BEHAVIORAL AND NEUROBIOLOGICAL CONSEQUENCES OF ALPRAZOLAM EXPOSURE DURING ADOLESCENCE

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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May 2021

Major Subject: Psychological Sciences

ABSTRACT

There is evidence of increased use and abuse of alprazolam (Xanax; ALP) during adolescence, yet most available neurobiological evidence has been derived from studies using adult organisms. This study was designed to investigate the behavioral and neurobiological consequences of ALP exposure during adolescence. For experiment 1, adolescent male mice [postnatal day PD 35]) were pretreated with ALP (0, 0.5, 1.0 mg/kg) once daily from PD 35-49. Changes in behavioral responsiveness to morphine (2.5 and 5.0 mg/kg), using the conditioned place preference paradigm (CPP), were assessed 24 h after the end of drug treatment. Findings showed that ALP pretreated mice developed strong preference to the compartment paired with subthreshold dose of morphine (2.5 mg/kg), while demonstrating aversion to the compartments paired with a moderate dose (5.0 mg/kg). In experiment 2, PD35 male mice were exposed to a single ALP injection (0.5 mg/kg). ERK1/2-related gene and protein expression changes were assessed within the ventral tegmental area (VTA) and nucleus accumbens (NAc), using qPCR and western blot analysis ninety-minutes after the drug treatment. Acute ALP exposure during adolescence decreased mRNA and protein expression of ERK1/2, its downstream target cAMP response element-binding protein (CREB), and protein kinase B (AKT) within the VTA. Within the NAc, ALP increased ERK1/2, CREB, and AKT mRNA expression while it had no effect on protein expression. In experiment 3, adolescent male mice were pretreated with ALP (0.5 mg/kg), once daily from PD 35-49. Gene expression changes within VTA and NAc, using qPCR and western blot, were assessed 24 h after the end of

drug treatment. Within the VTA repeated ALP exposure during adolescence decreased mRNA expression of ERK1/2, CREB, and AKT. Moreover, ALP exposure increased protein expression of ERK1 and AKT. Within the NAc, ALP increased mRNA and protein expression of ERK1/2, CREB, and AKT when compared to controls. These findings suggest that exposure to ALP during adolescence potentiates the rewarding effects of opiates such as morphine. ALP exposure during this period results in changes of ERK-signaling within the VTA and NAc, brain regions implicated in regulation of both drug-reward and mood-related disorders.

DEDICATION

To myself, this one is for you.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Bolaños, and my committee members, Dr. Dulin, Dr. Maren for their support, guidance, and understanding throughout the course of this research.

I would also like to thank my lab mates, Tamara Gnecco, Omar Sial and Ernesto Cardoso for their constant support in lab.

Finally, thanks to my husband and my parents for their unconditional love and encouragement.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Professor Carlos Bolaños, Professor Stephen Maren of the Psychological and Brain Sciences department, and Professor Jennifer Dulin of the Neuroscience department. All other work conducted for the thesis was completed by the student independently.

Funding Sources

This work was supported by funds from the College of Liberal Arts.

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1. INTRODUCTION

In the mid-1950s, tranquilizers, a new class of therapeutic agents were shown to have substantial clinical value, leading the pharmaceutical company Hoffmann-La Roche to embark on the journey to synthesize products of this type. Chemist Leo Sternabch serendipitously identified the first benzodiazepine (BDZ) after submitting one of his previously synthesized compounds for animal testing (Sternbach, 1978). The compound showed strong sedative, anticonvulsant, and muscle relaxant effects. These unexpected but impressive results led to the introduction of Chlordiazepoxide (Librium) into the market. The new tranquilizer produced similar therapeutic effects as the barbiturates and soon was advertised as a safer alternative. Consequently, the introduction of BDZs led to a significant decline in the prescription of barbiturates, and by the 1970's the BDZs had largely replaced them (Birdwood, 1975). Since then, the production of the new generation of tranquilizers has spurred with nearly thirty different BDZs currently approved for clinical use. Today, BDZs are widely prescribed for the treatment of insomnia, convulsive and anxiety-related disorders such as generalized anxiety, panic disorder, post-traumatic stress disorder, and obsessive-compulsive disorders, among others. Additionally, BDZs are used for other reasons such as presurgical sedation, detoxification from alcohol, and used off-label as treatment for major depression (Longo and Johnson, 2000). The BDZs are relatively safer as they cause less respiratory depression than barbiturates; however, they possess adverse side effects such as amnesia, tolerance, dependence, and high potential for addiction.

Concerning trends of BDZ abuse have been consistently reported in the past few decades implicating BDZs in approximately one-third of unintentional drug overdoses (Henderson and Pond, 1993; Jones et al., 2013). Furthermore, BDZ abuse often occurs in conjunction with other substances (e.g., alcohol, opioids) with about 33% of opioid overdose deaths in the U.S. involving BDZ co-ingestion (CDC, 2019).

Alprazolam (Xanax; ALP) is a highly potent and short-acting BDZ that is among the most prescribed psychotropic medications in the U.S. – of the approximately 92 million BDZ prescriptions dispensed in outpatient pharmacies in 2019, ALP was the most commonly (38%) prescribed (FDA, 2020). Its high prescription rates have persisted throughout the years despite exerting adverse effects as many other BDZs. In 2011, ALP accounted for about a third of total BDZ emergency department visits (DAWN, 2011) and it has consistently ranked in the top 10 drugs involved in drug overdose deaths in the U.S. (NVSS, 2018). A recent longitudinal cohort study revealed that primary care physicians prescribed BDZs more frequently to patients with at least some known risk factor for BDZ-related adverse events such as pulmonary diseases and substance use disorders, thus leading to a significant increase in healthcare utilization (Kroll et al., 2016). Interestingly, BDZ prescription among primary care physicians in ambulatory care has increased substantially as well, and this group accounted for about half of all BDZ visits (Agarwal and Landon, 2019). Perhaps not surprisingly, the U.S. Food and Drug Administration (FDA) recently announced a requirement of class-wide labeling changes (i.e., boxed warning) for BDZs to include the risks of abuse, addiction, physical dependence, and withdrawal reactions in hopes to improve their safe use (FDA, 2020).

Together, these findings suggest that most of BDZ prescriptions are sourced from non-psychiatric settings and highlight the need to closely monitor prescription patterns to avoid BDZ-related adverse consequences.

While research on the consequences of BDZ use has largely focused on the elderly and their co-prescribing with opioids, much less is known about their use and misuse in the adolescent population. A national projectable survey reported concerning trends in teen prescription drug abuse (PATS, 2013), indicating one in four teens as having misused/abused prescription medications at least once in their lifetime, and that about 20% of these had done so before the age of 14. Despite safety concerns surrounding BDZ treatment utilization, which have prompted guidelines to confine their use as a short-term treatment (Guina and Merril, 2018), long-term use of BDZ is rather common, including in pediatric settings (Murphy et al., 2015; Kurko et al., 2015; Yeh et al., 2011; Bushnell et al., 2018). This is cause for concerns because BDZ prescription treatment trends positively correlate with nonmedical use among adolescents, thus emphasizing the need to monitor BDZ use/abuse in this population (McCabe and West, 2014). And though it is estimated that only about 15% of drug users transition from recreational use to substance use disorder (SUD), the chances of developing SUD increase dramatically when the onset of drug use occurs at a younger age (Piazza and Deroche-Gamonet, 2013; Clark et al., 1998; Dawson et al., 2008). During adolescence the brain is believed to be exceptionally vulnerable to the effects of drugs of abuse as significant neural restructuring occurs during this developmental period (Fuhrmann et al., 2015). Hence, the consumption of drugs of abuse during this time can prime the

brain into other vulnerabilities and pose profound and long-lasting consequences into adulthood. Despite the increased abuse of BDZs during adolescence and its potential for adverse life-long consequences, most available neurobiological evidence of their use and abuse liability comes from studies using adult models. Indeed, a systematic review on the nonmedical use of prescription medications among adolescents identified sedative/tranquilizer use as an area of much needed research (Young et al., 2012). It is therefore imperative to investigate the neurobiological consequences of ALP use/abuse during adolescence as it is a sensitive period in which these drug experiences can result in neural adaptations that can influence life-long behavior.

1.1. Clinical Pharmacology

After oral administration, ALP reach peak plasma concentrations within 1-2 h (Greenblatt and Wright, 1993; Greenblatt et al., 1993), with approximately 90% of it absorbed and bound to plasma proteins. Following absorption, ALP is primarily metabolized by hepatic microsomal oxidation of cytochrome P4503A isoforms CYP3A4 and CYP3A5 (Yasui et al.,1996; Hirota et al., 2001). The volume of drug distribution ranges from 0.8-1.3 l/kg with an elimination half-life of 8-15h. ALP has two principal metabolites, 4-hydroxy-alprazolam, and α-hydroxy-alprazolam; however, they possess low BDZ receptor affinity and hence not likely to contribute to its clinical effects. It is estimated that about 80% of ALP is excreted by the kidneys unchanged and its pharmacokinetic parameters do not differ significantly between routes of administration (Scavone et al., 1987, 1992).

1.2. Toxicity

Patients who suffer a BDZ overdose present central nervous system (CNS) depression, slurred speech, altered mental status, and ataxia (Kang, 2020). It is commonly thought that when taken in high doses without other co-ingestants, BDZs rarely cause toxicity (Penninga et al., 2016; Höjer et al., 1989). However, various studies have found that ALP causes greater toxicity in overdose when compared to other BDZ poisonings. A recent study reported a 38.1 rate of deaths per million ALP prescriptions when compared to a rate of 5.3 from other sedatives/anxiolytics as a drug group in New Zealand (Reith et al., 2003). Another study found ALP as having significantly greater intensive care unit (ICU) admission rates correlating with an increase in use of interventions (e.g., mechanical ventilation, flumazenil administration) to prevent respiratory depression complications (Isbister et al., 2004). Indeed, when taken in sufficiently large doses, BDZs can cause coma, respiratory depression, and death (Henderson and Pond, 1993; Drummer and Ranson, 1996). Even more concerning is that the severity of BDZ toxicity is often related to co-ingestants such as opioids and alcohol, resulting in significantly worsened outcomes (Jann and Lopez, 2014; Linnoila, 1990).

1.3. Mechanism of Action

It has been demonstrated that ALP exerts its therapeutic effects by binding non-selectivity to the gamma-amino butyric acid-A ($GABA_A$) BDZ receptor complex. At the receptor complex, ALP increases the affinity of the amino acid neurotransmitter GABA for the $GABA_A$ receptor and facilitates its binding. This GABA binding further

increases the frequency of the chloride ion channel opening thus increasing the influx of chloride ions which in turn hyperpolarize the postsynaptic neuron depressing its excitability. Inhibition by GABA on other neurotransmitter systems results in the general slowdown of brain activity thereby exerting its sedative, anxiety-reducing therapeutic effects (Griffin et al., 2013).

1.4. Chemical Neurotransmission

GABA is the primary inhibitory neurotransmitter in the brain. In the human brain, GABA receptors are found with highest densities in cortical and limbic areas. They are found in intermediate densities in the basal ganglia, cerebellum, thalamic and hypothalamic nuclei, while found with lowest densities in the brainstem nuclei (Zezula et al., 1988). Due to the widespread and heterogenous distribution of GABA receptors, the effects of BDZs are mediated through the interaction of GABA receptor sites at various brain structures. The following section will focus on the role of BDZs on GABAergic and Dopamigergic systems.

1.4.1. GABAergic Systems

BDZs are positive allosteric modulators of the ligand-gated $GABA_A$ type receptors. $GABA_A$ receptors localize at synaptic and extra-synaptic sites in the plasma membrane to mediate phasic and tonic inhibition. During phasic inhibition, GABA released from the presynaptic neuron binds to postsynaptic $GABA_A$ receptors for fast signaling, whereas during tonic inhibition, extracellular ambient GABA binds to extra-

synaptic $GABA_A$ receptors to modulate cell excitability and resting membrane potential (Farrant and Nusser 2005; Nusser et al., 1998). $GABA_A$ receptors are heteropentameric structures comprised of five subunits derived from seven receptor subunit families (α, β, γ, δ, ε, π and θ). In the CNS, the most common type of GABA receptor is comprised of α1, β2, and γ2 subunits (Sieghart and Sperk, 2002). BDZs such as ALP specifically bind to a pocket between the α and γ subunits (Sigel and Buhr 1997; Sigel, 2002). Pharmacological studies have revealed that α_1 subunit-containing GABA receptors mediate the sedative and anterograde amnesic effects of BDZ, while α_2 subunit-containing receptors mediate the anxiolytic, and myorelaxant effects (Rudolph et al., 1999; McKernan et al., 2000; Löw et al., 2000). More recently, it has been shown that α_1-subunit containing receptors are required for the addictive properties of BDZs (Heikkinen et al., 2009; Tan et al., 2010). The latter findings demonstrated BDZ pharmacological effects on cell-type specific $GABA_A$ receptors within the ventral tegmental area (VTA), an important brain region in the mesolimbic dopamine pathway.

1.4.2. Dopaminergic Systems

The mesolimbic pathway and its forebrain targets have been studied extensively due to their critical role in regulating responses to drugs of abuse as well as natural rewards such as food, drink, sex and social interaction. All drugs of abuse share common characteristics despite their many distinct effects in the brain. Drugs of abuse activate the mesolimbic pathway by increasing the firing rate of midbrain dopamine (DA) neurons in the VTA leading to an increase in DA release into the nucleus accumbens (NAc), an

important area for the mediation of goal-directed behavior (Nestler and Carlezon 2006). The VTA is heterogeneous in neurochemical profile, comprising of DA neurons, GABA interneurons and glutamatergic neurons (Holly and Miczek., 2016). It has been demonstrated that BDZs have a stronger impact on GABA interneurons than on DA neurons. Indeed, the presence of BDZs in brain VTA slices increased spontaneous inhibitory postsynaptic current (IPSC) frequency in GABA interneurons and decreased it in DA neurons (Tan et al., 2010). These findings have led to the disinhibition hypothesis, which states that in the presence of BDZs, GABA interneurons are more strongly hyperpolarized and no longer inhibit DA neurons as seen under basal conditions (Tan et al., 2011). The disinhibition of DA neurons thus results in a greater release of DA onto the NAc. These findings are of great importance for the understanding of the neurobiological consequences of ALP exposure, as increase of DA neuron firing within the VTA-NAc network after exposure to drugs of abuse is thought to induce synaptic plasticity changes that aid in the development of addiction if drug use continues (Heikkinen et al., 2009; Di Chiara et al., 2004; Brown et al., 2010; Vaschinkina et al., 2014a).

1.5. Drug-Drug Interactions

It is well known that BDZ are commonly used in patients with opioid use disorder (CDC, 2019; SAMHSA, 2011) and preclinical research has shown that these drugs exert significant modulatory effects on each other. Opioid receptors and their endogenous peptide ligands are distributed throughout the CNS and peripheral system

(Kitchen et al., 1997; Wittert et al., 1996). Morphine and other opioid drugs are able to induce a wide array of pharmacological effects including strong analgesia, sedation, endocrine dysregulation and euphoria. Opioid receptors which include: μ (mu), δ (delta), and κ (kappa) receptors that belong to a superfamily of seven transmembrane domain G-protein coupled receptors that produce their cellular effects via coupling with GTP-binding proteins G_i/G_o (Waldhoer et al., 2004; Bodnar, 2016). At the cellular level, these actions lead to the inhibition of neuronal activity and a reduction in neurotransmitter release (Law et al., 2000). In terms of drug addiction, opioids interact predominantly with mu-opioid receptors, which have been shown to be responsible for the rewarding properties of morphine (Matthes et al., 1996). In line with the disinhibition hypothesis, opioid-induced rewarding effects are associated with the stimulation of mu-opioid receptors localized on GABAergic terminals within the VTA. Such stimulation inhibits GABA release that subsequently results in disinhibition of dopaminergic neurons leading to the release of DA into the NAc (Johnson and North., 1992; Hirose et al., 2005; Yoshida et al., 1999). Consequently, opioids induce a strong feeling of well-being and contentment. Indeed, the endogenous opioid system and opioid receptors have been shown to be involved in the rewarding properties and development of physical dependence to other drugs of abuse (Trigo et al., 2010; Boutrel, 2008; Acquas et al., 1993; Rukstalis et al., 2005). Clinical reports indicate that human opiate users often self-administer BDZs either prior to or concurrently with opiates (Stitzer et al., 1981; Preston et al., 1984). Studies of this co-administration have reported that the rewarding experience of opiates is potentiated by BDZ pretreatment (Navaratman and Foong, 1990;

Iguchi et al., 1993). Research using adult animal models have also investigated the opiate + BDZ interaction in order to better understand the modulatory properties of BDZs on opiate reward. Walker and Etternberg (2001) first demonstrated that a single administration of ALP could enhanced the rewarding properties of a low dose of heroin that by itself was not rewarding as measured by the conditioned place preference (CPP) paradigm. In a subsequent study, the authors found that ALP pretreatment enhanced the rewarding effects of intra-VTA heroin induced CPP, thus suggesting that the VTA might be a site where opiate + BDZ interaction occurs (Walker and Etternberg, 2005). The mechanism(s) underlying BDZ-induced enhancement of opiate reward has not been fully elucidated. Studies have shown that BDZs may alter the pharmacokinetics of opioids by inhibiting their CYP metabolism thereby increasing their serum concentrations (Jann et al., 2014; White and Irvine, 1999) and enhancing their rewarding effects (Jones et al., 2012). A study by Poisner et al. (2009) demonstrated that co-administration of buprenorphine, a partial opioid agonist, and low dose ALP raised the receptor number to near basal level in brain areas with the highest density of mu-opioid receptors. These findings suggest that pretreatment with ALP has the potential to induce adaptations that allow for the recruitment of more mu-opioid receptors than opioids alone. Such molecular adaptations could further contribute to the enhancement of sensitivity and reward observed in the co-administration of BDZ and opioids. Within the VTA, $GABA_A$ receptors are thought to be co-localized with opiate receptors on inhibitory interneurons (Xi and Stein., 1998), it is therefore feasible that BDZ exposure induces synaptic

alterations within the VTA and subsequently on its target regions, in a way that is similar to opioids.

1.6. Design and Hypothesis

Epidemiological reports indicate that BDZs are the most widely used medications worldwide (Donogue and Lader., 2010; Airagnes et al., 2019; Moore and Mattison, 2017) and are the pharmaceutical most commonly involved in opiate overdose deaths (Jones et al., 2013; Jann and Lopez, 2014). Patterns of BDZ prescription among adolescents follow nonmedical use (McCabe and West, 2014), thus emphasizing the need for neurobiological research using age-appropriate models. Basic research on the behavioral and neurobiological consequences of ALP, the most commonly misused BDZ during adolescence (Friedrich et al., 2020; Bachhuber et al., 2016; Hughes et al., 2016; Johnston et al., 2019), is severely lacking. Therefore, the central goal of the present project was to investigate the neurobiological consequences of ALP exposure during this critical period. Given that ALP exposure has been shown to increase reward sensitivity to opioids in adult rodent models (Walker and Ettenberg, 2001; Walker and Ettenberg, 2005), experiment 1 assessed whether repeated ALP exposure during adolescence (postnatal day [PD]35-49) would alter reward sensitivity to morphine in male mice. Based on previous work on the modulating properties of ALP on the opiate system, I hypothesized that ALP exposure during adolescence will increase the reward sensitivity to subthreshold doses of morphine when compared to control vehicle-pretreated mice. Because drugs targeting the GABAergic system are known to induce molecular

adaptations in the mesolimbic system (i.e., VTA and NAc) (Vashchinkina et al., 2014a), the goal of experiment 2 was to delineate the neurobiological consequences of a single ALP exposure in adolescent male mice (PD 35). Specifically, I measured drug-induced changes in the expression of extracellular regulated protein kinase-1/2 (ERK) and its downstream target cAMP response element-binding protein (CREB) within the VTA and NAc, which are neural substrates implicated in drug-reward and play a role in mood-related disorders (Iñiguez et al., 2010a; Ortiz et al., 1995; Iñiguez et al., 2014). In addition, I assessed changes in the expression of the protein kinase B (AKT) due to its role as molecular regulator of drug-reward as seen with repeated opioid administration (Russo et al., 2007). In experiment 3, I examined the effects of repeated ALP exposure during adolescence (PD35-49). As with experiment 2, I assessed ALP-induced changes in the expression of ERK1/2, CREB and AKT within the VTA and NAc to start laying the foundation of potential molecular changes that may underly enhanced reward sensitivity to opiates. Given, the mounting evidence of ERK's role in the neuroplasticity induced by stress, antidepressant treatment and drugs of abuse and its ability to influence downstream targets (Iñiguez et al., 2010b; Ortiz et al., 1995; Iñiguez et al., 2014), I hypothesized that single and repeated ALP exposure during adolescence will result in a decrease in ERK1/2 and CREB expression within the VTA. Conversely, ALP exposure will have the opposite effect: an increase in ERK1/2 and CREB expression within NAc. Moreover, I hypothesized that ALP treatment will induce a downregulation of the insulin receptor substrate 2 (IRS2)-AKT pathway within the VTA and induce the opposite effects within the NAc, further contributing to the dysregulation in reward sensitivity.

2. MATERIALS AND METHODS

2.1. Animals

C7BL/6J male adolescent mice [postnatal day 28 (PD28) on arrival] were used in this study (Jackson Laboratory; Bar Harbor, ME). Mice were housed (5 per cage) in clear polypropylene boxes containing wood shavings, located in a temperature-controlled (23-25 ˚C) vivarium maintained on a 12-h light-dark cycle in which the lights were on between 7:00 A.M. and 7:00 P.M. Food and water were provided ad libitum throughout the course of the experiments. The mice were allowed to habituate for 1 week before experimental manipulation. All experimental procedures were performed in strict accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003) and approved by the Texas A&M University Animal Care and Use committee.

2.2. Drugs

Alprazolam (ALP), a benzodiazepine (BDZ), was purchased from Spectrum Pharmaceuticals (Irvine, CA). Due to its insolubility in water, the vehicle solution consisted of 90% koliphor and 10% physiological sterile saline. Alprazolam was administered in a volume of 0.04 ml/kg intraperitoneal (i.p.). Morphine sulfate was obtained from Spectrum Pharmaceuticals (Irvine, CA) and was dissolved in 0.9% sterile saline. Morphine was delivered subcutaneously (s.c.) in a volume of 0.04 ml/kg.

2.3. Drug Treatment and Experimental Design

Three different experiments were performed. In the first experiment, mice received one daily injection of either vehicle or ALP (0.5, 1.0 mg/kg, i.p.) for 14 days. Twenty-four hours after the last injection, the mice's behavioral responsivity to the rewarding effects of subthreshold doses of morphine (0, 2.5, 5.0 mg/kg, s.c.) was measured using the conditioned place preference (CPP) paradigm. The CPP paradigm followed a 4-day protocol that involved a one-trial baseline (day 0), 3 days of drug conditioning (days 1-3) consisting of two sessions (morning and afternoon), and a one-trial test day (day 4). In the second experiment, a separate group of adolescent male mice received a single injection of either a vehicle or ALP (0.5 mg/kg, i.p.), were sacrificed ninety-minutes later, and brain tissue dissected for RT-qPCR and western blot analysis. In the third experiment, a different group of adolescent male mice received one daily injection of either vehicle or ALP (0.5 mg/kg) for 14 days, were sacrificed 24 h after the last injection and brain tissue was dissected for RT-qPCR and western blot analysis.

2.4. Conditioned Place Preference

Place preference conditioning to morphine was performed in a three-compartment apparatus where each compartment differed in wall coloring and floor texture. On the preconditioning day (day 0), mice were allowed to explore the entire apparatus for 30 min to obtain baseline preference to any of the three compartments (length by width by height: side compartments, 35×27×25 cm; middle compartment, 10×27×25 cm). Mice did not show any preference for either side compartment (before

morphine exposure). Conditioning trials occurred over three consecutive days. During conditioning days 1-3 the mice received vehicle injection in the morning and were confined to one of the side compartments of the apparatus for 1 hour. After an intermission period of 4 hours, mice received a morphine (2.5, 5.0 mg/kg, s.c) injection in the afternoon and were confined to the opposite side compartment of the apparatus (drug-paired compartment) for 1 hour. On the test day (day 4), mice were allowed to explore the entire apparatus for 30 min under a drug-free state and time spent in the drug-paired compartment was assessed. The test was performed in the middle of the day to control the mice from making potential associations with vehicle or drug injection based on time of day.

2.5. Quantitative Real-Time PCR

Mice were sacrificed ninety-minutes (short-term) after acute, and 24 h after repeated ALP exposure. Brains were extracted and sliced into 1-mm diameter coronal sections. A 14-gauge needle was used to collect VTA and NAc punches that were rapidly stored at -80°C until assayed. RNA was isolated using Illustra TriplePrep kit (GE Healthcare) according to the manufacturer's instructions and cDNA was then created from these samples using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo-Fisher). Quantitative real-time qPCRs were performed in triplicates using 384 well PCR plates and RealMasterMix (Eppendorf) with Eppendorf MasterCycler Realplex2 according to the manufacturer's instructions. Threshold cycle [C(t)] values were measured using the supplied software and analyzed using the $\Delta\Delta C(t)$

method as described previously (LaPlant et al. 2010; Vialou et al. 2010; Warren et al. 2013). Primer sequences for ERK1 *(Mapk3)*, ERK2 (*Mapk1*), CREB (*creb1*), AKT (*Akt*), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) are listed on Table 2.1.

Table 2.1 RT-qPCR primers

Gene	Primer Sequence	
	Forward	Reverse
Mapk3	5'-GTACATCGGAGAAGGCGCCTAC-3'	5'-TTGTAAAGGTCCGTCTCCAT-3'
Mapk1	5'-GGTTGTTCCCAAATGCTGACT-3'	5'-CAACTTCAATCCTCTTGTGAGGG-3'
Creb1	5'-AGTGACTGAGGAGCTTGTACCA-3'	5'-TGTGGCTGGGCTTGAAC-3'
Akt	5'-GCACCTTTATTGGCTACAAGGA-3'	5'-GGGGACTCTCGCTGATCCA-3'
Gapdh	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'

Mapk3: Mitogen activated protein kinase 2 (ERK1); *Mapk1*: Mitogen activated protein kinase 1 (ERK2); *Creb1*: cAMP response element binding protein 1 (CREB); *Akt:* Protein kinase B (AKT); *Gapdh:* glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.6. Western Blotting

Protein from VTA and NAc tissue punches was isolated using Illustra TriplePrep kit (GE Healthcare) according to the manufacturer's instructions and stored at -80°C until assayed. Fifteen micrograms of protein from each sample were treated with β-mercaptoethanol and electrophoresed on precast 4-20 % gradient gels (Bio-Rad), as described previously (Iñiguez et al. 2012; Warren et al. 2011). All antibodies were obtained from Cell Signaling (Beverly, Massachusetts). Blots were probed overnight at a

4°C with antibodies against the phosphorylated forms of CREB, and AKT. Membranes were stripped with Restore Pierce Biotechnology (Rockford, Illinois) and re-probed with antibodies against the phosphorylated forms of ERK1, ERK2, and GAPDH. On separate membranes, blots were probed with antibodies against total forms of CREB and AKT. Membranes were stripped as previously described, and re-probed with antibodies against the total forms of ERK1, ERK2, and GAPDH. All primary antibodies were diluted to a 1:1,000 concentration. Membranes were washed several times with TBST and were then incubated with peroxidase-labeled goat anti-rabbit IgG (1:10,000; Cell signaling, Beverly, Massachusetts). Bands were visualized with SuperSignal West Dura substrate (Pierce Biotechnology, Rockford, IL), quantified using ImageJ (NIH), and subsequently normalized tot GAPDH.

2.7. Statistical Analysis

The assignment of subjects to the various experimental conditions was random. The behavioral data was analyzed using a two-way analysis of variance (ANOVA) with ALP pre-treatment and MOR treatment as sources of variance. Post-hoc comparisons were analyzed using Tukey's test. When appropriate, Student's *t* tests were used to determine statistical significance. Data are expressed as the mean ± SEM. Statistical significance was defined as $p < 0.05$.

3. RESULTS

3.1. Body Weights

Body weight was measured every other day throughout repeated ALP administration (Figure 3.1). A two-way repeated measures ANOVA showed that all groups gained weight over time ($F_{(2,76)}$= 138.1, p< 0.0001) but did not differ from each other as a function of treatment exposure ($F_{(2,33)}$= 0.777, p= 0.468). The pattern of change was overall uniform across groups, and no fluctuations occurred (mean body weight = 21.14 g ±, 21.37 g ±, and 20.78 g ± for groups VEH, 0.5 mg/kg ALP, and 1.0 mg/kg ALP, respectively). As indicated, no significant differences were seen between groups by the beginning of the baseline (day 0) of CPP.

3.2. Effects of Repeated Alprazolam Administration on Morphine-Induced Conditioned Place Preference

To test for changes in behavioral sensitivity to morphine reward, conditioned place preference was assessed 24 h (short-term; n = 4-6/group) following repeated adolescent exposure to VEH, or ALP (Figure 3.2). Time spent in the morphine-paired compartment was not influenced by adolescent drug pretreatment, but did vary as a function of morphine ($F_{(2,45)}$= 8.012, p= 0.001), and by an interaction between the two variables ($F_{(4,45)}$= 8.786, p< 0.0001). Mice pretreated with 0.5 mg/kg ALP readily conditioned to the compartments paired with 2.5 mg/kg morphine when compared with the VEH-pretreated mice (p< 0.05). Likewise, mice pretreated with 1.0 mg/kg ALP also

showed preference for the compartment paired with 2.5 mg/kg morphine when compared to VEH-pretreated mice ($p< 0.01$). Interestingly, mice pretreated with 1.0 mg/kg ALP showed an aversion-like behavior for the compartment paired with 5.0 mg/kg morphine when compared to VEH-pretreated mice ($p< 0.05$). In addition, the magnitude of morphine-induced preference showed by the 0.5 or 1.0 mg/kg ALP-pretreated mice was not significantly different ($p> 0.05$), indicating that these doses similarly influence sensitivity to morphine.

Given behavioral findings indicating that either 0.5 or 1.0 increased the rewarding of morphine (2.5 mg/kg), I chose the 0.5 mg/kg ALP dose for the subsequent biochemistry experiments.

3.3. Effects of Acute Alprazolam Exposure on ERK-Related Gene Expression

ERK-related gene expression within the VTA was assessed ninety-minutes after a single exposure to VEH, or 0.5 mg/kg ALP using RT-qPCR (Figure 3.3*a-d*, n = 20; 8-10/group). ALP treatment significantly decreased ERK1 ($t_{(14)}= 4.400$, $p< 0.001$; Figure 3.3*a*), ERK2 ($t_{(14)}= 2.614$, $p< 0.05$; Figure 3.3*b*), CREB ($t_{(14)}= 4.039$, $p< 0.01$; Figure 3.3*c*), and AKT ($t_{(12)}= 2.635$, $p< 0.05$; Figure 3.3*d*) mRNA expression when compared to the VEH-treated controls.

ERK-related gene expression was also assessed within the NAc ninety-minutes after a single exposure to VEH, or 0.5 mg/kg ALP (Figure 3.4*a-d*, n = 20; 8-10/group). ALP treatment significantly increased ERK1 ($t_{(14)}= 5.581$, $p< 0.0001$; Figure 3.4*a*), ERK2 ($t_{(14)}= 3.716$, $p< 0.01$; Figure 3.4*b*), CREB ($t_{(12)}= 2.354$, $p< 0.05$; Figure 3.4*c*), and

AKT ($t_{(14)}$= 2.518, p< 0.05; Figure 3.4*d*) mRNA expression when compared to VEH-treated controls.

3.4. Effects of Acute Alprazolam Exposure on ERK-Related Protein Phosphorylation

The activity of ERK-related signaling within the VTA after acute VEH or 0.5 mg/kg ALP exposure during adolescence was further assessed as inferred from the phosphorylation of ERK protein and its downstream target CREB. Additionally, changes in phosphorylation of AKT protein were assessed (Figure 3.5*a-d*; n = 20; 8-10/group; all normalized to GAPDH and presented as phosphorylated form of protein). ALP treatment significantly decreased the phosphorylated forms of ERK1 ($t_{(13)}$= 2.477, p< 0.05; Figure 3.5*a*), ERK2 ($t_{(11)}$= 2.228, p< 0.05; Figure 3.5*b*), CREB ($t_{(13)}$= 3.216, p< 0.01; Figure 3.5*c*) and AKT ($t_{(11)}$= 2.464, p< 0.05; Figure 3.5*d*) protein when compared to the VEH-treated controls. In addition, ALP treatment decreased the ratio of phospho over total levels of protein of ERK1 ($t_{(10)}$= 3.104, p< 0.05), CREB ($t_{(13)}$= 2.221, p< 0.05) and AKT ($t_{(13)}$= 4.157, p< 0.01). No change in total ERK1, ERK2, CREB, AKT, or GAPDH protein levels were detected when compared with VEH-treated controls (p> 0.05). No change in the ratio of phospho over total levels of protein of ERK2 was detected when compared to VEH-treated controls (p> 0.05).

Protein expression of ERK-related signaling within the NAc was also assessed after acute VEH or ALP exposure (Figure 3.6*a-d*; n = 20; 8-10/group; all normalized to GAPDH and presented as phosphorylated form of protein). Surprisingly, ALP treatment

did not influence total, phospho, or the ratio of phospho over total levels of protein expression within the NAc ($p > 0.05$) for any of the targets mentioned above.

3.5. Effects of Repeated Alprazolam Exposure on ERK-Related Gene Expression

Gene expression was also assessed within the VTA (Figure 3.7*a-d*, n = 8-10/per group) and NAc (Figure 3.8*a-d*, n = 8-10/per group) 24 h after repeated VEH or 0.5 mg/kg ALP exposure in a separate group of adolescent mice. Within the VTA, ALP treatment significantly decreased ERK1 ($t_{(13)} = 2.571$, $p < 0.05$; Figure 3.7*a*), ERK2 ($t_{(13)} = 2.594$, $p < 0.05$; Figure 3.7*b*), CREB ($t_{(13)} = 2.438$, $p < 0.05$; Figure 3.7c), and AKT ($t_{(14)} = 3.549$, $p < 0.01$; Figure 3.7*d*) mRNA expression when compared to VEH-treated mice. Conversely within the NAc, ALP treatment increased ERK1 ($t_{(12)} = 2.468$, $p < 0.05$; Figure 3.8*a*), ERK2 ($t_{(13)} = 2.841$, $p < 0.05$; Figure 3.8*b*), CREB ($t_{(13)} = 2.239$, $p < 0.05$; Figure 3.8*c*), and AKT ($t_{(14)} = 4.287$, $p < 0.001$; Figure 3.8*d*) mRNA expression when compared to VEH-treated controls.

3.6. Effects of Repeated Alprazolam Exposure on ERK-Related Protein Phosphorylation

Assessment of ERK-related protein expression within the VTA and NAc (Figures 3.9*a-d* and 3.10*a-d* respectively; n = 20; 8-10/group; all normalized to GAPDH and presented as phosphorylated form) was performed after repeated VEH or 0.5 mg/kg ALP exposure in adolescent mice. Within the VTA, ALP treatment significantly increased phosphorylated forms of ERK1 ($t_{(16)} = 2.485$, $p < 0.05$; Figure 3.9*a*), and AKT

($t_{(16)}$= 2.855, p< 0.05; Figure 3.9*d*), while ERK2($t_{(18)}$= 0.178, p> 0.05; Figure 3.9*b*) and CREB ($t_{(18)}$= 0.207, p> 0.05; Figure 3.9*c*) remained unchanged when compared to VEH-treated controls. Interestingly, a significant increase in total CREB ($t_{(17)}$= 2.196, p< 0.05) protein levels was observed. No changes in total ERK1, ERK2 or AKT were observed (p> 0.05). Moreover, a significant increase in the ratio of phospho over total protein levels was observed in ERK1 ($t_{(17)}$= 2.196, p< 0.05), ERK2 ($t_{(17)}$= 2.407, p< 0.05), and AKT ($t_{(16)}$= 2.294, p< 0.05), while CREB remained unchanged (p> 0.05) when compared to VEH-treated mice.

Within the NAc, repeated ALP exposure induced an increase in ERK1($t_{(18)}$= 2.478, p< 0.05; Figure 3.10*a*), ERK2 ($t_{(17)}$= 2.553, p< 0.05; Figure 3.10*b*), CREB ($t_{(17)}$= 2.237, p< 0.05; Figure 3.10*c*), and AKT ($t_{(18)}$= 2.470, p< 0.05; Figure 3.10*d*) phosphorylated protein levels when compared to VEH-treated controls. Repeated ALP exposure also significantly increased total CREB protein levels ($t_{(18)}$= 2.566, p< 0.05), however no effects were observed in total protein levels of ERK1, ERK2, or AKT (p> 0.05) when compared to VEH-treated controls. In addition, ALP exposure induced an increase in the ratio of phospho over total protein levels of ERK1 ($t_{(17)}$= 2.363, p< 0.05), ERK2 ($t_{(18)}$= 3.344, p< 0.05), and AKT($t_{(14)}$= 2.222, p< 0.05), while CREB remained unchanged (p> 0.05) when compared to the VEH-treated mice.

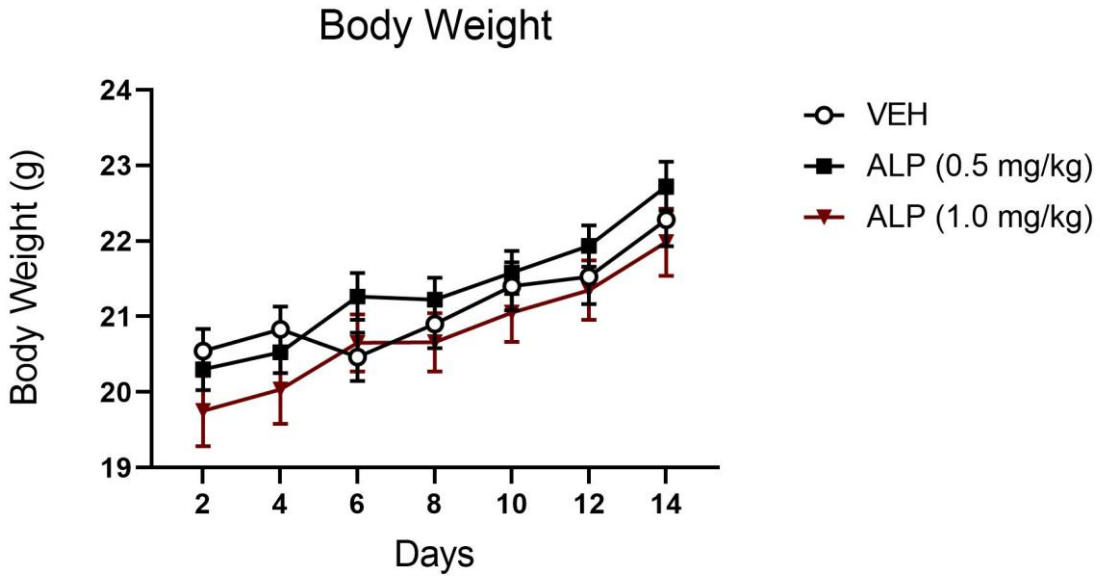

Figure 3.1. Body weights of male adolescent mice throughout repeated ALP exposure. Mice were habituated for one week (PD 30-35), then exposed to 14 days to either vehicle (VEH) or alprazolam (ALP; n= 12/group). Mice in all conditions gained weight over time ($p< 0.0001$) and no effect of treatment was observed ($p> 0.05$) throughout drug exposure.

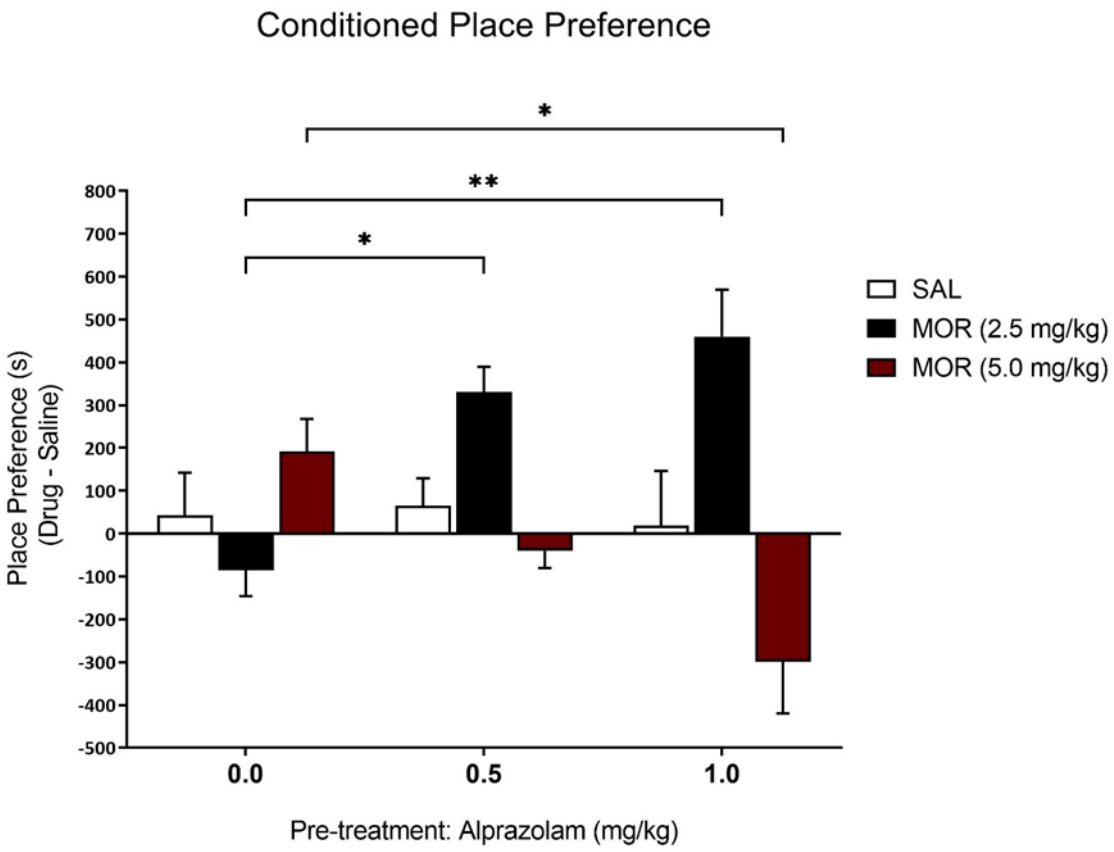

Figure 3.2. Effects of repeated exposure to vehicle (VEH) or alprazolam (ALP) during adolescence on morphine-induced conditioned place preference. Mice pretreated with 0.5 mg/kg ALP showed an increased preference for the subthreshold dose of morphine (MOR, 2.5 mg/kg) when compared to VEH-pretreated controls. Likewise, mice in the 1.0 mg/kg ALP condition showed an increased preference for the subthreshold dose of 2.5 mg/kg MOR when compared to VEH-pretreated mice. Conversely, mice pretreated with 1.0 mg/kg ALP showed an aversion-like behavior to subthreshold to 5.0 mg/kg MOR when compared to VEH-pretreated mice (n= 4-6 per group; *p< 0.05, **p< 0.01 when compared to VEH-pretreated controls).

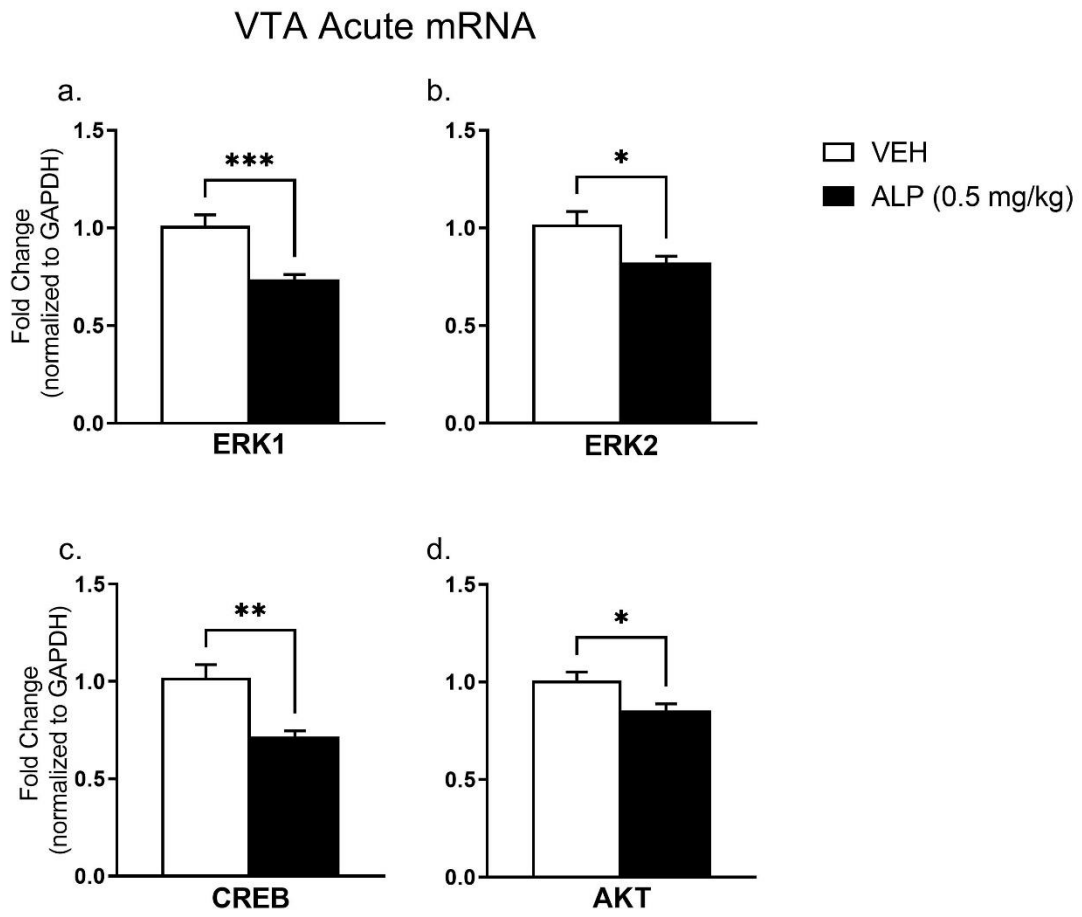

Figure 3.3. Effects of acute exposure to vehicle (VEH), or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on ERK-related gene expression within the ventral tegmental area (VTA) 90-min after a single injection. (a) ERK1 ($p< 0.001$); (b) ERK2 ($p< 0.05$); (c) CREB ($p< 0.01$); and (d) AKT ($p< 0.05$) mRNA levels were significantly reduced by ALP when compared to VEH-treated controls. *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$ compared to VEH-treated controls. Data are represented as fold change normalized to GAPDH (mean ± SEM).

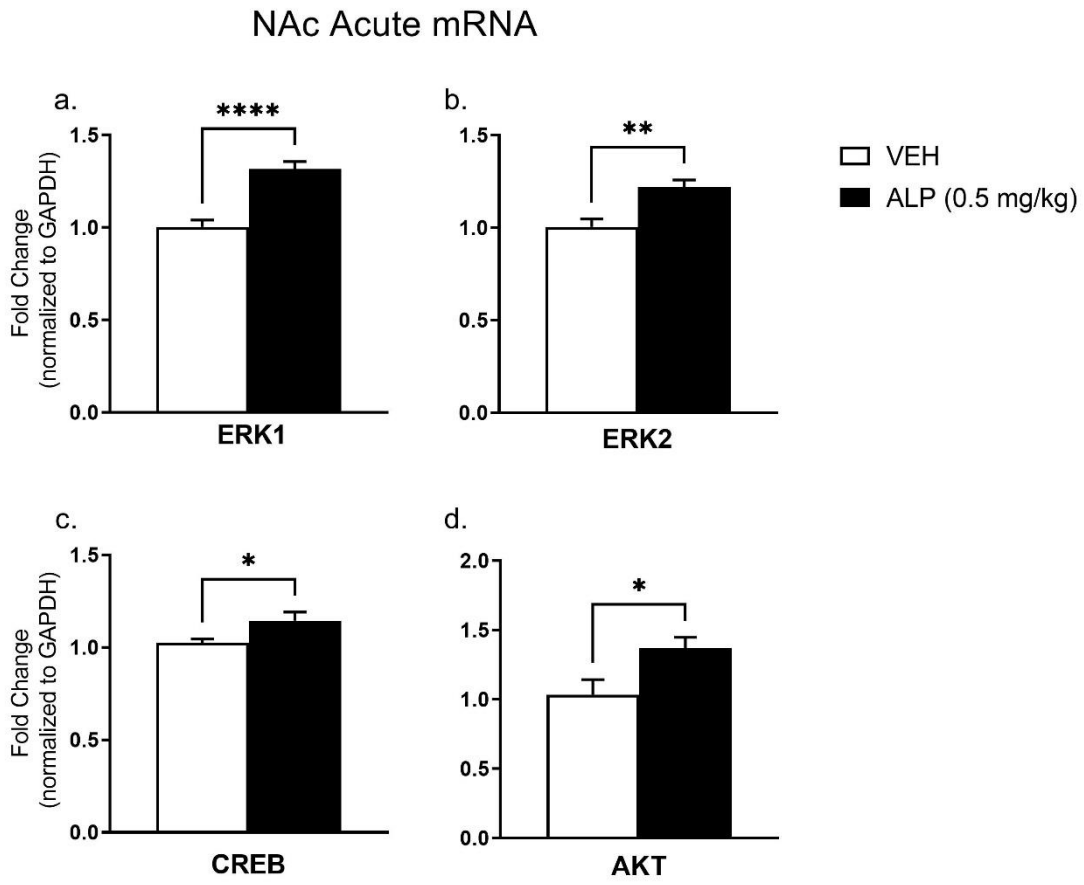

Figure 3.4. Effects of acute exposure to vehicle (VEH), or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on ERK-related gene expression within the nucleus accumbens (NAc) 90-min after a single injection. (a) ERK1 ($p< 0.0001$); (b) ERK2 ($p< 0.01$); (c) CREB ($p< 0.05$); and (d) AKT ($p< 0.05$) mRNA levels were significantly increased by ALP when compared to VEH-treated controls. *$p< 0.05$, **$p< 0.01$, ****$p< 0.0001$ compared to VEH-treated controls. Data are represented as fold change normalized to GAPDH (mean ± SEM).

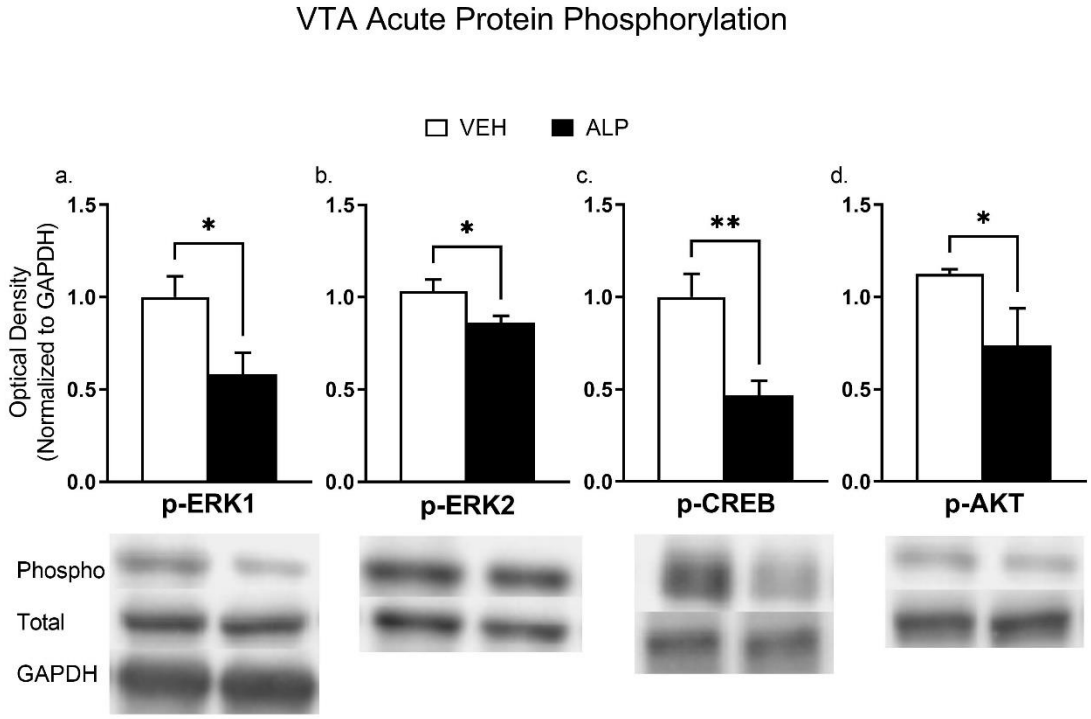

Figure 3.5. Effects of acute exposure to vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on protein phosphorylation within the ventral tegmental area (VTA) 90-min after a single injection. Treatment with ALP significantly decreased (a) ERK1, (b) ERK2, (c) CREB, and (d) AKT phosphorylated forms (mean ± SEM) when compared to VEH-treated controls. *$p < 0.05$, **$p < 0.01$ compared to VEH-treated controls.

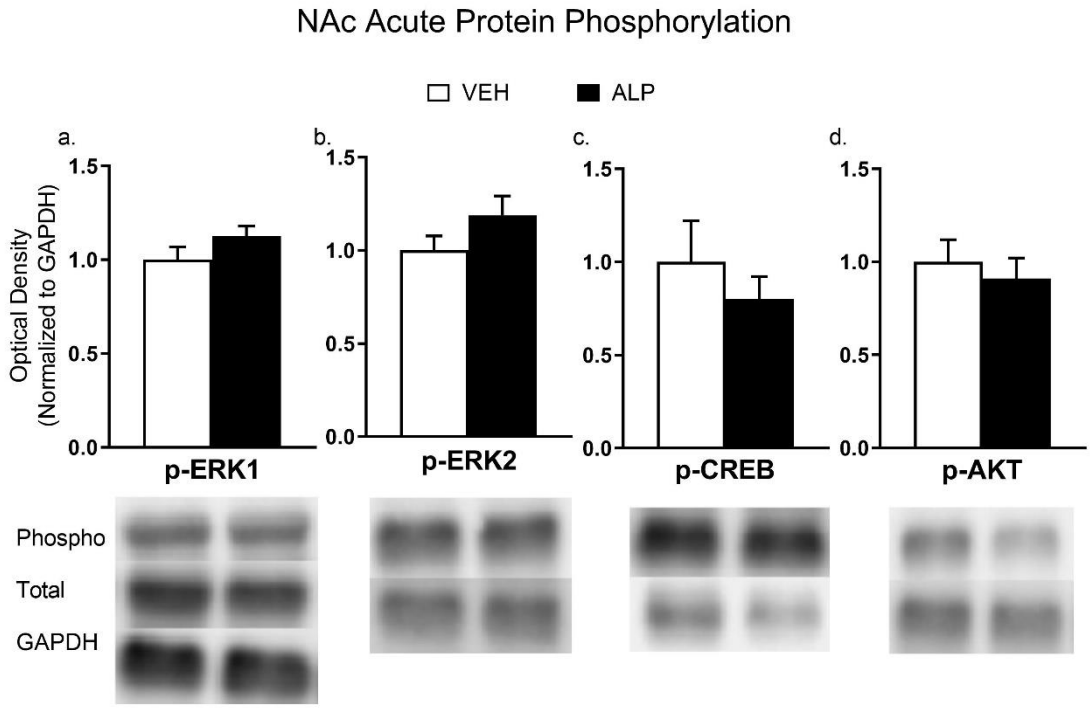

Figure 3.6. Effects of acute exposure to vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on protein phosphorylation within the nucleus accumbens (NAc) 90-min after a single injection. Treatment with ALP did not influence phosphorylated forms of (a) ERK1, (b) ERK2, (c) CREB, or (d) AKT (mean ± SEM). $p > 0.05$ when compared to VEH-treated controls.

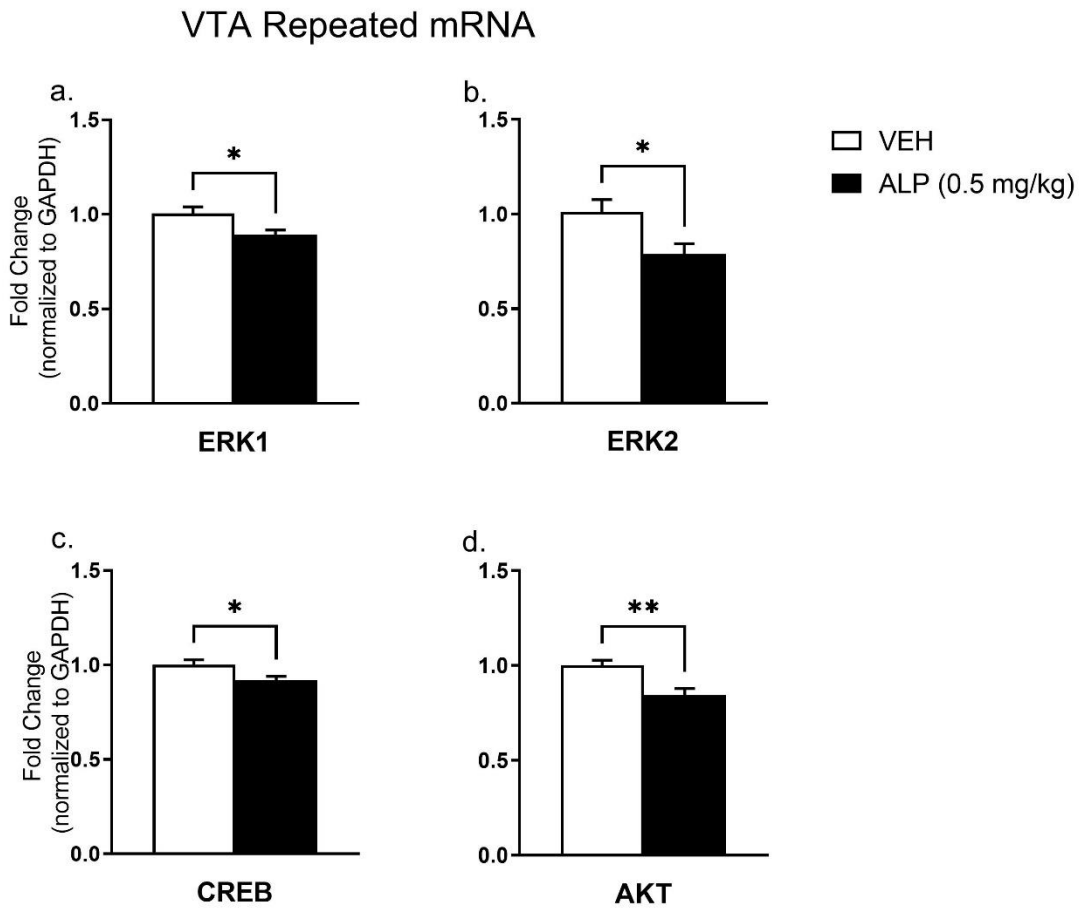

Figure 3.7. Effects of repeated exposure to vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on ERK-related gene expression within the ventral tegmental area (VTA) 24 h after the last injection. (a) ERK1 ($p< 0.05$); (b) ERK2 ($p< 0.05$); (c) CREB ($p< 0.05$); and (d) AKT ($p< 0.05$) mRNA levels were significantly decreased by ALP when compared to VEH-treated mice. *$p< 0.05$, **$p< 0.01$ compared to VEH-pretreated controls. Data are represented as fold change normalized to GAPDH (mean ± SEM).

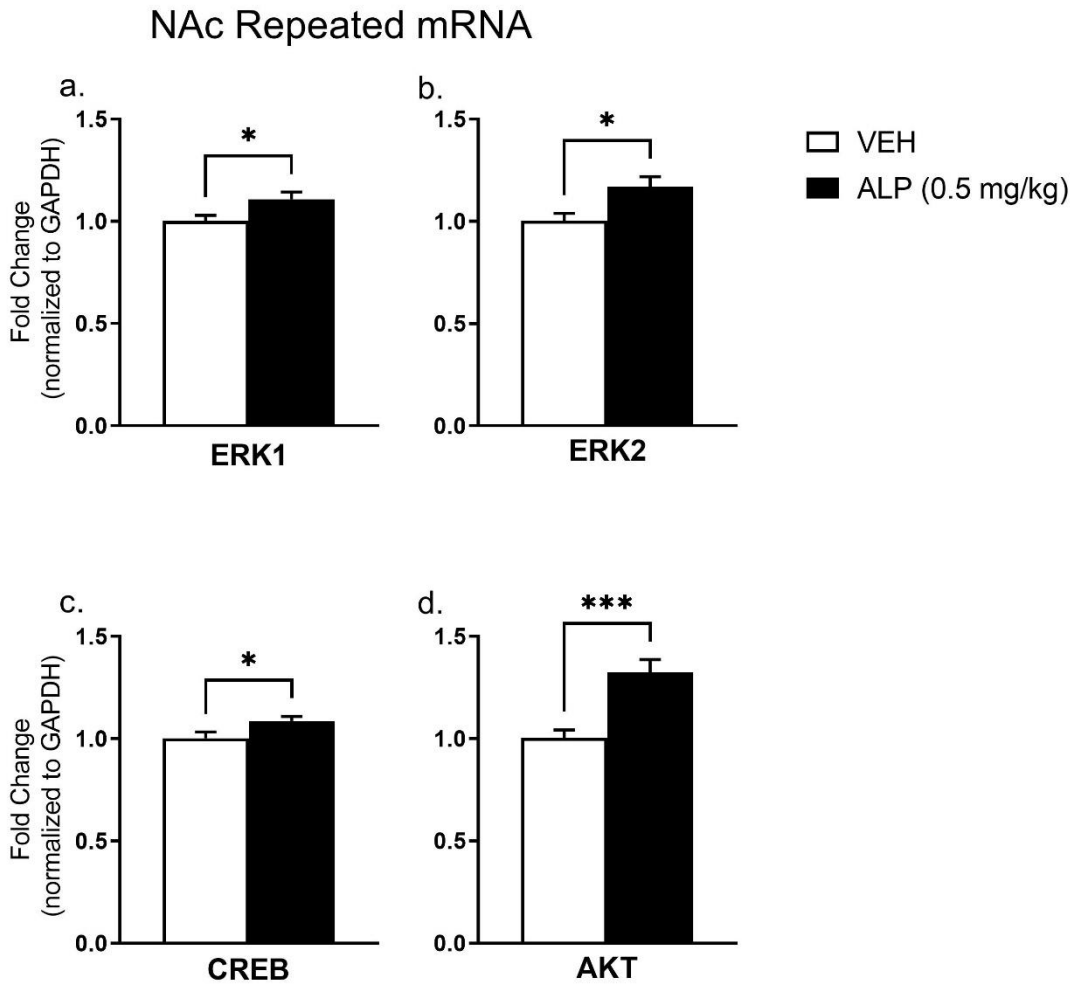

Figure 3.8. Effects of repeated vehicle (VEH) or alprazolam (ALP; 0.05 mg/kg) in adolescent male mice on ERK-related gene expression within the nucleus accumbens (NAc) 24 h after the last injection. (a) ERK1 ($p< 0.05$); (b) ERK2 ($p< 0.05$); (c) CREB ($p< 0.05$); and (d) AKT ($p< 0.001$) mRNA levels were significantly increased by ALP when compared to VEH-treated controls. *$p< 0.05$, ***$p< 0.001$ compared to VEH-treated controls. Data are represented as fold change normalized to GAPDH (mean ± SEM).

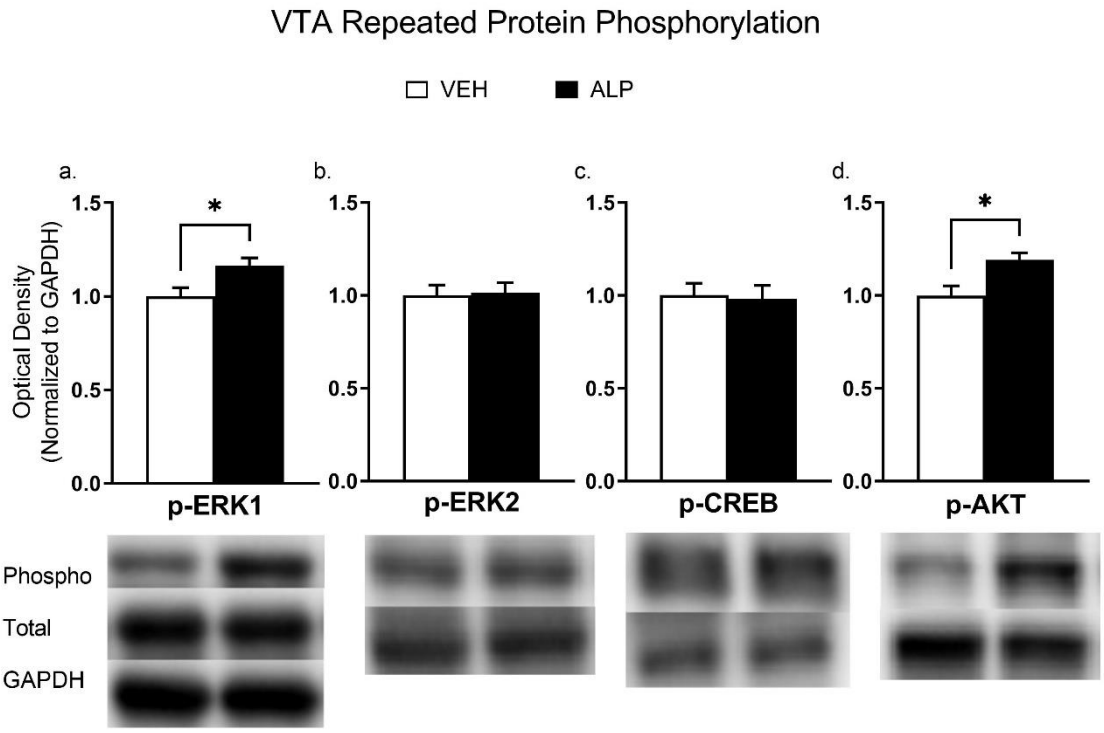

Figure 3.9. Effects of repeated vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) exposure in adolescent male mice on protein phosphorylation within the ventral tegmental area (VTA) 24 h after the last injection. Treatment with ALP significantly increased (a) ERK1, and (d) AKT phosphorylated forms (mean ± SEM) when compared to the VEH-treated controls. There was no change in (b) ERK2 or (c) CREB phosphorylation (mean ± SEM) when compared to VEH-treated mice. *p< 0.05 compared to VEH-treated controls.

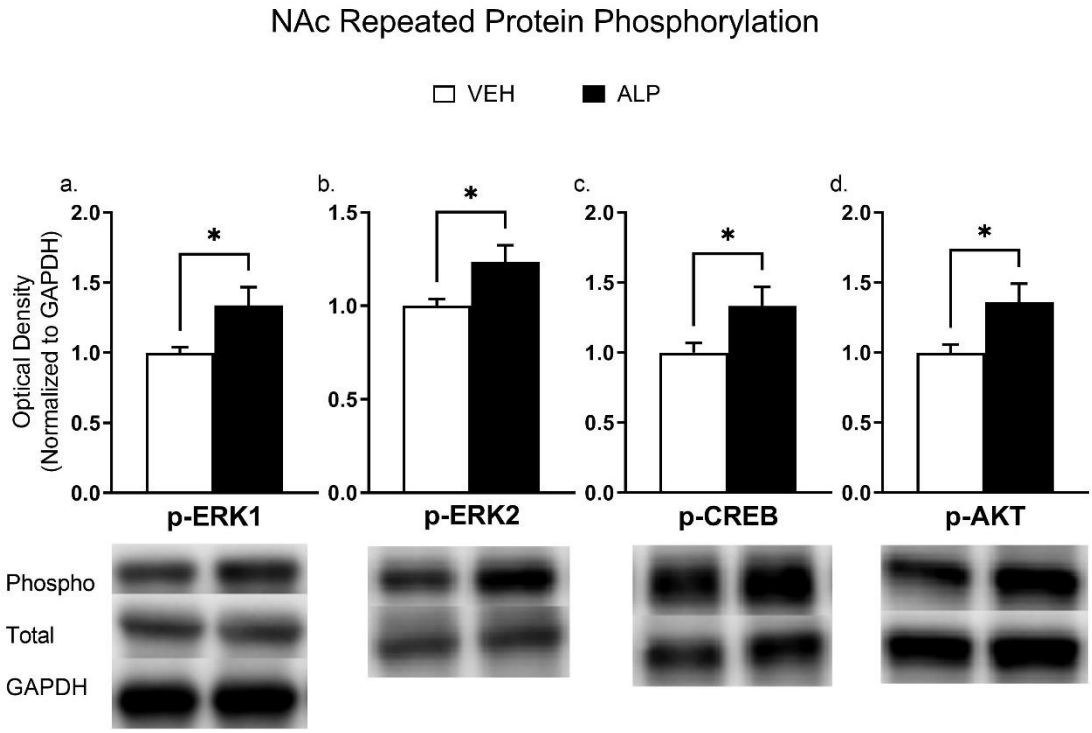

Figure 3.10. Effects of repeated vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) exposure in adolescent male mice on protein phosphorylation within the nucleus accumbens (NAc) 24 h after the last injection. Treatment with ALP significantly increased (a) ERK1, (b) ERK2, (c) CREB, and (d) AKT phosphorylated forms (mean ± SEM) when compared to VEH-treated controls. *p< 0.05 compared to VEH-pretreated controls.

4. DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

This study assessed the behavioral and neurobiological consequences of ALP exposure during adolescence, a drug that is both highly prescribed (Moore and Mattison, 2017; Bachhuber et al; 2016) and abused by the adolescent population in the U.S. (Friedrich et al., 2020; Hughes et al., 2016; Johnston et al., 2019). Here, I report that repeated ALP administration during adolescence (PD 35-49) results in enhanced reward sensitivity to other drugs of abuse such as morphine. In addition, both acute and repeated ALP administration results in dysregulation of the extracellular signaling-regulated kinase (ERK1/2) and related downstream signaling within the VTA and NAc, brain regions highly implicated in drug-reward and mood-related disorders (Iñiguez et al., 2010a; Ortiz et al., 1995; Iñiguez et al., 2014; Nestler and Carlezon, 2006).

I first assessed the effects of repeated ALP exposure during adolescence on morphine reward as measured by the conditioned place preference (CPP) paradigm. Adolescent mice pretreated with ALP (0.5, 1.0 mg/kg) showed a preference for environments previously paired with 2.5 mg/kg morphine, a dose that had no significant effects in the VEH-pretreated mice. This suggests that ALP exposure produces a leftward shift of the dose response curve for morphine as mice pretreated with ALP showed a significant preference for the morphine-paired compartments at a dose of the drug that by itself had no effects on CPP. These results are supported by previous work on the modulatory properties of ALP in adult rodent models (Walker and Ettenberg 2001; Walker and Ettenberg 2005). Interestingly, there were no differences in the

magnitude of morphine-induced place preference developed by the mice treated with the different ALP doses (0.5 and 1.0 mg/kg), suggesting that ALP similarly influenced the system to induce sensitivity to the low morphine dose.

Surprisingly, mice pretreated with the higher dose of ALP used (1.0 mg/kg) showed aversion-like behavior profile to the moderate dose of morphine (5.0 mg/kg). The mechanism(s) responsible for this effect is not known. However, a potential explanation for these behavioral effects is that although morphine primarily binds to μ-opioid receptors, it can also bind to other receptors including κ-opioid receptors (Suzuki et al., 2001). Studies have shown that application of κ-opioid agonists produce place aversion (Mucha and Herz, 1985). More recently, it has been shown that κ-opioid agonists directly inhibit VTA dopaminergic neurons and produce actions that are opposite to those produced by μ-opioid receptor agonists (Margolis et al., 2003). Therefore, the aversion-like behavior observed herein may be, at least partially, attributable to the binding of morphine to κ-opioid receptors. Nevertheless, a 5 mg/kg morphine dose by itself is not known to induce place aversion, thus it is possible that pretreatment with ALP is modulating the behavior observed. The effects of repeated BDZs administration on opioid receptor regulation have not been fully elucidated. A study showed that buprenorphine (a partial μ-opioid receptor agonist) promotes rapid desensitization and downregulation of receptors resulting in a reduction in agonist efficacy. However, ALP administration prior to buprenorphine exposure restored the density of μ-opioid receptors binding sites (Poisnel et al., 2009). It is possible that ALP exposure prior to morphine upregulates the density of μ-opioid receptors which may

enhance the binding of morphine once it is introduced and therefore intensifying its pharmacological effects. Previous literature has suggested that morphine possess biphasic effects (Randall et al., 1992). It is thus possible that ALP preexposure increased receptor number and/or affinity such that when coupled with a moderate dose of morphine will induce aversion-like behavior effects.

Given the behavioral findings from experiment 1 indicating that either 0.5 or 1.0 ALP increased reward sensitivity to subthreshold morphine, the 0.5 mg/kg ALP dose was chosen in subsequent experiments to assess the neurobiological consequences of acute and repeated ALP exposure during adolescence. In experiment 2, I measured the expression of ERK-related signaling molecules within the VTA and the NAc ninety minutes after a single ALP exposure. This brain region approach was taken because as mentioned, it has been hypothesized that BDZs exert their rewarding properties by interacting with the VTA and NAc (Vashchinkina et al., 2014b; Heikkinen et al., 2009), a neural circuit that is a major substrate for motivated behavior and responses to natural reinforcers (Koob and Le Moal, 2008; Kelley and Berridge, 2002). Intracellular pathways such as the ERK and IRS2-AKT are highly regulated by drugs of abuse, and more recently involved in regulation of mood-related disorders and drug-induced neuroplasticity (Russo et al., 2007; Wang et al., 2003; Wang and Mao, 2019; Krishnan et al., 2008; Berhow et al., 1996). To this end, I sought to measure the levels of gene expression of the molecular targets: ERK1/2 and its downstream target CREB and AKT. Within the VTA, it was observed that ERK1/2, CREB, and AKT mRNA was decreased after acute ALP exposure. Decreases in ERK1/2 and CREB mRNA were expected as

ALP has been found to produce behavioral effects that are comparable to those seen after antidepressant exposure (Flugy et al., 1992). Previous work supports this assumption as it has been demonstrated that after chronic stress ERK activity is significantly increased within the VTA (Iñiguez et al., 2010a), while ERK mRNA and protein phosphorylation levels are decreased after antidepressant treatment, molecular changes accompanied by suppression of depression-like behavior in rodent models of stress (Iñiguez et al., 2014). Moreover, ALP has been used off-label for the treatment of depression, and some studies have found ALP to be superior to placebo and effective when compared to other antidepressants for the treatment of moderate depression (Jonas and Cohon, 1993). Acutely, the functional significance of the observed decrease in AKT mRNA is unknown as most studies on the effects of drug-induced AKT signaling have been done using chronic drug administration (Mazei-Robison et al., 2011; Russo et al., 2007). Although speculative, a possible explanation for these effects is that AKT signaling would be influenced by ERK activity. The ERK and IRS2-AKT signaling pathways are activated by similar mechanisms, and previous work has shown evidence of crosstalk/inhibition. For instance, ERK phosphorylation of the adaptor GAB1 protein upstream of the IRS2-AKT pathway could subsequently inhibit the activation of AKT downstream (Yu et al., 2002; Mendoza et al., 2011; Moellinget al., 2002).

ALP-induced changes in gene expression within the NAc was also assessed after acute exposure. A single injection of ALP increased ERK1/2, CREB, and AKT mRNA expression. Although novel within the framework of adolescence drug exposure, these results were not surprising as a well-established neurobiological response to acute

administration of addictive drugs is the increase in DA levels within the NAc where the encoding of incentive-motivational valence of drugs supposedly occurs (Di Chiara et al., 2004). Activation of NAc by BDZs has been shown to be mediated by disinhibition of DA neurons projections from the VTA (Tan et al., 2010). More recently, Wolf and colleagues (Wolf et al., 2013) reported direct evidence of the effects of BDZs on the NAc in humans. In this study, it was shown that acute ALP increases cerebral blood flow (CFB) perfusion in the NAc and that these effects were accompanied with impulsive responding in a subsequent cognitive task thus reflecting the known effects of BDZs on addiction-related behaviors (i.e.., risk-taking, impulsivity, disinhibition). Increases in AKT mRNA in the NAc were expected as this pathway is implicated in the control of synaptic strength (Lin et al., 2001; Sanna et al., 2002) in several brain regions. Moreover, AKT activation has been directly implicated in increases in $GABA_A$ receptors on the plasma membrane. An increase in $GABA_A$ receptors leads to an enhancement in synaptic transmission between neurons (Wang et al., 2003). Not surprisingly, the rapid regulation of receptor numbers in the postsynaptic cell is an important means of strengthening of synapses and producing synaptic plasticity after initial drug exposure (Lüscher and Malenka, 2011). Indeed, it has been reported that activation of AKT in the NAc in response to acute alcohol exposure contributes to subsequent binge drinking behaviors (Neasta et al., 2011). ALP-induced AKT activation within the NAc may therefore contribute to the enhancement of synaptic transmission within the VTA-NAc pathway contributing to drug-induced synaptic plasticity.

Because the increase/decrease in gene expression does not necessarily correlate with similar results in protein levels (Mehra et al., 2003; Lee et al., 2003), protein phosphorylation after acute ALP exposure within the VTA and NAc were also assessed. Decreased ERK1/2, CREB and AKT phosphorylation within the VTA were found while total protein levels remained unchanged thus confirming the decrease activity of these enzymes. Decreased VTA-ERK-CREB activity further supports the notion that at acute exposure, ALP induces neurochemical effects that are comparable to those seen by antidepressants. Data from several studies have shown that the onset of antidepressant effect was significantly more rapid for ALP when compared to antidepressants at least short-term time points (Fawcett et al., 1987; Van Markwijk et al., 2012). However, the authors concluded that these findings should be interpreted with caution given the addictive properties of short-acting BDZs. Surprisingly, ALP exposure did not induce changes in phosphorylation in any of these molecular targets within the NAc. The lack of significant differences in protein phosphorylation after ALP exposure may be due to cell-specific kinetic parameters such as changes in concentration of total levels ribosomes and the availability of free ribosomes to initiate translation (Mehra et al., 2003; Lee et al., 2003). For instance, if the total amount of ribosomes remains constant and the amount of mRNA were to increase, the corresponding protein expression levels would be expected to decrease relative to the change in mRNA levels due to limitations in ribosome availability (Lee et al., 2003). Another reason for the discrepancy between gene and protein expression is the fact that transcription and translation are stochastic (McAdams and Arkin, 1997; Thattai and van Oudenaarden, 2004; Raj et al., 2006). Cell

transcription can be intermittent in which periods of strong mRNA production are followed by periods of silence where transcription is stopped (Cai et al., 2006; Tantale et al., 2016). Periods of inactivity may be due to incompletely formed activation complexes or a paused RNA polymerase, to name a few (Nicolas et al., 2017). Furthermore, the duration of periods of activity and inactivity are random and therefore the levels of protein expression may vary significantly within cell populations (Elowitz et al., 2002; Ferguson et al., 2012). Although speculative, it is possible that at the time of tissue dissection the NAc was undergoing a period of inactivation and consequently no changes in protein phosphorylation were detected due to a lack of protein translation.

Given that ALP is often prescribed and abused chronically, an additional goal was to assess this drug's neurochemical effects after repeated administration (14 days). Gene expression within the VTA and NAc was assessed 24 h after the last injection. Within the VTA, I found that ERK1/2, CREB and AKT mRNA was significantly decreased. These findings were not surprising given that ERK downregulation plays a critical role in the effects of chronic antidepressant treatment (Iñiguez et al., 2014). Though speculative, a possible explanation for these effects is that ALP treatment exerts antidepressant effects via sustained ERK down regulation within this region. In contrast, ALP induced the opposite effects within the NAc: increased ERK1/2, CREB, and AKT mRNA. Increases in ERK1/2 activity supports the notion that repeated ALP treatment dysregulates reward sensitivity as ERK is known to induce molecular adaptations that increase sensitivity to drugs of abuse within this brain region (Girault et al., 2007; Carlezon et al., 2005; Kim et al., 2011).

Protein phosphorylation levels within the VTA and NAc after repeated ALP exposure during adolescence were assessed to determine whether changes seen in gene expression were reproduced in protein phosphorylation. Increases in ERK1 and AKT phosphorylation were found within the VTA while ERK2 and CREB remained unchanged. An increase in CREB total protein levels was also observed, which would indicate that the activity levels of this protein were not affected by ALP treatment. Given that ERK2 and CREB are within the same pathway, it might be expected that increases in ERK1 phosphorylation would accompany increases in ERK2 and CREB, however ALP induced contrasting effects. Although ERK1 and ERK2 share about 83% amino acid identity, specific physiological functions have been determined to each. While ERK1 knockout mice are viable, ERK2 knockout mice die in utero (Pagès et al., 1999; Hatano et al., 2003). However more recently, Frémin and colleagues (Frémin et al., 2015) reported that transgenic ERK1 rescued all developmental defects resulting from the genetic inactivation of ERK2 suggesting a functional redundancy of the isoforms. Perhaps ERK1 activity compensates for the lack of activity in ERK2. In addition to this complexity, the significant increases in ERK1 and AKT phosphorylation contradict the findings in mRNA expression. The discrepancy between these findings might be due to several reasons including post-translational modifications (i.e, acetylation, hydroxylation, ubiquitination) that may change the functional state, catalytic activity, or signaling of these kinases (Karve and Cheema, 2011). Moreover, differences in neurochemical profile (GABAergic, dopaminergic and glutamatergic neurons) of the VTA may add another layer of complexity (Holly and Miczek, 2016). It is also possible

that the changes observed are region-specific which the technique used does not allow for more precise determination.

Repeated ALP exposure increased ERK1, ERK2, and AKT phosphorylation within the NAc while total protein levels remained unchanged, thus confirming increased activity of these enzymes. And though there was a significant increase in CREB phosphorylation, this change may have been driven by the increase in total protein levels. Taken together, the increases in ERK signaling observed within the VTA and NAc lend support to the notion that repeated ALP treatment induces a state of sensitization of the VTA-NAc pathway that may contribute to the enhancement of reward to other drugs of abuse. Repeated ALP exposure may result in an increase in the responsiveness of the VTA-NAc system such that rewarding stimuli (i.e., morphine) produce a greater increase in neurotransmission within these regions. Within the context of drug use and abuse, a neural system that is sensitized or "hypersensitive" is hypothesized to mediate psychosocial functions such as the increase in incentive salience of stimuli that may lead to the "wanting" of the drug (Robinson and Berridge, 2008; Berridge and Robinson, 2016). Previous studies have shown that ERK plays a critical role in the development of sensitization to drugs of abuse, and its blockade within the NAc inhibits the expression of sensitization (Kim et al., 2011). Alterations in glutamate signaling and plasticity within the VTA and NAc also play a critical role in the expression of psychomotor sensitization of stimulants (Pierce and Wolf, 2013; Thomas et al., 2008). Similarly, long-lasting modulation of glutamatergic transmission in VTA DA neurons have been observed after BDZ treatment, including the increase in ratio of

AMPA/NMDA receptors (Heikkinen et al., 2009), changes lasting for at least 3 days after initial drug exposure. Increased AKT activity within the VTA further supports this notion, as it has been reported that overexpression of the IRS2-AKT pathway results in an enhance CPP for cocaine, a drug that is well known to induce behavioral sensitization (Iñiguez et al., 2008; Steketee, 2005).

To summarize, repeated ALP exposure during adolescence increased the rewarding effects of a low dose of morphine, while inducing an aversion-like effect at a moderate morphine dose (5 mg/kg). Although speculative, the aversion-like behavior observed at the moderate morphine dose used in this study may be attributable to ALP's ability to regulate μ-opioid receptors therefore enhancing the behavioral effects of morphine. Under this assumption, these effects may also reflect the biphasic properties morphine, such that upregulation of μ-opioid receptors (e.g., number or sensitivity) would result in enhanced effects: low dose induces reward while a moderate dose induces aversion. Therefore, ALP exposure may be sensitizing the VTA-NAc neural system enhancing the effects of other drugs of abuse (i.e., morphine). Acute and repeated ALP exposure induced changes within brain pathways that are highly regulated by drugs of abuse and are substrate for drug-induce neuroplasticity. This data supports the notion that repeated administration of drugs of abuse, particularly early in life, may result in the dysregulation of second-messenger systems that may result in enduring aberrant behavioral consequences. These results add to the growing body of work on the BDZ-opioid interactions. Importantly, the current work has considerable relevance for understanding the behavior of opioid users who co-abuse BDZs and opioids regardless

of adverse consequences. It is important to note, however, that the findings reported here are derived from an adolescent model. Studies on the potential enduring effects of ALP exposure during adolescence in humans are severely lacking (Young et al., 2012), making interpretative parallels challenging. Nevertheless, it is possible to imagine that ALP exposure early in life may influence responsiveness to drugs of abuse and subsequent drug taking behavior later in adulthood.

The findings presented herein necessitate to be expanded to look into the effects of repeated ALP exposure during adolescence on functional outcomes in adulthood given that SUDs are more likely to emerge when drug exposure starts early in life (Clark et al., 1998; Dawson et al., 2008). One approach is to determine the long-term behavioral effects of ALP exposure during adolescence by assessing morphine CPP in adulthood. It would also be of interest to assess the long-term neurobiological effects of ALP exposure to determine whether the molecular changes observed within the VTA-NAc pathway persist into adulthood. It is also of great importance to replicate the current work in adolescent females given that females may respond differently to drugs of abuse when compared to males (Becker et al., 2001). Recent trends indicate that women are prescribed BDZs at higher rates than men, and overdose deaths among women, particularly involving BDZs, have increased substantially (Agarwal and Landon, 2019; VanHouten et al., 2019). Together, the findings from future work will help us better understand the behavioral and neurobiological effects of BDZs on opioid reward and provide insights into the development of better therapeutics that minimize the risk for developing SUDs/addiction.

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APPENDIX A

AUP IACUC PROTOCOL APPROVAL

DIVISION OF RESEARCH

Research Compliance and Biosafety

TEXAS A&M UNIVERSITY

March 19, 2020

MEMORANDUM

TO: Dr. Carlos Bolanos, Ph.D.
TAMU - TAMU - Psychology

FROM: Dr. Mark Westhusin, Chair
Institutional Animal Care and Use Committee

SUBJECT: **Approval of AUP IACUC 2019-0373**
Title: Neurobiology of Stress, Diet, and Drug Exposure
Reference Number: 101254
Funding Source: NIH(NIH), NIH/National Institute on Drug Abuse (NIDA)
AUP Approval Date: 03/19/2020
AUP Expiration Date: 03/18/2023
Species: rats, mice

The above referenced AUP has been approved by the IACUC for a period of 3 years. It is the responsibility of the principal investigator to assure all animal work is conducted in accordance with this AUP.

If you have indicated that you will be performing post procedural monitoring of animals at specific intervals, please provide documentation of your observations in the medical record or by using Animal Observation cards that are available through the Comparative Medicine Program.

A copy of this approval will be sent to the housing facility. ***You must consult with the housing facility manager prior to ordering animals to ensure that space is available.***

Pc: Comparative Medicine Program
Housing Facility Manager

750 Agronomy Road, Suite 2701
1186 TAMU
College Station, TX 77843-1186

Tel. 979.458.1467 Fax. 979.862.3176
http://rcb.tamu.edu

APPENDIX B

FIGURE CAPTIONS

Figure 4.1. Body weights of male adolescent mice throughout repeated ALP exposure. Mice were habituated for one week (PD 30-35), then exposed to 14 days to either vehicle (VEH) or alprazolam (ALP; n= 12/group). Mice in all conditions gained weight over time ($p< 0.0001$) and no effect of treatment was observed ($p> 0.05$) throughout drug exposure.

Figure 4.2. Effects of repeated exposure to vehicle (VEH) or alprazolam (ALP) during adolescence on morphine-induced conditioned place preference. Mice pretreated with 0.5 mg/kg ALP showed an increased preference for the subthreshold dose of morphine (MOR, 2.5 mg/kg) when compared to VEH-pretreated controls. Likewise, mice in the 1.0 mg/kg ALP condition showed an increased preference for the subthreshold dose of 2.5 mg/kg MOR when compared to VEH-pretreated mice. Conversely, mice pretreated with 1.0 mg/kg ALP showed an aversion-like behavior to subthreshold to 5.0 mg/kg MOR when compared to VEH-pretreated mice (n= 4-6 per group; $*p< 0.05$, $**p< 0.01$ when compared to VEH-pretreated controls).

Figure 4.3. Effects of acute exposure to vehicle (VEH), or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on ERK-related gene expression within the ventral tegmental area (VTA) 90-min after a single injection. (a) ERK1 ($p< 0.001$); (b) ERK2 ($p< 0.05$); (c) CREB ($p< 0.01$); and (d) AKT ($p< 0.05$) mRNA levels were significantly reduced by ALP when compared to VEH-treated controls. $*p< 0.05$,

**p< 0.01, ***p< 0.001 compared to VEH-treated controls. Data are represented as fold change normalized to GAPDH (mean ± SEM).

Figure 4.4. Effects of acute exposure to vehicle (VEH), or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on ERK-related gene expression within the nucleus accumbens (NAc) 90-min after a single injection. (a) ERK1 (p< 0.0001); (b) ERK2 (p< 0.01); (c) CREB (p< 0.05); and (d) AKT (p< 0.05) mRNA levels were significantly increased by ALP when compared to VEH-treated controls. *p< 0.05, **p< 0.01, ****p< 0.0001 compared to VEH-treated controls. Data are represented as fold change normalized to GAPDH (mean ± SEM).

Figure 4.5. Effects of acute exposure to vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on protein phosphorylation within the ventral tegmental area (VTA) 90-minutes after a single injection. Treatment with ALP significantly decreased (a) ERK1, (b) ERK2, (c) CREB, and (d) AKT phosphorylated forms (mean ± SEM) when compared to VEH-treated controls. *p< 0.05, **p< 0.01 compared to VEH-treated controls.

Figure 4.6. Effects of acute exposure to vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on protein phosphorylation within the nucleus accumbens (NAc) 90-minutes after a single injection. Treatment with ALP did not influence phosphorylated forms of (a) ERK1, (b) ERK2, (c) CREB, or (d) AKT (mean ± SEM). p> 0.05 when compared to VEH-treated controls.

Figure 4.7. Effects of repeated exposure to vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) in adolescent male mice on ERK-related gene expression within the

ventral tegmental area (VTA) 24 h after the last injection. (a) ERK1 ($p< 0.05$); (b) ERK2 ($p< 0.05$); (c) CREB ($p< 0.05$); and (d) AKT (p< 0.05) mRNA levels were significantly decreased by ALP when compared to VEH-treated mice. *$p<$ 0.05, **$p< 0.01$ compared to VEH-pretreated controls. Data are represented as fold change normalized to GAPDH (mean ± SEM).

Figure 4.8. Effects of repeated vehicle (VEH) or alprazolam (ALP; 0.05 mg/kg) in adolescent male mice on ERK-related gene expression within the nucleus accumbens (NAc) 24 h after the last injection. (a) ERK1 ($p< 0.05$); (b) ERK2 ($p<$ 0.05); (c) CREB ($p< 0.05$); and (d) AKT ($p< 0.001$) mRNA levels were significantly increased by ALP when compared to VEH-treated controls. *$p<$ 0.05, ***$p< 0.001$ compared to VEH-treated controls. Data are represented as fold change normalized to GAPDH (mean ± SEM).

Figure 4.9. Effects of repeated vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) exposure in adolescent male mice on protein phosphorylation within the ventral tegmental area (VTA) 24 h after the last injection. Treatment with ALP significantly increased (a) ERK1, and (d) AKT phosphorylated forms (mean ± SEM) when compared to the VEH-treated controls. There was no change in (b) ERK2 or (c) CREB phosphorylation (mean ± SEM) when compared to VEH-treated mice. *$p< 0.05$ compared to VEH-treated controls.

Figure 4.10. Effects of repeated vehicle (VEH) or alprazolam (ALP; 0.5 mg/kg) exposure in adolescent male mice on protein phosphorylation within the nucleus accumbens (NAc) 24 h after the last injection. Treatment with ALP significantly

increased (a) ERK1, (b) ERK2, (c) CREB, and (d) AKT phosphorylated forms (mean ± SEM) when compared to VEH-treated controls. *p< 0.05 compared to VEH-pretreated controls.

APPENDIX C

CURRICULUM VITAE

Astrid Mariela Cardona-Acosta

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RESEARCH INTERESTS

My research interest focuses on the study of the causal relationship between early life experiences (stress and use of psychotropic drugs), brain biochemistry and behavior. Currently, my research focus is on assessing the long-term neurobiological consequences of benzodiazepine (Alprazolam) drug exposure during adolescence as a primer for drug abuse/addiction liability in adulthood.

EDUCATION

Texas A&M University, College Station, Texas August 2019 – Present

Behavioral and Cellular Neuroscience

Graduate student

Cumulative GPR: 4.0; Major GPR: 4.0

Texas A&M University, College Station, Texas August 2014 – December 2018

Bachelor of Science in Psychology

Minor in Spanish and Neuroscience

Cumulative GPR: 3.45; Major GPR: 3.45

MANUSCRIPTS IN PREPERATION

1. Sial OK, Gnecco T, **Cardona-Acosta AM**, Vieregg EL, Cardoso E, Parise LF, Bolaños-Guzmán CA (Submitted) Exposure to vicarious social defeat stress and western-style diets during adolescence leads to physiological dysregulation, decreases in reward sensitivity and reduced antidepressant efficacy in adulthood.
2. Parise EM, Parise LF, Sial OK, **Cardona-Acosta AM**, Juarez A, Dipesh C, Ming-Hu H, Nestler EJ, Bolaños-Guzman CA (Submitted August 2020) The enduring resilient phenotype induced by ketamine exposure during adolescence is mediated by the VTA-NAC dopamine pathway.
3. Sial OK, Parise LF, **Cardona-Acosta AM**, Gnecco T, Vieregg EL, Skansi PN, Bolaños-Guzmán CA (to be submitted Summer 2021) Early-life adversity followed by western-style

diet leads to physiological dysregulation, depressive phenotype, decreases in reward sensitivity, and treatment resistance in adulthood.

4. Sial OK, Cardozo E, **Cardona-Acosta AM**, Parise EM, Parise LF, Iniguez S, Bolaños-Guzmán CA (to be submitted Fall 2021) Adolescent exposure to methamphetamine results in long-term physiological and behavioral consequences.

5. Parise LF, Sial OK, **Cardona-Acosta AM**, Vieregg EL, Skansi PN, Bolaños-Guzmán CA (to be submitted May 2021) Extracellular-regulated kinase 2 in the lateral habenula regulates reactivity to stress in adolescent male rats.

PUBLISHED ABSTRACTS/PRESENTATIONS

1. **Cardona-Acosta AM**, Sial OK, Gnecco T, Cardoso E, Bolaños-Guzman CA (2020) Alprazolam exposure during adolescence dysregulates second messenger signaling in the mesolimbic dopamine reward system. National Hispanic Science Network, virtual conference.

2. **Cardona-Acosta AM**, Parise LF, Sial OK, Vieregg EL, Rozofsky JP, Bolaños-Guzmán CA (2020) Alprazolam exposure during adolescence dysregulates reward sensitivity and second messenger signaling in adulthood., Biology, Behavior and Chemistry: Translational research in Addiction Conference, San Antonio, TX.

3. Sial OK, Parise LF, Gnecco T, **Cardona-Acosta AM**, Bolaños-Guzmán CA (2019) Social stress during adolescence followed by western-style diet leads to physiological dysregulation, depressive phenotype, and decreases in reward sensitivity in adulthood. American College of Neuropsychopharmacology, Orlando, FL.

4. Sial OK, Parise LF, Gnecco T, **Cardona-Acosta AM**, Bolaños-Guzmán CA (2019) Social stress during adolescence followed by western-style diet leads to physiological dysregulation, depressive phenotype, and decreases in reward sensitivity in adulthood. National Hispanic Science Network, New Orleans, LA.

5. Sial OK, Parise LF, Skansi PN, **Cardona-Acosta AM**, Vieregg EL, Gnecco T, Bolaños-Guzmán CA (2019) Social stress during adolescence followed by western-style diet leads to physiological dysregulation, depressive phenotype, and decreases in reward sensitivity in adulthood. International Behavioral Neuroscience Society, Cairns, Australia.

6. Sial OK, Parise LF, Skansi PN, **Cardona-Acosta AM**, Vieregg EL, Gnecco T, Bolaños-Guzmán CA (2019) Social stress during adolescence followed by western-style diet leads to physiological dysregulation, depressive phenotype, and decreases in reward sensitivity in adulthood., Biology, Behavior and Chemistry: Translational research in Addiction Conference, San Antonio, TX.

7. Sial OK, Parise LF, Skansi PN, **Cardona-Acosta AM**, Vieregg EL, Gnecco T, Bolaños-Guzmán CA (2018) Social stress during adolescence followed by western-style diet leads to physiological dysregulation, depressive phenotype, and decreases in reward sensitivity in adulthood. Society for Neuroscience, San Diego, CA.

8. **Cardona-Acosta AM**, Parise LF, Sial OK, Vieregg EL, Rozofsky JP, Bolaños-Guzmán CA (2018) Alprazolam exposure during adolescence dysregulates reward sensitivity and second messenger signaling in adulthood. Society for Neuroscience annual meeting, San Diego, CA.

9. **Cardona-Acosta AM,** Alcantara LF, Rozofsky JP, Bolaños-Guzmán CA (2018) Lasting neurobiological consequences of alprazolam exposure in adolescent C57BL/6J mice National Hispanic Science Network annual meeting, Rockville, MD.
10. Parise LF, Sial OK, **Cardona-Acosta AM,** Vieregg EL, Skansi PN, Bolaños-Guzmán CA (2018) Extracellular-regulated kinase 2 in the lateral habenula regulates reactivity to stress in adolescent male rats. Society for Neuroscience annual meeting, San Diego, CA.
11. Sial OK, Parise LF, Skansi PN, **Cardona-Acosta AM**, Vieregg EL, Gnecco T, Bolaños-Guzmán CA (2018) Social stress during adolescence followed by western-style diet leads to physiological dysregulation, depressive phenotype, and decreases in reward sensitivity in adulthood. Society for Neuroscience annual meeting, San Diego, CA.

PROFESSIONAL ASSOCIATIONS

- Society for Neuroscience**,** *Member* May 2018 – Present
- National Hispanic Science Network on Drug Addiction**,** *Member* May 2018 – Present

HONORS & AWARDS

- Scientific Development Travel Fellowship NHSN Fall 2020
- Opioid Overdose Education & Naloxone Administration Certificate Summer 2020
- eIRTI Fellow, University of Southern California Summer 2020
- Behavior, Biology and Chemistry Travel Award Spring 2020
- Professional Development G.R.A.D. Aggies Basic Certificate Spring 2020
- Texas A&M Society for Neuroscience Symposium- 2nd Place Fall 2018
- Scientific Development Travel Fellowship NHSN Fall 2018
- Houston Aggie Moms Scholarship Recipient Fall 2018
- Texas A&M Aggie Spirit Award Spring 2018
- Liberal Arts Dean's list Fall 2015