). The amygdala is largely known to be a center for the processing of emotions, particularly fear (Balleine & Killcross, 2006; Bonnet et al., 2015). However, the amygdala is also known to interact with the hippocampus to attribute emotional coding to memories and has been shown to interact with the NAC and VTA to modulate stress and addiction (Belujon & Grace, 2011; Chi Yiu & Mogenson, 1982; Hurd et al., 1999; C. Nguyen et al., 2021). The interconnectedness of these brain regions may indicate the potential for opioid induced changes in structure or function within one region to impact other regions as well. Understanding what effects drugs of abuse, such as opioids, can have on the normal development, structure, and function of the mesolimbic dopamine pathway and the CNS in general will be crucial to better understanding and treating substance abuse.

Drugs of Abuse Can Interfere with the Normal Development of Neurons in the CNS

Given the importance of the endogenous opioid system in development and the impact exogenous opioids can have on CNS function, the question of how opioids could potentially affect the development of the CNS naturally arises. In animal models, prenatal opioids have been shown to stunt the proliferation and development of neurons within the CNS. Levels of neuronal specific protein markers (Neu-N and MAP-2) as well as stem/progenitor biochemical markers of the neural lineage (nestin, Sox2, KLF4, and doublecortin) were reduced in 21-day-old rats that had been exposed to buprenorphine during prenatal development (Wu et al., 2014). This same group of 21-day-old rats also showed decreased brain-derived neurotrophic factor (BDNF) expression and signaling (Wu et al., 2014). BDNF is critical is critical in CNS development. In particular, signaling between BDNF and the receptor TrkB is vital for neurogenesis, axon growth, and synaptic plasticity (Cohen-Cory et al., 2010; Kramár et al., 2012). The relationship between BDNF and exogenous opioids is likely significant given that plasma levels of BDNF were found to be increased in 2-day-old human infants diagnosed with NAS compared to healthy infants (Subedi et al., 2017). Epigenetic modifications resulting from opioid exposure may explain this relationship, given that expression of BDNF is highly epigenetically regulated (Browne et al., 2020; K.-W. Chen & Chen, 2017).

Altered expression of growth factors and signaling molecules do not appear to be the only mechanism through which opioids affect neuronal development. In free-floating cultured rat cortical neurospheres, buprenorphine was shown to reduce proliferation of neural stem/progenitor cells (Wu et al., 2014). It has also been shown, within rats, that opioids alter neuronal expression of apoptotic proteins (namely caspase-3, Bcl-2, and Bax) particularly within

the hippocampus (Wang & Han, 2009). Dysregulation of apoptosis could lead to inappropriate cell death and potentially interfere with normal neuronal development.

In addition to affecting proliferation and overall numbers of neurons in the developing CNS, prenatal opioids also appear to influence neuronal growth and maturation. Neurons isolated from the somatosensory cortices of heroin-exposed 3-day-old mouse pups possessed fewer and shorter dendrites compared to neurons from control pups (Lu et al., 2012). This change in dendritic development could impede synaptic connectivity and inter-neuronal communication throughout the brains of these pups.

Drugs of Abuse Can Interfere with the Normal Function and Development of Glia in the CNS

While studies examining the effects of prenatal drug exposure in the CNS have predominantly focused on the development of neurons, the development of nonneuronal glial cells has also been shown to be influenced by both the endogenous opioid system (Stiene-Martin & Hauser, 1991) and by exogenous drugs of abuse (Narita et al., 2006). There are a variety of different glial cell types, each with distinct and vital roles in the development, function, maintenance, and repair of the CNS. Oligodendrocytes (Kettenmann & Verkhratsky, 2011; Sanchez et al., 2008; Tan et al., 2018; Vestal-Laborde et al., 2014) and microglia (Maduna et al., 2019; Schwarz & Bilbo, 2013; Zamani et al., 2022) have been implicated in prenatal opioid-induced changes in neurological development. However, an increasing amount of evidence points to astrocytes as being the glial cell type most involved in the changes within the CNS in response to prenatal drug exposure.

Astrocytes are the most numerous type of glia in the CNS and are involved in a number of vital processes (Sidoryk-Wegrzynowicz et al., 2011; Volman et al., 2012) including, but not

limited to, maintenance of the blood brain barrier (Allen & Lyons, 2018), regulation of extracellular ion balance (Volman et al., 2012), regulation of the concentrations of neurotransmitters in the extracellular space (Boddum et al., 2016; Minelli et al., 1996; A. Q. Nguyen et al., 2020), glutamate uptake and release (Parpura & Haydon, 2000), and regulation of synaptic development within the CNS (Allen & Lyons, 2018; Bosworth & Allen, 2017; Christopherson et al., 2005; Clarke & Barres, 2013; Cresto et al., 2019; Risher et al., 2018; Risher et al., 2014). Astrocytes are able to mediate both excitatory (Christopherson et al., 2005) and inhibitory synaptogenesis (Elmariah et al., 2005; A. Q. Nguyen et al., 2020) as well as mediate synapse elimination via phagocytosis involving cell surface receptors such as MEGF10 and MERTK (Chung et al., 2013). Astrocytes promote synaptogenesis between developing neurons by secreting a number of different factors (Allen et al., 2012; Baldwin & Eroglu, 2017; Kucukdereli et al., 2011), including a class of extracellular matrix glycoproteins known as thrombospondins (TSPs) (Christopherson et al., 2005; Risher & Eroglu, 2012). Of greatest interest are thrombospondin 1 and 2 (TSP-1/2), which exert their synaptogenic effect by binding to α2δ-1, a subunit of L-type voltage-gated Ca^{2+} channels found on the surface of neurons at pre- and postsynaptic terminals throughout the CNS (Risher et al., 2018) (**Figure 2**). This was further evidenced by the observation that the number of excitatory glutamatergic synapses and overall dendritic spine maturity were both decreased in the cerebral cortices of transgenic α2δ-1 knockout (α2δ-1 KO) mice (Risher et al., 2018). Given the known involvement of astrocytes in certain forms of neuronal dysfunction and neurodegenerative disease (Cresto et al., 2019; Sidoryk-Wegrzynowicz et al., 2011; Zoghbi & Bear, 2012), the effect of opioids on the modulation of astrocyte signaling, particularly astrocyte-mediated synaptogenesis, may explain some of the developmental aberrations associated with prenatal opioid exposure.

Opioid receptors (particularly MORs) have been confirmed on the surface of astrocytes (Hauser et al., 1996; Nam et al., 2018; Stiene-Martin & Hauser, 1991), making these cells susceptible to receptor-ligand interactions with both endogenous and exogenous opioids. Morphine and other MOR agonists have been shown to inhibit astrocyte growth and development by suppressing DNA synthesis *in vitro* (Hauser et al., 1996) and increase expression of glial fibrillary acidic protein (GFAP), an indicator of reactivity, in astrocytes (Beitner-Johnson et al., 1993). Opioids also appear to affect astrocyte-neuronal interactions, as cultured immortalized rat astrocytes subjected to prolonged treatment with μ-opioid receptor agonists, including morphine, showed decreased production of TSP-1 and 2 (Phamduong et al., 2014) (**Figure 2**). These findings can be seen *in vivo* as well, since rat pups repeatedly injected with morphine were shown to have decreased astrocyte expression of TSP-1 (Ikeda et al., 2010). The importance of astrocytes in the development of opioid addiction is also evidenced by morphine-treated mice that showed enhanced conditioned place preference, actively choosing to spend more time in a chamber paired with morphine injections over a chamber paired with saline injections, after receiving injections of astrocyte conditioned media into their brains (Narita et al., 2006). Greater understanding of the role of astrocytes in maintaining excitatory/inhibitory synaptic balance, coupled with increased awareness of the effects of drugs of abuse on astrocytes during development, may facilitate novel insight into how neurological function may be impacted in cases of early life drug exposure.

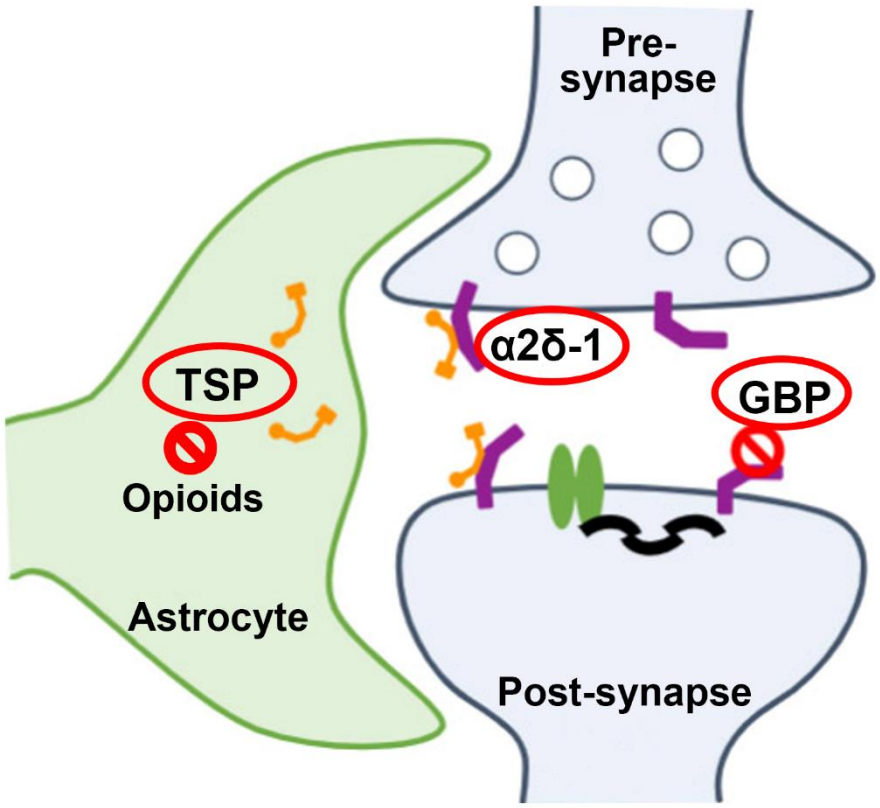

Figure 2: The Tripartite Synapse and the Interactions Between Thrombospondins (TSP), α2δ-1, and Gabapentin (GBP)
Astrocyte foot processes interact with pre- and post-synaptic terminals. Opioids and gabapentin have been shown, in different ways, to interfere with normal release of TSPs and their interaction with α2δ-1. Adapted from figure originally presented in review article by Baldwin and Eroglu (Baldwin & Eroglu, 2017).

Effects of Prenatal Gabapentin Exposure

Although the rise in rates of NAS over the past 20 years can largely be correlated with the rise in rates of OUD, opioids are not the only class of drugs commonly used by women during pregnancy. Co-abuse of multiple substances at once is common in these women and in substance use disorder patients in general (Ahmad et al., 2022; O'Donnell J, 2020). One prescription drug that has been shown to be increasingly co-abused along with opioids is gabapentin, an anticonvulsant drug commonly prescribed for the treatment of neuropathic pain

(Smith et al., 2016). Originally prescribed to treat partial seizures, gabapentin has been increasingly prescribed to treat neuropathic pain. Though originally designed as a structural analogue for the neurotransmitter γ-aminobutyric acid (GABA), gabapentin does not bind to $GABA_A$ or $GABA_B$ receptors. It has instead been proposed that gabapentin decreases neurotransmitter release from the presynaptic terminal by inhibiting Ca^{2+} influx through L-type channels (Fink et al., 2000; Patel & Dickenson, 2016). Although gabapentin was initially believed to have no potential for abuse or addiction (Bastiaens et al., 2016; Smith et al., 2016), surveys of OUD patients have found that as many as 26% of those interviewed reported abusing gabapentin for nonmedical reasons, often taking gabapentin in combination with opioids as a means to potentiate the experienced high (Baird et al., 2014; Bastiaens et al., 2016; Smith et al., 2016). Supporting their testimonies are studies demonstrating that gabapentin has also been shown to enhance the analgesia of morphine in a rat model (Meymandi et al., 2006). In addition, clinicians have observed a unique presentation of NAS in infants whose mothers abused both opioids and gabapentin while pregnant, with symptoms including tongue thrusting, back arching, and increased eye wandering (Loudin et al., 2017). Reports of gabapentin's potential for abuse has led many states to classify the drug as a Schedule V Controlled Substance. The mechanisms by which gabapentin and opioids may interact and the reasons for the unique presentation of NAS in infants with a history of exposure to a combination of both drugs remain unclear.

A possible explanation for the unique interaction between gabapentin and opioids in prenatally exposed infants may lie in the mechanism of action of gabapentin. Gabapentin is believed to primarily act through binding to α2δ-1, the same Ca^{2+} channel subunit that, as stated earlier, is also the binding site for TSP-1 and TSP-2 (Cole et al., 2005; Field et al., 2006; Nicolas S. Gee, 1996). Gabapentin appears to interfere with TSP binding to α2δ-1 as evidenced by the

disruption of normal astrocyte-induced synaptogenesis in cultured retinal ganglion cells in response to the addition of gabapentin (Eroglu et al., 2009). This interference in normal synaptogenesis could potentially explain the unique NAS phenotype associated with gabapentin and could indicate the potential for greater disruption of neurological development.

Changes in Size and Structure in the CNS Associated with Prenatal Drug Exposure

The changes in cellular function and development observed to result from exposure to drugs of abuse, particularly opioids and gabapentin, would be expected to be accompanied by observable changes in the size, structure, and function of both the entire CNS as a whole and of specific brain regions and the pathways that connect them. Within the brains of adults with a history of long term prescription opioid dependence, Magnetic Resonance Imaging (MRI) scans showed significant changes in functional connectivity have been observed, with the duration of opioid exposure positively correlated with the severity of those changes (Upadhyay et al., 2010). Additional MRI studies have shown reduced white matter and grey matter within the brains of adult opioid abusers (Upadhyay et al., 2010; Y. Yuan et al., 2009).

In comparison to the changes observed in the adult CNS in response to opioid exposure, prenatal opioid exposure tends to lead to even more dramatic effects. For decades, researchers have observed lower birth weights and smaller head circumferences in children prenatally exposed to opioids (H. Johnson et al., 1984; Asher Ornoy et al., 2001; Wilson et al., 1979). These low birth weights in children correlate with higher rates of mortality in the first two years of life (Ostrea et al., 1997) and have also been associated with poor performance in school and higher rates of behavioral problems (McCormick et al., 1990; Taylor et al., 2015).

Other structural deficits associated with prenatal opioids have been observed in studies utilizing medical imaging. Ultrasound studies have found that fetuses at 18-22 weeks gestation

whose mothers were undergoing methadone maintenance therapy had significantly larger thalamic diameter-to-head circumference ratios compared to a control group (Schulson et al., 2014). Another ultrasound study observed abnormally small, slit-like ventricles in NAS affected 1-month-old infants (Pasto et al., 1985). Other studies utilized MRI to examine the brains of children with a history of prenatal drug exposure. In both infants (Q. Yuan et al., 2014) and in children 10-14 years of age (Sirnes et al., 2017), certain brain regions (including the basal ganglia, thalamus, and cerebellar white matter) were observed to be significantly smaller compared to age-matched control children after adjusting for intracranial volume and birthweight. Another MRI study examining 17-22-year-olds whose mothers had abused multiple drugs, including heroin, while pregnant had significantly smaller whole brain, cerebral cortex, cerebral white matter, basal ganglia, pallidum, thalamus, and cerebellar white matter volumes compared to controls (Egil Nygaard et al., 2018). Taken together, these results show that changes in the sizes of brain region observed in drug-exposed infants can and do persist into adolescence and young adulthood.

Prenatal Drug Exposure is Associated with Changes in Behavior

The types of changes observed within the CNS, both in cellular development and systemic structure, could reasonably be expected to result in alterations to normal CNS function that would be reflected in behavior. Indeed, a variety of behavioral and cognitive deficits have been documented both in animal models of prenatal drug exposure and in human patients. Tests of anxiety-type behaviors in animal models have shown significant differences between drug-exposed and control subjects. Adult rats whose mothers were injected with morphine during pregnancy were shown to spend less time both in the light compartment of a light/dark box and while exploring the open arms of an elevated plus maze compared to rats whose mothers

received saline (Ahmadalipour et al., 2015; Klausz et al., 2011). Similar behaviors suggestive of more pronounced anxious phenotypes among these prenatal morphine-exposed rats were also observed in rats prenatally exposed to buprenorphine (Chen H, 2015). Exactly what structural and functional changes within the CNS would result in such anxious phenotypes have not yet been determined.

Given the importance of opioids in reward processing, it is not surprising that reward-seeking and depressive behaviors are affected in animals prenatally exposed to opioids. Adult rats exposed to morphine *in utero* showed a greater preference for a saccharin/water solution over pure water compared to control rats, indicating an elevated response to pleasurable stimuli (Gagin et al., 1996). Differences are also seen in exposed animals when faced with unpleasant stimuli. Rats prenatally exposed to buprenorphine and tested using the forced swimming and tail suspension tests showed reduced escape-oriented behaviors, which was interpreted as greater depressive behavior than control rats (Hung et al., 2013; Wu et al., 2014) Changes in connectivity or strength of the mesolimbic dopamine reward pathway caused by prenatal exposure to opioids could explain these trends of elevated response to rewarding stimuli and greater depressive behavior in response to unpleasant stimuli.

Studies utilizing memory tests have shown significantly worse performance in rodents prenatally exposed to opioids compared to control animals. Rats prenatally exposed to morphine, methadone, or buprenorphine subjected to tests of novel object recognition on postnatal day 44 were shown to have impaired recognition memory compared to control rats (Chen H, 2015). When tested with the Morris water maze on postnatal day 30, rats prenatally exposed to heroin took longer than unexposed rats to follow visual cues and swim to a platform they had previously located, indicating deficits in learning and memory (Wang & Han, 2009). Another study showed

a reduced tendency to explore a familiar object after it was moved to a novel location in 120-day-old mice that had been exposed to heroin during prenatal development (Lu et al., 2012).

Studies use a variety of scales and tests to evaluate human cognitive function at different developmental stages. The meta-analysis by Yeoh et al. (2019) of 26 human prenatal opioid exposure studies provides an extensive overview of the current knowledge concerning cognitive as well as motor related dysfunctions. Male infants with a history of prenatal opioid exposure have been shown to score higher than females on tests of habituation, which measure the ability of the infant to ignore extraneous environmental stimuli (Jones et al., 2010). One of the earliest studies to investigate prenatal drug exposure in humans showed that children aged 3 to 6 years old who were prenatally exposed to heroin performed worse on the General Cognitive Index and on the perceptual performance, quantitative, and memory subtests of the McCarthy Scales of Children's Abilities (Wilson et al., 1979). Another study compared a group of 12-month-old children with a history of prenatal methadone exposure to an age-matched control group by utilizing the Bayley Scales of Infant Development. It was found that 20% of the methadone-exposed group had Mental Development Index scores indicative of developmental delay, while only 4% of the unexposed children had such low scores (H. Johnson et al., 1984). A more recent study used the second edition of the Bayley Scales of Infant Development (Bayley-II) to evaluate a group of boys whose mothers had abused multiple drugs, including heroin, during pregnancy. The average scores for these children at years 1, 2, and 3 were, respectively, 9.7, 12.9, and 10.3 points lower than the average scores for an unexposed control group (Egil Nygaard et al., 2015). The same cohort scored 15.4 points lower than controls on the McCarthy Scales of Children's Abilities at age 4.5 and 14.8 points lower than controls on the Wechsler Intelligence Scale for Children at age 8.5, suggesting that diminished cognitive ability among prenatally the exposed

children did not improve over time. An analysis of standardized test scores in New South Wales, Australia also provides evidence that prenatally exposed children do not catch up with non-exposed peers in terms of cognitive ability. A cohort of children who had been diagnosed with NAS as infants scored lower on the National Assessment Program: Literacy and Numeracy (NAPLAN) than the statewide average and were more likely to not meet a minimum standard score in grades 3, 5, and 7 (ages 8–9, 10–11, and 12–13 years respectively) (Oei et al., 2017). These persistent trends imply that opioid exposure in early stages of development lead to lasting, detrimental cognitive changes.

While standardized tests can be used to reveal deficits in cognitive function associated with prenatal drug exposure, in some cases this exposure appears to negatively impact cognition to the point where the affected child develops a diagnosable disability. A study in Tennessee found that a group of school aged children (ages 3-8 years) with a history of NAS were more likely to have been evaluated for, diagnosed with, or treated for an educational disability compared to an age-matched control group of children (Fill et al., 2018). The authors of this study also noted that children in the NAS group were more likely to have required specialized classroom therapies or services than members of the control group. Another study used the Truven Health Analytics' Multi-State Medicaid Database to follow the psychiatric outcomes of over 1,000 children who had been diagnosed with NAS as infants. Within their first 5 years of life, these children were more than twice as likely to have been diagnosed with either disturbance of conduct, hyperkinetic syndrome of childhood, adjustment reaction disorder, or an intellectual disability compared to children with no history of NAS (Laura J. Sherman et al., 2019). Given the increasing rates of NAS nationwide, observations such as these highlight the importance of

research to an educational system that will have to accommodate the specific needs of the affected children.

The vast majority of studies comparing cognitive function and mental health between individuals with a history of prenatal drug exposure to non-exposed controls are focused on children and adolescents. The number of longitudinal studies following subjects into adulthood is limited. One study that included adult subjects examined a group of individuals 17-21 years of age whose mothers had abused heroin and other drugs during pregnancy. It was found that members of the prenatal drug-exposed group had significantly higher rates of attention deficit disorder/attention hyperactivity disorder (ADD/ADHD) and substance abuse disorder compared to a non-exposed age-matched control group (E. Nygaard et al., 2017). In addition, the exposed group performed significantly poorer on tests of general mental abilities and memory compared to the non-exposed control group (E. Nygaard et al., 2017).

Goals of this Dissertation

The combined results of these basic and clinical research studies, including my own study identifying significant trends among a local population of NAS patients, appear to indicate that there is an interaction between drugs of abuse, such as opioids and gabapentin, and astrocyte-mediated synaptogenic signaling. This interaction, predominantly centering on the association between astrocyte-secreted thrombospondin and α2δ-1 expressed on the cell surface of neurons at synaptic terminals, may be crucial to understanding the long-term effects of prenatal exposure to drugs of abuse. With this in mind, two specific aims were developed for this dissertation: 1. To test the hypothesis that exposure to opioids, gabapentin, or a polysubstance combination of the two drugs during early life development will lead to significant disruptions in astrocyte-regulated synaptic connectivity in brain regions associated with addiction and reward, and 2. To

test the hypothesis that such drug-exposed mice would demonstrate more pronounced addiction-related behavioral phenotypes compared to genetically similar drug-naïve mice.

To these aims, pregnant mice belonging to two separate lines transgenic for the expression of α2δ-1 were administered one of four treatments: the opioid drug buprenorphine, gabapentin, a combination of both drugs, or a vehicle control. α2δ-1 wild-type, heterozygous, and knockout littermate pups of both sexes at 21 days of age were used for studies involving immunohistochemistry and confocal fluorescence microscopy to determine the number of synapses within brain regions associated with reward. A second cohort of transgenic pups at 70 days of age was subjected to a regimen of experiments meant to assess anxiety, impulsivity, and reward-seeking type behaviors. By completing these studies, I hoped to contribute to a greater understanding of the effects of prenatal opioid exposure on neurological and behavioral development that would inform parents, healthcare providers, and educators looking to meet the unique developmental and educational needs of children impacted by drug abuse.

CHAPTER 2

AN INVESTIGATION INTO POTENTIAL TRENDS AMONG A LOCAL POPULATION OF NEONATAL ABSTINENCE SYNDROME PATIENTS

INTRODUCTION

West Virginia has been especially negatively impacted by rising rates of opioid abuse and neonatal abstinence syndrome (NAS) in recent years. The state routinely ranks among the highest in the country in terms of NAS diagnoses (AHRQ, 2021; CDC, 2020; Hayes & Brown, 2012; Scholl L, 2019; Umer et al., 2019). Historically, patients living in rural regions such as Appalachia have limited access to healthcare facilities and are therefore less likely to receive treatment for substance abuse or NAS (J. Brown et al., 2018; Hayes & Brown, 2012). Lily's Place, a non-profit NAS treatment center in Huntington, WV, founded in 2014, provides therapeutic and pharmacological care to newborns born at nearby hospitals diagnosed with NAS. Patient records from facilities such as Lily's Place may offer insight into the patient populations that benefit most from the services provided by these facilities. In this study, the charts of patients treated at Lily's Place who returned for follow-up appointments between 2015 and 2019 were reviewed in order to examine any emerging trends among the children and their mothers. The types of drugs abused by the mothers of patients, the number of patients to reach developmental milestones, the types of medical issues observed among patients and their mothers, the insurance status of the patients, and the plan for care at each patient appointment were all recorded and compiled in order to report any emerging trends and increase understanding of the patient population served by Lily's Place. The results described in this study are currently pending publication in the *Journal of Clinical Pediatrics and Neonatology*.

MATERIALS AND METHODS

Newborn patients from Huntington-area hospitals evaluated as showing symptoms of NAS were referred and transported to Lily's Place. During their stay at the facility each patient was treated in individual nursery rooms where their health was monitored. Pharmaceutical treatments, such as buprenorphine and/or clonidine, were administered to patients, if necessary, to help mitigate the process of weaning them from drugs of abuse. A monthly follow-up clinic was held for the parents of patients to bring their children for neurological and general health evaluations. The first follow-up appointments for all patients are scheduled 3 months following departure from the treatment center. Electronic charts (Allscripts Healthcare, LLC, Chicago, IL) from Lily's Place patients who appeared for these follow-up visits between the years 2015 to 2019 were compiled and analyzed using statistical software (Microsoft Excel, Redmond, WA and GraphPad Prism, San Diego, CA). Confidentiality of patient names and identifying information was maintained in line with HIPAA regulations.

RESULTS

Few Patients Returned for More than One Follow-Up Appointment

One hundred and twenty-two patients who appeared for follow-up appointments at Lily's Place between 2015 and 2019 were included in this study. These patients accounted for a total of 217 individual clinic visits. Of the 122 patients who appeared for follow-up appointments at Lily's Place, 64 (52.5%) were male and 58 (47.5%) were female. Fifteen patients were born via cesarian section while seven were born prematurely (before 37 weeks of pregnancy).

The majority of patients appeared at the clinic only once (67 patients or 54.9%), despite the encouragement for parents to schedule additional appointments (**Figure 3)**. The mean number of visits per patient was 1.92 with the highest number of visits for any one patient being

9. Seventy-five patients arrived in the custody of someone other than a biological parent, such as foster parents or grandparents, at the time of their follow-up appointment.

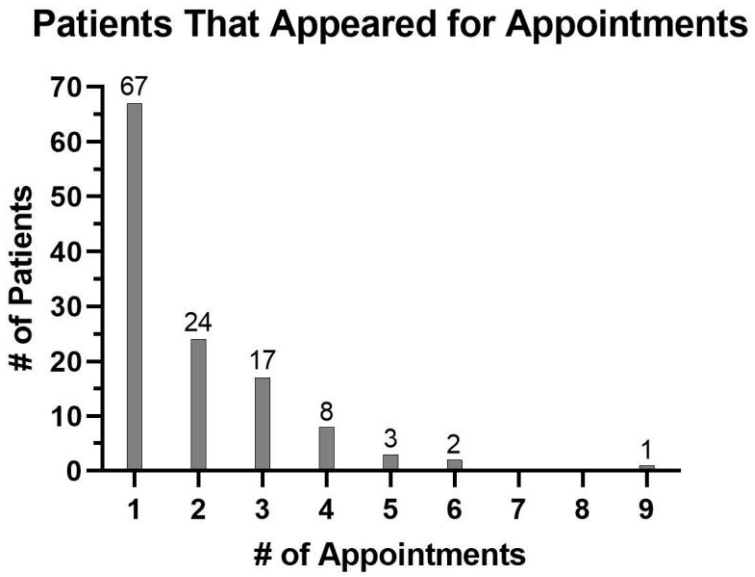

Figure 3: Patient Appearance for Follow-Up Appointments
Breakdown of the numbers of times patients appeared for follow-up appointments at Lily's Place in Huntington, WV.

Certain Drugs Were Commonly Misused by Patient Mothers

Drugs of abuse taken by the mothers of patients during their pregnancies were reported in the patient records through a combination of drug screenings at the time of birth or through self-report by the mothers (see **Table 1**). Opioid drugs of some form were confirmed to be used by 104 mothers of patients (85.2%) during their pregnancy. The single most commonly used drug among mothers of patients was buprenorphine, with 56 either currently taking the drug as a part of medication-assisted treatment for opioid abuse or with a history of using the drug without a prescription. 28 mothers were recorded as having used heroin. Tobacco products were the most

commonly used non-opioid drugs (31 mothers; 25.4%) followed by cannabis products and gabapentinoids (e.g. Neurontin®, Lyrica®) with 19 mothers (15.6%) each.

Drug	# Patient Mothers
Some Form of Opioid	104
Methadone	26
Buprenorphine	56
Heroin	28
Unknown/Other	39
Tobacco	31
Cocaine	11
Benzodiazepine	14
Methamphetamine	7
Cannabis/THC	19
Gabapentinoids	19
Alcohol	2
SSRIs	8
TCAs	2
Tramadol	2
Other	6

Table 1: Drugs Misused by the Mothers of Patients During Their Pregnancies

Many Patients Did Not Reach Developmental Milestones on Time

The development of each patient was assessed in each follow-up appointment based on the Centers for Disease Control and Prevention's (CDC) Developmental Milestones, a list of motor skills and behaviors that indicate typical normal and healthy development in young children ("CDC's developmental milestones," 2020). The milestones are separated into four categories: Social/Emotional, Language/Communication, Cognitive Milestones (learning, thinking, problem-solving), and Movement/Physical Development Milestones. The number of

patients to reach all milestones assigned for their age group was recorded for this study (**Table 2**). By two months of age, infants are expected to be able to perform such tasks as hold their head up when on lying on their stomach, move both arms and both legs, follow movement with their eyes, and smile in response to words or smiling faces. Only 17 of the 122 patients included in this study successfully reached all these developmental milestones at two months of age. At four months of age, milestones for infants include being able to hold their head steady without support when being held, hold a toy in their hands, bring their hands to their mouth, turn their head to follow the sound of a voice, and push up onto elbows/forearms when on stomach. Only 14 patients in this study managed to reach all of these developmental milestones on time. Milestones for an infant at 6 months of age include being able to recognize familiar people, reach to grab objects and place them in their mouth, make squealing noises, and push themselves up with their arms and roll over to their backs when lying on their stomachs. Only seven patients successfully reached all these developmental milestones at the designated age. These results reflect significant developmental delays among this population of NAS patients, and likely among NAS patients in general.

Developmental Milestone Age (months)	# Patients
2	17
4	14
6	7
9	5
12	6
18	1

Table 2: Number of Patients Who Reached Each CDC Developmental Milestone

Certain Medical Issues Were Frequently Observed Among the Patients

Medical issues commonly observed among Lily's Place patients during their follow-up appointments were recorded (see **Table 3**). The most common medical issues seen in patients were musculoskeletal, with 47 patients (38.5%) showing muscle and/or joint tightness, most commonly within the hips and legs. Twenty patients were diagnosed with torticollis and 10 showed muscle weakness or low tone in at least one major muscle group.

In terms of neurological symptoms, 38 patients (31.1%) exhibited tremors that could not be attributed to another neurological condition during their appointments. Among these patients, 25 patients exhibited tremors at their 3-month follow-up but not in subsequent clinic visits. The latest any patient demonstrated tremors during an examination was at 8 months of age. In addition, 6 patients exhibited clonus in at least one heel when tested during their neurological examinations.

The most common medical issue not involving the musculoskeletal or nervous systems was gastroesophageal reflux disease (GERD) with 40 patients diagnosed with the condition, the majority of which also had already been prescribed ranitidine (Zantac®) to reduce reflux by their primary care physicians. Five patients were diagnosed with asthma and had all been prescribed medications for its treatment.

Certain Medical Issues Were Common Among the Mothers of Patients

Significant health conditions of the mothers of patients were included in their children's medical histories if such a condition could have impacted either their pregnancy or the delivery (see **Table 4**). The most common medical condition among mothers was Hepatitis C. Forty-three mothers either tested positive at the time of delivery or had a recent history of testing positive.

Sexually transmitted diseases, hypertension, diabetes, and psychological conditions (including depression, anxiety, and bipolar disorder) were also observed in mothers.

Medical Issue	# Patients
Muscle/Joint Tightness	47
G.E.R.D.	40
Tremors	38
Torticollis	20
Low tone/muscle weakness	10
Clonus	6
Asthma	5

Table 3: Common Medical Issues Observed Among Lily's Place Patients at Follow-Up Appointments

(G.E.R.D.: gastroesophageal reflux disease)

Medical Issue	# Patient Mothers
Hepatitis C	43
Herpes Simplex	4
Other STD	3
Hypertension	4
Psychological Disorder	3
Diabetes	2

Table 4: Common Medical Diagnoses Among the Mothers of Lily's Place Patients

Medicaid was Primary Medical Insurance for Majority of Patients

The medical insurance status of each patient was recorded at each clinic visit. Primary medical insurance for 65 patients (53.3%) was listed as West Virginia Medicaid, making it the most common form of insurance among the patients included in this study. Ten patients had no

medical insurance or were listed as “Self-pay” at the time of their appointment. All other patients were insured by at least one other healthcare insurance company (e.g. Aetna®).

A Plan for Care was Developed to Meet the Needs of Each Patient

At the end of every patient encounter, in addition to scheduling their next appointment at the clinic, a plan was developed to attempt to meet the medical needs of each patient. Sixty-six of 217 visits to Lily’s Place resulted in a referral to developmental support services such as Birth to Three (BTT), a program developed by the West Virginia Department of Health and Human Resources (DHHR) through the Bureau for Public Health and the Office of Maternal, Child, and Family Health available for free to all children at risk for a developmental delay. Other services to which patients were referred included Head Start (a program overseen by the United States DHHR Office of the Administration for Children & Families), First Steps (overseen by the Kentucky Department for Public Health in the Cabinet for Health and Family Services), or one of Cabell Huntington Hospital’s Rehabilitation Services (including physical therapy, occupational therapy, and speech therapy). Sixty-one clinic visits included a demonstration of stretches or exercises designed to improve muscle/joint tightness or low tone/muscle weakness. Specific recommendations other than stretching or exercise, such as increasing the amount of time the patient spent on its stomach to promote motor skills or deliberately stimulating one side of the patient’s face in order to encourage head turning in a specific direction to treat torticollis, were made in 13 visits. In 11 visits, specific physical aids, such as supportive shoes, were encouraged to improve mobility development in struggling patients. Tests such as MRI scans or prescription drugs were ordered in only seven patient visits. Discussions of test results or general reassurance of patient parents/guardians occurred in 13 visits, while in 20 visits parents/guardians of patients were given encouragement to continue current therapies with the

goal of reaching developmental milestones. At the end of 13 separate visits, plans were made to consult with additional physician(s) (such as the patient's primary care physician) to discuss a patient's plan for treatment. Lastly, although written informational materials are always made available to patients and their families, during two separate interactions relevant informational handouts were shared and discussed with the patient's parents/guardians

Plan For Care at Visit	# Appointments
Encourage Developmental Milestones	20
Referral to Birth to 3, First Steps, PT, etc.	66
Reassurance or Discussion of Test Results	13
Specific Recommendation (Tummy time, stimulation of one side of face, etc.)	13
Prescription or Test Ordered (e.g. MRI)	7
Demonstrated Stretching or Exercises	61
Recommended Shoes or Other Physical Aid Device	11
Consult with Other Physician	13
Handout or Written Information Handed Out	2

Table 5: Plans for Care Developed for Patients at the End of Follow-Up Appointments

DISCUSSION

The results of this study of NAS patients treated at Lily's Place reveal some important information about trends among NAS patients in West Virginia and, potentially, in the wider United States. The high appearance of opioid drug use among the mothers of patients is consistent with national trends of increased rates of opioid (Hedegaard H., 2020; Scholl L, 2019; USDHHS, 2015) abuse. The long-term health and psychological effects of prenatal exposure to drugs of abuse, such as opioids, continue to be an area of intense scientific investigation. Many such studies suggest a correlation between prenatal drug exposure and diagnosis of an

intellectual disability or mental disorder (Fill et al., 2018; H. Johnson et al., 1984; Laura J. Sherman et al., 2019; Egil Nygaard et al., 2015; Egil Nygaard et al., 2018; A. Ornoy, 2003; Ross et al., 2015; Sirnes et al., 2017). Additional studies suggest a correlation between prenatal drug exposure and poor academic performance (Fill et al.; Oei et al., 2017).

Among the opioid drugs used by patients, the single most common drug was buprenorphine. Buprenorphine is increasingly prescribed to pregnant mothers as part of medication-assisted therapy to help them overcome opioid addiction and limit the exposure of the developing fetus to potentially more harmful opioids (Jansson & Velez, 2012). It has also been prescribed as a weaning therapeutic in place of methadone for infants with NAS prenatally exposed to opioids (Kraft, 2018). Therefore, it is not surprising that the most common drug taken by the mothers of the children in this study was some form of buprenorphine (as Subutex®, Suboxone®, Butrans®, etc.). However, this study agrees with previous research which shows that buprenorphine as a replacement therapy for other opioids does not eliminate the risk of NAS in exposed infants (Kayemba-Kay's & Laclyde, 2003; Kraft, 2018). Of the six patients included in this study that exhibited clonus, five had been exposed to buprenorphine *in utero* (the 6th was exposed to methadone and other unspecified opioid drugs), a possible indication that a greater understanding of the effects of prenatal exposure to opioids, even ones considered "less harmful" such as buprenorphine, is still needed.

Drugs besides opioids were also observed in significant numbers among the mothers of NAS patients. Tobacco products were found to be the second most common drugs of abuse. Products containing nicotine, including cigarettes or electronic vape devices are commonly used by OUD patients, and the cessation of such products is not often encouraged in patients undergoing opioid abuse treatment programs (Guydish et al., 2011). Gabapentinoids such as

gabapentin have increasingly been reported as being misused by substance abuse patients (Vickers Smith et al., 2018), both the drugs by themselves and in combination with opioids in order to potentiate the experienced high (Bastiaens et al., 2016). A unique set of NAS symptoms, such as tongue thrusting, back arching, and eye wandering, have also been reported among infants who were prenatally exposed to gabapentinoids (Loudin et al., 2017). Although the rise in rates of abuse of opioids is likely the driving factor for the rise in rates of NAS, these drugs of abuse are not the only ones commonly used by pregnant women and are often co-abused with other non-opioids.

Among the medical conditions observed among the patients, musculoskeletal issues were among the most common. The high rate of muscle and joint tightness seen among these patients can likely be attributed to the care they received during their initial stay at Lily's Place. Tightly wrapping the body of an infant suffering from NAS in blankets has been shown to be an effective method of soothing these frequently fussy patients (Blount et al., 2019). This swaddling keeps the limbs of the infant immobile and close to its body, potentially resulting in muscle and joint tightness that must be remedied by stretching later in the child's development to avoid long term difficulties.

Examining the different plans for care developed in each appointment also gives insight into the different needs of the patients served by Lily's Place. A large number of patient interactions involved only reassurance, guidance, or counseling on the part of the physician. Some of the most common issues seen among this patient group, namely muscle/joint stiffness, torticollis, and low tone/muscle weakness, could in fact be remedied through exercises, stretches, and specific recommendations (e.g. stimulating one side of the patient's face to promote head

turning) designed to promote musculoskeletal development (Carenzio et al., 2015; He et al., 2017).

This study also revealed important demographic information about patients most likely to receive services from facilities such as Lily's Place. Over half of all patients included in this study were insured by Medicaid, indicating that the majority of patients seen at Lily's Place could likely be described as lower socio-economic status. While substance abuse and socio-economic status are not necessarily correlated (Hanson & Chen, 2007; M. Patrick et al., 2012), families of a lower socio-economic status traditionally have limited access to quality healthcare (Becker & Newsom, 2003; Braveman & Gottlieb, 2014) and children of such families tend to do worse in academic settings (Thomson, 2018). In addition, the majority of patients in this study appeared at their follow-up appointments in the legal custody of someone other than their biological mothers. Stressful early life events and environments, such as those that might be found during a transition between biological and foster parent care, are correlated with poorer cognitive and physical development in children (Berry et al., 2016; Coley et al., 2015). Such considerations may make a significant difference for NAS patients who would benefit from the involvement of social welfare programs as well as the engagement of invested healthcare professionals.

While this study was successful in its attempts to describe trends observed among the chosen group of patients, there are several important limitations of this investigation that need to be considered. Given how many infants are diagnosed with NAS each year (AHRQ, 2021), this pool of 122 patients may be too small to accurately represent the tens of thousands of NAS patients currently living in the United States. In addition, while this study was primarily a review of the charts of patients diagnosed with NAS, the full medical histories of the mothers of these

patients were not reviewed. Only pertinent information concerning disease status and drug abuse history that was gained through interviews and examinations of mothers after they had been admitted to the hospital in the lead up to delivery was included in this study. Any demographical information concerning such factors as race or socio-economic status was also not included in this study in the interest of maintaining patient anonymity. Gathering this type of information may have revealed information about the circumstances of women most likely to give birth to children diagnosed with NAS. Additional outreach and prenatal care could be beneficial to such women and may even assist them in treating their substance use, thus lowering the risk of their children developing NAS. Future studies of similar patient populations may benefit from including a greater number of patient records from a wider geographical area as well as including information from the health histories of the mothers of the patients.

The results of this study may point towards important trends among NAS patients and their families in West Virginia and, potentially, in the wider United States. The data gained from this study should make apparent the importance of regular medical evaluation in the months immediately following treatment for NAS. It should also make apparent the need for facilities such as Lily's Place that seek to meet the healthcare needs of socio-economically disadvantaged populations. Increased understanding of the patient population served by a healthcare facility is likely to result in better patient outcomes and shape future policy to provide services better designed to meet the needs of those patients.

CHAPTER 3

AN INVESTIGATION OF ALTERATIONS IN SYNAPTIC DEVELOPMENT IN A MOUSE MODEL OF EARLY LIFE DRUG EXPOSURE

INTRODUCTION

Despite considerable research efforts to increase the understanding of the pathophysiology of NAS as well as methods to improve treatment of NAS symptoms (Bio et al., 2011; Grossman & Berkwitt, 2019; Jansson & Velez, 2012; Kocherlakota, 2014; Patrick et al., 2014), knowledge concerning the long-term effects of prenatal exposure to drugs of abuse on neurological development is limited (Boggess & Risher, 2020). One of the most commonly used opioid drugs among pregnant substance abuse patients is buprenorphine, a partial MOR agonist that is often prescribed to replace and prevent the reinforcing effects of more harmful and addictive opioid drugs, such as heroin or fentanyl. Buprenorphine is often used in medication-assisted treatment (MAT) to help treat OUD in pregnant women in the hopes of achieving better health outcomes for the mother and child. However, emerging evidence asserts that any opioid use during pregnancy, even when part of a medical treatment plan, can have deleterious effects on the developing fetus (Burke & Beckwith, 2017; Kayemba-Kay's & Laclyde, 2003).

While the increased prevalence of opioid abuse is often credited with the rise in incidence of NAS, it is important to note that many mothers of NAS patients abuse multiple drugs besides, or in combination with, opioids. One prescription drug that has been shown to be increasingly co-abused along with opioids is gabapentin (Smith et al., 2016). OUD patients have reported abusing gabapentin in combination with opioids as a means to potentiate the experienced high (Baird et al., 2014; Bastiaens et al., 2016; Smith et al., 2016). In addition, clinicians have

observed a unique presentation of NAS in infants whose mothers abused both opioids and gabapentin while pregnant (Loudin et al., 2017).

Although the mechanisms by which gabapentin and opioids may interact with each other are unknown, the addictive nature of such drugs of abuse can largely be attributed to their activity within the mesolimbic dopamine pathway (**Figure 1**) (Carlezon & Wise, 1996; Di Chiara et al., 2004; Wachtel et al., 1989). Examining the effects of these drugs of abuse on synaptogenesis within brain regions associated with the mesolimbic dopamine pathway may lead to insight on how these drugs affect neurological development in prenatally exposed children.

In this study, we used fluorescence immunohistochemistry (IHC) in a mouse model of early life drug exposure to investigate the effects of both buprenorphine and gabapentin on developmental synaptic connectivity. Our hypothesis was that exposure to buprenorphine, gabapentin, or a combination of both drugs during critical periods for synaptic development would lead to significant disruptions in excitatory and inhibitory synaptic connectivity in brain regions associated with addiction and reward. We further hypothesized that haploinsufficiency of the calcium channel subunit α2δ-1, the receptor for gabapentin and a critical player in developmental synapse formation and maturation, would result in further disruption of these synaptic populations. The results described in this study were previously published in *Frontiers in Pediatrics* (Boggess et al., 2021).

MATERIALS AND METHODS

Animals and Drug Treatments

All experiments were conducted in accordance with Marshall University's Institutional Animal Care and Use Committee (IACUC) guidelines (W.C.R. protocols 696 and 697). Adult

C57Bl/6J mice heterozygous for *Cacna2d1*, the gene encoding α2δ-1 (i.e. α2δ-1 +/-) (Risher et al., 2018) were a kind gift from Dr. Cagla Eroglu (Duke University). The mice were bred onsite at the Marshall University Animal Resource Facility. Virgin α2δ-1 +/- females and α2δ-1 +/- males were set up as mating pairs. Visual confirmation of a vaginal plug was counted as embryonic day 0 (E0) at which time males and females were separated. On E6, pregnant females were given access to 1 ml of a 1:1 sweetened condensed milk/water solution served in a plastic 35 mm dish. Starting on E7, pregnant females were given free access to a once daily 1 ml solution of 1:1 condensed milk/water containing either pharmaceutical grade buprenorphine hydrochloride (CIII) (5 mg/kg; Spectrum Chemical, Gardena, CA), gabapentin (30 mg/kg; Spectrum), a combination of both drug doses, or vehicle control. These doses were selected based on the results of previous rodent studies that used these drugs (Martin et al., 2001; Meymandi et al., 2006). Drug doses were calculated based on the weight of pregnant females on E7. Daily dosing continued through the birth of pups and ended on postnatal day 11 (P11). All animals were given ad libitum food (standard mouse chow milled on site) and water.

For wild-type (WT) mice, seven pups (three male and four female) from vehicle control treated litters, six (two male, four female) from gabapentin treated litters, six (three male, three female) from buprenorphine treated litters, and seven (four male, three female) from combined buprenorphine and gabapentin treated litters were included. For α2δ-1 +/- mice, 11 pups (five male, six female) from vehicle control treated litters, seven (three male, four female) from gabapentin treated litters, seven (four male, three female) from buprenorphine treated litters, and seven (four male, three female) from combined buprenorphine and gabapentin treated litters were included.

Mouse Brain Sample Drug Concentration Analysis

The brains of newborn WT pups from dams treated with 5 mg/kg buprenorphine (six pups), 30 mg/kg gabapentin (15 pups), or vehicle control (15 pups; two litters per treatment) were isolated and then shipped on dry ice to the Pharmaceutical Sciences Research Institute at the McWhorter School of Pharmacy (Samford University, Birmingham, AL) for drug concentration analysis. Mouse brain samples were homogenized 1:5 with 5 mM ammonium acetate buffer using an Omni THQ homogenizer (Atlanta, GA). Gabapentin calibration standards, blanks and QCs were prepared by spiking 20 µl of blank control brain homogenate with the appropriate amount of gabapentin to achieve concentrations in ranging from 25-10,000 ng/ml. Internal standard (1 ml of 100 ng/ml Gabapentin-d4 in methanol) was added to precipitate the proteins. After centrifugation for five minutes at 13,000 rpm, the supernatant was transferred to glass tubes and the solvent was evaporated under nitrogen at 40°C. The samples were then redissolved in 200 µl of 95/5 10 mM ammonium formate/methanol with 0.1% formic acid and transferred to limited volume autosampler vials and analyzed in positive ion mode by liquid chromatography with tandem mass spectrometry (LC/MS/MS).

Buprenorphine calibration standards, blanks, and quality controls (QCs) were prepared by spiking 100 µl of blank control brain homogenate with the appropriate amount of buprenorphine to achieve concentrations ranging from 0.2-200 ng/ml. Standards, blanks, QCs and samples were spiked with internal standard (10 µl of 50 ng/ml Terfenadine in acetonitrile). Acetonitrile (1 ml) was added to precipitate the proteins. After centrifugation for five minutes at 13,000 rpm, the supernatant was transferred to glass tubes and the solvent was evaporated under nitrogen at 50°C. The samples were then redissolved in 200 µl of 50/50 5 mM ammonium

acetate/acetonitrile and transferred to limited volume autosampler vials and analyzed in positive ion mode by LC/MS/MS.

The LC/MS/MS system consisted of Shimadzu system (Columbia, MD) equipped with LC20-AD dual HLPC pumps, an SIL20-AC HT autosampler, and a DGU-20A2 in-line degasser. Detection was performed using an Applied BioSystems 4000 QTRAP (Applied Biosystems, Foster City, CA) triple quadrupole mass spectrometer operated in the positive ion mode utilizing electrospray ionization. Mass calibration, data acquisition and quantitation were performed using Applied Biosystem Analyst 1.6.2 software (Applied Biosystems).

Separation of gabapentin and the gabapentin-d4 from the brain homogenate matrix was achieved from a 10 µl injection of the samples using a Phenomenex Polar C18, 100 X 2 mm 5 µm particle column. The mobile phase was delivered at a flow rate of 400 µl/min using a gradient elution profile consisting of 5 mM ammonium acetate (A) and acetonitrile with 0.1% formic acid (B). The analyte and internal standard were detected using multiple reaction monitoring (MRM) for the following transitions: Gabapentin (m/z 172.2 à 137.0), Gabapentin-d4 (m/z 176.2 à 141.0).

Separation of buprenorphine and terfenadine from the brain homogenate matrix was achieved from a 10 µl injection of the samples using a Phenomenex Luna C18, 100 X 2 mm 5 µm particle column. The mobile phase was delivered at a flow rate of 400 µl/min using a gradient elution profile consisting of 5 mM ammonium acetate with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The analyte and internal standard were detected using multiple reaction monitoring (MRM) for the following transitions: Buprenorphine (m/z 468.4 à 396.1), Terfenadine (m/z 472.4 à 436.3). Drug concentrations are summarized in **Table 6**.

Genotyping

At P7, the tails of the pups were clipped and collected. Toe pads were tattooed (AIMS NEO9 Animal Tattoo System, Hornell, NY) for later identification. Tissue digestion was performed on the clipped tails and DNA was isolated for PCR amplification and genotyping with a 2% agarose gel. Pups were identified as either α2δ-1 +/+ wildtype (WT), α2δ-1 +/- heterozygous (Het), or α2δ-1 -/- knockout (KO) using the following primers: F1 (WT forward, 5′-TCTCAGTTACAAGACTATGTGG-3′), F3 (KO forward, 5′-GGCTGTGTCCTTATTTATGG-3′), and LAF-Test (reverse, 5′-AGTAGGAGAAGGTACAATCGGC-3′) (Integrated DNA Technologies, Coralville, IA).

Perfusion, Freezing, and Cryosectioning

For the P21 mouse group, when a litter reached P21, the dam and pups were placed individually on a scale to be weighed (group data is included in **Table 7**) before being anesthetized with tribromoethanol/Avertin (250 mg/kg). Cardiac perfusion was performed, first with 0.24 μg/ml heparin salt in 0.1M phosphate buffered saline (PBS) for two minutes followed by 4% paraformaldehyde (PFA) for two-five minutes at a flow rate of 5 ml/min. The brains of the dam and pups were extracted and submerged in 4% PFA at 4°C for 24 hours. After 24 hours, the brains were rinsed in PBS and then placed in 30% sucrose:PBS solution for 48 hours. Brains were removed from sucrose solution, submerged in 2:1 sucrose solution:Tissue Freezing Medium (Cat. #72592-G, Electron Microscopy Sciences), and frozen within plastic embedding molds (Cat. #70182, Electron Microscopy Sciences, Hatfield, PA). All brains were stored at -80°C until they were cryosectioned into 20 μm slices using a Leica CM 1950 (Leica, Wetzlar, Germany) and stored in 50% glycerol:Tris-Buffered Saline (TBS) at -20°C.

Synaptic Labeling Immunohistochemistry

Three independent coronal sections per mouse containing the PFC (bregma, 2.96 to 2.58 mm; interaural, 6.76 to 6.38 mm) and three sections per mouse containing the ACC and NAC core/shell (bregma, 1.42 to 0.86 mm; interaural, 5.22 to 4.66 mm) were used for analyses (Paxinos, 2008). After selection, brain slices were washed in TBS + 0.2% Triton X-10% (Cat. #11332481001, Roche Diagnostics, Mannheim, Germany) w/v (TBST) solution at room temperature. The slices were then placed in a 5% normal goat serum (NGS; Cat. #005-000-121, Jackson Immuno Research Laboratories Inc., West Grove, PA):TBST solution for 1 hour to block nonspecific binding sites. After blocking was completed, the slices were then placed in 5% NGS:TBST containing primary antibodies against both pre- and postsynaptic proteins. Guinea pig anti-vesicular glutamate transporter 1 (VGluT1; Cat. #AB5905, EMD Millipore, Burlington, MA) at 1:2000 dilution was used to identify excitatory glutamatergic presynaptic axonal regions while rabbit anti-post synaptic density protein 95 (PSD95; Cat. #51-6900, Invitrogen, Carlsbad, CA) at 1:300 dilution was used to identify postsynaptic dendritic regions. To label inhibitory GABAergic synapses, guinea pig anti-vesicular GABA transporter (VGAT; Cat. #131004, Synaptic Systems, Göttingen, Germany) at 1:1000 dilution was used to identify presynaptic axonal regions and rabbit anti-gephyrin (Cat. #147002, Synaptic Systems) at 1:1000 dilution was used to identify postsynaptic dendritic regions. Slices were incubated overnight on an orbital shaker at 4°C.

After overnight incubation was completed, brain slices were removed from primary antibody solution and washed with TBST. Slices were then placed in 5% NGS:TBST containing fluorescent secondary antibodies against the primary antibodies. Alexa Fluor IgG (H+L) 594 goat anti-guinea pig (Cat. #A11076, Invitrogen, Carlsbad, CA) at 1:200 dilution was used to

stain against presynaptic antibodies (either VGluT1 for excitatory or VGAT for inhibitory) and Alexa Fluor IgG (H+L) 488 goat anti-rabbit (Cat. #A11034, Invitrogen) at 1:200 dilution was used to stain against postsynaptic antibodies (either PSD95 for excitatory or gephyrin for inhibitory). Slices were incubated for two hours at room temperature in darkness. After incubation was complete, the slices were washed with TBST.

After the final washing step, the slices were rinsed once in 2/3 TBS:1/3 dH20 solution. Then, the slices were transferred onto glass slides and excess liquid was suctioned off with a pipette. One drop of mounting media containing DAPI (Cat. #H-1200; VectaShield, Burlingame, CA) was applied to each slice prior to placement of coverslip and sealing with clear nail polish. The glass slides were allowed to dry overnight in darkness before being stored at -20°C.

Confocal Fluorescence Microscopy for Synaptic Protein Imaging

Images of the slides were captured using the Leica SP5 Confocal Fluorescence Microscope housed and maintained by the Marshall University Molecular Biological and Imaging Core. Argon visible light laser (at 30% final filter power) was used with emission windows set at 493 nm – 550 nm for blue excitation and 595 nm – 647 nm for green excitation. Leica LAS AF software was used to capture 5 μm z-stacks with 15 steps (0.33μm distance between each step) within the brain regions of interest (ROI) across all treatment groups (imaged area/scan = 19,036 μm2; 63× oil objective, 1.4 NA) (**Figure 1B-D**).

Synaptic Puncta Image Analysis

The saved confocal images were analyzed using ImageJ (NIH) software. The custom plug-in "ProjectZ_Triple" was used to average and convert the 15 separate images of each z-stack into five separate maximum projections, each representing a 1 μm "mini-stack". Puncta Analyzer (Dr. Cagla Eroglu, Duke University, Durham, NC) was then used to quantify the

number of discrete puncta for the 493 nm – 550 nm wavelength channel (corresponding to PSD95 in brain slices stained for excitatory synapses or Gephyrin for inhibitory synapses), the 595 nm – 647 nm wavelength channel (corresponding to VGluT1 in brain slices stained for excitatory synapses or VGAT for inhibitory synapses), and the co-localized puncta, where puncta from both channels overlapped within four pixels of each other (**Figure 4**). These regions of co-localization of pre- and postsynaptic markers were designated as synapses (Ippolito & Eroglu, 2010). The counted synapses were compiled into spreadsheets (Microsoft Excel, Redmond, WA) and analyzed with GraphPad Prism (San Diego, CA).

Statistical Analysis

For body mass comparisons, dams belonging to different treatment groups were compared via One-Way ANOVA with Tukey's multiple comparisons post hoc test. For pups, a Three-Way ANOVA (treatment × genotype × sex) was performed with Tukey's multiple comparisons post hoc test. The D'Agostino and Pearson omnibus normality test confirmed that most synapse counts were not normally distributed; therefore, statistical differences were analyzed using the nonparametric Kruskal-Wallis test to detect differences in mean number of synapses amongst drug treatments or between genotypes. Post-hoc Dunn's multiple comparisons tests were performed to detect significant differences ($p<0.05$) amongst the mean values of each treatment group. Analyses were performed using GraphPad Prism.

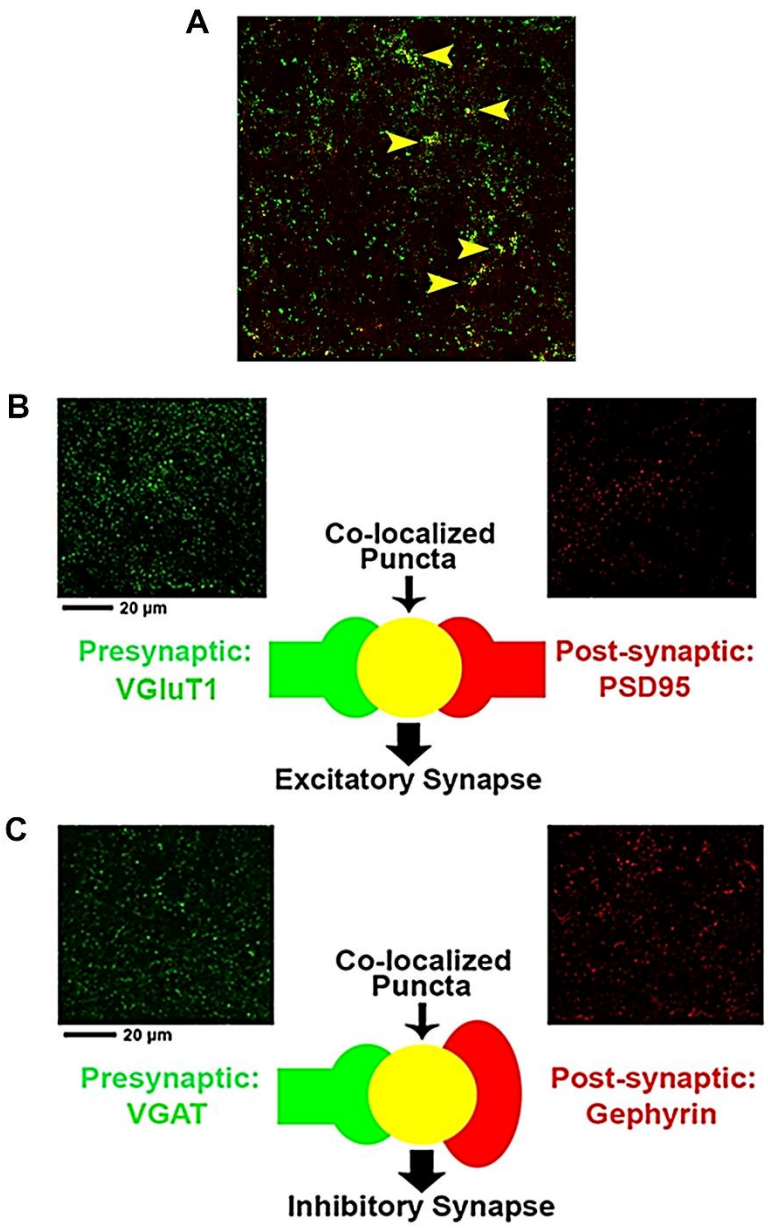

Figure 4: Visual Representation of Immunohistochemistry Staining
Representative images taken by confocal fluorescent microscope along with graphic representations of pre- (green) and post-synaptic (red) terminals meeting at puncta of co-localization (yellow). (A) Representative example merged image of red and green channels with arrows indicating colocalization. (B) Representative images for VGluT1 and PSD95 staining with graphic representation of excitatory glutamatergic puncta. (C) Representative images for VGAT and Gephyrin staining with graphic representation of inhibitory GABAergic puncta.

RESULTS

Drug Accumulation was Confirmed in Neonatal Mouse Brain Tissue

From E7 until P11, pregnant C57Bl/6J mice were administered drug treatments within 1 ml of a 1:1 sweetened condensed milk/water solution. It is important to note here that since drug treatments continued after the birth of pups and could therefore still be transferred from the dam to the pups via the breast milk, the treatments included in these studies are described as "early life" rather than "prenatal". The plastic dishes in which those drug treatments were served were consistently observed to be empty on the following day, confirming that the dams routinely consumed the entirety of the treatment solution. Concentrations of buprenorphine and gabapentin within the brains of adult rodents following administration have previously been demonstrated at detectable levels (Errante & Petroff, 2003; Rudeck et al., 2020). To confirm drug passage from dam to pups in this study, the brains of newborn pups from litters treated with buprenorphine, gabapentin, or a vehicle control were isolated, lysed, and subjected to liquid chromatography-tandem mass spectrometry to determine brain tissue concentrations for each drug. Both buprenorphine and gabapentin were detected in the brains of the exposed pups (**Table 6**).

Drug Concentrations in Brain Tissue Lysates from Drug Exposed Pups				
Treatment	**Maternal Dose**	**n**	**Concentration**	**Pup:Maternal Concentration Ratio**
Buprenorphine	5 mg/kg	6	1.85 ± 0.30 nM	0.054 ± 0.009
Gabapentin	30 mg/kg	15	3.29 ± 0.84 μM	0.259 ± 0.037
Vehicle	0 mg/kg	15	0 μM (both drugs)	N/A

Table 6: Drug Concentrations (Shown as Mean ± SEM) Within Brain Tissue Lysates from Drug-Exposed Newborn C57Bl/6/J Pups Determined via Liquid Chromatography-Tandem Mass Spectrometry

Body Mass of Dams and Pups Impacted by Drug Treatment, Sex, and Expression of α2δ-1

In order to produce the required number of WT and Het pups for IHC in this study, the following litter numbers were required: four litters dosed with vehicle control, five litters dosed with 30mg/kg gabapentin, four litters dosed with 5mg/kg buprenorphine, and five litters dosed with both drugs. Immediately before anesthetization and perfusion at P21, dams and all pups from each litter were weighed and their weights were recorded. The mean masses for the dams of the different litters and the pups in each sex-genotype-treatment group were calculated (**Table 7**). The mean body masses for dams revealed a significant main effect of treatment, [$F(3, 14) = 50.21$, $p < 0.0001$]. Dams treated with buprenorphine ($p = 0.0122$), gabapentin ($p = 0.0006$), and buprenorphine + gabapentin ($p < 0.0001$) were all significantly lower than the mean body mass of the vehicle control dams. Significant differences were also observed among the various pup groupings across treatment [$F(3, 42) = 140.4$, $p < 0.0001$], genotype [$F(1, 42) = 12.66$, $p =0.0009$], treatment × sex [$F(3, 42) = 4.487$, $p = 0.0081$], and genotype × sex [$F(1, 42) = 5.627$, $p = 0.0223$]. No significance was found for the main effect of sex [$F(1, 42) = 0.5268$, $p = 0.4720$] or the interactions of treatment × genotype [$F(3, 42) = 1.301$, $p = 0.2866$] or treatment × genotype × sex [$F(3, 42) = 2.039$, $p = 0.1229$]. Of note, key differences between treatments that reached significance included: WT male vehicle vs. either WT male buprenorphine (Bup) or WT male buprenorphine plus gabapentin (BupGBP), WT female vehicle vs. WT female BupGBP; Het male vehicle vs. either Het male Bup or Het male BupGBP, and Het female vehicle vs. Het female BupGBP.

Body Mass of Mice at P21 Sacrifice Date									
Treatment		**Vehicle Control**		**30 mg/kg Gabapentin**		**5 mg/kg Buprenorphine**		**5 mg/kg Buprenorphine + 30 mg/kg Gabapentin**	
Wildtype (WT)	**Sex**	Male (n = 3)	Female (n = 4)	Male (n = 2)	Female (n = 4)	Male (n = 3)	Female (n = 3)	Male (n = 4)	Female (n = 3)
	Mass (g)	10.00 ± 0.12	9.03 ± 0.22	9.00 ± 0.35	9.98 ± 0.38	7.20 ± 0.62	7.80 ± 0.31	11.73 ± 0.31	12.05 ± 0.09
Heterozygous (α2δ-1 +/-/Het)	**Sex**	Male (n = 5)	Female (n = 6)	Male (n = 3)	Female (n = 4)	Male (n = 4)	Female (n = 3)	Male (n = 4)	Female (n = 3)
	Mass (g)	10.15 ± 0.25	9.33 ± 0.14	10.30 ± 0.55	9.53 ± 0.25	8.33 ± 0.11	8.60 ± 0.07	12.50 ± 0.06	12.07 ± 0.13
Dams		n = 4		n = 5		n = 4		n = 5	
	Mass (g)	33.30 ± 0.35		30.63 ± 0.25		31.35 ± 0.48		27.30 ± 0.36	

Table 7: Body Mass of Dams and Pups (Shown as Mean ± SEM) in Each Treatment and Genotype Group at P21 Perfusion Date

Early Life Exposure to Buprenorphine Increases Excitatory Synapses in the Mesolimbic Dopamine Pathway

Fluorescence IHC followed by confocal imaging was used to distinguish and quantify excitatory glutamatergic synapses within three different brain regions of WT mouse pups at P21 (**Figure 5**) in order to determine whether excitatory synaptic development within the mesolimbic dopamine pathway was significantly impacted by the presence of buprenorphine and/or gabapentin *in utero*. Synapses were identified by the co-localization of presynaptic VGluT1 and postsynaptic PSD95 (see **Figure 4**). This type of imaging-based synapse analysis relies on the fact that these markers are expressed in completely different neuronal compartments (i.e. VGluT1 in axons, PSD95 in dendrites), and would only appear co-localized in confocal microscopy when directly opposed at sites of synaptic contact. Beginning with excitatory glutamatergic synapses within the ACC (**Figure 5A**), a significant main effect between groups

(H = 99.00, p < .0001) was observed. Mice treated with either buprenorphine or buprenorphine in combination with gabapentin had a significantly increased mean number of synapses than mice treated with vehicle control. Interestingly, gabapentin treatment on its own did not significantly impact excitatory synapse number compared to vehicle, despite its known role as an inhibitor of prominent synaptogenic pathways (Eroglu et al., 2009). Similar results were also observed within the NAC (**Figure 5B**; H = 119.2, p < .0001), with buprenorphine and buprenorphine together with gabapentin having significantly more synapses than vehicle, with no significant effect of gabapentin alone. However, in the NAC, the combination drug treatment group also exhibited a significantly higher number of synapses than the gabapentin-only group. In contrast, within the PFC (**Figure 5C**; H = 165.0, p < .0001), no significant differences were observed among any of the treatment groups when compared to vehicle.

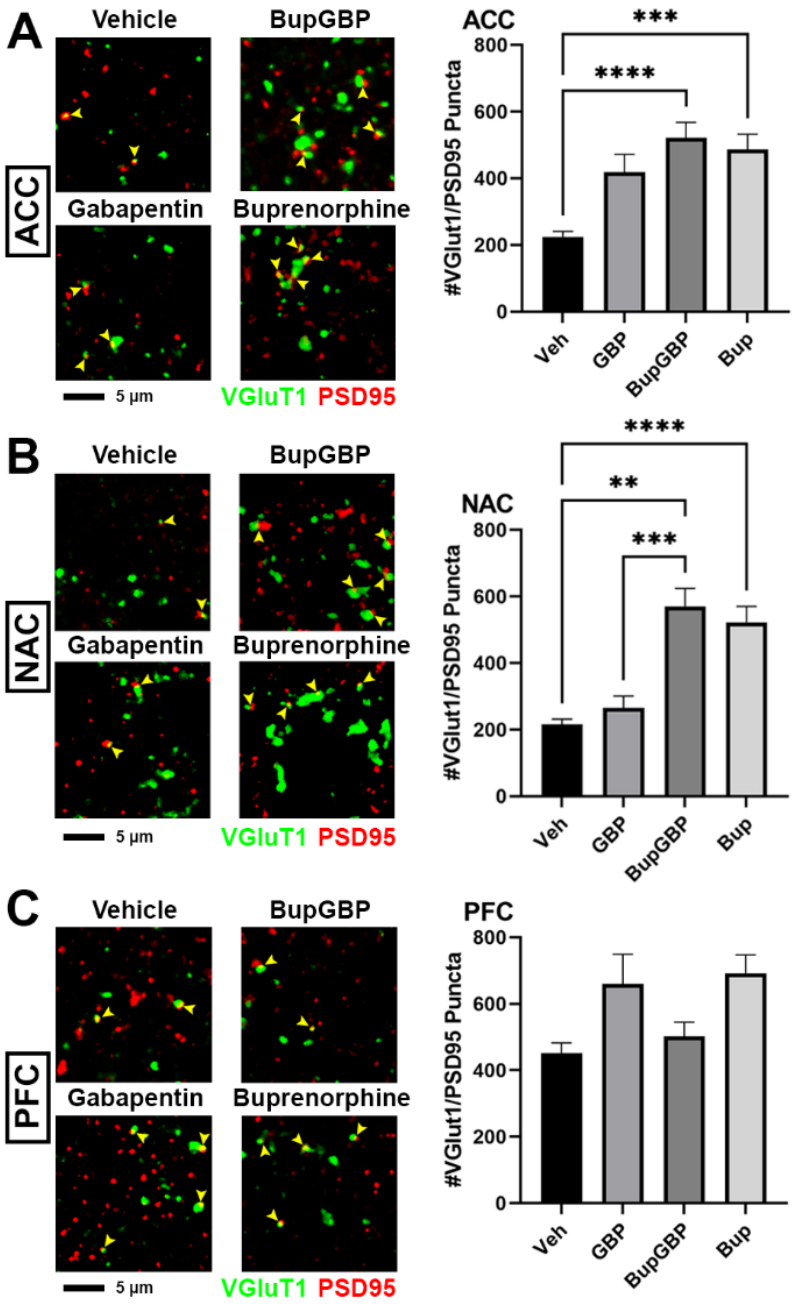

Figure 5: Increased Excitatory Synapses with Early Life Buprenorphine Exposure
(A–C) Representative IHC images (left) and quantification (right) of co-localized VGluT1 (green) and PSD95 (red) excitatory synaptic puncta (yellow arrowheads) from early life drug-exposed WT C57Bl/6J P21 mouse brain within (B) the anterior cingulate cortex (ACC), (C) nucleus accumbens (NAC), and (D) prefrontal cortex (PFC). n = 7 (vehicle control), 6 (GBP), 7 (Bup+GBP), 6 (Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Combined Buprenorphine/Gabapentin Exposure Decreases Inhibitory Synapse Number

The same IHC procedure used for glutamatergic excitatory synapses was next used to stain for GABAergic inhibitory synapses (identified by the co-localization of presynaptic VGAT and postsynaptic gephyrin; **Figure 6**) within the same brain regions of the previously analyzed WT pups. As with excitatory synapses, a main effect of treatment ($H = 196.5$, $p < .0001$) was observed for inhibitory synapses within the ACC (**Figure 6A**). A significant decrease in mean number of inhibitory synapses was observed in the combination drug treated mice compared to all other treatment groups. As with excitatory synapses, similar observations were made in the NAC (**Figure 6B**; $H = 219.8$, $p < .0001$), with the buprenorphine plus gabapentin group having significantly fewer inhibitory synapses than the other groups. The PFC, however, was the only region that exhibited an increase in inhibitory synapse number with any of the drug treatments (**Figure 6C**; $H = 121.8$, $p < .0001$), with the buprenorphine treatment group having significantly more VGAT/gephyrin co-localized puncta than the vehicle control group. Yet, as in the ACC and NAC, the combination treated mice had significantly fewer synapses within the PFC than mice from either of the single drug treatment groups, but not compared to vehicle control.

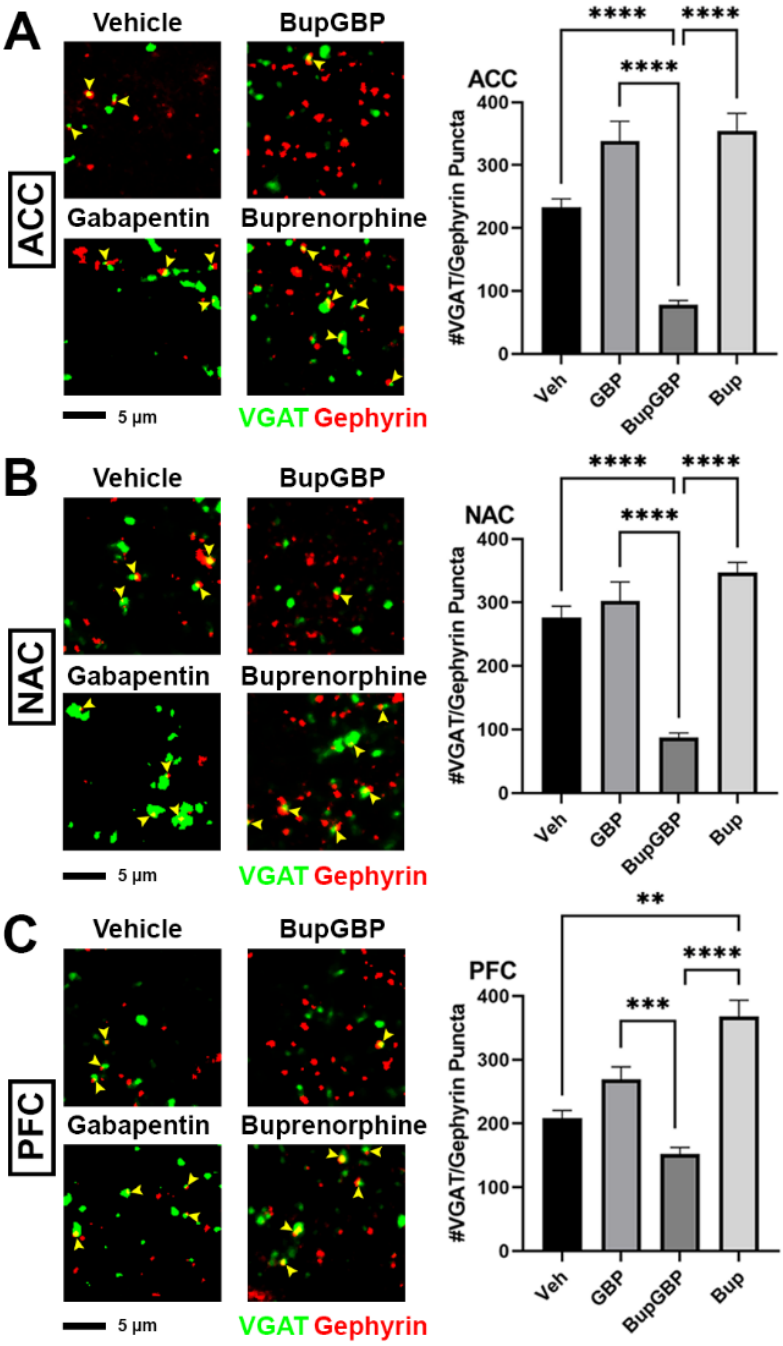

Figure 6: Dual Exposure to Buprenorphine and Gabapentin Decreased Inhibitory Synapses at P21

(A–C) Representative IHC images (left) and quantification (right) of co-localized VGAT (green) and gephyrin (red) inhibitory synaptic puncta (yellow arrowheads) from early life drug-exposed WT C57Bl/6J P21 mouse brain within (A) ACC, (B) NAC, and (C) PFC. n = 7 (vehicle control), 6 (GBP), 7 (Bup+GBP), 6 (Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

α2δ-1 Haploinsufficiency Results in Region- and Treatment-Specific Differences in Synaptic Connectivity

Gabapentin has previously been shown to inhibit excitatory synapse formation by binding to the neuronal calcium channel subunit α2δ-1 (Eroglu et al., 2009), and this mechanism is proposed to underlie the efficacy of gabapentin in the alleviation of symptoms in both epilepsy and neuropathic pain (Chen H, 2015; Field et al., 2006). Deficits in synaptic connectivity have previously been observed in the brains of young mice lacking α2δ-1 (Risher et al., 2018). To determine whether α2δ-1-mediated synaptic development is impacted by early life drug exposure, synapse analysis was expanded to compare WT synapse numbers with those in mice that were haploinsufficient for α2δ-1 (**Figure 7**). Comparing excitatory synaptic development, within the ACC (**Figure 7A**; $H = 99.00$, $p < .0001$), only the gabapentin treated α2δ-1 +/- mice showed a significantly greater number of synapses compared with WT mice that received the same treatment. A similar gabapentin-induced increase was observed within the NAC (**Figure 7B**; $H = 119.2$, $p < .0001$). The same held true for the α2δ-1 +/- PFC (**Figure 7C**; $H = 165.0$, $p < .0001$), which also exhibited more synapses than WT mice with buprenorphine treatment.

DISCUSSION

Glutamatergic signaling has been shown to be critical to many different aspects of opioid abuse and addiction (Heinsbroek et al., 2021). Previous studies have shown that morphine-induced activation of dopaminergic neurons cannot occur within the VTA without glutamatergic associated with heroin addiction and reinstatement of heroin-seeking behavior in rats (LaLumiere & Kalivas, 2008). In addition, GABAergic synaptic activity has also been heavily

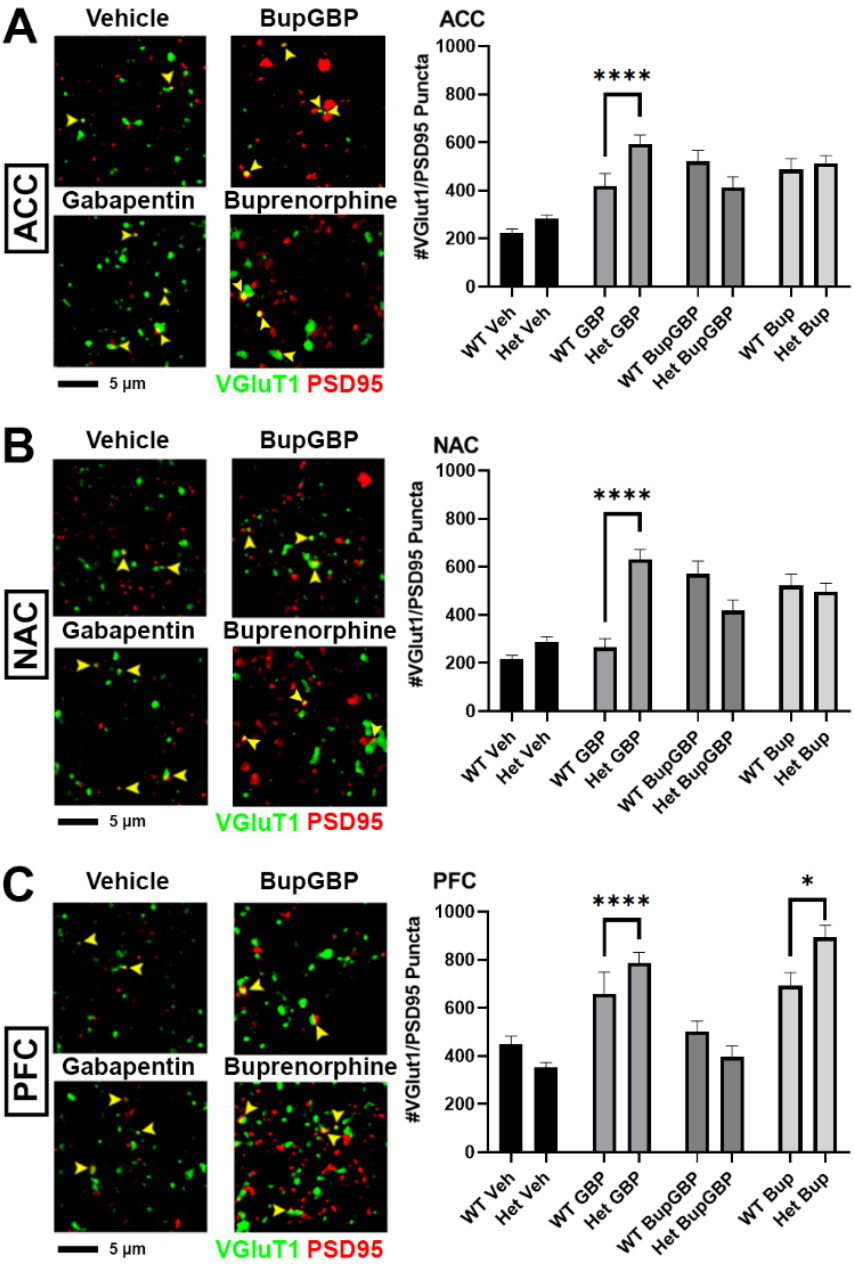

Figure 7: Altered Excitatory Synaptic Connectivity Following Early Life Drug Treatment in α2δ-1 Haploinsufficient Mice

Representative IHC images (left) of co-localized VGluT1 (green) and PSD95 (red) excitatory synaptic puncta (yellow arrowheads) from early life drug-exposed α2δ-1 +/- (Het) C57Bl/6J P21 mouse brain with quantification (right) compared to WT C57Bl/6J P21 mouse brains with the same early life treatment within (A) ACC, (B) NAC, and (C) PFC. Het: n = 11 (vehicle control), 7 (GBP), 7 (Bup+GBP), 7 (Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

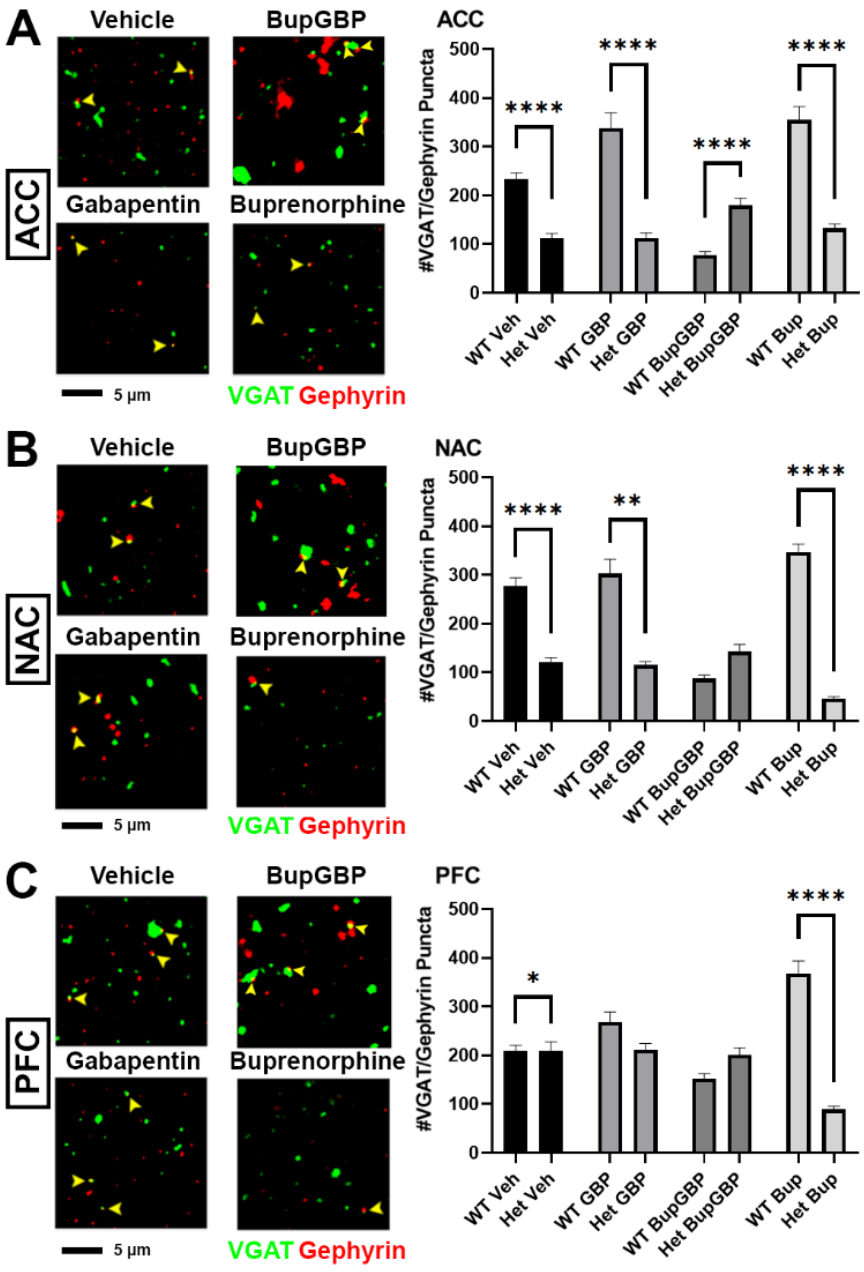

Figure 8: α2δ-1-Influenced Changes in Inhibitory Synapse Development in Drug-Exposed Pups

Representative IHC images (left) of co-localized VGAT (green) and gephyrin (red) inhibitory synaptic puncta (yellow arrowheads) from early life drug-exposed α2δ-1 Het C57Bl/6J P21 mouse brain and quantification (right) compared to WT C57Bl/6J P21 mouse brains with the same early life treatment within (A) ACC, (B) NAC, and (C) PFC. Het: n = 11 (vehicle control), 7 (GBP), 7 (Bup+GBP), 7 (Bup); *$p < 0.05$; **$p < 0.01$; **$p < 0.001$; ****$p < 0.0001$

implicated in the acute response to drugs and the reinforcement of drug seeking behavior (Gardner, 2011). However, relatively little is known about how exposure to drugs of abuse during critical developmental periods affects the trajectories of these synaptic populations. The goal of this study was to investigate the effects of early life drug exposure on synaptic development within brain regions associated with addiction and reward, as well as to explore the role of the gabapentin receptor, α2δ-1, in these alterations of synaptogenesis. Results indicate that excitatory and inhibitory synaptic populations are significantly disrupted by early life exposure to buprenorphine and gabapentin, separately or in combination, and that these disruptions are strongly influenced by α2δ-1 function.

Significant increases in excitatory glutamatergic synapses were observed in multiple regions associated with the mesolimbic dopamine reward pathway in WT mice that had undergone early life exposure to only buprenorphine or buprenorphine in combination with gabapentin. Concomitantly, mice exposed to the combination buprenorphine-gabapentin treatment *in utero* showed significant decreases in inhibitory GABAergic synapses compared to the vehicle control group within these same regions. In addition, the combined impact of buprenorphine and gabapentin resulted in significantly lower numbers of inhibitory synapses compared to either single drug treatment in all three brain regions, suggesting a synergistic effect of polysubstance use on synaptic development. Given the increased prevalence of polysubstance abuse in mothers of infants born with NAS, as well as the worsened clinical conditions observed in many of these children (Labella et al., 2021; Egil Nygaard et al., 2018), this finding is of particular concern.

Taken together, these results indicate a net increase in excitatory glutamatergic signaling capability within the mesolimbic dopamine pathway of mice exposed to buprenorphine and

gabapentin. Glutamatergic neurotransmission is heavily implicated in many aspects of drug use, including acute effects, consolidation, craving/seeking, withdrawal, and relapse (Heinsbroek et al., 2021). Whether the synaptic population shifts observed in this study result in greater dopamine release in response to rewarding stimuli, such as drugs of abuse, or a greater risk for developing substance abuse disorder later in life remains to be elucidated. Studies examining functionality of dopaminergic neurons and addiction-related behaviors in a similar mouse model (see **Chapter 4**) will likely be important parts of future investigations.

Drug Dosing Paradigm Correlates

The dosing for buprenorphine in this study was in line with a study by Martin et al. that showed 5 mg/kg to be capable of producing significant increases in pain threshold in rats (Martin et al., 2001). The gabapentin dosage was chosen based on the finding that 30 mg/kg gabapentin was sufficient to produce analgesia in mice (Kilic et al., 2012). The dosing schedule, starting on E7 and ending on P11, was chosen to correspond to a period of significant synaptogenesis and astrocyte development in mice (Farhy-Tselnicker & Allen, 2018). This period also roughly correlates to the second trimester of human fetal development (Christopherson et al., 2005; De Felipe et al., 1997; Farhy-Tselnicker & Allen, 2018; Hill, 2020; Otis & Brent, 1954). At birth, a mouse pup is considered to be at a developmental stage similar to that of a human fetus in the late second trimester. Despite the fact that newborn mouse pups are no longer receiving direct exposure to drugs of abuse via the placental blood supply, there were still precedents in the literature for postnatal drug transference to the brains of pups being nursed by drug-exposed dams. It has previously been demonstrated that buprenorphine accumulates and remains in the brain tissue of newborn rodents for several days following birth (Kongstorp et al., 2019). In addition, both buprenorphine and gabapentin have been shown to pass from mother to infant via

breast milk, albeit in low concentrations compared to the mothers' blood plasma levels (Kristensen et al., 2006; Lindemalm et al., 2009; Ohman et al., 2005). The presence of both buprenorphine and gabapentin in the brains of drug exposed pups was confirmed, though the levels in our study were admittedly at the lower ends of the ranges reported in the referenced works. Regardless, the results of this study show that the chosen treatment paradigm was capable of disrupting normal CNS synaptic development in mouse pups. The fact that relatively lower concentrations were detected in these animals may even suggest that the observed deficits may have been further exacerbated if the accumulated drug levels had been higher.

Gabapentin, α2δ-1, and Astrocytes

Unexpectedly, single drug treatment with gabapentin did not result in any significant differences in excitatory or inhibitory synapses within any of the examined brain regions in the WT. This finding would appear to contradict the findings of previous studies showing that gabapentin inhibits normal excitatory synapse development by interfering with pathways mediated by α2δ-1. It may be possible that, despite interference by gabapentin in normal TSP/α2δ-1 mediated synaptic development, the net level of synaptogenesis is maintained via other signaling mechanisms (Allen et al., 2012; Baldwin & Eroglu, 2017; Kucukdereli et al., 2011; Walker et al., 2020) as a means to compensate for the gabapentin-induced decrease in glutamate release. There is evidence that gabapentin may even have a separate mechanism of action in addition to its binding to α2δ-1 and interference with normal interaction between TSP and α2δ-1 (Lana et al., 2016). It has also been shown that gabapentin binding to α2δ-1 can instead interfere with binding between α2δ-1 and NMDA-type glutamate receptors (J. Chen et al., 2018), inhibiting glutamate signaling in the synapse. A mechanism such as this may

contribute to the overall changes in excitatory-inhibitory synapse balance seen in gabapentin treated mice.

In this study, a mouse model of early life drug exposure was used to examine the effects such exposure may have on synaptic development within brain regions traditionally associated with reward and addiction. Though there were key regional differences, an overall increase in glutamatergic excitatory synapses as well as a general decrease in GABAergic inhibitory synapses was observed in the mesolimbic dopamine pathway of mice exposed to buprenorphine or buprenorphine in combination with gabapentin. Such changes in synapse number may indicate a general increase in net excitation in response to rewarding stimuli. α2δ-1, which binds to gabapentin and is involved in critical synaptogenic pathways mediated by astrocytes, was also shown to have an important role in the changes in synapse formation induced by early life drug exposure, as evidenced by the significant differences observed between WT and α2δ-1 haploinsufficient animals. This work may serve to inform future studies examining the effects of these changes in synapse formation as well as more longitudinal studies that may link history of early life drug exposure with altered reward circuitry and addiction-like behavior in adolescents and adults.

CHAPTER 4

AN INVESTIGATION OF ALTERATIONS IN SYNAPTIC DEVELOPMENT AND ADDICTION RELEVANT BEHAVIORS IN A BRAIN SPECIFIC A2Δ-1 KNOCKOUT MODEL OF EARLY LIFE DRUG EXPOSURE

INTRODUCTION

In the previous chapter, an attempt was made to elucidate the role of α2δ-1 in synaptic development within a drug exposed brain. The goal of this study was, initially, to compare littermate pups with three possible α2δ-1 genotypes, α2δ-1 +/+ (WT), α2δ-1 +/- (Het), and α2δ-1 -/- (knockout; KO), and four different possible early life treatments: 5 mg/kg buprenorphine, 30 mg/kg gabapentin, a combination of both drug doses, or a vehicle control. However, after several attempts to breed α2δ-1 KO pups, it was found that such pups were either not being born or did not survive to age P21, particularly in the buprenorphine or gabapentin treatment groups. At least four α2δ-1 KO pups that were present at P7 tattoo date were observed as missing or dead before or at P21 weaning date. Given the widespread presence of α2δ-1 throughout the mouse CNS (Cole et al., 2005; Nieto-Rostro et al., 2018), skeletal muscle (Catterall et al., 1988; Tanabe et al., 1987), and cardiac muscle (Klugbauer et al., 1999), the global knockout of this Ca^{2+} channel subunit, in combination with exposure to drugs of abuse during fetal development, may have been potentially lethal.

Despite the difficulty in generating the necessary number of drug exposed α2δ-1 KO pups for this study, significant drug treatment effects on synaptic development were still observed with the loss of just a single copy of α2δ-1, which may indicate that astrocyte-mediated synaptogenic pathways were disrupted with the drug treatment paradigms. In order to improve survival rates among α2δ-1 KO pups, a new transgenic conditional α2δ-1^{Thy1} mouse line on

mixed C57Bl/6J-FVB/NJ background was generated to repeat the IHC studies performed in the C57Bl/6J mice (**Figure 9**). As in the C57Bl/6J mouse IHC study, it was hypothesized that early life exposure to buprenorphine, gabapentin, or a combination of both drugs would lead to significant disruptions in excitatory and inhibitory synaptic connectivity in brain regions associated with addiction and reward and that reduced expression of α2δ-1, either as haploinsufficiency or as a brain specific knockout, would result in further disruption of these synaptic populations.

Even if changes in synaptic development do occur as a result of early life drug exposure, the changes will not be expected to significantly impact the long-term development of the CNS if they are not permanent and are naturally corrected/repaired early in life. To assess whether the changes in synaptogenesis persist throughout life, this study included a separate cohort of postnatal day 70 (P70) mice, an age at which mice have reached full sexual maturity and adulthood, to compare against the P21 adolescent (pre-sexual maturity) mice.

While IHC studies can show changes in molecular expression and are useful in studying changes in synaptic development due to early life drug exposure in a mouse model, they can not demonstrate how these developmental changes might translate into changes in behavior in that mouse. To date, few studies have investigated whether early life drug exposure might predispose an individual towards developing a substance use disorder later in life. Although it has been shown that some forms of addiction carry a level of heritability (Ducci & Goldman, 2012), only a small number of studies have attempted to isolate prenatal drug exposure as the sole potential contributing factor to increased risk of developing a substance use disorder. In one such study, rats prenatally exposed to morphine were shown to demonstrate increased morphine self-

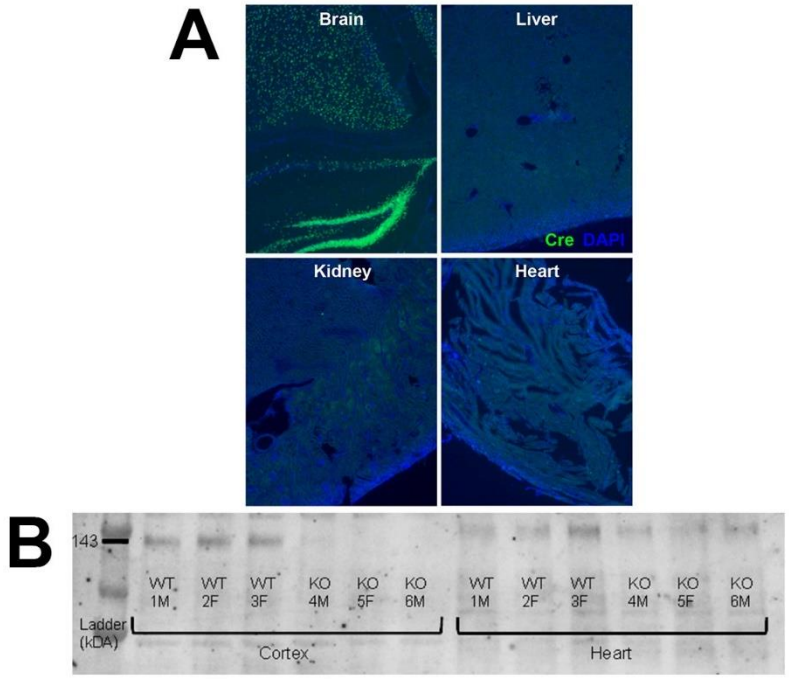

Figure 9: Confirmation of Forebrain-Specific Conditional α2δ-1 Knockout Mouse
(A) Representative images of Cre expression (green) under the *Thy1* promoter. Positive staining can be seen in P14 brain (cortex and hippocampus shown) but not in liver, kidney, or heart tissue (DAPI = blue). (B) Western blot against α2δ-1 (143 kDa) in P14 tissue lysates showing loss of α2δ-1 expression in cortex (lanes 2-7) but not heart (lanes 8-13). n = 3 WT, 3 KO (i.e. α2δ-1^{Thy1} cKO)

administration (Hovious & Peters, 1985) as well as enhanced conditioned place preference when treated with morphine as adults (Gagin et al., 1997). In a similar study, rats prenatally exposed to heroin demonstrated increased self-administration of both heroin and cocaine, a stimulant drug with a different mechanism of action to that of opioids (Ramsey et al., 1993). Given the involvement of opioids in the development of the reward pathway and the effects astrocytes can have on synaptogenesis in these regions, it is possible that prenatal exposure to opioids may predispose the brain towards addiction to other rewarding stimuli including other drugs of abuse.

Findings such as these as well as the general lack of longitudinal data examining humans with a history of prenatal drug exposure are what prompted this study's inclusion of several different behavioral assays.

This study attempted to connect changes in synapse number within the mesolimbic dopamine pathway to changes in behavior through the use of a seven-week behavioral study regimen. Light/Dark chamber, Open Field, Conditioned Place Preference (CPP), and Vapor Chamber Self-Administration were chosen for this regimen due to them previously being used in studies assessing behaviors traditionally associated with anxiety, reward seeking, and impulsivity (Avelar et al., 2019; Bourin & Hascoët, 2003; Chen H, 2015; Cooper et al., 2020; Henderson & Cooper, 2021; Leite Junior et al., 2019; Seibenhener & Wooten, 2015). All of these behaviors can in some way be connected to synaptic signaling within the mesolimbic dopamine pathway. It was hypothesized that mice exposed to drugs of abuse during prenatal and early life development would show heightened anxiety. This would manifest as fewer entrances and less time spent in the light compartment of Light/Dark chamber, less time spent in the center of the Open Field, and more time spent in thigmotaxis around the edges of the Open Field. It was also hypothesized that early life drug exposure would increase preference for the chamber associated with nicotine injections in CPP experiments and would cause mice to initiate a greater number of nose pokes in order to self-administer vaporized nicotine compared to drug-naïve animals. In addition, it was expected that reduced expression of α2δ-1 within the brain would dysregulate these behavioral phenotypes.

MATERIALS AND METHODS

Animals and Drug Treatments

Experiments were conducted in accordance with Marshall University's Institutional Animal Care and Use Committee (IACUC) guidelines (W.C.R. protocols 696 and 697). Adult C57Bl/6J mice heterozygous for *Cacna2d1* were a kind gift from Dr. Cagla Eroglu (Duke University). These mice were crossed with the FVB/N-Tg(Thy1-cre)1Vln/J line (The Jackson Laboratory, Bar Harbor, Maine), which express Cre recombinase under the control of the forebrain neuron-specific *Thy1* promoter. The mice were bred onsite at the Marshall University Animal Resource Facility. Confirmation of the resulting brain-specific knockout of α2δ-1 in these mice was obtained via IHC and Western blotting (**Figure 9**). Methods used in setting mouse mating pairs and caring for the animals were identical to those used in the C57Bl/6J mouse studies. The same four treatments (5mg/kg buprenorphine, 30mg/kg gabapentin, 5mg/kg buprenorphine + 30mg/kg gabapentin, and vehicle control) and the treatment schedule were also used.

Before any experiments were performed or statistical analyses were conducted, a power analysis was conducted using the software G*Power (Version 3.1.9.6, Franz Faul Universität Kiel, Germany) to determine appropriate sample sizes for each age-genotype-treatment group that would still possess power $(1 - \beta)$ equal to or greater than 0.8. Effect size was set at four and the α error probability was set at 0.05. It was determined that approximately four mice per group would be sufficient to reliably produce results reaching level of statistical significance in the planned IHC studies. For the P21 mouse group treated with the vehicle control, five litters were produced from which five wild-type (WT) mice (three male, two female), five α2δ-1 +/flox conditional heterozygous (cHet) mice (three male, two female), and five α2δ-1 flox/flox

conditional knockout (cKO) mice (two male, three female) were included. Five litters were treated with gabapentin in order to produce 5 WT mice (two male, three female), five cHet mice (three male, two female), and five cKO mice (three male, two female). Four litters were treated with the combination of buprenorphine and gabapentin in order to produce six WT mice (two male, four female), six cHet mice (three male, three female), and five cKO mice (three male, two female). Five litters were treated with buprenorphine in order to produce four WT mice (two male, two female), five cHet mice (two male, three female), and five cKO mice (three male, two female) (**Table 8**).

For the P70 mouse group, the number of mice per group was increased to more closely align with methods described in previous literature. Two litters were treated with the vehicle control from which six WT mice (three male, three female) and seven cHet mice (three male, four female) were included in studies. Four litters were treated with gabapentin in order to produce eight WT mice (four male, four female) and six cHet mice (three male, three female). Three litters were treated with the combination of buprenorphine and gabapentin in order to produce seven WT mice (two male, five female) and seven cHet mice (three male, four female). three litters were treated with buprenorphine in order to produce eight WT mice (four male, four female) and seven cHet mice (three male, four female) (**Table 9**). Dams were not weighed and were instead euthanized following weaning at P21.

Genotyping

At P7, the tails of the pups were clipped and collected. Toe pads were tattooed (AIMS NEO9 Animal Tattoo System, Hornell, NY) for later identification. Tissue digestion was performed on the clipped tails and DNA was isolated for PCR amplification and genotyping with a 2% agarose gel. C57Bl/6J-FVB/NJ $\alpha 2\delta\text{-}1^{Thy1}$ pups were first identified as either Cre positive or

negative using the following primers: Cre sense (5'-GAACCTGATGGACATGTTCAGG-3'), Cre anti-sense (5'-AGTGCGTTCGAACGCTAGAGCCTGT-3'), myogenin sense (5'-TTACGTCCATCGTGGACAGC-3'), and myogenin anti-sense (5'TGGGCTGGGTGTTAGCCTTA-3') (Integrated DNA Technologies, Coralville, IA). All pups identified as Cre positive were then further identified as one of three genotypes: α2δ-1 +/+ wild-type (WT), α2δ-1 +/flox heterozygous (cHet), or α2δ-1 flox/flox knockout (cKO). The primers that were used in the genotyping of C57Bl/6J pups were used again: F1 (WT forward, 5'-TCTCAGTTACAAGACTATGTGG-3'), F3 (KO forward, 5'-GGCTGTGTCCTTATTTATGG-3'), and LAF-Test (reverse, 5'-AGTAGGAGAAGGTACAATCGGC-3') (Integrated DNA Technologies).

Immunohistochemistry Studies

For the P21 mouse group, when a litter reached P21, the dam and pups were placed individually on a scale to be weighed (group data is included in **Table 8**) The same techniques for brain perfusion, freezing, sectioning, and immunostaining that were used for C57Bl/6J mice were used again for this study (**Figures 1 and 4**). For each litter in the P70 mouse group, pups were weaned at P21, placed in new cages (two to five mice per cage and separated by sex), and the dam was euthanized via carbon dioxide inhalation. At P70, the mice were weighed, anesthetized, and perfused and their brains were frozen and cryosectioned as described above (**Table 9**). The same procedures for immunohistochemistry, confocal microscopy, and image analysis were also used.

Behavioral Studies

Handling

Procedures for the behavioral experiments chosen for this study are based on those previously used in studies of addiction and drug exposure (Avelar et al., 2019; Bourin & Hascoët, 2003; Buccafusco, 2009; Chen H, 2015; Henderson & Cooper, 2021; Leite Junior et al., 2019; Seibenhener & Wooten, 2015). Behavioral studies followed a seven-week schedule (as shown in **Figure 10).** At least 24 hours following weaning, mice designated to participate in behavioral studies were handled in the room in which behavioral trials were set to take place. Every morning for five days, each mouse was held in gloved hands for 10 minutes in order for the mice to acclimatize to the handler and the lab environment. The time of day and the order in which mice were handled was maintained for all five days. Gloves were cleaned and disinfected in between mice. Mice were allowed to habituate for at least 30 minutes after being transported to the testing room prior to behavioral testing.

Light/Dark Testing

Three days after their last handling session (at approximately P30), mice underwent light/dark testing. Mice were placed in the Light zone chamber (ambient light 60 lux) of a Light/Dark chamber and allowed to move freely between Light and Dark zone (ambient light < 10 lux) chambers (both 8”x16” in area) via a single, small opening (Bourin & Hascoët, 2003). Mice were video recorded for 10 minutes. Videos were analyzed with ANY-maze software (Wood Dale, IL) to track the number of entries to the Light zone and total time spent in the Light zone. Both chambers were cleaned and disinfected in between trials. Mice were allowed to habituate for at least 30 minutes after being transported to the testing room prior to behavioral testing.

Open Field

Four days following light/dark testing (at approximately P34), mice underwent Open Field testing. Mice were placed in the center of a 37 cm × 37 cm Open Field chamber where ambient light was adjusted to 60 lux. Mice were allowed to move freely in the chamber while being video recorded for 10 minutes. Videos were analyzed with ANYmaze to track total time spent in the center zone of the field, total entries to the center zone, total time spent in thigmotaxis near the walls at the edge of the field, and total distance traveled. The chamber was cleaned and disinfected in between trials. Mice were allowed to habituate for at least 30 minutes after being transported to the testing room prior to behavioral testing.

Conditioned Place Preference (CPP)

Three days after Open Field testing (at approximately P37), mice began conditioned place preference (CPP) testing. CPP assays were performed with a three-chamber spatial place-preference chamber (Harvard Apparatus, PanLab Item #76-0278, dimensions: 47.5 × 27.5 × 47.5 cm) and largely followed previously published methods (Avelar et al., 2019; Avelar et al., 2022; Lee et al., 2020). An unbiased 10-day protocol was used with a Pretest session held on Day One. Mice were placed in the neutral adjoining chamber connecting the two separate larger chambers, each with a distinct wall pattern and floor texture, and allowed to travel freely between the two chambers as they were video recorded for 10 minutes. On Days Two, Four, Six, and Eight, mice received a 0.5 mg/kg intraperitoneal injection of nicotine ditartrate dihydrate 98% (Thermo Fisher Scientific Waltham, MA) dissolved in normal saline before being placed in the drug-paired chamber for 20 minutes. On Days Three, Five, Seven, and Nine, mice received an injection of saline (equal in volume to the previous day's nicotine injection) before being placed in the saline-paired chamber for 20 minutes. On Day Ten, the Post-test session was held with

mice will again being placed in the neutral adjoining chamber and allowed to travel freely between the drug-paired and saline-paired chambers while being video recorded for 10 minutes. The time of day and the order in which mice were placed in chambers were consistent for all 10 days. All chambers were cleaned and disinfected in between trials. Mice were allowed to habituate for at least 30 minutes after being transported to the testing room prior to behavioral testing. Videos of the Pre- and Post-tests were analyzed with ANYmaze to record the amount of time spent in each chamber during the two testing phases and CPP scores were calculated using the following formula:

$\text{CPP score} = (A - B)_{\text{post-test}} - (A - B)_{\text{pretest}}$
A = Drug-paired B = Saline-paired

Self-Administration

Two days following the CPP Post-test session (at approximately P50), mice began the acclimation phase of vapor self-administration testing. Testing followed slight modifications of the established protocol and utilized the self-administration chambers described in previous work by Dr. Skylar Cooper and Dr. Brandon Henderson (Cooper et al., 2020; Henderson & Cooper, 2021). On Days One, Three, and Five, mice were placed in an acclimating vapor chamber for one hour. A three second puff of vaporized solution containing 3mg/ml nicotine-salt dissolved in 1:1 propylene glycol: vegetable glycerin (PGVG) was released once every five minutes. On Day Six, mice began operant training. Mice were placed in an operant chamber containing an active and inactive nose poke. Each active nose-poke delivered a three second vapor puff on a fixed ratio 1 (FR1) schedule and was immediately followed by the illumination of a cue-light inside the nose-poke hole that remained illuminated for a 60 second timeout. Inactive nose-pokes, which elicited no response, were also recorded. Mice participated in daily one-hour FR1 sessions

for 10 days. At the end of these 10 days, mice were assessed for reinforcement. If a mouse showed consistently high numbers of nose pokes, if the number increased gradually over time, or if the number increased suddenly after being relatively low for several days, these were taken as potential signs of reinforcement. Mice that demonstrated reinforcement were transitioned to a daily one-hour fixed ratio 3 (FR3) schedule (three active nose-pokes required for a three second vapor puff to be delivered followed by a 60-second timeout) for three days starting on the day immediately following the final FR1 session. The mean number of active nose-pokes from the final two FR3 sessions was calculated (Henderson & Cooper, 2021). The time of day and the order in which mice were placed in chambers was consistent and all chambers were cleaned and disinfected in between sessions. Mice were allowed to habituate for at least 30 minutes after being transported to the testing room prior to behavioral testing.

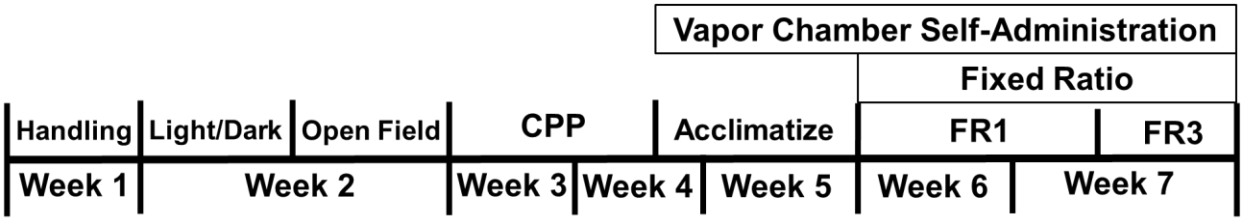

Figure 10: Schedule for Behavioral Studies for Each Mouse in the P70 Group, Beginning at P22 and Ending at P70

Statistical Analysis

For body mass comparisons, dams belonging to different treatment groups were compared via One-Way ANOVA with Tukey's multiple comparisons post hoc test. For pups, a Three-Way ANOVA (treatment × genotype × sex) was performed with Tukey's multiple comparisons post hoc test. For IHC studies, the D'Agostino and Pearson omnibus normality test confirmed that most synapse counts were not normally distributed. Therefore, statistical

differences between synapse counts were analyzed using the nonparametric Kruskal-Wallis test to detect differences in mean number of synapses amongst drug treatments or between genotypes. Post-hoc Dunn's multiple comparisons tests were performed to detect significant differences ($p < 0.05$) amongst the mean values of each treatment group.

Multiple statistical methods were required for behavioral studies. D'Agostino and Pearson omnibus normality tests were performed for each data set to determine whether or not nonparametric statistical testing was necessary. For light/dark testing, the nonparametric Kruskal-Wallis test and post-hoc Dunn's multiple comparisons tests were performed. For analysis of the Open Field studies examining center zone entries, time spent in the center zone, and time spent freezing, Kruskal-Wallis test and post-hoc Dunn's multiple comparisons tests were performed. For analyses of time spent in thigmotaxis and total distance traveled within the Open Field, however, Two-way ANOVA (treatment × genotype) with Tukey's multiple comparisons post hoc tests were used. In the CPP analyses, the CPP scores among WT mice were analyzed using One-way ANOVA with Tukey's multiple comparisons post hoc test, whereas the comparisons between WT and Het mice were analyzed with Two-way ANOVA (treatment × genotype) and Tukey's multiple comparisons post hoc test. The mean number of nose pokes for each treatment-genotype mouse group for each of the 10 days of FR1 self-administration testing were analyzed with Kruskal-Wallis test and post-hoc Dunn's multiple comparisons test. The mean averages of the last two days of FR3 for each treatment-genotype group of mice that demonstrated reinforcement during FR1 were analyzed with Kruskal-Wallis test and post-hoc Dunn's multiple comparisons test. All analyses were performed using GraphPad Prism and all graphs, where applicable, were shown with bars representing Standard Error of the Mean (SEM).

RESULTS

Body Mass of Pups Impacted by Drug Treatment and Expression of α2δ-1

The mean masses for the dams of the different litters and the pups in each age-sex-genotype-treatment group were calculated and compared (**Tables 8 and 9**). Among the P21 group, significant main effect was observed in treatment × genotype [$F(11, 80) = 4.070$, $p < 0.0001$]. One notable finding was that among pups with the cKO genotype, those treated with gabapentin ($p = 0.014$) and those treated with buprenorphine ($p < 0.0001$) had significantly lower average body masses compared to the cKO vehicle control. No other interactions among the pups rose to the level of significance, nor were any significant differences observed among the dams.

In the P70 group, a significant main effect was again observed in treatment × genotype [$F(7, 45) = 3.842$, $p = 0.0024$]. WT mice treated with gabapentin ($p = 0.0036$) and WT mice treated with buprenorphine ($p = 0.0125$) had average body masses significantly greater than vehicle treated WT mice. A significant main effect was also observed in treatment × genotype × sex [$F(15, 41) = 10.18$, $p < 0.0001$]. Gabapentin treated WT females had a significantly greater average body mass than vehicle treated WT females ($p = 0.0028$), vehicle treated Het males were significantly greater than vehicle treated Het females ($p = 0.0015$), and buprenorphine treated WT males were significantly greater than buprenorphine treated WT females ($p < 0.0001$).
In addition to these body mass measurements, there were a number of observational findings not reflected in the data. The plastic dishes in which those treatments were served were, as in the C57Bl/6J mice, consistently observed to be empty on the day following administration, confirming that the dams routinely consumed the entirety of the treatment solution. One finding of note was that 4 pups from gabapentin treated litters and 1 pup from a buprenorphine +

gabapentin treated litter were either observed to be dead or were missing between the P7 tattoo date and P21 wean date. It was believed that this group of pups was a combination of both males and females and genotyping later confirmed that they were all cKO. It was also observed that pups from gabapentin treated litters appeared hyperactive on their P21 wean dates. They would attempt to jump out of their cages and often bit experimenters who attempted to pick them up. While these observations can not be quantified, they may point to a unique and, potentially, harmful interaction with gabapentin in the developing CNS.

P21 Immunohistochemistry

Early Life Exposure to Gabapentin Decreased Excitatory Synapses in the Mesolimbic Dopamine Pathway

Fluorescence IHC followed by confocal imaging was used, as it was in the C57Bl/6J mouse study, to distinguish and quantify excitatory glutamatergic synapses, identified by co-localization of presynaptic VGluT1 and post-synaptic PSD95 (see **Figure 1**), within the ACC, NAC, and PFC of $\alpha 2\delta\text{-}1^{Thy1}$ WT mouse pups at P21 (**Figure 11**) in order to determine whether excitatory synaptic development within the mesolimbic dopamine pathway was significantly impacted by any of the three drug treatments. First examining the ACC, a significant main effect between groups ($H = 338.8$, $p < 0.0001$) was observed. Mice treated with gabapentin showed a significantly lower number of synapses compared to the vehicle control treated mice (**Figure 11A**). A similar trend was observed within the NAC (**Figure 11B**; $H = 162.1$, $p < 0.0001$) except that the gabapentin treatment group showed a significantly lower number of synapses compared to the vehicle and the combination drug (buprenorphine + gabapentin) treatment groups. The buprenorphine treated group was also significantly lower than the vehicle. This trend appeared to reverse within the PFC (**Figure 11C**; $H = 231.7$, $p < 0.0001$) with the buprenorphine and

Body Mass of Mice at P21 Sacrifice Date							
Treatment	**Vehicle Control**						
M/F Ratio	0.76						
Genotype		**WT**		**cHet**		**cKO**	
Sex	**Dams**	**M**	**F**	**M**	**F**	**M**	**F**
n	5	3	2	3	2	2	3
Mean Body Mass (g)	31.04	12.55	12.70	12.81	12.11	15.30	14.56
Standard Deviation	2.59	1.18	0.96	1.37	0.87	2.55	2.50
Combined Mean Body Mass (g)		13.33		12.88		13.63	
Standard Deviation		1.00		1.16		2.32	
Treatment	**30 mg/kg Gabapentin**						
M/F Ratio	1						
Genotype		**WT**		**cHet**		**cKO**	
Sex	**Dams**	**M**	**F**	**M**	**F**	**M**	**F**
n	5	2	3	3	2	3	2
Mean Body Mass (g)	31.56	10.40	11.44	12.50	11.78	11.28	11.38
Standard Deviation	3.96	2.69	1.08	1.51	1.40	1.73	0.56
Combined Mean Body Mass (g)		11.28		11.95		12.13	
Standard Deviation		1.41		1.45		1.27	
Treatment	**5 mg/kg Buprenorphine + 30 mg/kg Gabapentin**						
M/F Ratio	1.18						
Genotype		**WT**		**cHet**		**cKO**	
Sex	**Dams**	**M**	**F**	**M**	**F**	**M**	**F**
n	4	2	4	3	3	3	2
Mean Body Mass (g)	31.05	11.78	11.97	13.02	11.83	11.30	10.35
Standard Deviation	2.66	0.93	1.34	1.13	1.21	3.29	0.07
Combined Mean Body Mass (g)		12.33		12.18		10.00	
Standard Deviation		1.02		1.24		2.59	
Treatment	**5 mg/kg Buprenorphine**						
M/F Ratio	0.67						
Genotype		**WT**		**cHet**		**cKO**	
Sex	**Dams**	**M**	**F**	**M**	**F**	**M**	**F**
n	5	2	2	2	3	3	2
Mean Body Mass (g)	31.06	9.45	10.30	10.75	11.69	10.22	7.20
Standard Deviation	4.36	0.64	1.84	1.34	2.66	2.31	0.99
Combined Mean Body Mass (g)		9.88		9.18		7.80	
Standard Deviation		1.23		2.47		2.43	

Table 8: Body Mass of α2δ-1^{Thy1} Dams and P21 Pups in Each Treatment and Genotype Group at Perfusion Date

Body Mass of Mice at P70 Sacrifice Date				
Treatment	**Vehicle Control**			
M/F Ratio	0.86			
Genotype	**WT**		**cHet**	
Sex	**M**	**F**	**M**	**F**
n	3	3	3	4
Mean Body Mass (g)	23.13	18.17	25.07	19.78
Standard Deviation	1.71	0.81	0.67	2.45
Combined Mean Body Mass (g)	20.65		22.04	
Standard Deviation	2.97		3.34	
Treatment	**30 mg/kg Gabapentin**			
M/F Ratio	1			
Genotype	**WT**		**cHet**	
Sex	**M**	**F**	**M**	**F**
n	4	4	3	3
Mean Body Mass (g)	25.53	23.44	24.80	23.53
Standard Deviation	2.00	1.91	0.87	0.70
Combined Mean Body Mass (g)	24.37		24.17	
Standard Deviation	2.13		0.99	
Treatment	**5 mg/kg Buprenorphine + 30 mg/kg Gabapentin**			
M/F Ratio	0.5			
Genotype	**WT**		**cHet**	
Sex	**M**	**F**	**M**	**F**
n	2	5	3	4
Mean Body Mass (g)	24.25	21.15	24.50	20.86
Standard Deviation	1.63	1.48	2.76	1.39
Combined Mean Body Mass (g)	21.93		22.23	
Standard Deviation	2.00		2.62	
Treatment	**5 mg/kg Buprenorphine**			
M/F Ratio	0.93			
Genotype	**WT**		**cHet**	
Sex	**M**	**F**	**M**	**F**
n	4	4	3	4
Mean Body Mass (g)	26.46	20.82	25.67	22.06
Standard Deviation	1.59	1.03	1.44	0.61
Combined Mean Body Mass (g)	23.64		23.41	
Standard Deviation	3.23		2.07	

Table 9: Body Mass of P70 α2δ-1^{Thy1} Mice in Each Treatment and Genotype Group at Perfusion Date

combination treatments being significantly greater than the vehicle control and the gabapentin.

Decreased Expression of α2δ-1 Significantly Decreased the Number of Excitatory Synapses

By comparing synapse numbers between three different genotypes for α2δ-1, the role of this channel subunit in drug influenced synaptogenesis can be elucidated. Within the ACC (**Figure 12**), in α2δ-1^{Thy1} mice within the vehicle control, gabapentin, and buprenorphine + gabapentin treatment groups, decreased forebrain expression of α2δ-1 corresponded with a decrease in glutamatergic excitatory synapses. This trend was not observed in buprenorphine treated mice, however. A decrease in synapse number corresponding with decreased expression of α2δ-1 was again seen among the vehicle and the buprenorphine + gabapentin treatment groups within the NAC (**Figure 13**). Intriguingly, though, cKO mice treated with gabapentin actually showed an increase in synapse number compared to WT or Het mice administered the same treatment. This unique finding appeared to be isolated to the NAC, however, as within the PFC (**Figure 14**), mice treated with the vehicle control and the buprenorphine + gabapentin combination showed a decrease in glutamatergic excitatory synapses, corresponding with decreased expression of α2δ-1. Buprenorphine treated mice also followed this trend.

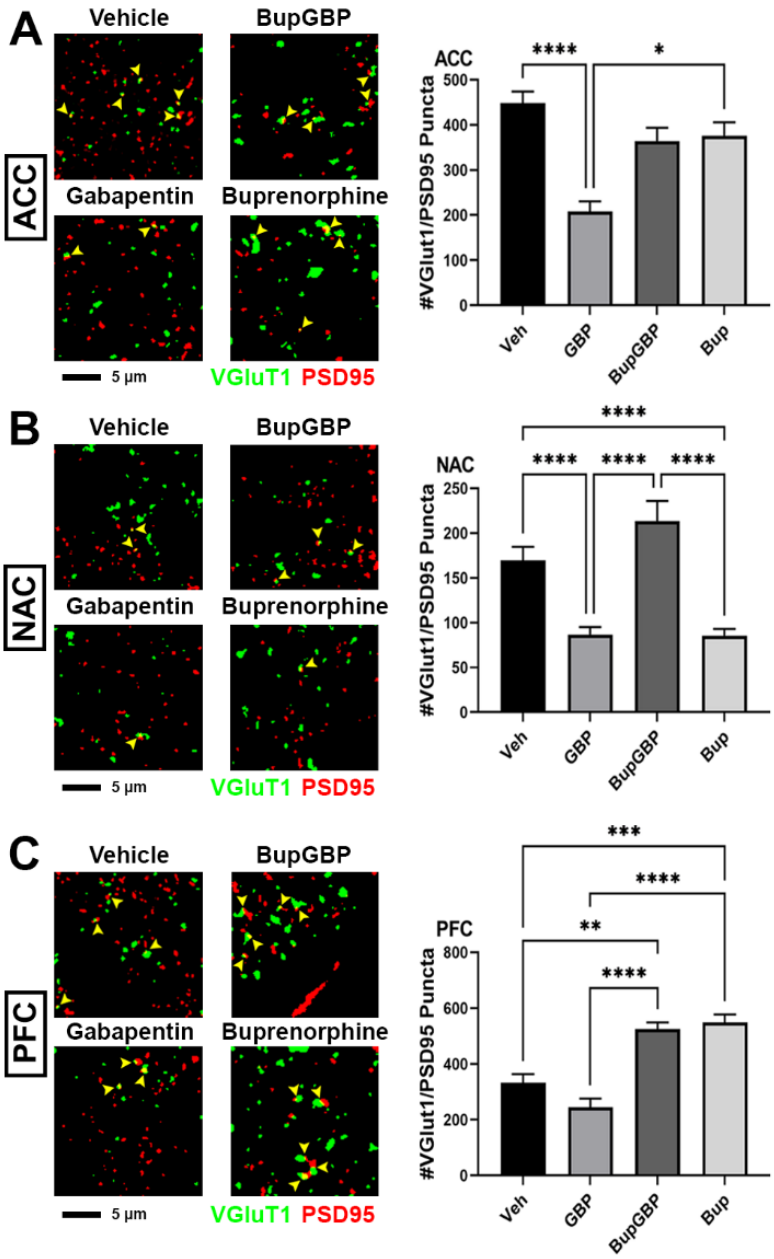

Figure 11: Formation of Excitatory Synapses Decreased with Early Life Gabapentin Exposure

(A–C) Representative IHC images (left) and quantification (right) of co-localized VGluT1 (green) and PSD95 (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} WT mice at P21 within (A) the anterior cingulate cortex (ACC), (B) nucleus accumbens (NAC), and (C) prefrontal cortex (PFC). n = 5 (vehicle control), 5 (GBP), 6 (Bup+GBP), 4 (Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

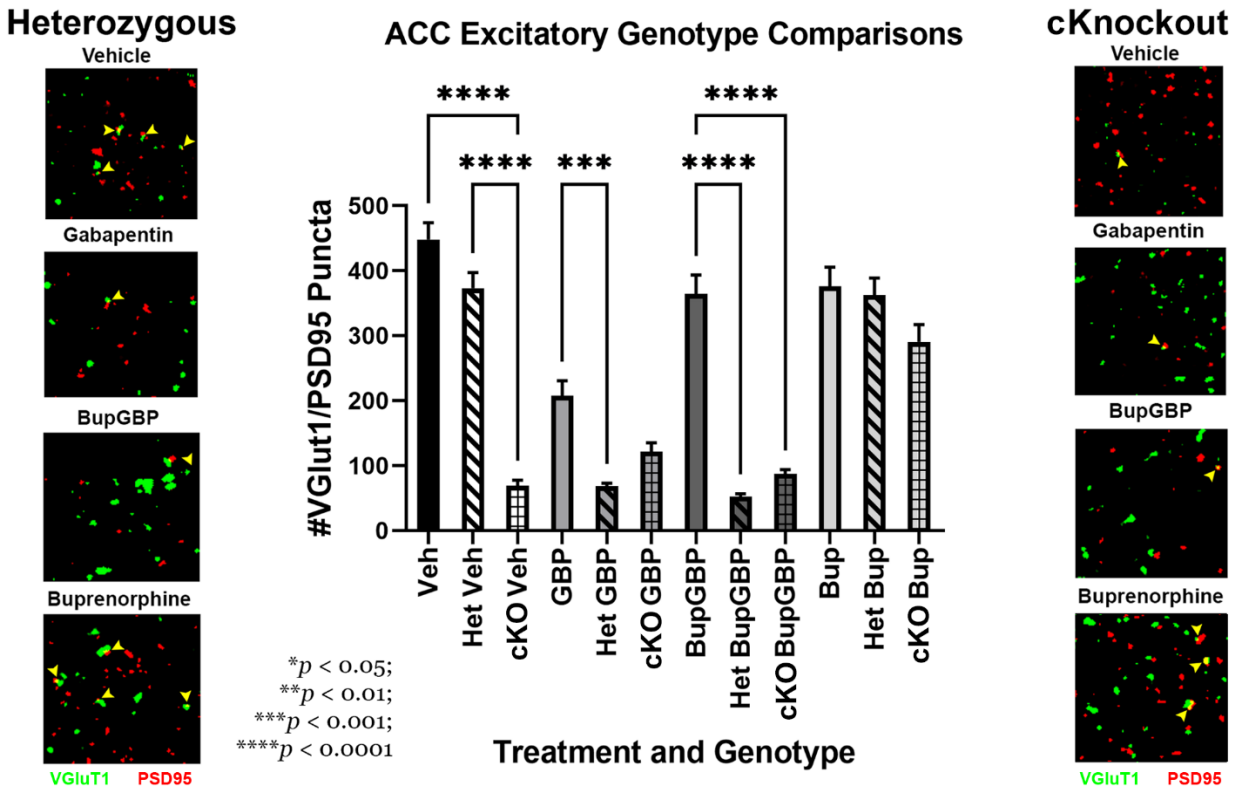

Figure 12: Presence of α2δ-1 Significantly Impacted Excitatory Synapse Number Within the Anterior Cingulate Cortex

(A–C) Representative IHC images (forebrain specific α2δ-1 heterozygous left and knockout right) and quantification of co-localized VGluT1 (green) and PSD95 (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} mice at P21 within the ACC. n = 5 (cHet Veh), 5 (cHet GBP), 6 (cHet Bup+GBP), 5 (cHet Bup), 5 (cKO Veh), 5 (cKO GBP), 5 (cKO Bup+GBP), 5 (cKO Bup). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

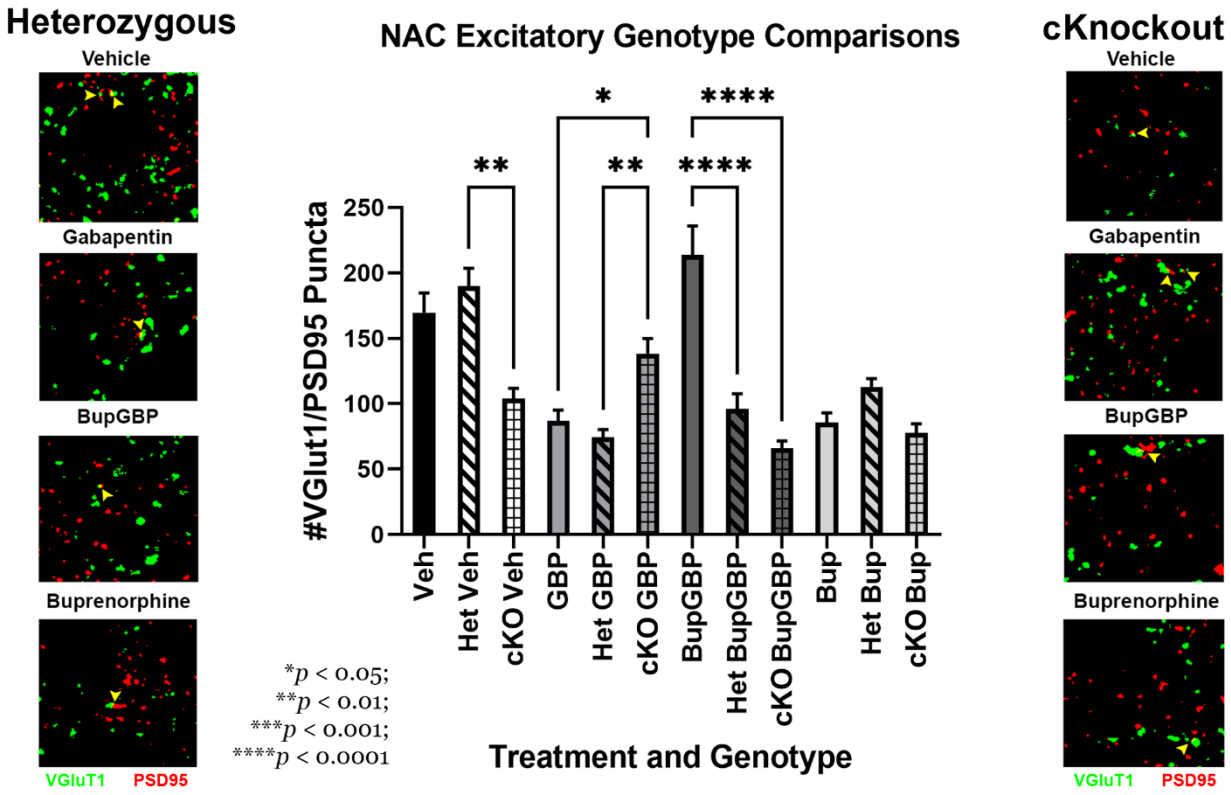

Figure 13: Response to the Combination of Buprenorphine and Gabapentin Determined by Presence of α2δ-1 Within the Nucleus Accumbens

(A–C) Representative IHC images (forebrain specific α2δ-1 heterozygous left and knockout right) and quantification of co-localized VGluT1 (green) and PSD95 (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} mice at P21 within the NAC. n = 5 (cHet Veh), 5 (cHet GBP), 6 (cHet Bup+GBP), 5 (cHet Bup), 5 (cKO Veh), 5 (cKO GBP), 5 (cKO Bup+GBP), 5 (cKO Bup). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

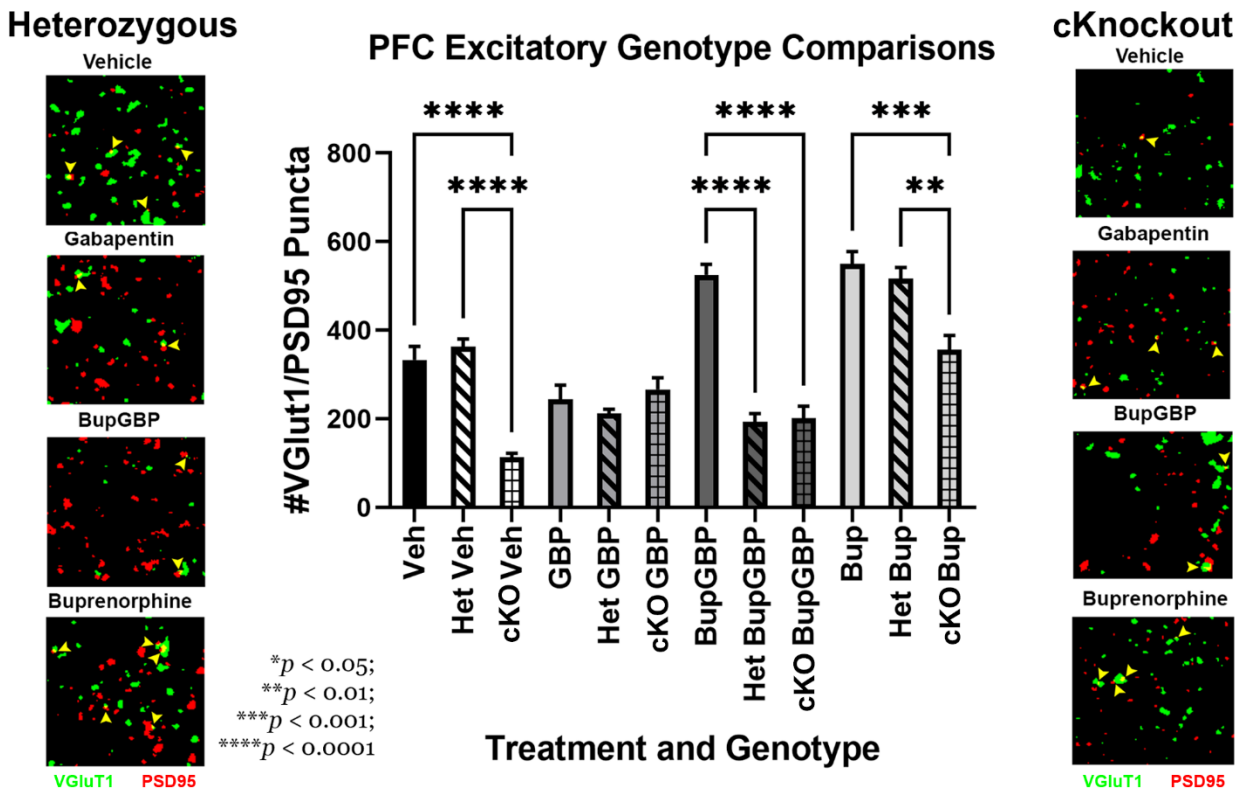

Figure 14: Response to Buprenorphine Impacted by the Presence of α2δ-1 in Prefrontal Cortex

(A–C) Representative IHC images (α2δ-1 cHet left and cKO right) and quantification of co-localized VGluT1 (green) and PSD95 (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} mice at P21 within the PFC. n = 5 (cHet Veh), 5 (cHet GBP), 6 (cHet Bup+GBP), 5 (cHet Bup), 5 (cKO Veh), 5 (cKO GBP), 5 (cKO Bup+GBP), 5 (cKO Bup). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

Inhibitory GABAergic Synapses Were Significantly Impacted by Early Life Exposure to Buprenorphine, as a Single Treatment and in Combination with Gabapentin

Fluorescence IHC of presynaptic VGAT and post-synaptic Gephyrin was again used to distinguish and quantify inhibitory GABAergic synapses (**Figure 1**) within the ACC, NAC, and PFC of P21 α2δ-1^{Thy1} WT pups (**Figure 15**). No significant differences were observed between treatment groups within the ACC (**Figure 15A**; H = 188.5, p < 0.0001). Within the NAC,

however, the combination buprenorphine + gabapentin treatment group had a significantly greater number of inhibitory synapses compared to the vehicle control group as well as the gabapentin solo treatment group (**Figure 15B**; $H = 111.3$, $p < 0.0001$). Within the PFC (**Figure 15C**; $H = 183.2$, $p < 0.0001$), the only significant difference in synapse number was a decrease in the buprenorphine treatment group compared to the vehicle control group.

Decreased Expression of α2δ-1 Significantly Decreased the Number of Inhibitory Synapses, But Not in Mice Treated with Gabapentin

Differences in the number of inhibitory synapses were also observed between the three different genotypes for α2δ-1 (**Figure 16**). Within the ACC, similarly to what was observed in excitatory synapses, there was a decrease in synapse number corresponding with decreased expression of α2δ-1 in the vehicle control, buprenorphine, and buprenorphine + gabapentin treatment groups. This trend was also observed within the NAC (**Figure 17**). No significant differences were observed between the three genotype groups of mice treated with gabapentin in either the ACC or NAC. However, within the PFC (**Figure 18**), similarly to what was seen with excitatory synapses within the NAC, cKO mice treated with gabapentin had a greater number of inhibitory synapses than either the WT or cHet mice that received the same treatment (although this difference only rose to the level of significance in the comparison between cKO and cHet).

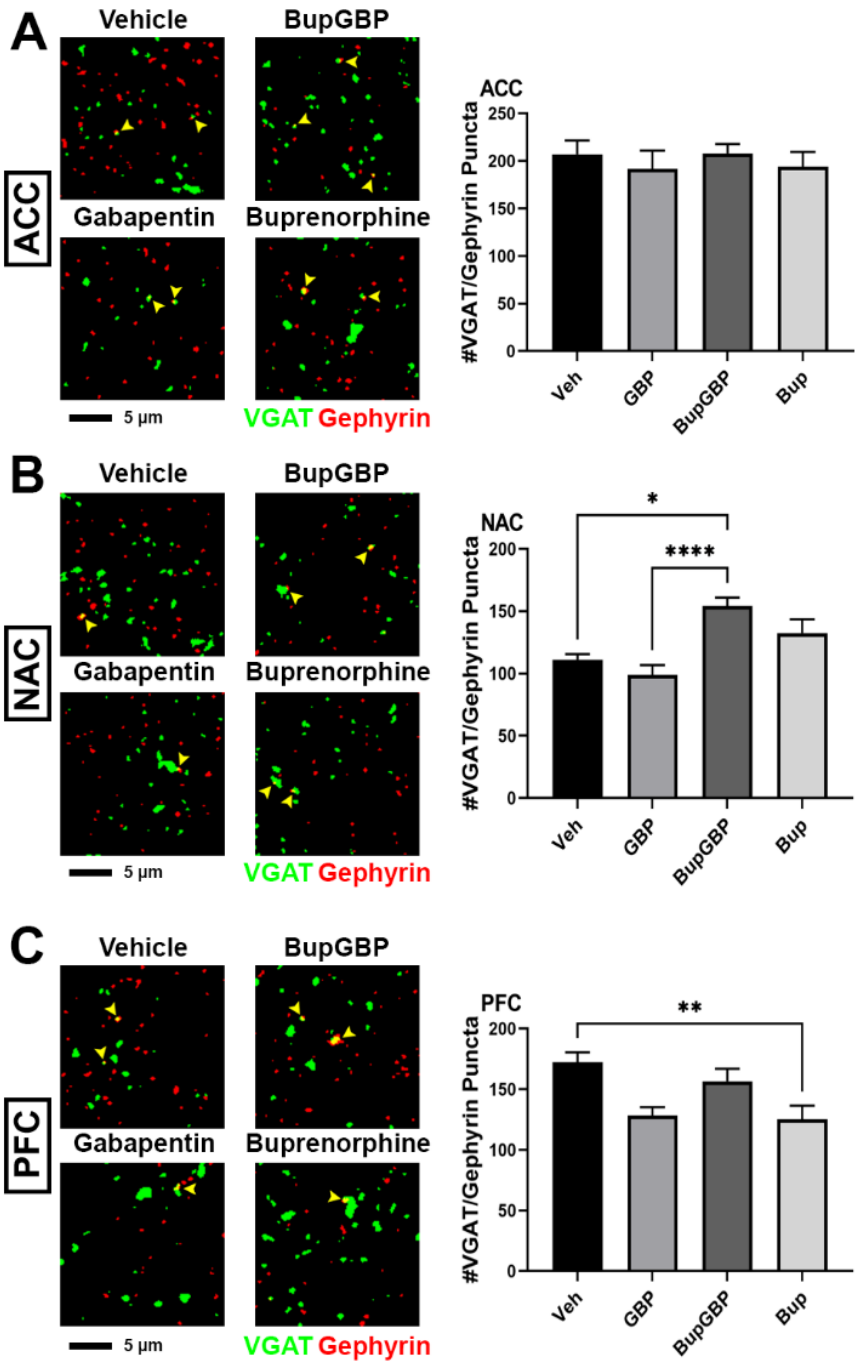

Figure 15: Development of Inhibitory Synapses Impacted by Buprenorphine
(A–C) Representative IHC images (left) and quantification (right) of co-localized VGAT (green) and Gephyrin (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} WT mice at P21 within (A) the ACC, (B) NAC, and (C) PFC. n = 5 (Veh), 5 (GBP), 6 (Bup+GBP), 4 (Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

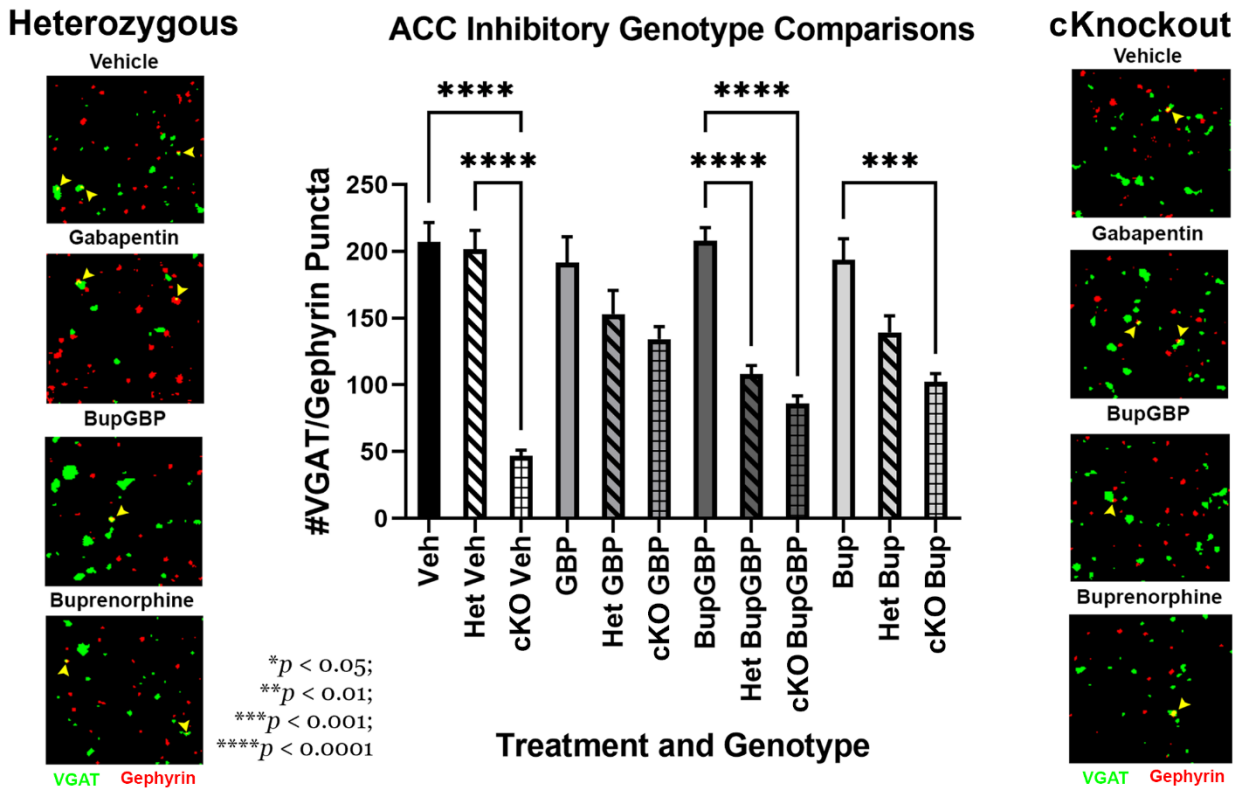

Figure 16: Presence of α2δ-1 Significantly Impacted Inhibitory Synapse Number Within the Anterior Cingulate Cortex

(A–C) Representative IHC images (α2δ-1 cHet left and cKO right) and quantification of co-localized VGAT (green) and Gephyrin (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} mice at P21 within the ACC. n = 5 (cHet Veh), 5 (cHet GBP), 6 (cHet Bup+GBP), 5 (cHet Bup), 5 (cKO Veh), 5 (cKO GBP), 5 (cKO Bup+GBP), 5 (cKO Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

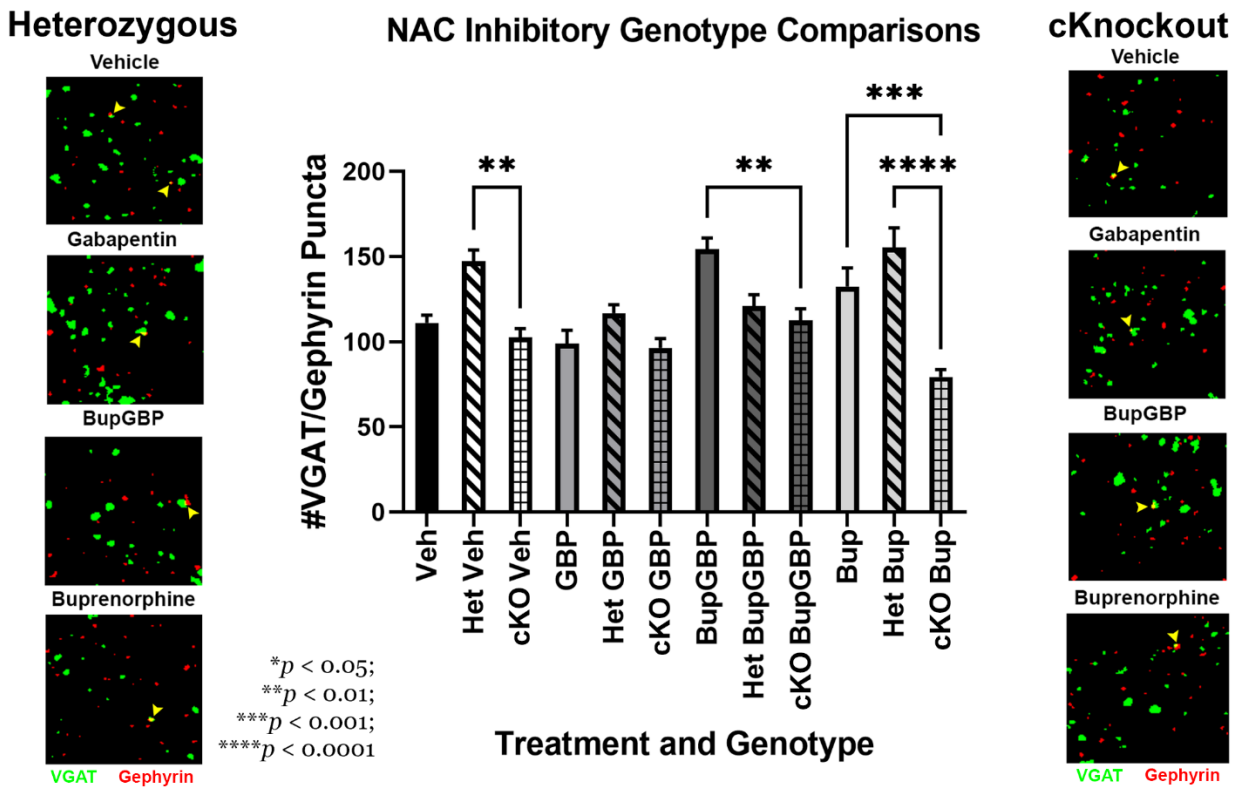

Figure 17: Buprenorphine Can Affect Changes in Inhibitory Synapse Number Within the Nucleus Accumbens

(A–C) Representative IHC images (α2δ-1 cHet left and cKO right) and quantification of co-localized VGAT (green) and Gephyrin (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} mice at P21 within the NAC. n = 5 (cHet Veh), 5 (cHet GBP), 6 (cHet Bup+GBP), 5 (cHet Bup), 5 (cKO Veh), 5 (cKO GBP), 5 (cKO Bup+GBP), 5 (cKO Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

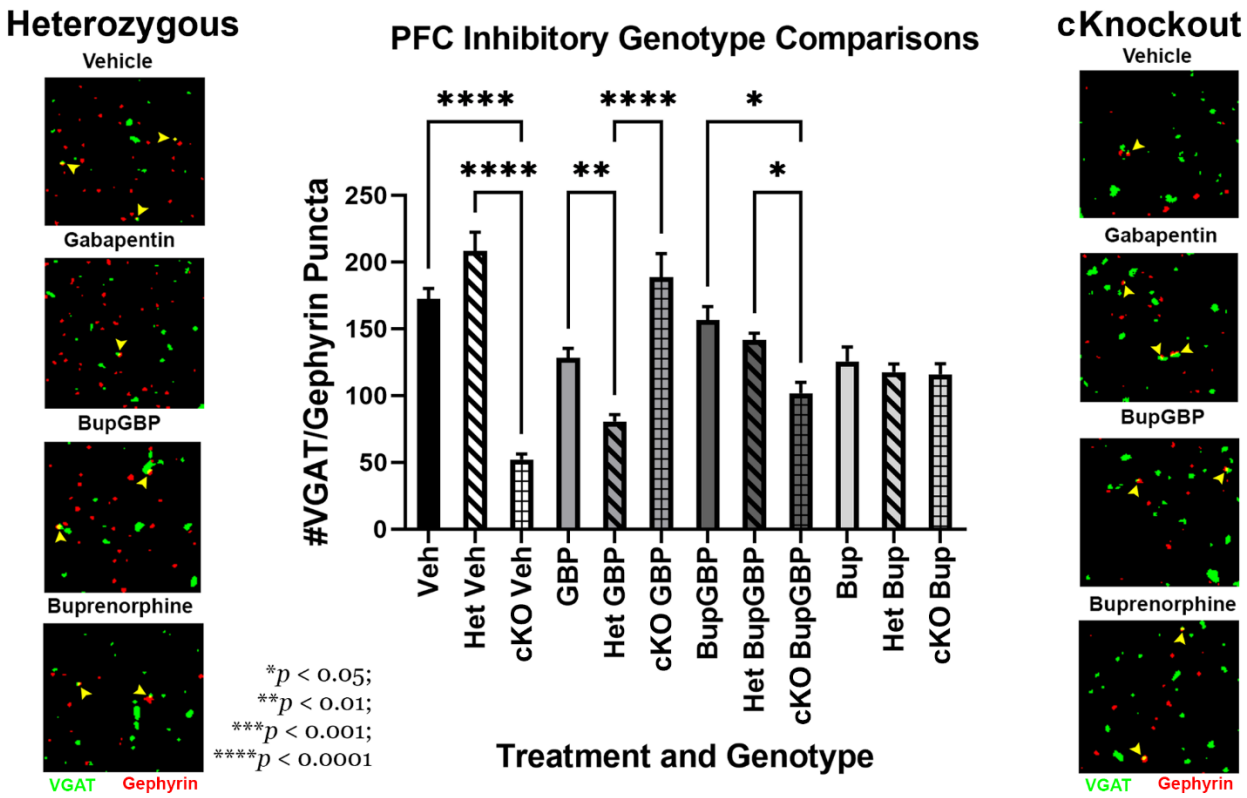

Figure 18: Gabapentin Capable of Interfering with α2δ-1 Related Decrease in Inhibitory Synapse Number Within the Prefrontal Cortex

(A–C) Representative IHC images (α2δ-1 cHet and cKO right) and quantification of co-localized VGAT (green) and Gephyrin (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} mice at P21 within the PFC. n = 5 (cHet Veh), 5 (cHet GBP), 6 (cHet Bup+GBP), 5 (cHet Bup), 5 (cKO Veh), 5 (cKO GBP), 5 (cKO Bup+GBP), 5 (cKO Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

P70 Immunohistochemistry

Changes in Excitatory Synapses Resulting from Early Life Drug Exposure Persisted into Adulthood

To determine whether the types of changes in synapse formation observed in mice at P21 persist throughout life, a separate cohort of α2δ-1^{Thy1} mice were sacrificed and perfused at P70 for the same IHC methods as were used in the P21 group. Within the ACC (**Figure 19A**),

staining for VGluT1 and PSD95 to examine excitatory glutamatergic synapses showed a significant decrease in synapse number in mice exposed to gabapentin and a significant increase in mice treated with both gabapentin and buprenorphine compared to vehicle control treated mice ($H = 81.53$, $p < 0.0001$). This combination treatment group also had a significantly higher number of synapses than the buprenorphine single drug group. The buprenorphine treatment group had a significantly lower number of synapses compared to each of the other treatment groups within the NAC ($H = 97.86$, $p < 0.0001$; **Figure 19B**). Within the PFC (**Figure 19C**) both the gabapentin and the buprenorphine + gabapentin treatment groups showed significantly greater numbers of synapses than either the vehicle control or buprenorphine groups, yet the buprenorphine group was significantly lower than the vehicle control ($H = 139.7$, $p < 0.0001$).

Persistent Effects of Early Life Drug Exposure on Excitatory Synapses Influenced by Expression of α2δ-1

The responses to different early life treatments in α2δ-1^{Thy1} WT P70 mice were compared to those of α2δ-1^{Thy1} cHet mice with the goal of determining whether significant differences in excitatory synapses related to genotype persisted into adulthood. Loss of a single copy of α2δ-1 did not appear to play as significant a role in excitatory synapse formation as it had in P21 mice. Within the ACC (**Figure 20A**), no significant differences were observed between the two genotypes. Within the NAC (**Figure 20B**) and PFC (**Figure 20C**), the only significant differences observed were in the buprenorphine treatment groups with cHet mice showing significantly greater numbers of excitatory synapses compared to WT mice in their respective brain regions.

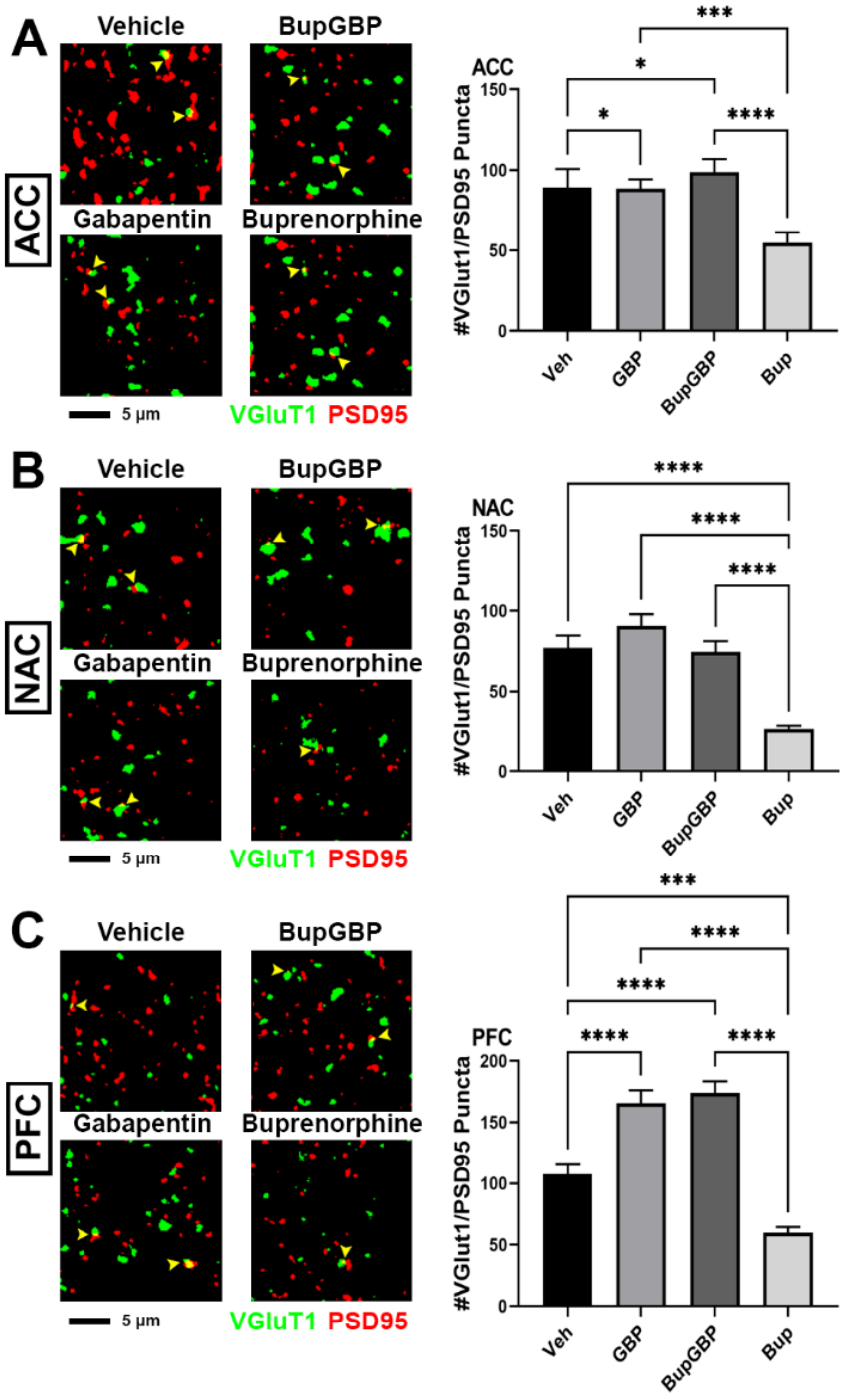

Figure 19: Drug Induced Changes in Excitatory Synapse Number Persist into Adulthood
(A–C) Representative IHC images (left) and quantification (right) of co-localized VGluT1 (green) and PSD95 (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} WT mice at P70 within (A) ACC, (B) NAC, and (C) PFC. n = 6 (Veh), 8 (GBP), 7 (Bup+GBP), 8 (Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

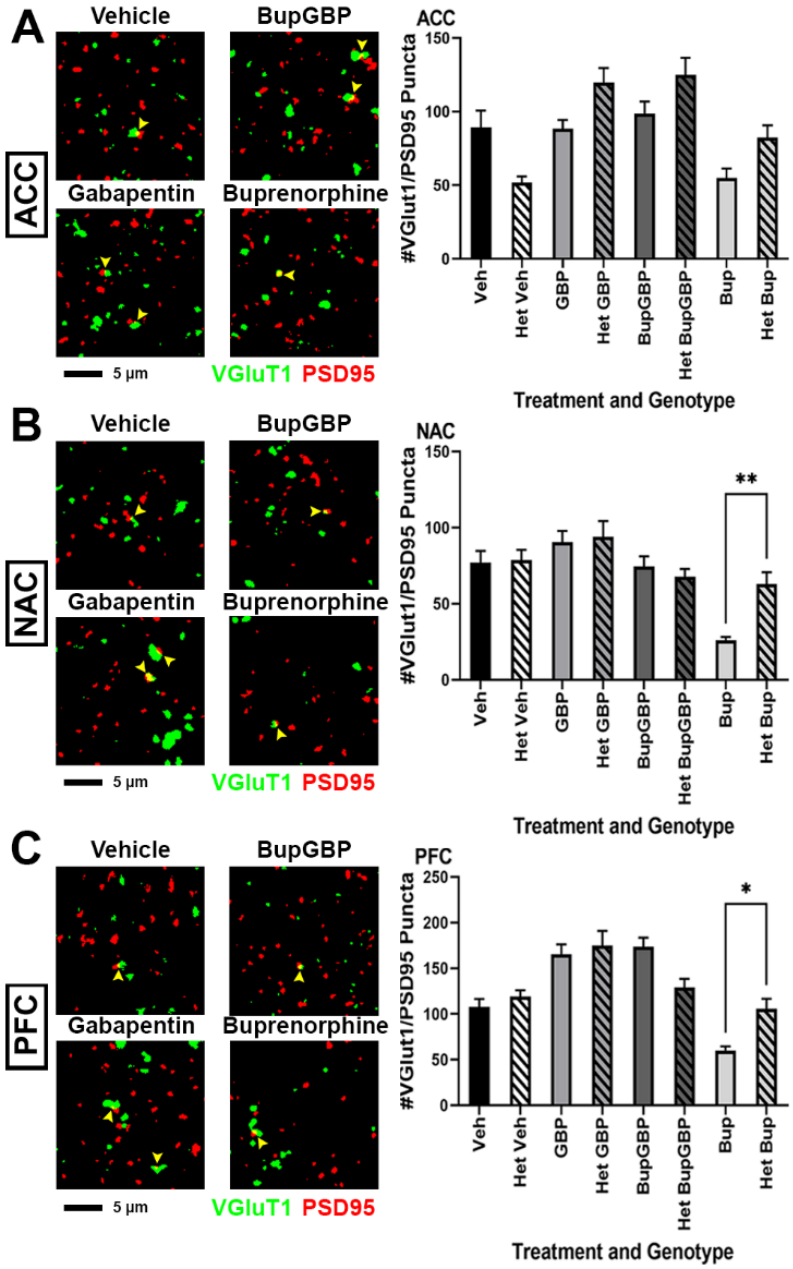

Figure 20: Persistent Effects of Early Life Buprenorphine Exposure Influenced by Haploinsufficiency of α2δ-1

(A–C) Representative IHC images (left) and quantification (right) of co-localized VGluT1 (green) and PSD95 (red) excitatory synaptic puncta (yellow arrowheads) from the brains of α2δ-1^{Thy1} cHet mice at P70 compared with WT mice of the same age within the (A) ACC, (B) NAC, and (C) PFC. n = 6 (Veh), 7 (cHet Veh), 8 (GBP), 6 (cHet GBP), 7 (Bup+GBP), 7 (cHet Bup+GBP), 8 (Bup), 7 (cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Inhibitory Synapses Were Significantly Impacted by Early Life Exposure to Both Buprenorphine and Gabapentin

As in the P21 mouse group, IHC staining for VGAT and Gephyrin was performed in P70 mice to determine the number of inhibitory GABAergic synapses within the three brain regions of interest. Early life buprenorphine treatment, whether by itself or in combination with gabapentin, was associated with a significantly lower number of synapses within the ACC (**Figure 21A**; $H = 209.9$, $p < 0.0001$). Within the NAC, gabapentin treated mice had a significantly greater number of inhibitory synapses than any other treatment (**Figure 21B**; $H = 303.8$, $p < 0.0001$). In addition, the buprenorphine + gabapentin combination treatment group had a significantly lower number of synapses compared to either the vehicle control or the gabapentin single treatment groups. Gabapentin treatment was also associated with a significantly greater number of synapses compared to any other treatment group within the PFC (**Figure 21C**; $H = 234.2$, $p < 0.0001$).

Influence of α2δ-1 Expression on Inhibitory Synapses not Observed in Mice with Early Life Drug Exposure

α2δ-1^{Thy1} WT P70 mice were also compared to α2δ-1^{Thy1} cHet mice determining whether significant differences in inhibitory synapses related to genotype persisted into adulthood. It was apparent that cHet mice, in all three brain regions, had significantly lower numbers of GABAergic synapses, as evidenced in the vehicle control groups (**Figure 22**). However, while there was a significant difference in synapse numbers within the ACC of WT vs cHet mice treated with gabapentin (**Figure 22A**), no significant differences were observed within the NAC (**Figure 22B**) or PFC (**Figure 22C**) in any drug treatment comparisons of these two mouse groups.

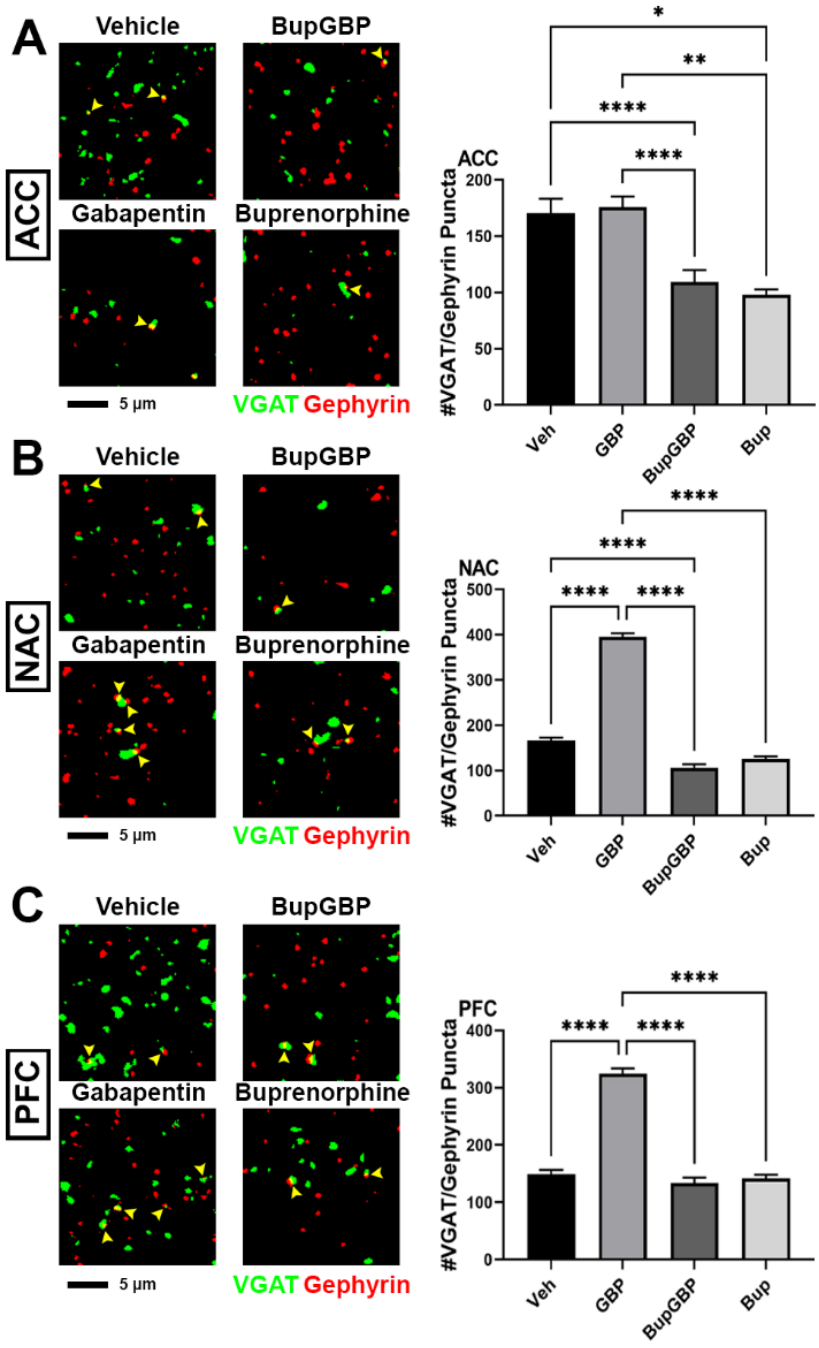

Figure 21: Early Life Drug Exposure Produces Persistent Changes in Inhibitory Synapse Number

(A–C) Representative IHC images (left) and quantification (right) of co-localized VGAT (green) and Gephyrin (red) excitatory synaptic puncta (yellow arrowheads) from the brains of early life drug-exposed α2δ-1^{Thy1} WT mice at P70 within (A) the ACC, (B) NAC, and (C) PFC. n = 6 (Veh), 8 (GBP), 7 (Bup+GBP), 8 (Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

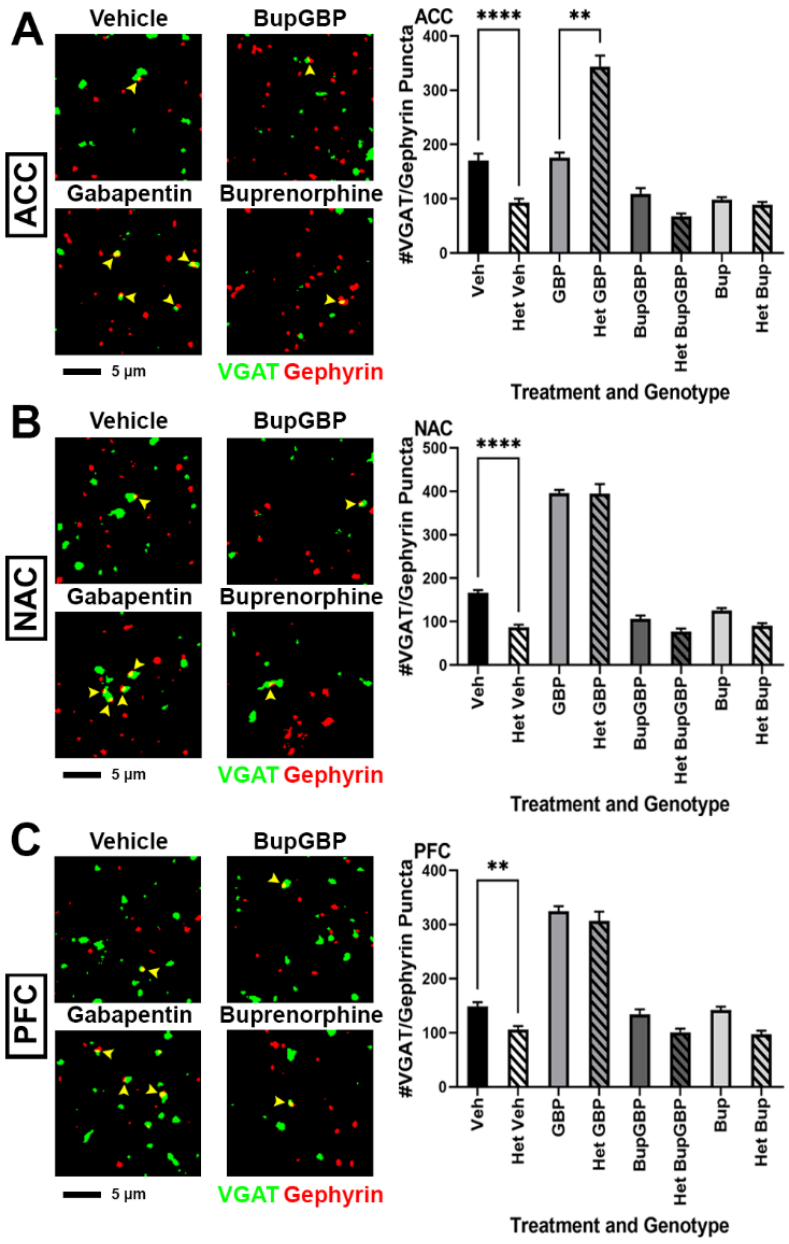

Figure 22: Effects of Early Life Drug Exposure on Inhibitory Synapse Number is Less Impacted by Haploinsufficiency of α2δ-1 Over Time

(A–C) Representative IHC images (left) and quantification (right) of co-localized VGAT (green) and Gephyrin (red) excitatory synaptic puncta (yellow arrowheads) from the brains of α2δ-1^{Thy1} cHet mice at P70 compared with WT mice of the same age within the (A) ACC, (B) NAC, and (C) PFC n = 6 (Veh), 7 (cHet Veh), 8 (GBP), 6 (cHet GBP), 7 (Bup+GBP), 7 (cHet Bup+GBP), 8 (Bup), 7 (cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Excitatory Synapses Generally Decrease with Age

Although differences in the numbers of synapses were observed in early life drug exposed mice at both P21 and at P70, how these differences may have changed over time was not yet clear. To this end, synapse numbers in each brain region in mice from each age group were compared within each treatment-genotype. IHC staining for glutamatergic excitatory synapses within the ACC (**Figure 23**; $H = 515.8$, $p < 0.0001$) showed a general decrease in synapse number with age in most treatment-genotype groups (excluding gabapentin and buprenorphine + gabapentin in cHet mice). A similar trend was observed within the NAC (**Figure 24**; $H = 311.7$, $p < 0.0001$), though not in the gabapentin treated groups in either genotype nor the buprenorphine + gabapentin in cHet mice. Results very similar to those observed within the NAC were seen within the PFC (**Figure 25**; $H = 559.0$, $p < 0.0001$) as well.

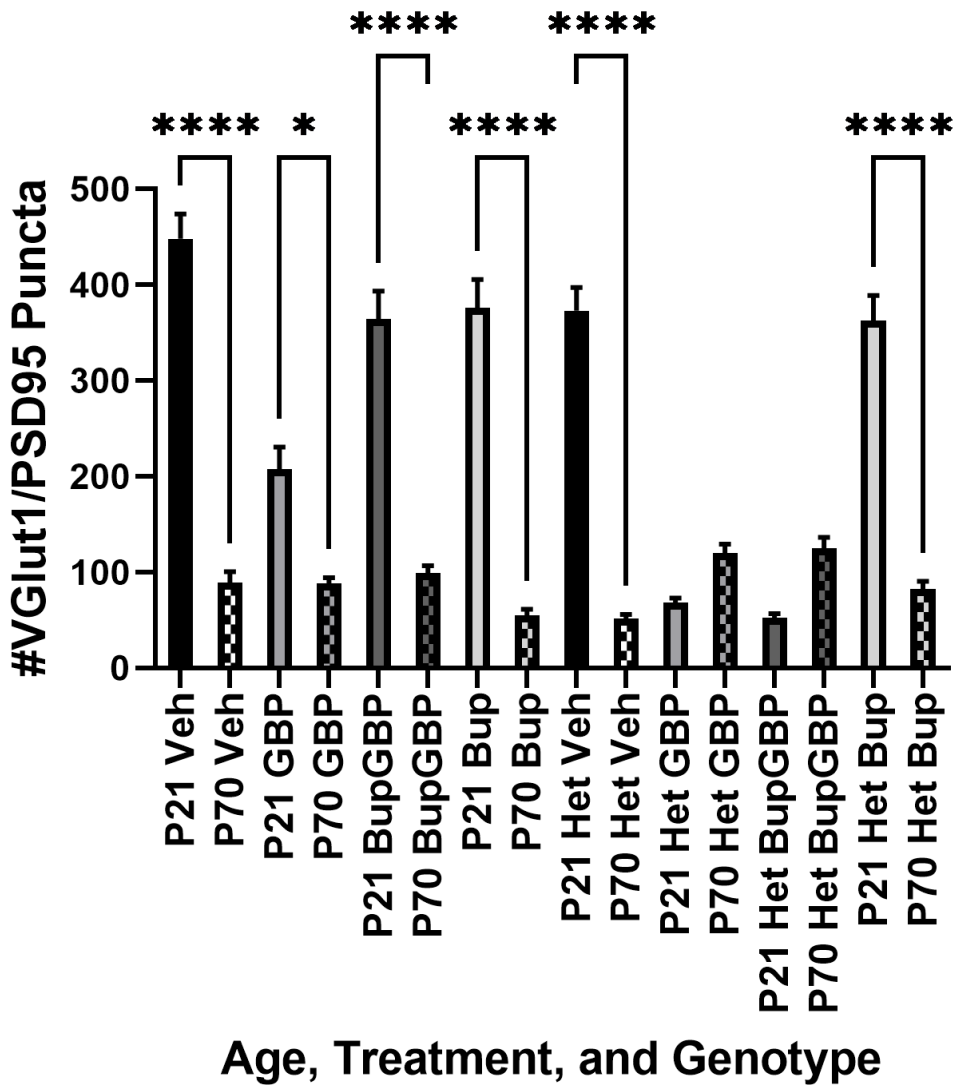

Figure 23: Increase in Age Generally Associated with Decrease in Excitatory Synapse Number Within the Anterior Cingulate Cortex

Comparison of excitatory synapse number between ages with the same genotype and early life treatment within the ACC. n = 5 (P21 Veh), 5 (P21 cHet Veh), 5 (P21 GBP), 5 (P21 cHet GBP), 6 (P21 Bup+GBP), 6 (P21 cHet Bup+GBP), 4 (P21 Bup), 5 (P21 cHet Bup), 6 (P70 Veh), 7 (P70 cHet Veh), 8 (P70 GBP), 6 (P70 cHet GBP), 7 (P70 Bup+GBP), 7 (P70 cHet Bup+GBP), 8 (P70 Bup), 7 (P70 cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

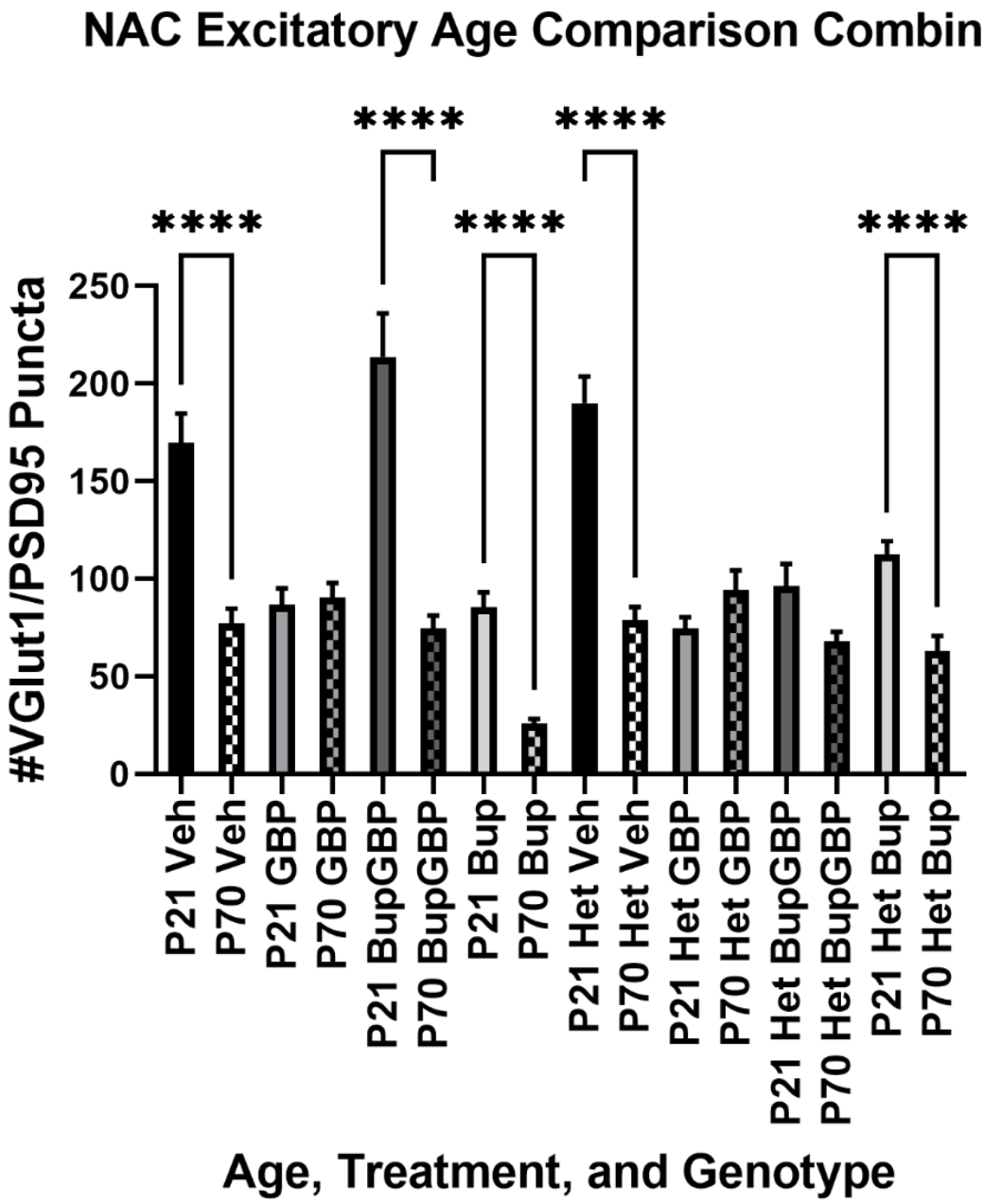

Figure 24: Age Related Decrease in Excitatory Synapse Number Not Equal Across Drug Treatment Groups Within the Nucleus Accumbens

Comparison of excitatory synapse number between ages with the same genotype and early life treatment within the NAC. n = 5 (P21 Veh), 5 (P21 cHet Veh), 5 (P21 GBP), 5 (P21 cHet GBP), 6 (P21 Bup+GBP), 6 (P21 cHet Bup+GBP), 4 (P21 Bup), 5 (P21 cHet Bup), 6 (P70 Veh), 7 (P70 cHet Veh), 8 (P70 GBP), 6 (P70 cHet GBP), 7 (P70 Bup+GBP), 7 (P70 cHet Bup+GBP), 8 (P70 Bup), 7 (P70 cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

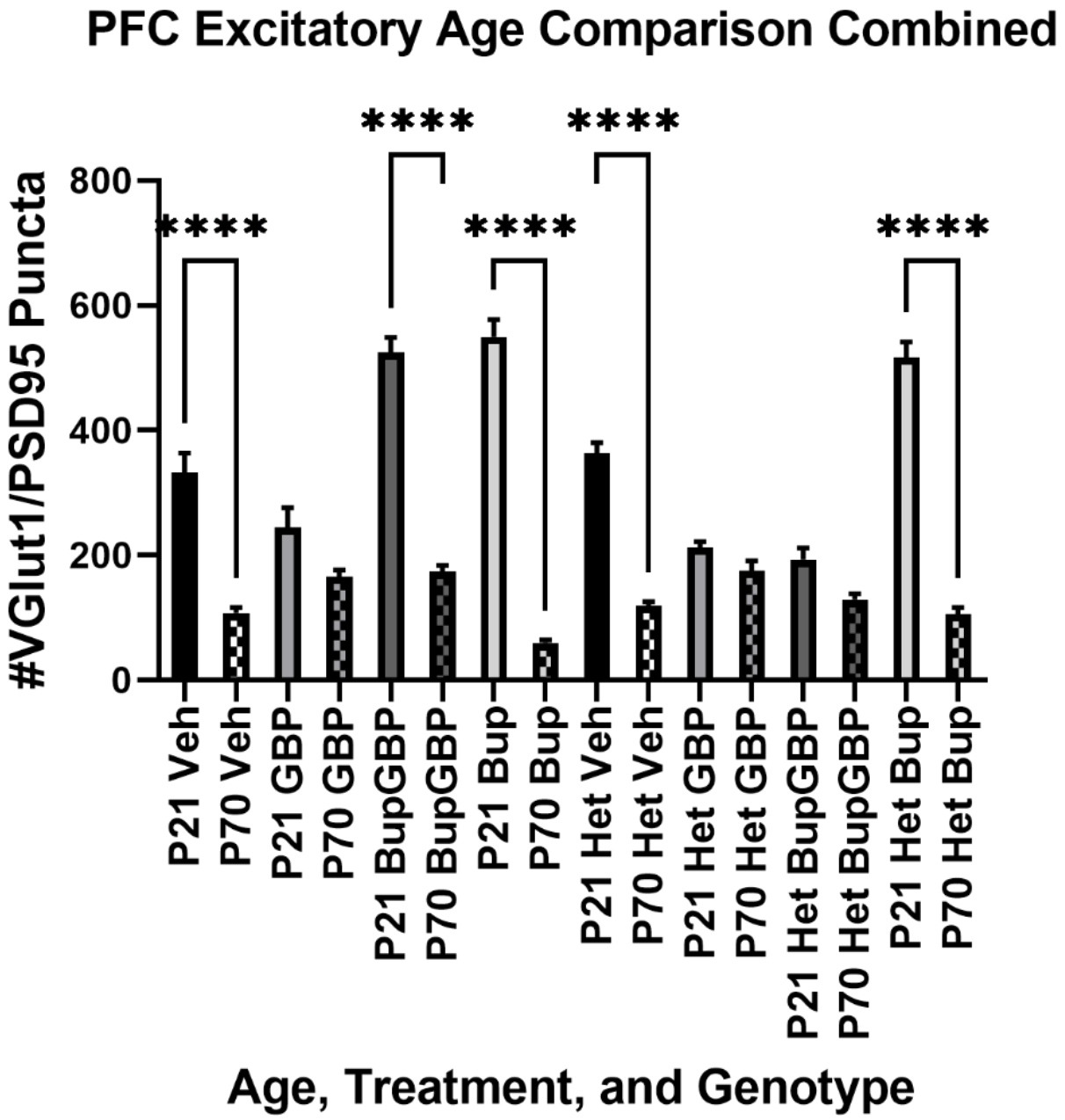

Figure 25: Changes in Excitatory Synapse Number in Gabapentin Treatment in Haploinsufficent Mice not Altered by Age Within the Prefrontal Cortex
Comparison of excitatory synapse number between ages with the same genotype and early life treatment within the PFC. n = 5 (P21 Veh), 5 (P21 cHet Veh), 5 (P21 GBP), 5 (P21 cHet GBP), 6 (P21 Bup+GBP), 6 (P21 cHet Bup+GBP), 4 (P21 Bup), 5 (P21 cHet Bup), 6 (P70 Veh), 7 (P70 cHet Veh), 8 (P70 GBP), 6 (P70 cHet GBP), 7 (P70 Bup+GBP), 7 (P70 cHet Bup+GBP), 8 (P70 Bup), 7 (P70 cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Inhibitory Synapses Generally Decrease with Age, But Not in Gabapentin Treated Mice

Despite observing in excitatory IHC staining that synapse numbers tend to generally decrease with age, IHC staining for GABAergic inhibitory synapses did not necessarily follow this same trend. Significantly lower numbers of synapses were observed within the ACC (**Figure 26**; $H = 327.8$, $p < 0.0001$) in P70 WT mice treated with either buprenorphine or buprenorphine in combination with gabapentin as well as in P70 cHet mice treated with vehicle control compared with their respective P21 counterparts. However, a significantly greater number of synapses were observed within the ACC in gabapentin treated P70 cHet mice compared with P21 mice given the same treatment. A greater synapse number was also observed between these mouse groups within the NAC as well (**Figure 27**; $H = 402.9$, $p < 0.0001$). Significant increases of this type were also observed between P70 and P21 WT mice treated with either gabapentin or with vehicle control, yet decreases were observed in vehicle control treated cHet mice and in buprenorphine + gabapentin treated WT and cHet mice. Within the PFC (**Figure 28**; $H = 334.5$, $p < 0.0001$), P70 WT and cHet mice showed significantly greater numbers of synapses compared to their P21 counterparts. It was only in cHet mice that significantly lower synapse numbers were observed between P70 counterparts P21 and only in the vehicle control and buprenorphine + gabapentin treatment groups.

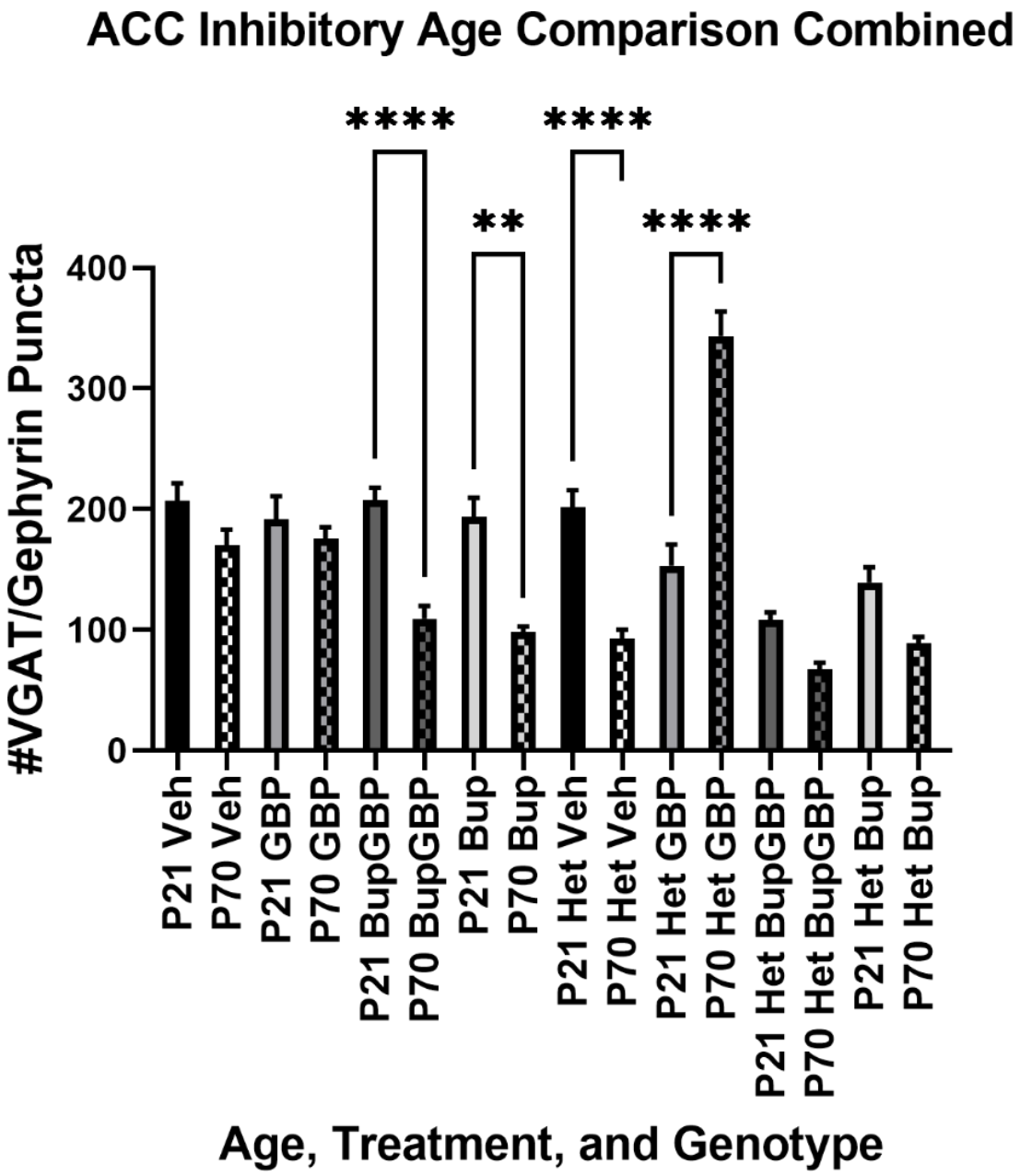

Figure 26: Expression Level of α2δ-1 Influences Age-Related Changes in Inhibitory Synapses Within Anterior Cingulate Cortex

Comparison of inhibitory synapse number between ages with the same genotype and early life treatment within the ACC. n = 5 (P21 Veh), 5 (P21 cHet Veh), 5 (P21 GBP), 5 (P21 cHet GBP), 6 (P21 Bup+GBP), 6 (P21 cHet Bup+GBP), 4 (P21 Bup), 5 (P21 cHet Bup), 6 (P70 Veh), 7 (P70 cHet Veh), 8 (P70 GBP), 6 (P70 cHet GBP), 7 (P70 Bup+GBP), 7 (P70 cHet Bup+GBP), 8 (P70 Bup), 7 (P70 cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

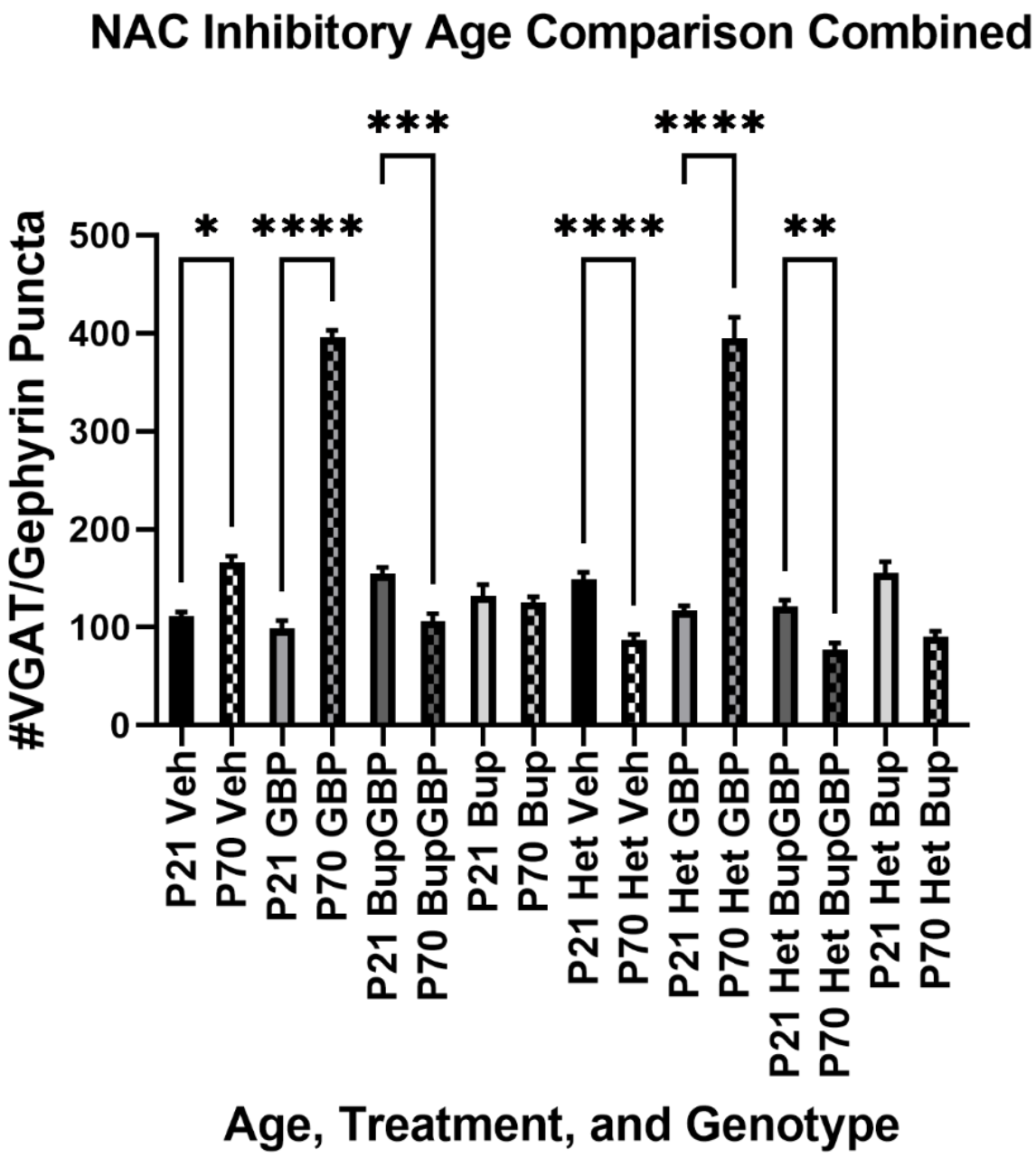

Figure 27: Inhibitory Synapses Increase with Age in Gabapentin Treated Mice Within the Nucleus Accumbens

Comparison of inhibitory synapse number between ages with the same genotype and early life treatment within the NAC. n = 5 (P21 Veh), 5 (P21 cHet Veh), 5 (P21 GBP), 5 (P21 cHet GBP), 6 (P21 Bup+GBP), 6 (P21 cHet Bup+GBP), 4 (P21 Bup), 5 (P21 cHet Bup), 6 (P70 Veh), 7 (P70 cHet Veh), 8 (P70 GBP), 6 (P70 cHet GBP), 7 (P70 Bup+GBP), 7 (P70 cHet Bup+GBP), 8 (P70 Bup), 7 (P70 cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

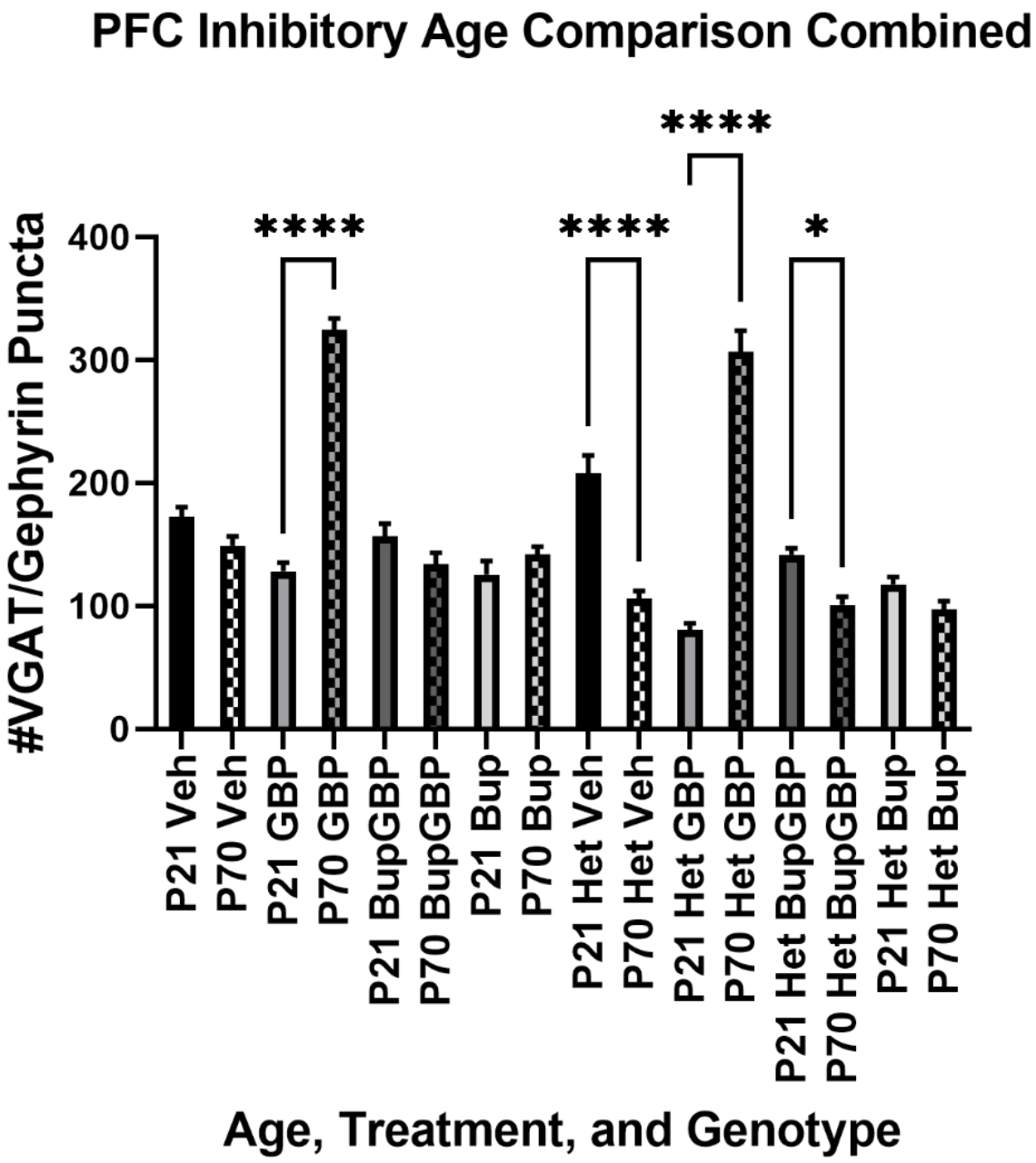

Figure 28: Inhibitory Synapses Increase with Age in Gabapentin Treated Mice Within the Prefrontal Cortex

Comparison of inhibitory synapse number between ages with the same genotype and early life treatment within the PFC. n = 5 (P21 Veh), 5 (P21 cHet Veh), 5 (P21 GBP), 5 (P21 cHet GBP), 6 (P21 Bup+GBP), 6 (P21 cHet Bup+GBP), 4 (P21 Bup), 5 (P21 cHet Bup), 6 (P70 Veh), 7 (P70 cHet Veh), 8 (P70 GBP), 6 (P70 cHet GBP), 7 (P70 Bup+GBP), 7 (P70 cHet Bup+GBP), 8 (P70 Bup), 7 (P70 cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Behavioral Studies

Early Life Drug Exposure Did Not Have Any Significant Effect on Performance During Light/Dark Testing

The Light/Dark chamber is a commonly used tool for the study of anxiety-like behaviors in rodents (Buccafusco, 2009). As part of a larger regimen of behavioral studies over the course of seven weeks (see **Figure 10**), Light/Dark testing was conducted with male and female mice all at the same age with one of two different genotypes from one of four different early life treatments. Each mouse was video recorded for 10 minutes and allowed to pass freely between two chambers: a Light zone chamber brightly lit from above and an unlit Dark zone chamber covered by a roof. To first examine the possible effects the different early life drug treatments might have on behavior, the number of entries into the Light zone from the Dark zone (**Figure 29A**) and the amount of time spent in the Light zone (**Figure 29B**) were recorded for each mouse and the means for each treatment-genotype group were calculated. No significant differences were observed between treatment groups in the mean number of Light Zone entries ($H = 4.146$, $p = 0.2461$) among WT $\alpha2\delta\text{-}1^{Thy1}$ mice, nor were any observed in the mean amount of time spent in the Light zone ($H = 2.247$, $p = 0.5228$). When WT mice were compared with cHet $\alpha2\delta\text{-}1^{Thy1}$ mice to determine what effect, if any, α2δ-1 genotype would have on testing behavior, no significant differences were observed in number of entries (**Figure 29C**; $H = 6.678$, $p = 0.4631$) or in time spent in the zone (**Figure 29D**; $H = 10.86$, $p = 0.1447$).

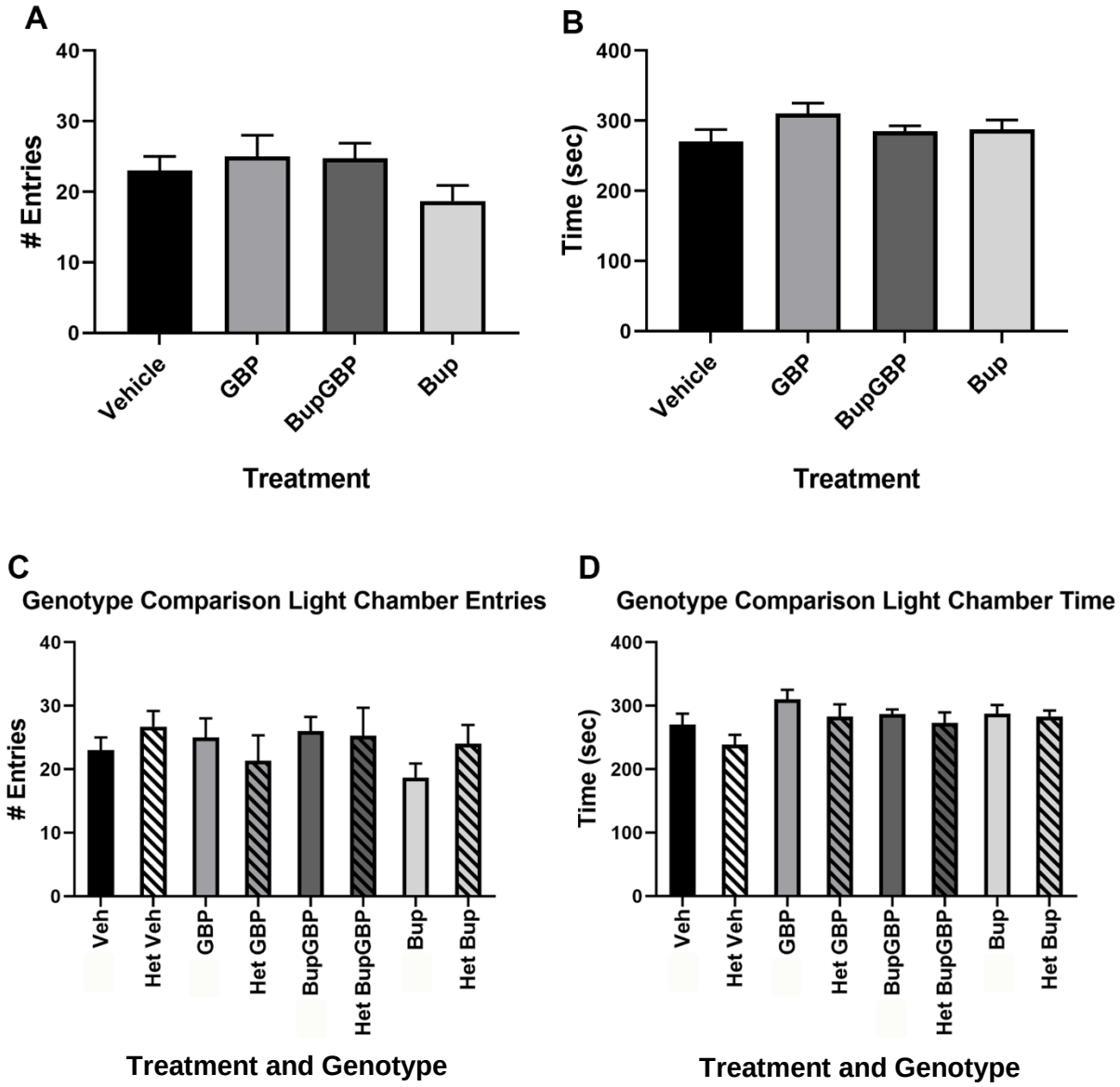

Figure 29: Light/Dark Testing Performance Not Impacted by Early Life Drug Exposure or Expression Level of α2δ-1

(A) Mean number of entries into the Light zone and (B) mean time spent in the Light zone for each treatment group of α2δ-1^{Thy1} WT mice. (C) Mean number of entries into the Light zone and (D) mean time spent in the Light zone for α2δ-1^{Thy1} cHet mice compared with WT mice of the same age. n = 6 (Veh), 7 (cHet Veh), 8 (GBP), 6 (cHet GBP), 7 (Bup+GBP), 7 (cHet Bup+GBP), 8 (Bup), 7 (cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Possible Changes in Locomotor Function and Freezing Behavior in Open Field

Open Field testing can be used to study several different behavioral phenotypes in animals, including those related to anxiety and locomotor function (Buccafusco, 2009; Sestakova et al., 2013). In this study, mice were placed in an Open Field chamber where their movement was recorded for 10 minutes. The number of entries into the center zone area of the field were recorded for each mouse and the means for each treatment-genotype group were calculated (**Figure 30A**). No significant differences were observed between the different treatment groups ($H = 5.101$, $p = 0.1646$) in $\alpha2\delta\text{-}1^{Thy1}$ WT mice, nor were any significant differences observed in the mean amount of time spent in the center zone (**Figure 30B**; $H = 1.764$, $p = 0.6228$). The amount of time each mouse spent in thigmotaxis near the walls of the chamber, another indicator of anxiety-type behavior, was also recorded (**Figure 30C**). No significant differences were observed between the mean times of each treatment group ($F = 1.764$, $p = 0.3329$). To assess whether early life drug exposure impacted locomotor function, the total distance that each mouse traveled during their time in the chamber was recorded (**Figure 30D**). The mean distance traveled by mice treated with buprenorphine was significantly less than that of the mean distance traveled by mice treated with buprenorphine + gabapentin ($p = 0.0430$), though neither treatment was significantly different from the vehicle control. A difference was also seen in gabapentin treated mice who spent significantly less time frozen (not moving in one place) than the vehicle control (**Figure 30E**, $p = 0.0130$) and the mice treated with buprenorphine + gabapentin spent a nearly significantly less amount of time frozen as well ($p = 0.0527$). These same measures were recorded for $\alpha2\delta\text{-}1^{Thy1}$ cHet mice as well and mean values were compared to those of the WT mice for each treatment group (**Figure 31**). In the number of entries to the center zone (**Figure 31A**), time spent in the center zone (**Figure 31B**), time spent in thigmotaxis (**Figure 31C**), mean

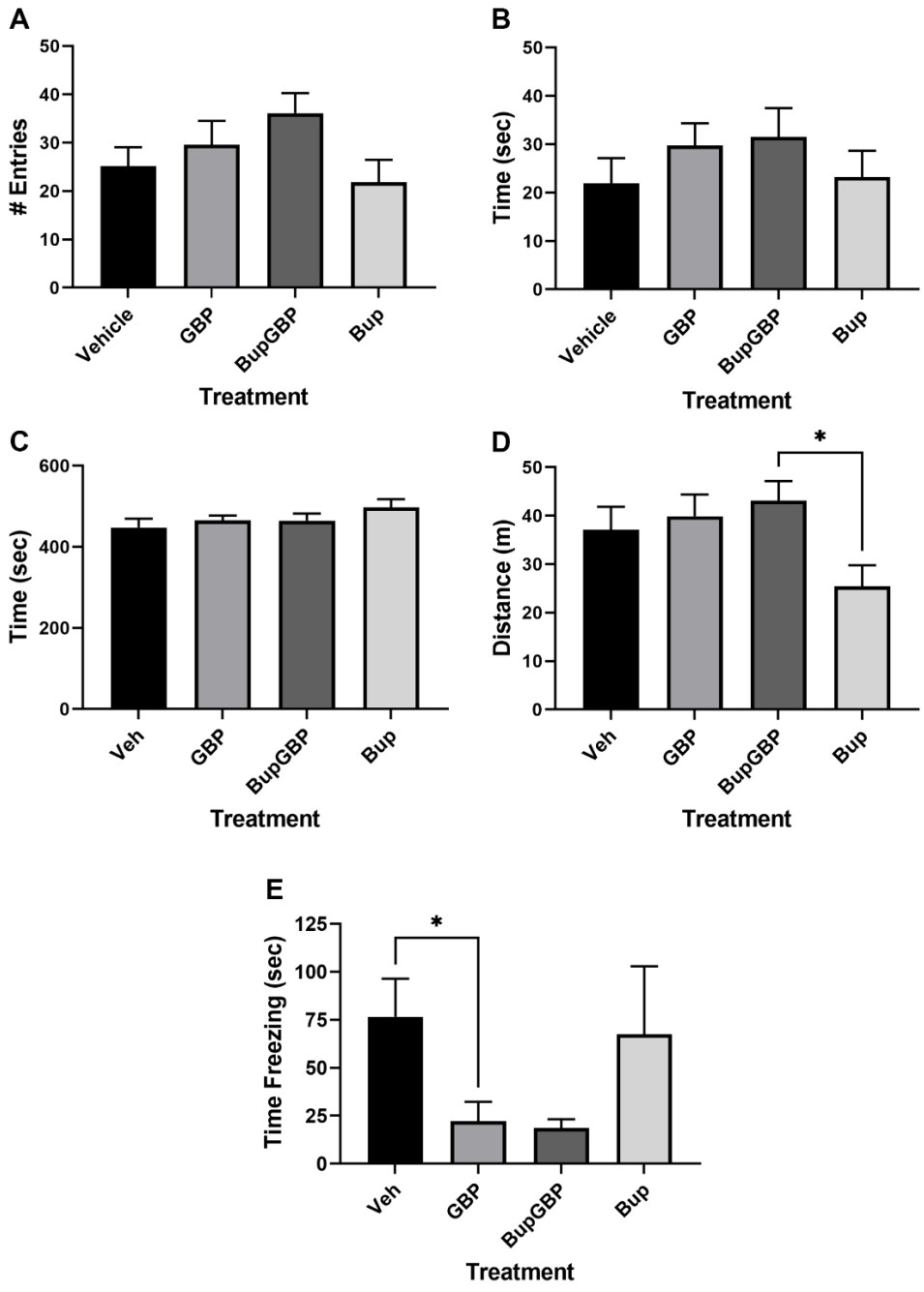

Figure 30: Anxiety and Locomotor Activity in Open Field Testing Potentially Impacted by Early Life Drug Exposure

Open Field performance metrics, including (A) mean number of entries into the center zone, (B) mean time spent in the center zone, (C) mean time spent in thigmotaxis, (D) mean total distance traveled, and (E) time spent freezing, for $\alpha 2\delta\text{-}1^{Thy1}$ WT mice. n = 6 (Veh), 7 (cHet Veh), 8 (GBP), 6 (cHet GBP), 7 (Bup+GBP), 7 (cHet Bup+GBP), 8 (Bup), 7 (cHet Bup); $*p < 0.05$

total distance traveled (**Figure 31D**), and total time spent frozen (**Figure 31E**), no significant differences were observed between the genotypes ([H = 7.868, p = 0.344], [H = 4.988, p = 0.6614], [F = 0.9016, p = 0.5129], [F = 1.616, p = 0.1525], and [H = 21.51, p = 0.0059] respectively).

Enhancement of Conditioned Place Preference Not Observed in Drug Treated Mice

Nicotine has been previously shown to produce conditioned place preference (CPP) in rodents (Buccafusco, 2009). In this study, intraperitoneal injections of 0.5mg/kg doses of nicotine were administered and paired with a chamber with a distinct wall pattern and floor texture while a different chamber with a different wall pattern and floor texture was paired with saline injections. CPP scores were calculated for each mouse based on the amount of time spent in each chamber before and after 8 days of daily injections alternating between saline and nicotine. The mean CPP scores for α2δ-1^{Thy1} WT mice from each treatment group were compared to determine if any drug treatment produced enhanced CPP (**Figure 32A**). Although each treatment group showed a positive average CPP score, indicating preference for the nicotine paired chamber, there were no significant differences observed between treatment groups (F = 0.5944, p = 0.6243). Mean CPP scores for α2δ-1^{Thy1} cHet mice were then compared to WT in each treatment group (**Figure 32B**). No significant differences were observed between genotypes in any treatment group (F = 0.8514, p = 0.5509).

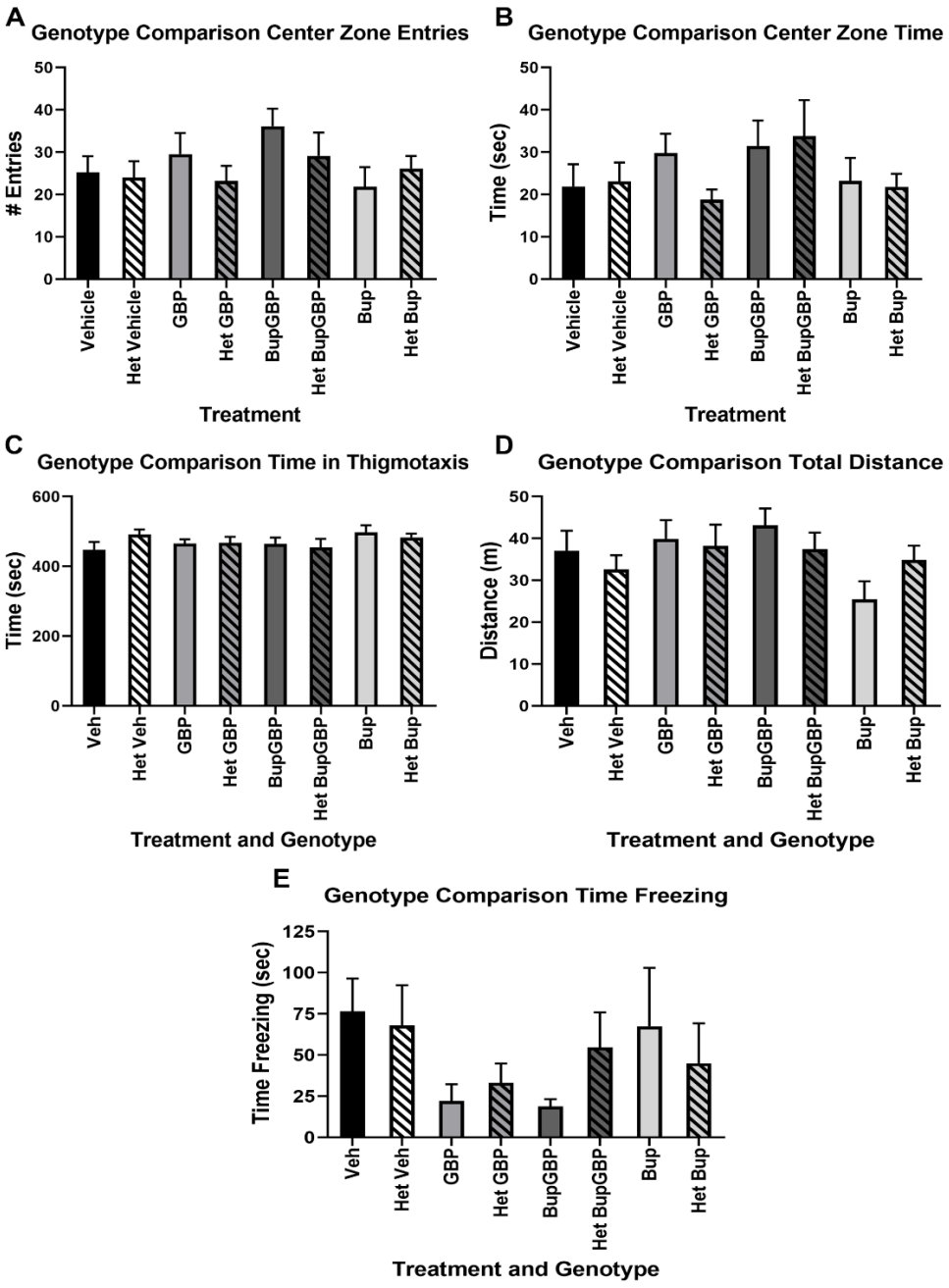

Figure 31: Performance in Open Field Not Impacted by Expression Level of α2δ-1
Open Field performance metrics, including (A) mean number of entries into the center zone, (B) mean time spent in the center zone, (C) mean time spent in thigmotaxis, (D) mean total distance traveled, and (E) time spent freezing, for each treatment group of α2δ-1^{Thy1} cHet mice compared with those of WT mice of the same age. n = 6 (Veh), 7 (cHet Veh), 8 (GBP), 6 (cHet GBP), 7 (Bup+GBP), 7 (cHet Bup+GBP), 8 (Bup), 7 (cHet Bup); *$p < 0.05$

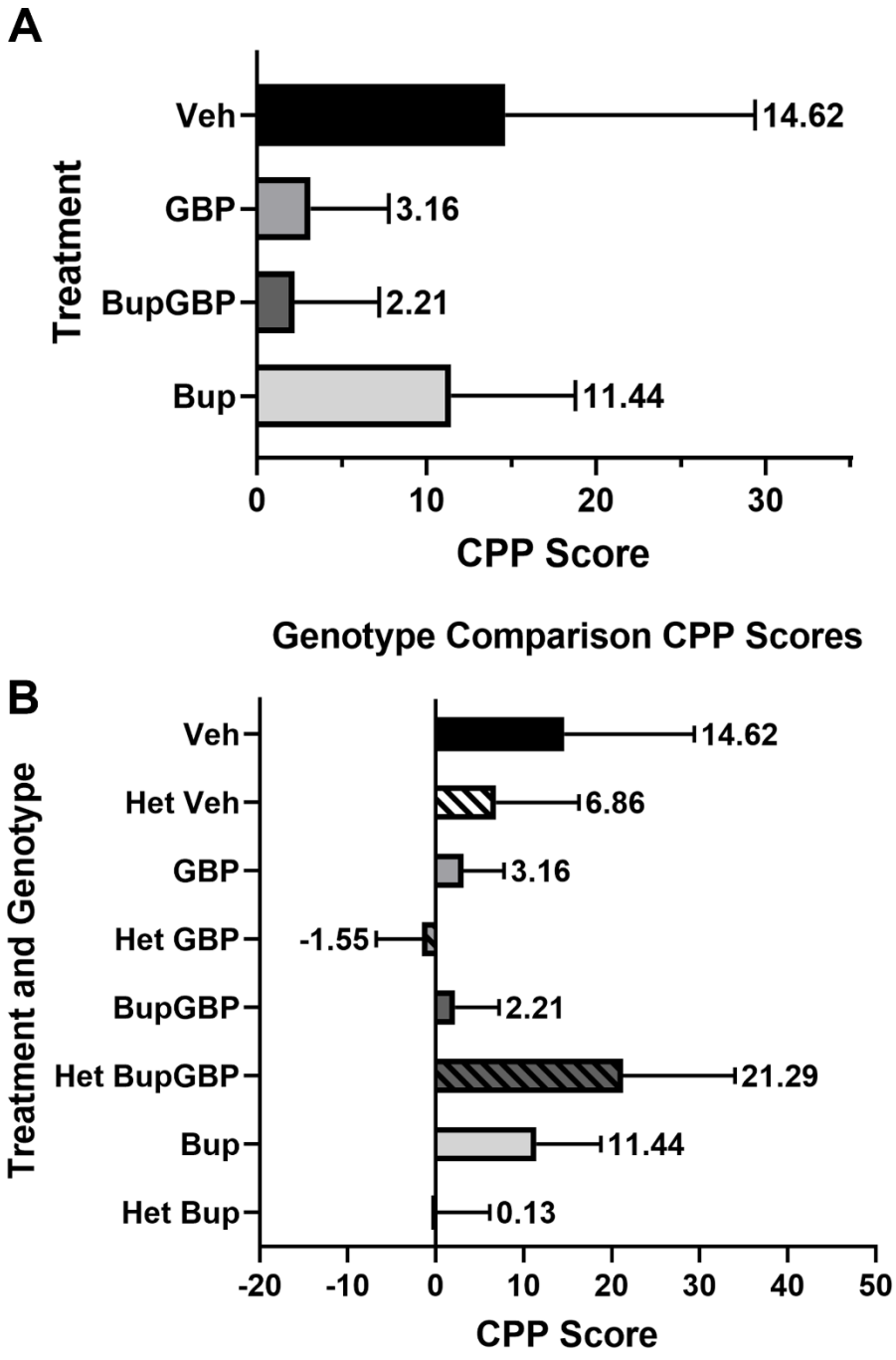

Figure 32: Neither Drug Treatment Nor α2δ-1 Expression Significantly Affected Conditioned Place Preference

(A) Mean CPP scores for α2δ-1^{Thy1} WT mice across 4 early life treatment groups. (B) Mean CPP scores for α2δ-1^{Thy1} cHet mice compared to WT across 4 treatment groups; n = 6 (Veh), 7 (cHet Veh), 8 (GBP), 6 (cHet GBP), 7 (Bup+GBP), 7 (cHet Bup+GBP), 8 (Bup), 7 (cHet Bup); *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

Decline After Day 1 Performance in FR1 Schedule Self-Administration

Self-administration of vaporized substances of abuse, including nicotine and alcohol, has previously been used as a tool to study reinforcement-related behaviors in rodents (Cooper et al., 2020; de Guglielmo et al., 2017; Henderson & Cooper, 2021). In this study, mice were placed in an operant vapor chamber for one hour each day for 10 days and allowed to self-administer a 3mg/ml nicotine vapor via nose poke on a FR1 schedule (**Figure 33**). On Day One of the daily sessions, $\alpha2\delta\text{-}1^{Thy1}$ cHet mice that had received a vehicle control treatment demonstrated the highest mean number of nose pokes compared to any other treatment-genotype group and had the highest one-day mean value of any mouse group. This difference, however, did not rise to the level of significance ($H = 4.175$, $p = 0.7594$) and was lost on the following day. No other significant differences in mean number of nose pokes between genotype × treatment groups were observed during the 10 days of testing.

FR3 Schedule Self-Administration Not Enhanced In Mice that Demonstrated Possible Reinforcement

At the end of the 10 days of FR1 self-administration, the daily results for total number of nose pokes for each mouse was reexamined to determine whether it was likely that reinforcement for nicotine vapor self-administration was achieved. The mice chosen to participate in the 3-day FR3 schedule were as follows: three of the six WT vehicle control (two male, one female), three of the seven cHet vehicle control (one male, two female), five of the eight WT gabapentin (one male, four female), three of the seven WT buprenorphine + gabapentin (one male, two female), four of the seven cHet buprenorphine + gabapentin (one male, three female), and four of the seven cHet buprenorphine (one male, three female). Mice from cHet gabapentin (two male) and WT buprenorphine (two female) were not included as n was too low for statistical analysis. The

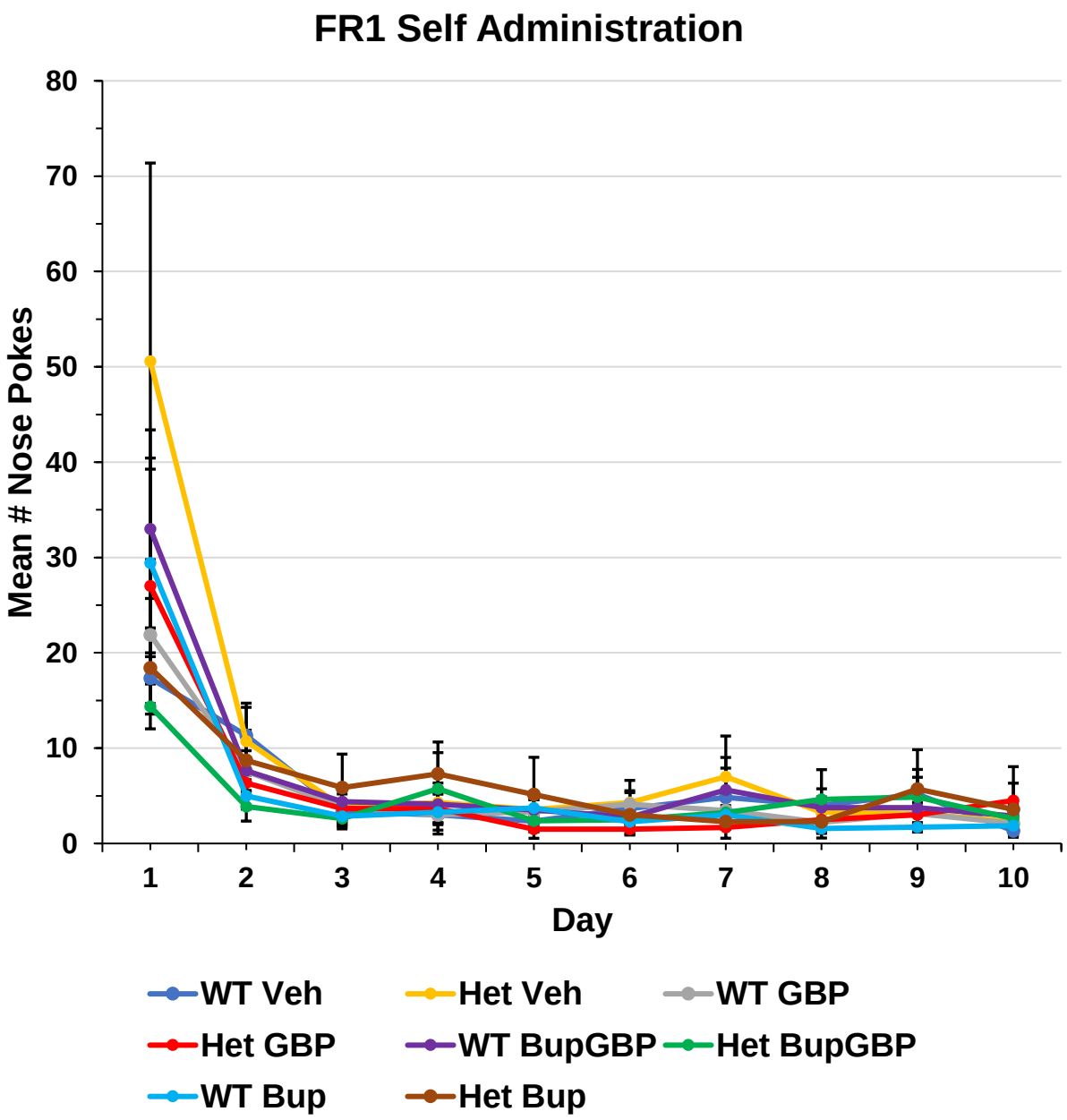

Figure 33: Number of Nose Pokes Declined After First Day of 10-Day FR1 Self-Administration

Mean number of nose pokes for each treatment-genotype mouse group each day of 10-day FR1 self-administration testing. Vehicle control treated α2δ-1 cHet mice showed a nonsignificant trend towards a greater number of nose pokes only on Day 1 of FR1 self-administration testing. n = 6 (Veh), 7 (cHet Veh), 8 (GBP), 6 (cHet GBP), 7 (Bup+GBP), 7 (cHet Bup+GBP), 8 (Bup), 7 (cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

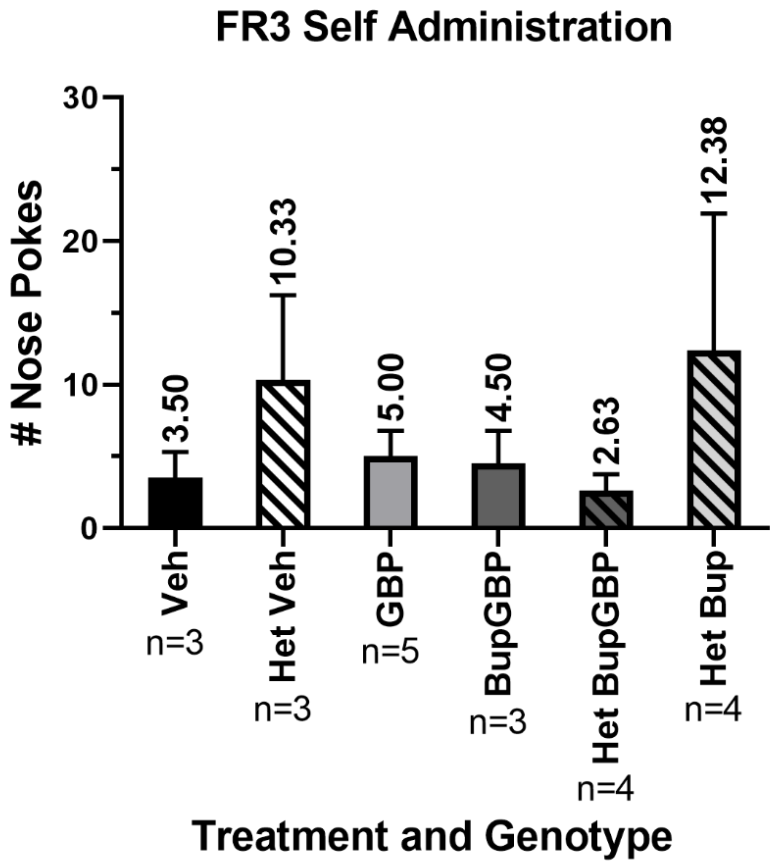

Figure 34: No Significant Differences Observed Among the Mean Number of Nose Pokes of from Last 2 Days of FR3 Self-Administration in Mice That Demonstrated Reinforcement
Mean average number of nose pokes across Days 2 and 3 of FR3 self-administration testing (listed above standard error bars) in mice that demonstrated reinforcement during FR1 self-administration testing. n = 3 (Veh), 3 (cHet Veh), 5 (GBP), 3 (Bup+GBP), 4 (cHet Bup+GBP), 4 (cHet Bup); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

mean number of nose pokes for each mouse for the last two days of FR3 self-administration were calculated. Then from those values, the mean for each treatment-genotype group was calculated (**Figure 34**). No significant differences were observed between any of the treatment genotype groups ($H = 3.402$, $p = 0.7569$).

DISCUSSION

Early Life Drug Exposure Significantly Impacts Astrocyte-Mediated Synaptogenesis via α2δ-1

A major goal of this study was to investigate disruptions in normal synaptic development within the mesolimbic dopamine pathway resulting from early life exposure to drugs of abuse. To this aim, a mouse model of early life drug exposure with brain-specific knockout of α2δ-1, the neuronal receptor for astrocyte-secreted synaptogenic thrombospondins, was developed. Four different treatments and three possible genotypes for *Cacna2d1*, the gene encoding α2δ-1, within two different age groups were tested. Average body masses for the pups were recorded and compared across sex, treatment, and expression of α2δ-1. It was noteworthy that there were few differences among the body masses of pups at P21. In the C57Bl/6J mouse study (**see Chapter 3**), several significant main effects were observed in treatment, genotype, and genotype × sex. The C57Bl/6J dams as well showed significant differences in body mass between treatment groups. Yet in the α2δ-1^{Thy1} dams, no significant differences were observed. Given that the primary difference between these two mouse lines was brain specificity of the decreased expression α2δ-1 versus throughout the whole body (Catterall et al., 1988; Cole et al., 2005; Klugbauer et al., 1999; Nieto-Rostro et al., 2018; Tanabe et al., 1987), this could indicate that whole body knockout of α2δ-1 led to greater susceptibility to drugs of abuse in terms of interruptions of normal development.

The results of this study's IHC experiments, much like those of the C57Bl/6J mouse study, indicate that excitatory glutamatergic and inhibitory GABAergic synaptic populations are significantly disrupted by early life exposure to buprenorphine and gabapentin, separately or in combination, and that these disruptions are strongly influenced by α2δ-1 function. While

haploinsufficiency of α2δ-1 was associated with significantly lower numbers of synapses in some cases, brain specific knockout of α2δ-1 correlated with lower numbers of both excitatory and inhibitory synapses within all three brain regions. This may indicate that, even when expression of α2δ-1 was decreased, synaptogenesis induced by thrombospondins can still occur, potentially by compensatory astrocyte-mediated signaling pathways (Baldwin & Eroglu, 2017; Risher & Eroglu, 2020).

Early life exposure to buprenorphine in combination with gabapentin was associated with an increase in net excitation within the mesolimbic dopamine pathway, with increased numbers of excitatory synapses and largely unaffected numbers of inhibitory synapses. Gabapentin in isolation, however, tended to be associated with a decrease in excitatory synapse number. These findings are in agreement with previous research showing that normal astrocyte mediated synaptogenesis via interaction between thrombospondin and α2δ-1 is interrupted by gabapentin (Eroglu et al., 2009). These findings also provide further evidence that a synergistic effect occurs between gabapentin and opioids, such as buprenorphine, that is not seen in either individual treatment. While the precise mechanism for this interaction is not yet known, it does agree with previous studies and patient reports of gabapentin increasing the effects of opioids (Baird et al., 2014; Bastiaens et al., 2016; Meymandi et al., 2006) as well as the clinical finding of a unique presentation of NAS in infants known to have been exposed to both gabapentin and opioids (Loudin et al., 2017).

A novel finding of this study concerned the role of α2δ-1 in regulation of GABAergic synapses. The ability of astrocytes to regulate synapse formation has largely been demonstrated with excitatory glutamatergic synapses, and signaling involving TSP binding to α2δ-1 has been one of the primary mechanisms through which it has been demonstrated (Christopherson et al.,

2005; Eroglu et al., 2009; Eroglu & Barres, 2010; Risher & Eroglu, 2012, 2020). The results of this study, however, showed that the number of inhibitory synapses was significantly decreased in α2δ-1 ^{Thy1} cKO mice across all three brain regions, indicating that α2δ-1 likely plays an important role in inhibitory synaptogenesis. Previous studies have shown that astrocytes are able to promote GABAergic synaptogenesis via secreted proteins other than TSPs (Hughes et al., 2010) and have attempted to identify possible mechanisms by which astrocytes might promote the development of GABAergic synapses. Such mechanisms include enhancing signaling between BDNF and its receptor TrkB to promote $GABA_A$ receptor clustering on the surface of post-synaptic neurons (Elmariah et al., 2005) and releasing transforming growth factor beta 1 (TGF-β1) to increase activity of NMDA glutamate receptors while initiating a kinase signaling pathway to induce the localization and cluster formation of synaptic adhesion proteins associated with $GABA_A$ receptor subunits (Diniz et al., 2014). Other studies have demonstrated that the same signaling pathways responsible for increasing expression of glutamate receptors are also responsible for increasing expression of GABA receptors (Marsden et al., 2007), highlighting the balance between excitatory and inhibitory synaptic signaling necessary for normal CNS development. Whether it is an absence of signaling involving α2δ-1 specifically that leads to this decrease in inhibitory synapse formation, or whether it is a result of a balancing response to a decrease in excitatory synapses is a subject for future investigations.

Effects of Early Life Drug Exposure on Synaptogenesis Are Not Fully Ameliorated with Age

To determine whether the changes in synapse number induced by early life drug exposure persist through life, IHC studies were performed with exposed α2δ-1^{Thy1} mice at P70 and compared to mice at P21. Significant differences were observed between the treatment groups of

P70 mice in both excitatory and inhibitory synapse numbers. In addition, it appeared that changes in synaptogenesis that resulted from altered expression of α2δ-1 were not nearly as pronounced in P70 mice as they were in P21 mice, with few significant differences being observed between α2δ-1^{Thy1} WT and α2δ-1^{Thy1} cHet P70 mice. While the reasons for this are not entirely known, it is possible that α2δ-1 mediated synaptogenesis primarily occurs early in life, with its role in synaptic maintenance diminishing after a certain age. In addition, it is typically expected for the number of synapses throughout the brain to decrease somewhat with age as those formed early in life are removed through such mechanisms as synaptic pruning (Petralia et al., 2014). Taken together, these results observed in P70 mice indicate that changes in synaptic development resulting from early life drug exposure have the potential to persist throughout life well into adulthood.

Early Life Drug Exposure Resulted in Few Significant Changes in Anxiety-Type or Locomotor Behaviors

Another major goal of this study was to not only investigate the role of α2δ-1 in alterations of synaptogenesis induced by early life drug exposure, but to observe what effects these alterations may have on behaviors associated with addiction. Measures of anxiety-type behavior included Light/Dark testing and Open Field. In behavioral tests that use a Light/Dark chamber, it is assumed that an anxious mouse will generally prefer darker and more enclosed spaces and avoid brightly lit, open spaces where it may feel vulnerable. For anxiety testing in an Open Field, it is assumed that an anxious mouse will generally avoid the center of the field where it may feel the most exposed or vulnerable and will instead spend most of its time moving around the edge of the field close to the walls (i.e. thigmotaxis) (Simon et al., 1994). Despite previous animal models of early life drug exposure demonstrating increased anxiety in these

types of experiments (Ahmadalipour et al., 2015; Andersen et al., 2020; Chen H, 2015; Daly et al., 2012; Seibenhener & Wooten, 2015), no significant differences were observed among the mice in this study.

The Open Field was also used to measure locomotor activity to determine if any of the chosen drug treatments were associated with motor impairments. Total distance traveled can also be an indicator of anxiety-type behavior with more anxious mice presumed to travel a shorter distance. It was observed that the buprenorphine treated mice did travel a significantly shorter distance while moving around the field. Previous studies have associated changes in locomotor activity to opioids in the past (Niikura et al., 2013; Seibenhener & Wooten, 2015; Zamani et al., 2022) and multiple clinical studies (Burke & Beckwith, 2017; Monnelly et al., 2019) have shown deficits in motor skills in children with a history of prenatal opioid exposure. My own study of NAS patients (see **Chapter 2**) showed that the most common medical issues among the children were musculoskeletal in nature and that the majority of those children were delayed in reaching developmental milestones for motor skills and coordination. Clearly then, there is a potential for prenatal drug exposure to affect the development of motor skills.

In addition, the total time spent frozen by each mouse was recorded during their Open Field session. As another measure of anxiety, mice that spend a greater amount of time frozen in place are assumed to be more anxious (Sestakova et al., 2013). Intriguingly, gabapentin treated WT mice were shown to have spent significantly less time frozen (and the time spent by buprenorphine + gabapentin WT mice was nearly significant less as well). There has been some data to suggest gabapentin has anxiolytic properties, with male mice having been shown to spend less time frozen in an Open Field after receiving a 30mg/kg dose injection (Sethi et al., 2005). Intriguingly, the primary neurotransmitter whose dysregulation is most associated with disorders

of anxiety is GABA (Nuss, 2015). Given that the glutamatergic-GABAergic synapse balance was shown to be disrupted in mice with early life exposure to gabapentin and/or buprenorphine (**Figures 11 and 15**), there is the possibility that drug-induced changes in synaptic development and function can manifest later in life as changes in anxiety-related motor behaviors.

Reward Seeking Behaviors were not Significantly Affected by Early Life Drug Exposure or Expression of α2δ-1

To study reward seeking behavior, mice in this study were subjected to a 10-day CPP protocol with injections of nicotine as the reward stimulus. It was predicted that the alterations in synaptic development within the mesolimbic dopamine pathway that have been shown to result from early life exposure to opioids and/or gabapentin would predispose mice to experience greater reinforcement of a rewarding stimulus (Pierce & Kumaresan, 2006). Given the high rates of nicotine use among opioid disorder patients (Chun et al., 2009; Kohut, 2017; Streck et al., 2020; Young-Wolff et al., 2017), it was also predicted that nicotine would be an effective rewarding stimulus for a CPP study involving these mice. Although all but one treatment-genotype mouse group demonstrated preference for the drug paired chamber, as evidenced by a positive mean CPP score, no significant differences were observed between the different mouse groups. In fact, many mice demonstrated negative CPP scores, particularly the cHet gabapentin treated group, indicating that an aversion was developed rather than a preference. Conditioned place aversion (CPA) is possible with nicotine, but typically only when administered in high doses (Buccafusco, 2009). Recently, however, coadministration of morphine with nicotine was actually shown to decrease nicotine reinforcement (Avelar et al., 2022). Clearly, the interaction between nicotine and opioids with or without gabapentin is more complicated than this study was able to characterize.

The complex interaction between nicotine and early life exposure to drugs of abuse, such as opioids, was also relevant to the vapor chamber self-administration behavioral experiments include in this study. Mice from each treatment-genotype group were placed in nicotine vapor self-administration chambers for daily one-hour FR1 sessions for 10 days. It was expected, based on previous research using the same technique (Cooper et al., 2020; Henderson & Cooper, 2021) that mice would initiate a greater number of nose pokes over time as nicotine reinforcement occurred. However, despite most treatment-genotype groups having a high mean number of nose pokes on Day One, especially the cHet vehicle group, daily nose poke means for all groups declined shortly afterwards and remained relatively low over time.

Despite the lack of significant differences in daily number of nose poke between the different treatment-genotype groups, there were still mice from six different treatment-genotype groups that demonstrated the possibility of reinforcement. These mice were included in the follow-up three-day one-hour FR3 self-administration sessions. No significant differences were observed between the two-day mean number of nose pokes of each group. One potentially noteworthy finding from this FR3 study, however, was that the majority of the mice that demonstrated possible reinforcement were female. This may indicate that sex-related differences in the response to rewarding substances could play a significant role in drug self-administration studies.

There are several possible reasons for this lack of significant differences in these self-administration studies, aside from the possibility that drug exposure may actually decrease nicotine reinforcement (Avelar et al., 2022). In previous nicotine vapor self-administration studies, the concentration of nicotine in the administered puffs of vaporized PGVG were 6mg/ml nicotine salt, double the concentration used in this study. In addition, previous studies placed

mice in vapor chambers for two-hour or three-hour FR1 sessions each day rather than the one-hour sessions used in this study (Cooper et al., 2020; Henderson & Cooper, 2021). Given that reinforcement had already been seen in mice that followed the procedures of previous studies, nicotine concentrations and session times were lower in this study as a means to assess whether reinforcement was enhanced in mice that had been exposed to drugs of abuse during early life development. It was reasoned that if any group of mice in this study demonstrated and significant reinforcement, it could be taken as a sign that that group had shown enhancement in the reinforcement of nicotine vapor self-administration.

Another possible explanation for why mice failed to show significant reinforcement for self-administration may have been related to the procedure through which it was tested. Mice were required to learn to associate the action of poking their nose into one of two holes and the turning on of a light with the administration of a three-second puff of nicotine vapor within their chamber. Previous studies have demonstrated, however, that learning and memory may be impaired in both humans and rodents with a history of prenatal exposure to drugs of (Ahmadalipour et al., 2015; Chen H, 2015; Egil Nygaard et al., 2015; Oei et al., 2017; A. Ornoy, 2003) abuse. Therefore, it is possible that any behavioral studies that require mice to learn relatively complex behaviors may not be accurate measures unless the impaired learning and memory in those mice can be controlled for or removed as a potentially confounding factor.

Overall, this study demonstrated the impact of early life drug exposure, α2δ-1 expression, and age on synaptic development within three brain regions associated with the mesolimbic dopamine pathway. It achieved this through the use of α2δ-1^{Thy1} mice and multiple IHC studies designed to examine and compare numbers of excitatory and inhibitory synapses between different early life drug treatments, α2δ-1 genotypes, and ages. This study also attempted to

connect the findings from these IHC to the results of multiple behavioral experiments examining behaviors related to addiction and activity within the mesolimbic dopamine pathway. Although few significant differences were observed between mice belonging to different groups based on treatment or genotype, future studies may be informed by the behavioral results of these mice. A greater understanding of the long-term effects of early life drug exposure on synaptic development and behavior will hopefully lead to improved health outcomes for human patients impacted by drug abuse.

CHAPTER 5

CONCLUSIONS

Reflection on Major Findings from These Investigations

The results of the studies included in this dissertation collectively paint a larger picture about the effects of prenatal exposure to drugs of abuse. First, a chart review of NAS patients who appeared at a treatment center for follow-up clinic appointments was carried out. This study observed several trends among these patients describing, among other factors, the drugs most commonly abused by patient mothers, the health problems most commonly seen among the patients, and the extent of the developmental delays seen in the patients.

Second, IHC and confocal fluorescent microscopy studies examined the numbers of excitatory glutamatergic and inhibitory GABAergic synapses within the ACC, NAC, and PFC of mice in two different age groups (P21 and P70) with one of three possible genotypes for expression of the Ca^{2+} channel subunit α2δ-1 (WT, Het, or cKO) exposed to one of four different treatments during early life development (5 mg/kg buprenorphine, 30 mg/kg gabapentin, a combination of both drug doses, or a vehicle control). It was demonstrated that excitatory and inhibitory synaptic populations are significantly disrupted by early life exposure to buprenorphine and gabapentin, separately or in combination, and that these disruptions are strongly influenced by the level of expression of α2δ-1 and by age.

Third, a regimen of behavioral experiments was carried out in early life drug exposed mice to determine what effects the different drug treatments and the different expression levels of α2δ-1 might have on behaviors related to addiction. There were indications that locomotor activity may be impacted by early life exposure to buprenorphine as well as indications that anxiety-related activity may be lessened in mice exposed to gabapentin during early life

development. Although few significant differences were observed between mice belonging to different groups based on treatment or genotype, future studies looking at other behaviors or utilizing different experimental methods may be informed by the behavioral results of these mice.

Potential Weaknesses and Directions for Future Studies

Although the experiments included in this dissertation were planned and carried out as carefully and methodically as possible, there were limitations and potential weaknesses to these studies that may have influenced the results. It is important to acknowledge for the mouse studies included in this dissertation that the timeline for rodent neurodevelopment does not correlate directly with that of humans. A newborn mouse is approximately neurodevelopmentally equivalent to a human fetus in the late second trimester (Ross et al., 2015). This was accounted for when planning the drug dosing schedule, for while efforts were made to replicate prenatal drug exposure in human patients as closely as possible, the period of time during which a great deal of synaptogenesis occurs in the developing mouse brain also needed to be captured within the timeframe.

The significant differences between rodent and human fetal development warrant the need for noninvasive, ethical methods of studying human tissues at early developmental stages. Tissue from post-mortem humans, for example, has been effectively and reproducibly used to study opioid receptor gene expression (Peng et al., 2012). Recent advances have also been made in three-dimensional *in vitro* culture techniques have allowed for the production of brain organoids derived from induced human pluripotent stem cells that have been used to study changes in neuronal protein expression and cell maturation. This model system is non-invasive and allows for the study of living, functional human tissue, making it a potentially invaluable

tool in the study of the effects of drug exposure on the developing human brain (Di Lullo & Kriegstein, 2017). Recent models such as these could lead to a more accurate understanding of the natural processes disrupted by exogenous opioids, or other drugs of abuse such as gabapentin, and could elucidate the role of glial cells in these processes. Going forward, it would be constructive to pursue the opportunities that are now available to explore the effects of opioid exposure on human CNS development at the cellular and molecular level.

In the future, the data gained from the studies described in this dissertation will need to be expanded to account for other important variables with the potential to influence either astrocyte-mediated synaptogenesis or the effects of drugs of abuse on the developing CNS. The inclusion of α2δ-1 knockouts in the P70 timepoint IHC studies as well as in the behavioral studies would more completely demonstrate the importance of α2δ-1 in determining the effects of early life drug exposure on synaptic development and behavior. In these studies, neither C57Bl/6J α2δ-1 KO nor α2δ-1^{Thy1} cKO pups were born and/or survived to P21 in sufficient numbers to be included in this study. This is likely due to a lack of α2δ-1 being deleterious to the health of mice, especially in those treated with buprenorphine and/or gabapentin. Given enough time, sufficient numbers of α2δ-1^{Thy1} cKO pups could likely have been produced and utilized in behavioral and IHC studies. In an effort to reduce the number of mice needed to complete the studies described in this dissertation, all mice used for behavioral studies were used for every experiment listed in the seven-week regimen in the order in which they are listed. These same mice were sacrificed and perfused at the end of those studies (at P70) to then be used in IHC studies. The various forms of stress experienced by those mice and the repeated nicotine treatments they received over the course of those behavioral studies may have impacted their performance in behavioral studies occurring later in the schedule and may have affected synapse

numbers within the mesolimbic dopamine pathway. One way in which this potential confounding factor could be addressed is by allowing for a separate cohort of mice for behavioral studies. Without the need to carry out all behavioral studies within the seven-week period between P21 weaning and P70 sacrifice dates, studies could be spread over a longer period of time, with significant "down-time" breaks in between each study. This could allow mice in this cohort to recover from the effects of stress and nicotine treatments before beginning another potentially stressful behavioral trial.

Another significant change that could have been made to this study would have been to examine sex-related differences between male and female mice. There has been significant evidence to suggest that not only are the brains between males and female different in key structural areas, including potential sex-specific synaptogenic responses to astrocyte-secreted TSP-2 (Mazur et al., 2021; Uhl et al., 2022), there are important differences in their prenatal development. During fetal development, the presence of male or female specific hormones is necessary for sex-dimorphic behaviors and sex-specific differences in the size and structure of certain brain regions (Ehrhardt & Meyer-Bahlburg, 1979; Xin et al., 2019).

Sex hormones may also modify the effects of prenatal drug exposure on neurological development. The expression of opioid receptors within the brains of rats has been shown to be influenced by the presence of estrogen and progesterone (Cruz et al., 2015), but the exact role of ovarian hormones in the development of the endogenous opioid system remains unclear. Animal studies have shown that the presence of estrogen and progesterone can influence the expression of mu and kappa opioid receptors (Cruz et al., 2015). One study examining learning and memory showed significant sex-related differences in the ability of rats prenatally exposed to morphine to solve the mazes. The presence or absence of ovarian hormones was implicated as the reason for

these sex-related differences (Šlamberová et al., 2001). Another investigation into learning and memory deficits used a symmetrical maze and a radial arm maze (RAM) to show significant sex-related differences in the ability of prenatal morphine-exposed rats to solve the mazes (Šlamberová et al., 2001). Male rats required less time to complete the symmetrical maze, while both sexes showed deficits in completing the RAM in a similar amount of time to sex-matched controls. The presence or absence of ovarian hormones was implicated as the reason for these sex-related differences, since ovariectomized females prenatally exposed to opioids exhibited poor performance that was rescued by hormone replacement injections.

Additional evidence for an interaction between sex hormones and opioids during development can be observed in the presentation of human infants with NAS. Multiple clinical studies have shown that rates of NAS are higher in male than in female newborns, and that male NAS patients are more likely than females to require treatment for their symptoms (Charles et al., 2017; Stevens et al., 2018). Differences in head circumference and types of symptoms between males and females have also been observed (Stevens et al., 2018). In terms of behavior, male infants with a history of prenatal opioid exposure have been shown to score higher than females on cognitive tests of habituation (Jones et al., 2010). Studies such as these highlight the need to consider sex-related differences both in animal model basic research and in clinical studies.

In clinical studies, such as the one performed in this dissertation, it is important to consider environmental factors that can indirectly influence the health and wellbeing of children and can also be potential confounders when investigating incidence of NAS or drug abuse in general. Such factors can include race (Becker & Newsom, 2003), geographic location (Berry et al., 2016; J. Brown et al., 2018), socio-economic status (M. Kim et al., 2018), and stability of

home life (Berry et al., 2016; Asher Ornoy et al., 2001). Lower socio-economic status has long been known to be associated with poorer health in general (Braveman & Gottlieb, 2014). It has been found that improving the economic resources available to infants born into families with low socio-economic status can significantly improve brain function within key regions (Troller-Renfree et al., 2022). However, there is evidence to suggest that prenatal drug exposure can impact neurological development even when social factors are controlled for in children raised in different, more stable households than the ones into which they were born (Egil Nygaard et al., 2015; Egil Nygaard et al., 2018; A. Ornoy, 2003; Asher Ornoy et al., 2001). In studies of a medical condition as inextricably linked to environmental factors as NAS, it will always be important to attempt to isolate the effects of drug exposure from the effects of the surrounding environment as much as possible.

Aside from the behaviors studied and the behavioral experiments utilized in this dissertation, there are several other aspects of behavior with the potential to be affected by early life drug exposure and/or alterations in synaptic development within the mesolimbic dopamine pathway. Studies designed to examine memory and learning, such as social interaction, novel object recognition, and passive avoidance test, have been used in rat models of prenatal opioid exposure and have shown significant differences between drug treated and control subjects (Ahmadalipour et al., 2015; Chen H, 2015). The learning difficulties and generally poorer performance in school seen in children with a history of prenatal drug exposure may also be attributed to deficits in learning and memory (Egil Nygaard et al., 2015; Oei et al., 2017; A. Ornoy, 2003). An investigation into these types of behaviors may also prompt examinations of the hippocampus, the brain region predominantly involved in learning and memory (Rubin et al., 2014), which has been shown to interact with the mesolimbic dopamine pathway and to have

altered development and function as a result of exposure to drugs of abuse (Belujon & Grace, 2011; Lisman & Grace, 2005). There are many opportunities for additional studies utilizing this mouse model and experimental paradigm.

Final Remarks

Neonatal abstinence syndrome has negatively impacted the lives an untold number of children and their families. Increasing the collective understanding of this condition and the factors that cause it will be critical to improving methods for preventing and treating it as well as meeting the unique developmental needs of the children who develop it. This dissertation is intended to add to that body of knowledge by identifying a potential molecular target in α2δ-1. This protein, as well as the broader mechanism of astrocyte-mediated synaptogenesis, may become the focus of studies exploring treatments and preventative methods designed to drive down the incidence rates of NAS that have risen to epidemic levels in the United States in recent years. It is the intention of the author that this dissertation further the cause of using scientific and clinical research towards improving the health and wellbeing of patients and the vulnerable populations most impacted by NAS and substance use disorders.

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APPENDIX A: APPROVAL LETTER

MARSHALL UNIVERSITY.
www.marshall.edu

Office of Research Integrity

April 28, 2020

Taylor Boggess
945 4th Avenue, Apt. 205
Huntington, WV 25701

Dear Taylor:

This letter is in response to the submitted dissertation abstract entitled *"Investigation of the Effects of Prenatal Drug Exposure on Astrocyte-Mediated Synaptogenic Signaling."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #696 and #697. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director
Office of Research Integrity