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DEDICATION

I would like to dedicate this dissertation to my family and friends who have supported me through this long journey.

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ABSTRACT

Zolpidem and benzodiazepines (BZs) potentiate the inhibitory action of gamma-Aminobutyric acid (GABA) by allosterically binding to $GABA_A$ receptors ($GABA_AR$). Prolonged use of $GABA_AR$ positive allosteric modulators (PAM) can lead to behavioral tolerance, the diminished response to the same drug dose with repeated use, and withdrawal, a group of symptoms that occur due to abrupt end of drug treatment. Zolpidem is a short-acting, non-BZ $GABA_AR$ PAM whose potential for tolerance and withdrawal is unclear. Zolpidem demonstrates sedative efficacy similar to BZs and has become a main treatment of insomnia in lieu of BZs. Zolpidem replaced BZs due to lower incidences of tolerance and withdrawal after prolonged treatment and discontinuation. Despite reported lower incidences, some studies find the occurrence of tolerance and withdrawal similar between zolpidem and BZs. Tolerance and withdrawal symptoms are likely caused by drug-induced neuroadaptive changes in central nervous system (CNS) functioning, and these alterations may be similar between zolpidem and BZ. Past rodent research suggests that long term use of zolpidem and BZs may produce alterations in normal inhibitory GABAergic and excitatory glutamatergic functioning in the cortex, hippocampus, amygdala, and PFC and that these alterations may underlie sedative tolerance and withdrawal symptoms.

The purpose of this project was to examine the molecular mechanisms involved in the tolerance cross-tolerance, and withdrawal of zolpidem and diazepam in C57/BL6J mice after different treatment durations. Elucidating the mechanisms behind zolpidem tolerance and withdrawal is necessary due to the ongoing usage of subunit specific $GABA_AR$ PAMs and, to a broader extent, an understanding of $GABA_ARs$ themselves.

In Study 1, we measured sedative tolerance, cross-tolerance, and $GABA_AR$ associated mRNA levels in 4 regions of interest (ROI; the cortex, prefrontal cortex (PFC), hippocampus, and amygdala) after 3 days of intraperitoneal (i.p.) injections of diazepam and zolpidem in comparison to vehicle. We expected that this "short-term" exposure duration to diazepam and zolpidem would not result is tolerance, cross-tolerance, or changes in mRNA levels. Study 2 examined the same measures as in Study 1, in addition to AMPAR subunits, NDMAR subunits mRNA levels in the ROI, and total, surface, and intracellular $GABA_AR$ subunits protein expression due to 7 days of i.p. injections of diazepam and zolpidem compared to vehicle. Based on previous research both groups should become tolerant to zolpidem's sedative effects and show decreases of $GABA_AR$ subunits and increases in NMDAR subunits in the ROI. It is also expected that there will be decreases in total $\alpha 1$ and $\gamma 2$ in the cortex, a decrease of surface $\alpha 1$ in the cortex, and increases in GluR1 in the hippocampus after zolpidem and diazepam treatment. Study 3 measured the same measures as in Study 1 due to 30 days of i.p. injections of diazepam and zolpidem compared to vehicle. It was expected that both groups would become tolerant to zolpidem's sedative effects and show decreases of $GABA_AR$ subunits in the cortex, PFC, and hippocampus.

The development of sedative tolerance and cross-tolerance to the locomotor impairing effects (measure of sedation) of zolpidem was measured by activity in the open field. Spontaneous withdrawal was also measured by activity and anxiety like behavior in the open field. Flumazenil-induced withdrawal was measured by anxiety-like behaviors in the elevated plus maze (EPM), activity, and anxiety like behavior in the open field. Messenger RNA levels were measured by quantitative real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and protein expression was measured by western blot. The surface and intracellular proteins were separated using bissulfosuccinimidyl suberate (BS3) cross-linking.

Three days of diazepam but not zolpidem resulted in cross-tolerance to zolpidem in Study 1. Three days of zolpidem but not diazepam resulted in a decrease in the mRNA level of the $\alpha 5$ subunit in the hippocampus in Study 1. After 7 days of zolpidem or diazepam, mice were tolerant and cross-tolerant to zolpidem's sedative effects. Spontaneous withdrawal resulted in anxiety-like behavior and decreased locomotor activity. Flumazenil did induce a robust withdrawal syndrome as measured in the EPM or open field. Seven days of zolpidem and diazepam caused significant decreases in the mRNA expression of $\alpha 1$, $\alpha 3$, $\beta 2$, and δ GABA$_A$R subunits in the cortex. Diazepam groups had significant decreases in the mRNA expression of $\alpha 4$, $\beta 1$, $\gamma 2$ subunits, GAT, and gephyrin in the cortex and significant decreases of $\alpha 5$- and $\beta 3$-GABA$_A$R subunits, and the GluN2A subunit in the hippocampus. Seven days of zolpidem resulted in a decrease in total $\alpha 2$ subunit protein level and 7 days of diazepam decreased total $\gamma 2$ subunit protein levels. Thirty days of diazepam but not zolpidem resulted in cross-tolerance to zolpidem in Study 3. Thirty days of zolpidem but not diazepam resulted in a decrease in the mRNA levels of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$, and $\gamma 2$ subunits in the PFC.

These results suggest that there is a window of time in which sedative tolerance to zolpidem is observed. The lack of zolpidem tolerance and minimal mRNA changes due to 3 days of zolpidem treatment may be due to its pharmacokinetic profile, zolpidem may not be in the system long enough to cause any changes. This may mean that sedative tolerance gradually develops and reaches detectable levels at later time points. Sedative tolerance and cross-tolerance to zolpidem is in line with other studies, however the spontaneous withdrawal is unique. Anxiety- like behavior and decreased activity were observed in our studies unlike other studies. The anxiety-like behavior is a common symptom of BZ withdrawal however the decrease in activity that was observed is not. It is unknown why this occurs though it may be due to a carryover sedative effect or more likely a placebo effect. Few studies have examined changes in protein levels. Study 2 found decreased protein expression of $\alpha 2$ and $\gamma 2$ in the cortex due to zolpidem and diazepam respectively, indicate GABA$_A$Rs containing those subunits are associated with tolerance and cross-tolerance to zolpidem's sedative effects. This implies that the sedative effects of zolpidem is mediated by $\alpha 1$-GABA$_A$Rs, the development of tolerance is mediated by $\alpha 2$-GABA$_A$Rs due to zolpidem binding to both. There was also a decrease in the intracellular $\alpha 1$ subunit which may indicate degradation of an intracellular pool of $\alpha 1$ subunits or $\alpha 1$-GABA$_A$Rs. The lack of zolpidem tolerance due to 30 days of zolpidem may be due to increased metabolism displayed as an increase in CYP3A enzymes. It is unclear what effect the decrease in GABA$_A$R subunits in the PFC due to 30 days of

zolpidem implicate. These may affect tolerance to zolpidem's other effects such as amnesia.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACSF   Artificial cerebrospinal fluid

αx-GABA$_A$Rs   αx-containing GABA$_A$Rs

AMPA   α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AUC   Area under the curve

BDNF   Brain-derived neurotrophic factor

BS3   Bissulfosuccinimidyl suberate

BLA   Basolateral amygdala

BZ   Benzodiazepine

CDP   Chlordiazepoxide

CeA   Central amygdala

cDNA   Complementary DNA

Cmax   Maximum serum concentration

CNS   Central nervous system

CPP   Conditioned place preference

Ct   Threshold value

CYP450   Cytochrome P450

DEA   Drug Enforcement Administration

DSM-V   Diagnostic and Statistical Manual of Mental Disorders, 5th Edition

ED50   Median effective dose

EMG   Electromyography

EPM   Elevated plus maze.

FDA   Food and Drug Administration

GABA   Gamma aminobutyric acid

GABA$_A$R   Gamma aminobutyric A Receptors

GABARAP   GABA receptor-associated protein

GAT-1   GABA transporter 1

GluN2A   NMDAR 2A

ICER   Inducible cAMP early repressor

ICSS   Intracranial self-stimulation

I.P.   Intraperitoneal

I.V.   Intravenously

KCC2   K-Cl cotransporter 2

KO   Knockout mouse

LC   Locus caeruleus

LORR   Loss of righting reflex

MeCP2   Methyl-CPG binding protein 2

mIPSC   Miniature inhibitory post-synaptic currents

mGluR   Metabotropic glutamate receptors

MnPO   Median preoptic nucleus

nAChR   Nicotinic acetylcholine receptors

NKCC2   Na-K-Cl cotransporter 2

NMDA   N-methyl-D-aspartate

NMDAR   N-methyl-D-aspartate receptor

NREM	Non-rapid eye movement
PAS	Precipitated withdrawal score
PFC	Prefrontal Cortex
PM-DAT	Plus maze discriminative avoidance task
PPN	Pedunculopontine nucleus
PTZ	Pentylenetetrazol
qRT-PCR	Quantitative real-time polymerase chain reaction
SICI	Short interval intracortical inhibition
SNr	Substantia nigra pars reticulate
SWS	Slow wave sleep
$T_{1/2}$	Half-life
T_{max}	Time of peak concentration
V_d	Volume of distribution
vLPO	Ventrolateral preoptic area

CHAPTER 1.   INSOMNIA TREATMENT ISSUES

Clinically, the sleep disorder insomnia is defined as difficulty initiating or maintaining sleep [1]. Treatment of insomnia is of particular importance because the estimated prevalence within the United States (U.S.) adult population is 10% to 30%. Insomnia is also often comorbid with the psychiatric disorders anxiety and depression [2, 3]. Until recently, traditional benzodiazepines (BZs) diazepam, alprazolam, and clonazepam were the first-line clinical medications for insomnia. However, the unwanted side effects of daytime drowsiness, dizziness, confusion and nausea often resulted from BZ use. In addition to unwanted side-effects, long term use of BZs could cause tolerance (defined as the need to markedly increase the amount of the substance to achieve the desired effect) and withdrawal symptoms (anxiety, insomnia, and seizures that develop after cessation of long term BZ treatment) [4, 5]. Tolerance and withdrawal are commonly associated with dependence (a desire often strong, sometimes overpowering to take psychoactive drugs) and addiction (a compulsive drive to take rewarding stimuli regardless of risk) [1, 4, 6-8]. Dependence and withdrawal are serious issues that can impede the treatment of insomnia and research into safer alternatives has been ongoing.

Over the past decade, the imidazopyridine zolpidem (brand name Ambien) has replaced BZs as the most commonly prescribed drug for patients suffering from insomnia in the U.S. Both zolpidem and BZs enhance the actions of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) at $GABA_A$ receptors ($GABA_ARs$). BZs exhibit non-preferential binding to $\alpha 1$-, $\alpha 2$-, $\alpha 3$, and $\alpha 5$-containing $GABA_ARs$ ($\alpha 1$-, $\alpha 2$-, $\alpha 3$-, and $\alpha 5$-$GABA_ARs$). Zolpidem, however, exhibits preferential binding and efficacy at $\alpha 1$-$GABA_ARs$ alone and produces therapeutic sedative effects. Zolpidem is purportedly safer and has fewer side effects than traditionally used BZs when used properly. However, recent studies have shown that zolpidem may be more similar to BZs in terms of behavioral tolerance, withdrawal, abuse potential, and physical dependence liability. In fact, the frequency of zolpidem abuse and dependence is similar to that of BZs within the population that has a prior history of BZ and alcohol abuse.

There has been conflicting evidence concerning zolpidem's ability to induce behavioral tolerance and withdrawal in animal models after various treatment doses and durations. Also unclear is the degree to which repeated administration of zolpidem can produce neuroadaptive changes in GABAergic and glutamatergic function that may underlie tolerance and withdrawal. Initial rodent studies indicated little evidence of sedative tolerance to zolpidem, however more recent studies present evidence that tolerance can develop to zolpidem's sedative effects. It is known that diazepam tolerance and withdrawal can been detected as early as 1 day and as late as 30 days. It is hypothesized that BZ tolerance and withdrawal may be caused by overlapping neuroadaptive changes. The purpose of this project is to examine tolerance, cross-tolerance, withdrawal, and molecular alterations in zolpidem and diazepam-treated C57/BL6J mice. Elucidating evidence of tolerance, withdrawal, and neuroadaptive changes after repeated zolpidem use is necessary due to the ongoing usage of subunit specific $GABA_AR$ positive allosteric modulators (PAMs) like zolpidem and, to a broader

extent, an understanding of GABA$_A$Rs. Furthermore, given the use of genetically engineered animal models for understanding GABA$_A$R function, knowledge about the mechanisms underlying tolerance in common background strains will be helpful in interpreting knockout and transgenic mouse studies that attempt to identify genetic factors that can play a role in drug abuse susceptibility.

CHAPTER 2.   LITERATURE REVIEW*

History of Hypnotics

The earliest known sedative-hypnotics (drugs used to induce or maintain sleep) were alcohol and opium. In the 17th century, the combination of opium and alcohol was packaged by the name laudanum for the treatment of insomnia and other medical conditions. Laudanum was discontinued due to high incidences of dependence, addiction, and death [9]. Other drugs would be developed to treat insomnia. Of those treatments barbiturates were of prominent use. Barbiturates were developed in the late 1800s and used throughout the 1950s. The first barbiturates were synthesized in 1864 by Adolph Von Baeyer and refined by Edouard Grimaux [10]. The first barbiturate approved for clinical use was diethyl-barbituric acid, known by barbital. Barbital was synthesized in 1881 by Max Conrad and M. Guthzeit and marketed to consumers as a sedative-hypnotic in 1904 [10]. After barbital, over 2500 agents based on barbital were created, including phenobarbital, occasionally used to treat insomnia to this day. The use of barbiturates as sedative-hypnotics peaked in the 1930s and 1940s, but usage declined due to high occurrences of addiction and death by overdose.

BZs replaced barbiturates as hypnotics because of their lower incidences of addiction and overdose compared to barbiturates. The safer alternatives to barbiturates, BZs were created by Dr. Leo Sternbach in the 1930s'. The first BZ, Ro 5-0690 or chlordiazepoxide (CDP), had hypnotic, sedative, and anticonvulsant effects. CDP was marketed for anxiety in 1960 followed by diazepam, marketed for multiple uses including the treatment for insomnia in 1963. As more BZs were developed, research into BZ function found that BZs are positive modulators of GABA$_A$ receptors (GABA$_A$Rs) like barbiturates and bind to $\omega 1$ ($\alpha 1$-containing) and $\omega 2$ ($\alpha 2$-, $\alpha 3$-, $\alpha 5$-containing) GABA$_A$RS [11, 12]. It was thought that preferential binding to this site made BZs safer and resulted in the absence of the abuse and addiction patients developed with barbiturates.

Unfortunately BZs displayed the same propensity for dependence and addiction as barbiturates [5, 13, 14]. Long term BZ use in humans, defined as greater than one year, could induce tolerance and withdrawal symptoms. The development of tolerance and withdrawal symptoms are more likely to occur in patients with a history of previous BZ, opioid, or ethanol abuse and patients with anxiety or depression [15]. The development of safer alternatives that have low to no incidences of tolerance and withdrawal effects have been ongoing and have led to the recent development of sedative-hypnotic non-BZs that should have a reduced capacity for tolerance.

Zolpidem as an Alternative

The ongoing issue of dependence to sedative-hypnotics (e.g. barbiturate and BZs) has motivated research into safer alternatives. These alternatives include zolpidem, zopiclone, zaleplon, and eszopiclone, non-BZs of distinct structural, pharmacokinetic and pharmacodynamic properties. Zolpidem was synthesized in 1978 by Jean-Pierre Kaplan and George Pascal at Synthélabo Laboratories and patented in the U.S. in 1983. The pharmaceutical company Sanofi marketed zolpidem as a sleep aid in the European market in 1988 and the U.S. market in 1992. Compared to BZs, zolpidem is thought to be a superior sedative-hypnotic due to its preferential binding and efficacy of $GABA_A$Rs containing the $\alpha1$ subunit ($\alpha1$-$GABA_A$R) and lower affinity to $GABA_A$Rs containing $\alpha2$, $\alpha3$, and $\alpha5$ subunits. In contrast, BZs have similar binding and efficacy at $\alpha1$-, $\alpha2$-, $\alpha3$-, and $\alpha5$-$GABA_A$Rs [17, 18]. Zolpidem's unique pharmacology results in fast acting sedative-hypnotic effects that are therapeutically advantageous for inducing sleep [4]. Past research has revealed that positive modulation of $\alpha1$-$GABA_A$Rs induces sedation, sleep, amnesia, and anticonvulsant effects [18, 19]. Zolpidem is currently one of the most widely used sedative-hypnotics in the U.S., and under the brand name Ambien©, has become a popular sedative-hypnotic with yearly sales of more than 2 billion dollars in the U.S. [20, 21]. However some studies suggest the propensity for tolerance and withdrawal symptoms to zolpidem is similar to BZs [22, 23].

Limits to Current Alternatives

The preferential affinity and binding of zolpidem to $\alpha1$-$GABA_A$Rs was believed to decrease the development of tolerance and withdrawal symptoms produced by long term use. To date, there have been conflicting evidence of development of tolerance and withdrawal to zolpidem- in both human and rodent studies [24-27]. Early evidence in rodents suggested that chronic zolpidem administration at relatively high dose levels (e.g. 60 mg/kg) does not induce tolerance [24]. However, recent studies suggest lower dose levels of zolpidem (e.g. 1.25-10 mg/kg) in rodents can induce tolerance [24, 28, 29]. Compared to humans, clinical studies do not find evidence of tolerance and withdrawal at clinical dose levels (e.g. 5-10 mg/kg), but case studies do find evidence of tolerance and withdrawal in humans at large dose levels (e.g.200 mg/kg) of zolpidem [27, 30]. Thus, numerous studies have focused on how zolpidem, BZs, other $GABA_A$R modulators affect GABAergic transmission might lead to tolerance and withdrawal.

Introduction to Zolpidem

Under the brand name Ambien©, zolpidem has largely replaced BZs for the treatment of insomnia [20]. The chemical name for zolpidem is N, N, 6-trimethyl-2-(4-methylphenyl) imidazo [1, 2-a] pyridine-3-acetamide hemitartrate and is an imidazopyridine, structurally distinct from BZs (**Figure 2-1**) [31, 32]. Zolpidem is widely available in most countries and known commercially by many names (Ambien©, Intermezzo©, Stilnox©, and Zonadin©). Zolpidem is defined by the Drug Enforcement
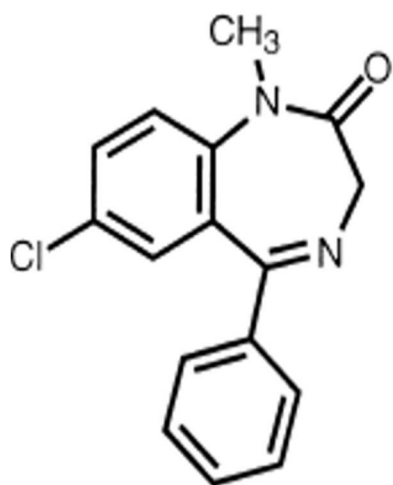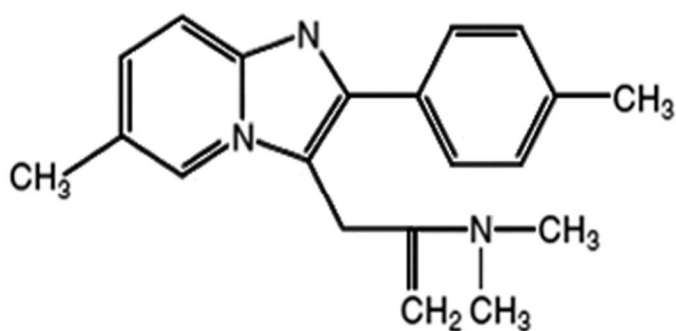
Figure 2-1. **Comparison of zolpidem and diazepam structures**
Diazepam as a BZ is a two ring heterocyclic compound with a benzene ring fused to a
diazepine ring, whereas zolpidem is an imidazopyridine which have an imidazole ring
fused to a pyridine ring. Modified with permission. Drover, D.R., *Comparative
pharmacokinetics and pharmacodynamics of short-acting hypnosedatives: zaleplon,
zolpidem and zopiclone.* Clin Pharmacokinet, 2004. **43**(4): p. 227-38. and Richards, J.G.
and Martin, J.R., *Binding profiles and physical dependence liabilities of selected
benzodiazepine receptor ligands.* Brain Research Bulletin. 45(4): p.381-387 [31, 32].

Administration (DEA) as a schedule IV drug (a drug with low potential and risk of abuse and dependence). The hemitartrate salt form of zolpidem is commonly taken as a solid pill and is commonly ingested orally, but can be administered intravenously (i.v.). Pharmacological studies indicate that zolpidem has different pharmacokinetic and pharmacodynamics properties than BZs that contribute to its efficacy as a sedative-hypnotic.

Pharmacokinetics

Preclinical Studies

Pharmacokinetics describes the absorption, distribution, metabolism, and elimination of a drug from the body. The pharmacokinetics of a drug determines the onset, duration, and intensity of a drug's effect. In rodents, a 5-10 mg dose of zolpidem has a peak concentration (C_{max}) of about 2341 ng/ml and a time of peak concentration (T_{max}) of 5-15 min [33]. Zolpidem has a volume of distribution (V_d) of 104.9 ml, no active metabolites, and has a half-life ($t_{1/2}$) of approximately 20-90 min [33-35]. In rodents, a 5-20 mg dose of diazepam has a C_{max} of about 297 ng/ml and a T_{max} of 72 min [36]. Diazepam has a V_d of 920 ml and a $t_{1/2}$ of 52 minutes [37]. Diazepam has an active metabolite, desmethyldiazepam, whose half-life is 11.1 hours and has sedative effects [37]. In rodents, zolpidem has a higher peak concentration and reaches it in a shorter amount of time compared to diazepam. Zolpidem also has a lower volume of distribution and a lower $t_{1/2}$ than diazepam. In contrast to diazepam, the degradation of zolpidem does not lead to the accumulation of active metabolites that can sustain drug action [31, 33, 38, 39].

Clinical Studies

In humans, a 10 mg dose of zolpidem or diazepam initiates sleep [40-42]. A 10 mg dose of zolpidem has a T_{max} between 1.2-1.6 hours similar. A 10 mg dose of diazepam has a T_{max} between 1.3-1.4 hours, similar to zolpidem. Zolpidem has a C_{max} of 120-140 ng/ml while diazepam at the same dose has a C_{max} of 253-885 ng/ml [31, 43-45]. Zolpidem has a bioavailability (fraction of unchanged drug that reaches the systemic circulation) of about 70% after first pass hepatic metabolism with 92% bound to protein [31]. Diazepam has a bioavailability of about 100% with around 97% bound to protein [31, 43, 46].

The main cytochrome P450 (CYP450) enzyme, isozymes responsible for the biotransformation of drugs, that metabolize zolpidem into inactive metabolites is CYP3A4 accounting for about 60% of clearance, the amount of drug removed from the body per unit time [47]. The other CYP450s that metabolize zolpidem are CYP2C9, accounting for 22%; CYP1A2, accounting for 14%; and CYP2D6/CYP2C19, accounting for < 3% of clearance [47]. Diazepam is metabolized by CYP2C9, 2C19, 2B6, 3A4, 3A5,

3A4 and 2C19 into three active metabolites, desmethyldiazepam, temazepam, and oxazepam [31, 43, 44]. Zolpidem has an $t_{1/2}$ of about 3 hours in comparison to diazepam with a long $t_{1/2}$ (~100 hours due to active metabolites) [48]. Zolpidem has a lower bioavailability and a shorter $t_{1/2}$ than diazepam. In comparison to diazepam's many active metabolites, zolpidem does not have active metabolites.

Recent evidence suggest that gender and age influences the pharmacokinetics of zolpidem. There is evidence that women have a lower rate of clearance of zolpidem and have higher blood plasma concentrations of zolpidem than men [49-51]. This may be due to a tempering effect of testosterone as elderly men also have a lower clearance that is correlated with reduced testosterone (a CYP3A4 substrate) in men [52]. These finding prompted the U.S. Food and Drug Administration (FDA) to recommend lowering the dose of zolpidem for women, as the dose for the elderly was already reduced [53]

Pharmacodynamics

Preclinical Studies

Pharmacodynamics is the study of the biochemical and physiological effects of drugs on the body and the relationship between drug concentration and effect. These effects include receptor affinity, receptor efficacy, and drug effect on behavior.

At clinical doses (5-10 mg), zolpidem has preferential affinity for α1-GABA$_A$Rs, lower affinity for α2- and α3-GABA$_A$Rs, and no affinity for α5-GABA$_A$Rs [34, 54]. It has been shown that increasing the concentration of zolpidem does cause affinity to shift towards α2- and α3-GABA$_A$Rs [55, 56]. Diazepam (10-20 mg) in comparison, has similar affinity at α1-, α2-, α3-, and α5-GABA$_A$Rs [57]. Multiple *in-vitro* and *in-vivo* studies have discerned the effects of both drugs on GABA$_A$R functioning. The amount of zolpidem required to potentiate α1-GABA$_A$Rs is lower than to potentiate α2-, α3-, and α5-GABA$_A$Rs [17, 57-60]. Zolpidem at high doses (> 10 nM) enhances the amplitude of miniature inhibitory post-synaptic currents (mIPSCs), beta wave frequency (low amplitude beta waves associated with increased brain activity), and mIPSC duration in the cortex [61, 62]. At low doses (< 10 nM), zolpidem decreases the beta wave frequency [62]. Diazepam at concentrations of 10 nM-1 μM potentiated Cl$^-$ currents and enhanced IPSCs in HEK cells, the hippocampus, and the basolateral amygdala (BLA) and central amygdala (CeA) [63-65].

The activation or positive modulation of GABA$_A$Rs generally depresses motor behavior. Zolpidem and diazepam at doses of 1-100mg/kg cause reductions in measures of locomotor activity, ataxia, muscle relaxant properties, and loss of righting reflex (LORR) [66-72]. In addition to depressing motor behavior, zolpidem and diazepam increases the threshold of pentylenetetrazole, electroconvulsive, and isoniazid induced convulsions [69, 70]. Zolpidem and diazepam's effects on sleep structure and associated

behaviors show some similarities, both increase slow wave sleep (SWS) and decrease body temperature in rodents, though diazepam causes tachycardia and hypertension [73].

Knockout (KO) mice are mice that are missing a particular GABA$_A$R subunit. KO mice have been used to discover how different GABA$_A$R subtypes modulate behaviors like sedation and hypnosis. KO mice that have the α1 subunit deleted, show a reduced hypnotic effect compared to wild-type (no deletion of α1 subunit) counterparts when given zolpidem though they show increased hypnotic effect when given diazepam [18]. The α1-GABA$_A$Rs mainly mediate zolpidem's hypnotic effects while α2-GABA$_A$Rs are responsible for diazepam's hypnotic effects [18, 74]. However an issue with KO mice is that results gained from them may be due to compensatory increases in other GABA$_A$Rs. Another tool used to examine how different GABA$_A$R subtypes modulate behavior are point mutation mice. The mice have a single point mutation in the α1-subunit protein, allowing for the receptor to be BZ and zolpidem-insensitive. Another benefit is that unlike KO mice, there are no compensatory increases in α2, α3-, α5-GABA$_A$Rs that may account for potential effects. Through the use of point mutation mice it has been found that α1-GABA$_A$Rs mediate the locomotor impairing effect (measure of sedation) and anticonvulsant effects of zolpidem and the locomotor impairing effects of diazepam [71]. This indicates α1-GABA$_A$Rs mediate zolpidem's sedative and hypnotic effects and partially mediate diazepam's sedative effects as well [71]. Since α1-GABA$_A$Rs modulation is important for sedation and hypnosis, it is possible that it also plays a part in the development of tolerance and withdrawal to these behaviors.

Clinical Studies

As in rodents, zolpidem has a greater binding affinity for α1-GABA$_A$Rs [75, 76]. Diazepam shows equal affinity for α1-, α2-, α3-, and α5-GABA$_A$Rs with human recombinant GABA$_A$Rs [17, 77, 78]. Both diazepam and zolpidem positively modulate α1-, α2-, and α3-GABA$_A$Rs with maximum potentiation > 70% [78]. Zolpidem has a GABA$_A$R occupancy in humans of about 27% in the neocortex indicating that zolpidem is effective at low occupancy [75].

Zolpidem at clinical doses (5 and 10 mg) exerts a hypnotic effect by shortening the latency to sleep, maintaining non-rapid eye movement (NREM) sleep, and increasing sleep duration in humans [31, 79]. Zolpidem is highly effective in initiating sleep, but is not as effective in maintaining sleep. In humans zolpidem induces sedation, impairs memory and psychomotor performance [49, 50, 80, 81]. Zolpidem seems to have increased adverse effects when compared to hypnotic alternatives like zaleplon, but lower adverse effects than diazepam [80, 82]. Although zolpidem is mainly used as a hypnotic, it also has weak anxiolytic, anticonvulsant, and amnesic effects. In humans zolpidem has weak anticonvulsant activity against pentylenetetrazole (PTZ) and electroshock convulsions in a dose dependent manner [69]. Intraseptal injections of zolpidem impairs spatial working memory and induces retrograde memory deficits [83]. Acute zolpidem injections also impair associative and contextual memory [84-86]. Diazepam but not zolpidem increases inhibition in the human motor cortex despite sedation by both drugs

8

as measured by short interval intracortical inhibition (SICI; a GABA$_A$R mediated inhibition in the motor cortex) [87].

GABA$_A$R Overview

GABAergic System

GABA is the main inhibitory neurotransmitter of the CNS. GABA binds to two different types of receptors, GABA$_A$Rs and GABA$_B$Rs. GABA$_A$Rs are ligand gated chloride (Cl⁻) ion channels (transmembrane channels that facilitate the passage of ions through a membrane when a ligand binds to the receptor) and GABA$_B$Rs are metabotropic receptors (a receptor that acts through secondary messengers). The GABA$_A$R (**Figure 2-2**) facilitates the passage of Cl⁻ ions, hyperpolarizing neuron [88-91]. The GABA$_A$R is heteropentameric (a receptor made up of five subunits) with 19 known subunits. The subunits, α1-6, β1-3, γ1-3, ρ1-3, δ ,ε, θ, and π, can occupy the five positions of the GABA$_A$R [88]. The GABA$_A$R is commonly composed of two α subunits, two β subunits, and a γ subunit. GABA$_A$Rs composed of two α1 subunits, two β2 subunits, and a γ2 subunit represent about 60% of the GABA$_A$Rs in the rodent brain and are widely expressed in the brain [90, 92-94]..

GABA$_A$R Subunit Localization and Distribution

The α1-, α2-, and α3-GABA$_A$Rs are often located directly opposite the presynaptic terminal and are referred to as synaptic receptors based on this localization. Synaptic GABA$_A$Rs modulate phasic (transient/fast) inhibition (< 500 μsecs) in the presence of high concentrations (> 50 uM) of GABA released into the synapse. The α4-, α5, and α6-GABA$_A$Rs are mostly located outside the synapse and are referred to as extra-synaptic receptors based on this localization. Extra-synaptic GABA$_A$Rs modulate tonic (persistent/slow) inhibition (> 500 μsecs) in the presence of low concentrations (< 50 uM) of GABA that diffuse outside the synapse (**Figure 2-3**) [92, 95].

The distribution of GABA$_A$R subunit mRNAs and proteins vary from region to region in the mouse brain **(Figure 2-4)** [50, 96, 97]. The distribution of GABA$_A$R subunits in the mouse brain show that the cortex and hippocampus contain most of the subunits with the exception of α6 and γ1 [93]. The amygdala contains a majority of the subunits with the exception of α6 [93]. The cortex has high levels of α1, α2, α3, β2, β3, and γ2 subunits. The hippocampus has high levels of α1, and β2 subunits, and very high levels of α2, α5, β3, and γ2 subunits. The amygdala has high levels of α2, α3, β3, and γ2 subunits with high levels of the α1-subunit mRNA expression in the BLA [94].
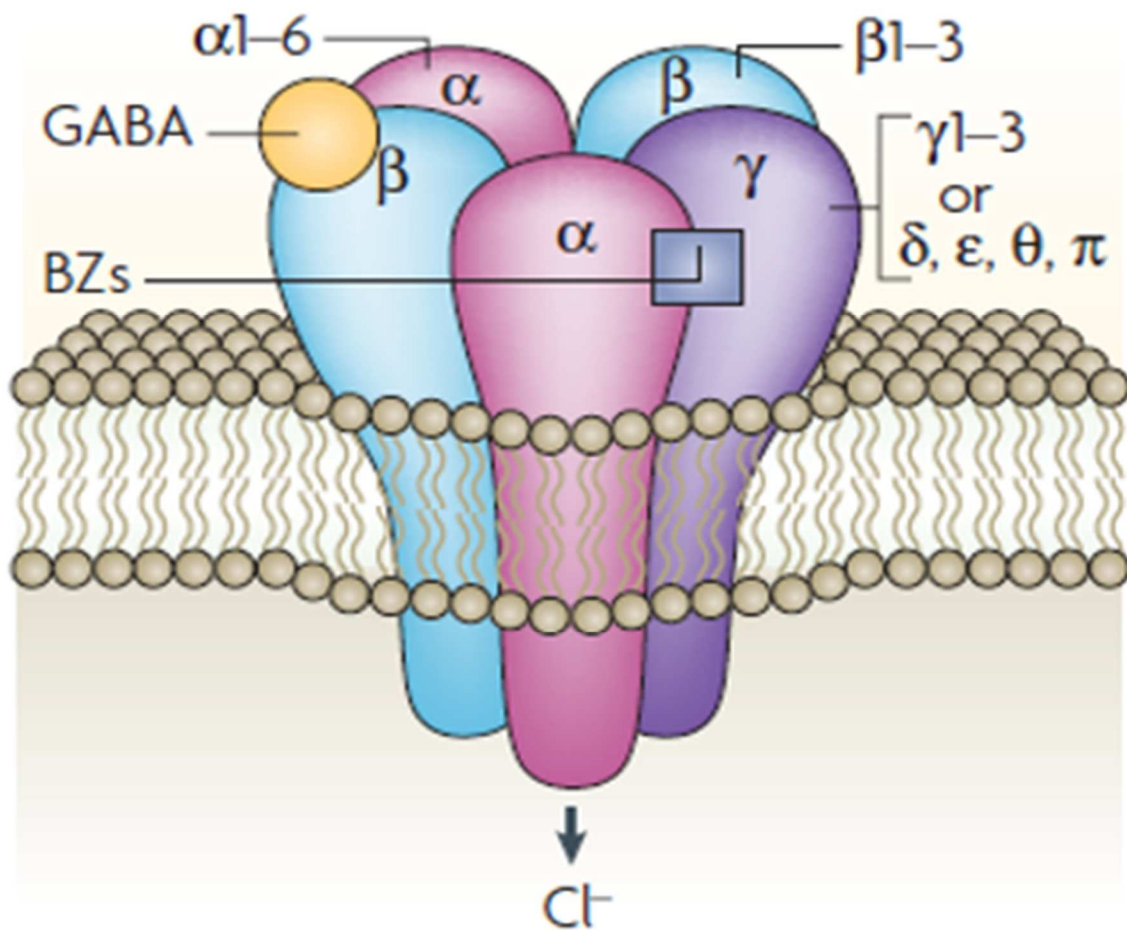
Figure 2-2.   Example of a typical GABA$_A$R complex

The GABA$_A$R is an ion channel that allows for entry of the Cl ion composed of 5 subunits. GABA$_A$Rs are usually composed of 2α subunits, 2β subunits and a γ subunit with the interface between α and β subunits contain a GABA binding site and the interface of α and γ subunits contains the BZ/non-BZ binding site. Reprinted with permission. Jacob, T.C., S.J. Moss, and R. Jurd, *GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition.* Nat Rev Neurosci, 2008. **9**(5): p. 331-43 [91].
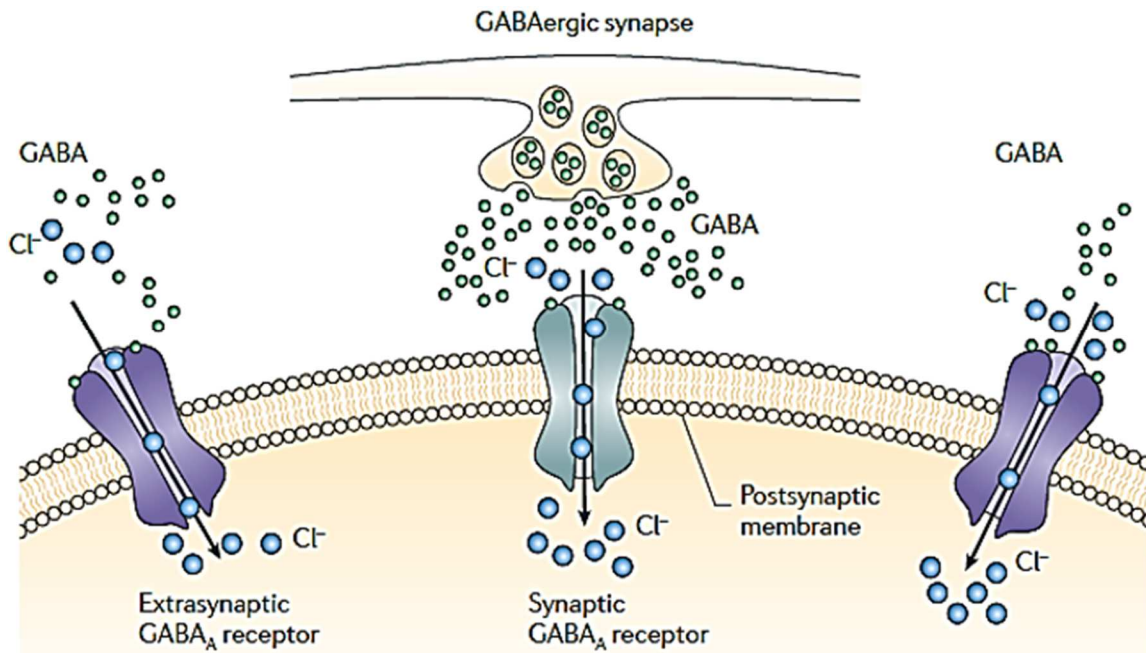
Figure 2-3.   Example of the localization of synaptic and extrasynaptic GABA$_A$R opposite the presynaptic cleft
At the GABAergic synapse presynaptic vesicles release GABA onto synaptic and extrasynaptic GABA$_A$ receptors. Reprinted with permission Rudolph, U. and F. Knoflach, *Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes.* Nat Rev Drug Discov, 2011. **10**(9): p. 685-97 [90].
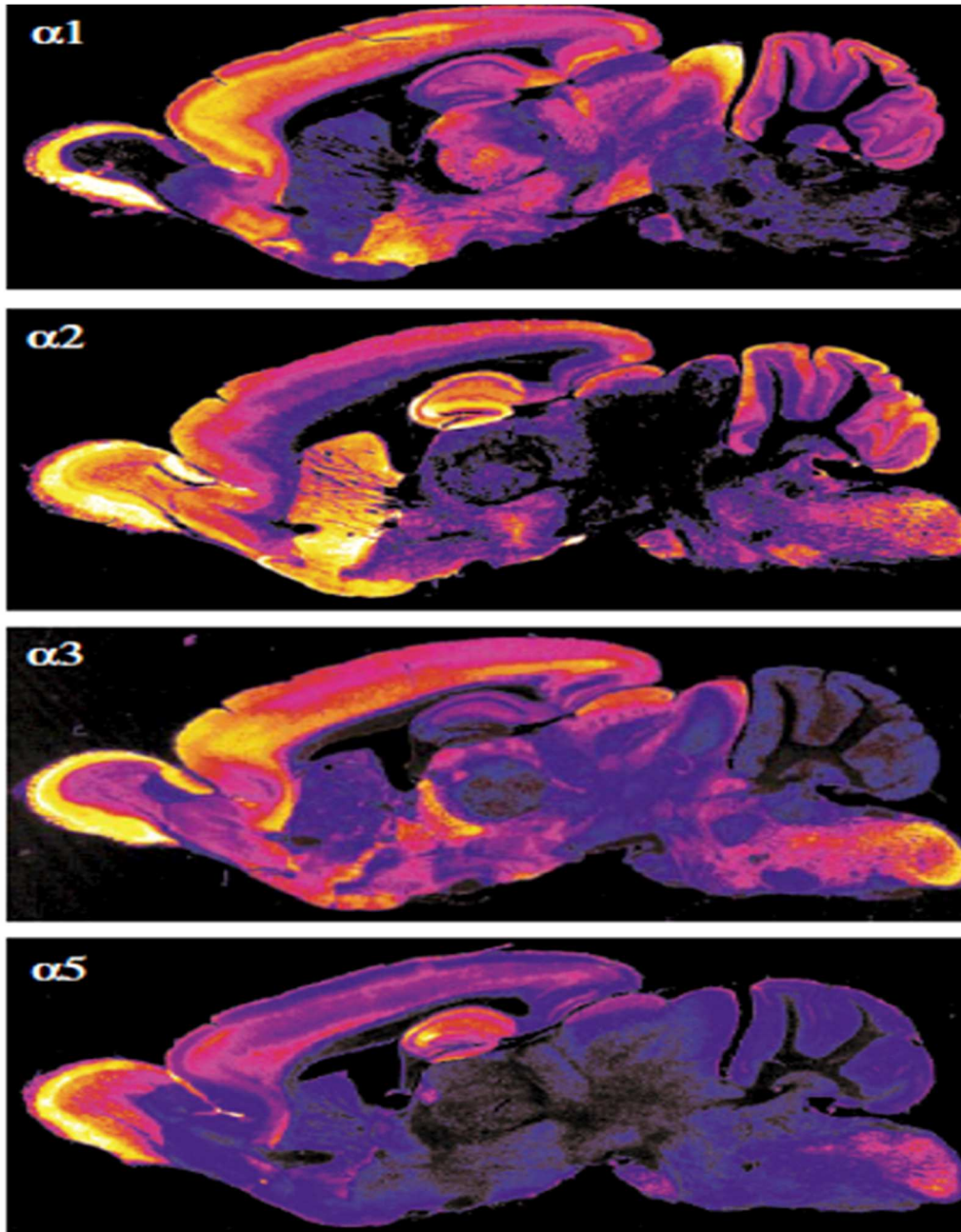
Figure 2-4.   Example of differential regional distribution of α subunits proteins in rat brain sensitive to BZs

The α1 subunit is expressed in most brain regions with high expression in the cortex. The α2 subunit has high expression in the hippocampus and amygdala. The α3 subunit has high expression in the amygdala, brain stem, basal forebrain and the thalamus. The α5 subunit is mainly expressed in the hippocampus. Reprinted with permission. Rudolph, U., F. Crestani, and H. Mohler, *GABA(A) receptor subtypes: dissecting their pharmacological functions.* Trends Pharmacol Sci, 2001. **22**(4): p. 188-94 [97].

GABA$_A$R Subtypes and Modulatory Effects

GABA$_A$Rs subtypes modulate various behaviors. The positive modulation of α1-GABA$_A$Rs mainly cause sedation, hypnosis, partial anticonvulsant effects, anterograde amnesia and addiction [71, 90, 98]. The α2- and α3-GABA$_A$Rs mainly mediate anxiety and myorelaxation [98]. Most BZs bind to α1-, α2-, α3-, and α5-GABA$_A$Rs, while zolpidem binds preferentially to α1- GABA$_A$Rs, has some affinity for the α2- and α3-GABA$_A$Rs, and no affinity to the α5-GABA$_A$Rs [99].

Contributions of GABA and Glutamate mRNA and Protein Alterations in the Cortex, PFC, Hippocampus, and Amygdala to Sedative Tolerance and Withdrawal Symptoms

Although it is known that both BZ and zolpidem can induce tolerance in mice, it is unclear whether specific alterations in brain regions mediate tolerance because the alterations after repeated BZ and zolpidem on brain regions are inconsistent. While BZs and zolpidem affect many brain regions, this project is focusing on the cortex, which modulates locomotion as well as the PFC, hippocampus, and amygdala. As stated above the cortex, hippocampus, amygdala, and PFC have high expressions of BZ-sensitive GABA$_A$Rs which may play a role in tolerance and withdrawal, particularly withdrawal-induced anxiety [87, 94, 100, 101]. Symptoms of tolerance and withdrawal have also been associated with changes in glutamatergic receptor systems [102-104]. At present, there are few studies that measure the effects of repeated zolpidem on GABA$_A$R and glutamatergic receptors, however studies that have examined the effects of repeated BZ can shed light on the potential changes after zolpidem use.

There are inconsistencies in the changes caused by BZs and zolpidem treatment. One consistent finding is that repeated BZ and zolpidem reduced the expression of α1 GABA$_A$R subunit mRNA and protein and increased the expression of α4 GABA$_A$R subunit mRNA and protein in the cortex, although some studies do not see these changes [105-114]. However some studies have found that in the rat cortex after diazepam treatment (7 and 14 days) there were no significant differences in α1-subunit mRNA, but increases in α3-, α4-, and α5-subunit mRNA [107, 111, 115, 116]. There were no changes in the β2-subunit but there was a decrease in γ2- subunit mRNA in the cortex [111]. Repeated BZ and zolpidem also reduce α1 mRNA and protein expression in the PFC [105]. Diazepam treatment caused an increase in the α5-subunit in the PFC [105]. In the hippocampus, repeated diazepam has caused a decrease in in α1- and α5-subunit mRNA [105, 107, 113, 116]. In the cortex, diazepam withdrawal also reportedly caused an increase in GluR1, GluN1, and GluN2B subunit mRNA [104, 117]. In the hippocampus, BZ withdrawal induced increases in GluR1, GluN1, and GluN2B subunit mRNA and protein and increased AMPAR responses [102, 118, 119]. In addition, diazepam withdrawal caused a decrease in GluR1 and GluR2 subunit mRNA expression in the amygdala [53, 117].

The changes found in all of the regions reflect an imbalance in the excitatory and inhibitory tone. A reduction of BZ-sensitive $GABA_A Rs$ seem to be more related to the development of tolerance, while the upregulation of AMPARs and NMDARs in these regions appear to be more associated with withdrawal induced regulation.

Adverse Consequences of Zolpidem Use

Tolerance

Preclinical Studies. BZs are known to induce dependence in rodents and primates [120]. In rodent and primate models dependence is usually manifested as tolerance and withdrawal. As defined in the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V), tolerance is operationally defined as a loss of efficacy to a given effect at a given dose or the need for higher doses for the same effect [1]. Tolerance due to chronic BZ treatment is well documented in rodents [109, 112, 120-131]. A main BZ that is used to investigate sedative tolerance is diazepam. Chronic diazepam treatment results in a robust display of sedative tolerance presented as decreased sensitivity to its own locomotor-impairing effects [119, 123, 124, 132]. However, there is conflicting evidence concerning the development of tolerance to chronic zolpidem in mice, with more evidence of dependence occurring in primates [24, 38, 133]. The variability in the development of tolerance may be affected by the type of behavior measured [28, 112]. Earlier rodent studies found that tolerance did not develop to zolpidem's sedative effects [24]. Some rodent studies do not show that zolpidem can cause tolerance to the anticonvulsant and motor-sedative effects while others show tolerance to the ataxic and hypothermic effects [29, 38]. Recent studies show that tolerance to zolpidem's sedative effects can occur [25, 105]. In addition to tolerance to its own effects zolpidem shows cross-tolerance, development of tolerance to a drug that causes tolerance to develop to another drug, to other BZs. It has been found that mice previously treated with 28 days of zolpidem were tolerant to the effects of diazepam's hypothermic effect, with partial tolerance to the anxiolytic effect, and no tolerance to the sedative effect [112]. Zolpidem had a decreased potency to induce locomotor impairment and antagonize isoniazid-induced convulsions in mice treated with 10 days of midazolam [24].

Clinical Studies. In human and non-human primates chronic, use of zolpidem has been shown to lead to dependence accompanied by withdrawal, though there are some studies that find zolpidem maintains a low abuse potential in humans [26, 134-138]. Many human case studies show common themes between the patients that abuse zolpidem. These themes are tolerance and withdrawal (**Table 2-1**) [26, 139, 140].

Patients who become dependent on zolpidem tend to use zolpidem at supratherapeutic doses (> 20 mg); though there are those who maintain dependence

Table 2-1.   **Case reports on dependence/abuse of zolpidem**

No.	Age (years)	Gender	Highest daily dose (mg/day)	Daytime Intake	Dose increase	Tolerance	Withdrawal symptoms	Former dependence	Psychiatric complications	Reference
1	67	F	100	+	+	+	+	+	+	Madrak & Rosenberg 2001
2	43	F	450–600	–	+	+	+	–	nr	Aragona 2000
3	39	M	120–480b	nr	+	nr	+	–	+	Golden & Vagnoni 2000
4	42	F	300	nr	+	+	+	+	+	Vartzpopulos et al. 2000
5	30	F	400–500	nr	+	+	+	+	+	Vartzpopulos et al. 2000
6	26	F	160–200	nr	+	+	+	+	+	Vartzpopulos et al. 2000
7	33	M	120	nr	+	+	+	+	+	Vartzpopulos et al. 2000
8	45	F	40 (+zopiclone)	+	+	+	+	–	+	Harter 1999[a]
9	49	M	50–80	+	+	+	+	–	–	Ströhle 1999
10	50	F	= 600	+	+	+	+	+	+	Hofmann & Eichmann 1998
11	44	F	30–300	+	+	+	+	nr	+	Sakkas 1999
12	63	F	300	+	+	+	+	nr	?	Courtet 1999
13	28	F	800	–	+	–	+	nr	?	Courtet 1999
14	69	F	200	+	+	+	–	nr	?	Courtet 1999
15	32	M	60	+	+	+	–	+	?	Courtet 1999
16	45	M	120	+	+	+	+	nr	?	Courtet 1999
17	40	M	200	+	+	nr	nr	+	?	Courtet 1999
18	35	M	100	+	+	nr	nr	nr	?	Courtet 1999
19	55	F	= 200	nr	+	+	+	nr	+	Ravishankar 1998
20	28	M	= 100	nr	+	nr	+	nr	+	Ravishankar 1998
21	53	M	140	+	+	+	+	+	–	Bottlender 1997
22	37	M	130	+	+	+	+	–	–	Gilbert & Stats 1997
23	35	M	200–300	–	+	+	+	–	+	Chamorro-Garcia 1996
24	33	M	300–400	+	+	+	+	–	nr	Sanchez 1996
25	75	M	70	+	+	+	+	–	nr	Thome 1995
26	33	M	150–280	+	+	+	+	–	+	Gericke 1994
27	42	F	nr	nr	nr	nr	+	+	+	Bruun 1993
28	52	M	60–70	nr	+	+	+	+	+	Bruun 1993
29	55	F	= 120	+	+	+	+	+	nr	Bruun 1993
30	60	F	100	nr	+	+	+	+	+	Cavallaro 1993
31	31	F	70–80	+	+	+	+	nr	nr	Cavallaro 1993
32	67	F	100	nr	+	+	+	–	+	Göder et al. 2001
33	50	F	450	+	+	nr	+	–	nr	Barrero-Hernandez et al. 2002
34	51	M	1200	+	+	nr	+	+	nr	Correas Lauffer et al. 2002
35	46	F	200	nr	+	nr	+	+	+	Correas Lauffer et al. 2002
36	40	F	200	+	+	nr	+	+	+	Correas Lauffer et al. 2002

M = male, F = female; nr = not reported. [a]Concomitant abuse of zopiclone and zolpidem.
[b]10–40 quaque hora somni (qhs; every hour of sleep).
The use of doses outside the therapeutic range (> 10mg/kg), increase in dose over time, tolerance, withdrawal, as well as usage during the daytime are common in those who abuse zolpidem. Reprinteda nd modified with permission. Hajak, G., et al., *Abuse and dependence potential for the non-benzodiazepine hypnotics zolpidem and zopiclone: a review of case reports and epidemiological data.* Addiction, 2003. **98**(10): p. 1371-8 [26].

below this dose) [26, 135, 141]. Another common theme are that those who become dependent previously abused BZs, opioid, and alcohol, or had psychiatric disorders [26]. Though some studies looking at extended use of zolpidem do not find escalation or tolerance to zolpidem's sedative effects in insomniacs and "normal" patients [40].

Withdrawal

Preclinical Studies. As defined in the DSM-V, withdrawal is the typical effects associated with the sudden stoppage of a drug[1]. For BZs and zolpidem the withdrawal syndrome is displayed as hyper-excited activity, like increased anxiety and insomnia and in severe cases seizures and hallucinations. Tolerance and withdrawal tend to be associated with each other, but they do not always occur together. Tolerance and withdrawal may have similar mechanisms. Withdrawal effects in rodents and primates can take many forms. Rats given chronic lorazepam experienced spontaneous and precipitated withdrawal, measured by motor activity within the home cage and increased electromyography (EMG) activity compared to control [38]. Chronic zolpidem compared to lorazepam did not display withdrawal symptoms [38]. Increased precipitated withdrawal scores (PAS) which include convulsions, motor signs, dyskinesia, autonomic signs, and affective signs were seen after chronic diazepam [142]. Mice given chronic diazepam and subjected to either repeated withdrawals or a single withdrawal, have a lower dose threshold for pentylenetetrazole (PTZ) after flumazenil precipitated withdrawal [143]. Many studies have found that BZs induce hyperactivity and anxiety-like effects in rodents. Of note is the duration between cessation of BZ treatment and the development of withdrawal symptoms. Some studies identify symptoms within 24 hours of drug termination while others saw symptoms after 96 hours [104]. Other studies have found that mice treated with zolpidem do not have clonic seizures when given the β-carboline inverse agonist FG-7142 after 4 days of treatment [144].

Clinical Studies. Initially zolpidem was thought to be absent of negative effects. Emerging case studies have found that patients increase zolpidem dosage beyond clinical doses. Once these patients stop taking these high doses, withdrawal occurs. Human withdrawal from BZs are well documented and tend to be severe, as dosage increases resulting in severe insomnia, seizures, anxiety, sweating, tremors, and irritation [139, 145, 146]. Patients tend to take supratherapeutic doses of these drugs due to the prescribed doses losing efficacy (tolerance) or to prevent withdrawal symptoms [30].

Relationship Between Duration of Zolpidem Use and the Emergence of Tolerance and Withdrawal

Most controlled studies investigating the effects of repeated zolpidem use in humans have not found tolerance and withdrawal. These studies allowed participants to self-administer and did not show an increase in dose level across time, suggesting

tolerance to the sedative effects do not occur. The studies also found there were no withdrawal effects such as next day insomnia or cognitive dysfunction [27, 40]. These data indicate zolpidem is a safe drug with a low propensity for tolerance and withdrawal. However, most of these controlled studies were done in healthy adults with or without insomnia and with no prior BZ, opioid, or alcohol abuse. Unlike controlled studies, case studies have recorded numerous cases of zolpidem dependency primarily in patients with prior BZ, opioid, or alcohol abuse and psychiatric disorders. The amount of time it takes these patients to become tolerant to zolpidem varies. Usually patients become tolerant within 1-6 months of starting zolpidem, increasing the dose substantially from 10 mg to > 100 mg [147, 148]. The amount of time between cessation of zolpidem and the emergence and severity of withdrawal symptoms also varies. In humans mild withdrawal symptoms, like anxiety and tremors occurs within hours of the last dose [149]. More severe withdrawal symptoms (e.g. seizures and hallucinations) can occur up to 3 days after the last dose of zolpidem [139, 145]. It should be noted that these symptoms usually occur after cessation of extremely high doses of zolpidem that taken for up to two years.

Along with humans, non-human primates can display tolerance to zolpidem. Interestingly primates show tolerance to the sedative effects of zolpidem at relatively low doses of 3.2 and 5.6 mg/kg over the course of 7 days of treatment [138]. Non-human primates also reliably show withdrawal symptoms after the abrupt end of zolpidem treatment. At increasing doses from 1-32 mg/kg, baboons showed withdrawal symptoms after abrupt end of zolpidem treatment after 78 days of treatment [6]. Acute flumazenil given 35 days after continuous zolpidem self-injections also induced physical symptoms typically seen after BZ withdrawal in baboons, including decreased eating, increased aggression, tremors, vomiting, and abdominal discomfort [150]. The termination of long-term zolpidem administration (> 60 days) also caused spontaneous withdrawal symptoms comparable to BZs and barbiturates [133, 150].

In comparison to humans and non-human primates, rodents can become tolerant to zolpidem and BZs after shorter treatment durations. Rodents can become tolerant to the sedative effects of BZs as early as 1-3 days of treatment at doses of 5-20 mg/kg [122, 127, 130, 131, 151]. Rodents can also exhibit mild withdrawal symptoms similar to humans 1-3 days after cessation of BZs [104, 144]. Some studies show at doses of 5-10 mg/kg, rodents become tolerant to zolpidem after 7-14 days of repeated zolpidem compared to humans after > 1 month at doses greater than 10 mg [25, 29, 105]. However, unlike repeated zolpidem use in humans and primates, there are few studies that examine zolpidem withdrawal, with only one study reporting withdrawal symptoms after cessation of zolpidem [24, 38, 144, 152].

Usefulness of Rodent Models of Sedation and Limitations

Rodent models of sedation are useful tools to understand the neuromechanisms underlying sedation in humans. These models are valuable due to the ability to control factors that might underlie tolerance in humans. In addition, rapid development of tolerance can be achieved in rodents compared to human and primate. As stated above,

rodents can become tolerant to zolpidem after 7 days of drug treatment compared to humans, which take > 1 month to become tolerant to the effects of zolpidem. While the rodent model of sedation may be a useful tool to study zolpidem tolerance, they may be limited in detecting withdrawal symptoms due to species differences in the pharmacokinetics and pharmacodynamics of zolpidem, in addition to the differential distribution of GABA$_A$Rs in the brain.

Abuse, Dependence, and Addiction

Preclinical Studies. Few studies evaluate the addictive properties of zolpidem in contrast to the many studies evaluating the addictive properties of BZs [153, 154]. The primary measure used to evaluate addictive behavior in primates and rodents is self-administration. Reinforcement studies have found that primates and rodents self-administer BZs, but not at the rate of abusive drugs like cocaine [155-159]. Due to the sedative effects of BZs at higher doses, an inverted U-shaped response curve is seen. The U-shaped curve shows that response rates (function of reinforcement) as dose increase but there is a downturn in response rate due to the locomotor-impairing effects of higher doses.

Unfortunately, it is difficult to discern between physical dependence and addiction, as both tend to accompany one another. Physical dependence is characterized by the body's adaption to a given drug while addiction is the compulsive need for a drug. It has been found however that dependence can occur without reinforcement [160]. Most addictive drugs increase glutamatergic transmission in the mesolimbic dopamine system. Dependence and abuse have been behaviorally demonstrated, as baboons self-administer zolpidem indicating abuse potential but this has not been found in rodents [133, 138, 150, 161, 162]. Zolpidem has similar rates of self-injection to barbiturates and cocaine and has a higher rate of self-injection than triazolam [138, 162].

Abuse and dependence in mice are related and usually measured after discontinuation of drug treatment. In mice intracranial self-stimulation (ICSS) and conditioned place preference can be used as measures of a drug's reinforcing effects. BZs and other drugs of abuse reduce the amount of stimulation required for ICSS [163, 164]. It has been found that diazepam but not zolpidem dose dependently reduced reward threshold for ICSS in C57BL/6J mice [165]. This reward enhancing effect was also found in α1(H101R) mice, but not α2(H101R) or α3(H101R) mice, indicating that α2- and α3-GABA$_A$Rs play more of a role than α1-GABA$_A$Rs in the rewarding effects of BZs. Conditioned place preference (CPP), a paradigm where rodents develop a preference for one of two distinctive compartments that was previously associated with the BZs, has also been used to identify the reinforcing effects of BZs, but not zolpidem [161, 166].

Though studies suggests that α1-GABA$_A$Rs may not mediate rewarding effects in rodents, recent studies challenge this view. A single dose of zolpidem and diazepam increase firing of mesolimbic dopamine neurons via modulation of α1-GABA$_A$Rs in the

ventral tegmental area (VTA) [167]. Wild-type but not α1 (H101R) mice develop a preference for a sucrose solution with midazolam indicating a role of α1-GABA$_A$Rs in the reinforcing effects of BZs [168, 169]. Though there may be a partial role of α1-GABA$_A$Rs in the rewarding effects of BZs, there are no studies that show zolpidem alone produces these effects in mice. The differences in rewarding effects between rodents and primates may be due to the differences in GABA$_A$Rs subtypes and/or binding characteristics [76]. Primate studies raise concerns about the possible rewarding effects of zolpidem and the potential for the development of abuse in humans [140]. Drugs with positive reinforcing effects have been implicated as causes for abuse and dependence, thus more research into the rewarding effects of zolpidem may provide insights into the neural mechanisms underlying drug abuse by other drugs acting through GABA$_A$RS [170].

Clinical Studies. Several agencies state that along with BZs, zolpidem is a highly prescribed drug of abuse [171]. In addition, there has been a 136% increase in emergency room visits from 2004 and 2011 due to nonmedical usage of zolpidem [172]. According to the World Health Organization it is thought that abuse of zolpidem is similar in rate to other hypnotic BZs, though some studies report it as significantly lower [26, 134, 135, 173]. Like hypnotic BZs, there appears to be some propensity for abuse among humans and baboons [133, 135-138]. Griffith and Weerts' review [6] focusing on human over usage of BZs found that those who used for more than 4 months tend to fall into two categories: recreational users and "quasi-therapeutic" users. Those who used BZs as hypnotics tend to make up 23% of BZ users yet account for the majority of sales of hypnotics. Such long-term usage may indicate a propensity for abuse that is marked by dependence, tolerance, and possibly withdrawal.

Zolpidem's propensity for abuse has been recorded through case and clinical studies. A similar characteristic of those who tend to abuse zolpidem is their prior abuse of other BZs and/or opioids [145, 174]. The cases of zolpidem abuse usually contain a withdrawal syndrome. This syndrome comes after abrupt ending of zolpidem at doses from 20 mg to 2000 mg a day. The sudden disuse of zolpidem at these doses result in anxiety, sweating, palpitations, seizures [139, 145, 147]. Drug naïve humans show similar potential to abuse to both zolpidem and triazolam, with zolpidem at its highest dose (20mg) increasing feelings associated with abuse potential like happy and good effects [135]. Another study with drug naïve humans found a similar profile with zolpidem at its highest dose (20 mg) increasing feelings associated with abuse, such as high, like, and good effects [137]. Most case studies define this abuse of zolpidem as dependence not addiction. There is a case in which zolpidem displayed addictive properties such as intense craving and drug seeking to the point of harming themselves to attain the drug [146, 148]. Along with the negative effects associated with abuse many of these studies state that patients abuse zolpidem for stimulation, euphoria, and relaxation properties [146, 148, 175].

Side Effects/Toxicology

Like most sedatives zolpidem has side effects that interfere with next day performance. Zolpidem in rodents has amnesic effects on a variety of memory tasks like passive avoidance (1-2 mg/kg), contextual fear conditioning (1, 3, and 10 mg/kg), and the plus maze discriminative avoidance task (PM-DAT) [85, 176, 177].

There are few incidences of toxicity in humans at therapeutic doses and fewer instances of overdose compared to BZs. A review of zolpidem's side effects found they come in the form of lightheadedness, dizziness, somnolence, headache, fatigue, memory deficits, nightmares, confusion, and depression, nausea, and vomiting [178-180]. In some instances delirium, nightmares, and hallucinations can occur, though these are uncommon [181]. The most common side effects appear to be amnesia and parasomnias (sleep walking/eating/driving) and in rare occasions attempted para-suicide [180, 182, 183]. Zolpidem is also associated with increased major injury due to unintentional falls and vehicle incidents [184].

Neuropharmacology and Neurocircuitry of Adverse Effects

Modulation of GABA$_A$Rs

Studies have looked into possible neuroadaptive mechanisms that may account for tolerance and withdrawal to BZs and zolpidem, focusing mainly on changes in GABA$_A$R subunits in *in-vitro* and *in-vivo* assays. Most studies investigate changes after long-term BZ treatment, but fewer have looked into the changes that may occur after long-term zolpidem treatment [92, 116, 185]. A main finding is that α1 subunit mRNA levels are decreased after chronic use and withdrawal from zolpidem and BZs in the cortex [106, 107, 109, 114]. Zolpidem and diazepam causes decreases in α1 subunit mRNA levels in the prefrontal cortex [105]. Also it appears that some studies find decreases in subunits that make up α1-GABA$_A$Rs (β2 and γ2 subunits) and increase subunits the α4 subunit [186]. This is in contrast to a study of recombinant α1β2γ2S GABA$_A$Rs in HEK293 cells, finding increases in α1 and γ2S mRNA expression after a 96 hour withdrawal from zolpidem [187].

There are differences in the time frame these changes occur and also differences in if they occur during chronic treatment or during withdrawal [92]. In cerebellar granule cells it was found that long term diazepam treatment and withdrawal caused a reduction of α1 and both γ2 mRNA [186, 188]. This same study and others found that long term diazepam causes an increase in the α4 subunit mRNA and that withdrawal from diazepam caused an increase in α4 subunit protein [188, 189]. In the rat cortex after 7 or 14 days of diazepam treatment there were no significant differences in amount of α1 subunit mRNA, but there were increases in α3, α 4, and α5 subunit mRNA, as well as an increase in the α5 subunit in the PFC [105, 111]. There were no changes in the β2 subunit but there was a decrease in γ2 subunit mRNA [111]. Alterations in protein expression also occur after

BZ and zolpidem treatment and may be concurrent or an effect of the decrease in mRNA expression.

Uncoupling is another mechanism by which BZs and zolpidem may induce tolerance and possibly withdrawal. Uncoupling is defined as a disassociation of the GABA and BZ binding interface. Chronic diazepam and zolpidem caused a decrease in the GABA potentiation by [H]flunitrazepam binding (a measure of uncoupling) in rat cortical membranes and HEK cells containing recombinant GABA$_A$Rs [190, 191]. Flurazepam also decreases GABA potentiation by [H]flunitrazepam binding [192]. Zolpidem treatment also increases [H]flunitrazepam binding sites and increases α1 and γ2 subunit mRNA alongside inducing uncoupling [191, 193]. Another measure of uncoupling is the reduced ability of BZs to enhance GABA induced IPSPs [194, 195]. Chronic diazepam decreases potentiation by GABA, indicating reduced GABA$_A$R function [188]. It appears uncoupling does not involve decreasing GABA$_A$Rs. It has been hypothesized that uncoupling involves altering the expression of α subunits and not affecting the amount of GABA$_A$Rs. Internalization has been thought to mediate this change in expression by increasing internalization of the α1 subunit and increasing surface expression of α4 after chronic BZ treatment [196-199].

BZs acting on the reward pathway may play a role in development of dependence. Many BZs can induce addiction after long periods of use and some case studies of zolpidem find patients experiencing positive effects like euphoria and increased energy. There is some conflicting evidence on the role of α1-GABA$_A$R in mediating reward. It has been found that α1-GABA$_A$Rs may mediate dependence and abuse through activating the VTA. BZs and zolpidem modulate the VTA through activation of α1-GABA$_A$Rs on local interneurons [168]. The local interneurons no longer inhibit dopaminergic neurons in the VTA, allowing for increased firing. Though it has been found that zolpidem does not cause reward enhancement and that reward enhancement was not abolished in α1 point mutation mice (mice with a H101R mutation) making their α1-GABA$_A$R BZ binding pocket non-functional [165]

Glutamate Receptors

NMDA/AMPA. While changes in GABA$_A$Rs likely play a major role in mediating tolerance and withdrawal to BZs and zolpidem, changes in other neurotransmitter systems are also likely involved. GABA$_A$Rs are commonly located on excitatory neurons, inhibiting these neurons to maintain optimal excitatory neurotransmission. Prolonged GABA inhibition caused by repeated BZ or zolpidem treatments may produce compensatory increases in NMDARs, AMPARs, and metabotropic glutamate receptors (mGluRs). Increases in these glutamate receptors may influence the development of tolerance and withdrawal to zolpidem and BZs.

In support of compensatory increases, the NMDAR antagonist MK-801 has been shown to block tolerance and withdrawal to various BZs [126, 200, 201]. Chronic BZs

have been found to increase *in-vitro* glutamate release and NMDA-stimulated cGMP efflux [127, 202]. Chronic use of BZs have been found to increase the mRNA and protein levels of NMDAR and AMPAR subunits in the cortex and hippocampus and also decrease AMPAR subunits in the amygdala. In rats it has been found that the mRNA and protein levels of GluN1 and GluN2B subunits in the dentate gyrus are increased in rats tolerant to diazepam 24 hours after last injection [118, 119]. In the CA1 and CA2 regions of the hippocampus, there were decreased NMDA-mediated currents as well as decreased GluN2B subunit mRNA and protein levels in hippocampal slices [119, 203]. GluN1 and GluN2B subunit protein expression has been found to be increased in the rat cerebral cortex as well [117].

GluR1 subunit mRNA expression was increased in the frontal cortex and hippocampus but the GluN1 subunit mRNA expression was not changed in either region during withdrawal 96 hours after diazepam cessation nor while tolerant[104]. In hippocampal slices there are also increases in AMPAR function after flurazepam withdrawal and increases in GluR1 subunit protein expression [102]. Withdrawal from chronic s.c. administration of diazepam caused an increase in AMPARs (increase in [^{3}H] Ro48-8487) in the hippocampus and thalamus [53, 201]. Diazepam withdrawal lead to a decrease in GluR1 and GluR2 subunit mRNA expression in the amygdala and a decrease in GluR1 subunit mRNA expression in the cortex [53, 117]. Most of the studies find that AMPAR induced changes in mRNA and protein expression occur during withdrawal. AMPAR may have an effect on tolerance as GluR1-/- mice show reduced tolerance to but increased withdrawal effects to flurazepam[121]. Based on the previous studies it appears that changes in NMDAR and AMPAR expression due to chronic BZ usage primarily occur in the hippocampus.

Metabotropic Glutamate Receptors. mGlu5 when activated causes intracellular calcium (Ca$^+$) to be released allowing for the activation of multiple kinases. mGlu5 has been found to be increased after 7 days of zolpidem treatment (30 mg/kg) in mice along with downstream proteins like phospholipase Cβ and calcium/calmodulin-dependent protein kinase Iiα [204]. mGlu5 is co-localized and co-expressed with the α1-GABA$_A$R subunit in the hippocampus [205].

BDNF

It has been found that brain-derived neurotrophic factor (BDNF) may play a role in BZ and zolpidem tolerance. Acute triazolam and zolpidem decrease BDNF protein levels in the hippocampus [206]. Acute zolpidem causes an increase in association of methyl-CpG binding protein 2 (MeCP2) with BDNF promoter IV and an increase in the association of phosphorylated cyclic adenosine monophosphate (cAMP) response element binding protein (pCREB) with BDNF promoter I, though long-term treatment did not affect any of these factors [206]. It is known that BDNF decreases mIPSC. BDNF decreases both the surface expression of GABA$_A$Rs and modulates of mRNA expression. BDNF decreases GABA$_A$R surface expression in general and specifically by the

JAK/STAT pathway BDNF downregulates α1-GABA$_A$Rs by activation of inducible cAMP early repressor (ICER) which binds to pCREB to decrease mRNA expression [207, 208].

Kinases

The cAMP –dependent protein kinase (PKA) and Ca2+/phospholipid-dependent protein kinase (PKC) modulate GABAergic functioning. Both kinases modulate mIPSCs in opposing ways in cortex, hippocampus and other brain regions. PKA decreases mIPSC amplitudes in pyramidal cells in the hippocampus whereas PKC increases mIPSC peak amplitudes in granules cells [209]. PKA activates ICER to regulate α1 subunit mRNA expression by preventing the binding of pCREB with the GABRA1 promoter leading to a decrease in α1-GABA$_A$Rs [210]. Chronic zolpidem has been found to increase phosphorylated PKCγ in the limbic forebrain [211].

Influence of GABA Transmission on Sleep, Sedation, Motor Inhibition, and Reinforcement: Neurocircuitry

Sleep/Sedation

Most sedative-hypnotics are positive modulators of GABA$_A$Rs. Sleep is a naturally occurring state of unconsciousness accompanied by altered consciousness, reduced reactivity to external stimuli, and reduced voluntary movement, while sedation is an artificially induced state where the level of arousal is suppressed [212]. Both states are accompanied by decreases in voluntary movements allowing motor activity to be used as an index for both behaviors as seen in many studies evaluating BZ hypnotics and sedatives [213, 214]. Major regions have been identified that modulate circadian rhythms, REM, non-REM sleep, and sleep-homeostasis. The preoptic area appears promote sleep as the ventrolateral preoptic area (vLPO) and the median preoptic nucleus (MnPO) provide GABAergic inhibitory projections to the basal forebrain and brainstem arousal systems. The GABAergic projections to the arousal system inhibit the release of histamine, 5-HT, dopamine, acetylcholine, and orexin to various cortical regions. Most of these regions with the exception of the LC express α1-, α2-, and α3-GABA$_A$Rs and are subject to zolpidem and diazepam, inducing sedation or sleep (**Figure 2-5**) [215, 216]. While the circuitry of sleep has been well defined this is not the case with sedation though there is some evidence sedation works along the same sleep pathway [217].

There is overlap in the behavioral aspects of sleep and sedation and work done to disseminate how modulation of GABA$_A$Rs affect both. In α1(H101R) and α3(H126R) mice diazepam do not affect sleep latency or sleep duration compared to control mice [74, 218]. When zolpidem is given to α1(H101R) at a hypnotic dose (same dose used for measures of sedation, 5-10mg/kg) non-REM sleep is no different than wild-type mice[219]. However diazepam's and zolpidem's sedative effect is missing in α1(H101R)

Cortex
$\alpha_1\beta_2\gamma_2$
$\alpha_2\beta_3\gamma_2$
$\alpha_3\beta_x\gamma_2$
δ-subtype
$\alpha_3\beta_x\gamma_2$, $\alpha_5\beta_3\gamma_2$
$\alpha_3\beta_x\gamma_2$

Hippocampus
$\alpha_1\beta_2\gamma_2$
$\alpha_2\beta_3\gamma_2$
$\alpha_5\beta_3\gamma_2$
$\alpha_4\beta_x\gamma_2$
δ-subtype

Main GABA$_A$ subtype(s)
Minor GABA$_A$ subtype(s)
* Tonic conductance

Rt (GABA)
$\alpha_3\beta_3\gamma_2$

Thalamus
$\alpha_1\beta_2\gamma_2$
$\alpha_4\beta_{2,3}\gamma_2$
$\alpha_4\beta_{2,3}\delta$ *

Basal Forebrain
$\alpha_1\beta_2\gamma_2$
$\alpha_3\beta_x\gamma_2$

Hypothalamus
$\alpha_1\beta_2\gamma_2$
$\alpha_2\beta_3\gamma_2$
ε-subtype
$\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$, ε-subtype
$\alpha_1\beta_2\gamma_2$
$\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$, $\alpha_5\beta_3\gamma_2$, ε-subtype

Brainstem
$\alpha_1\beta_2\gamma_2$
$\alpha_3\beta_x\gamma_2$
$\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_x\gamma_2$
$\alpha_1\beta_2\gamma_2$, $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_x\gamma_2$
$\alpha_3\beta_x\gamma_2$
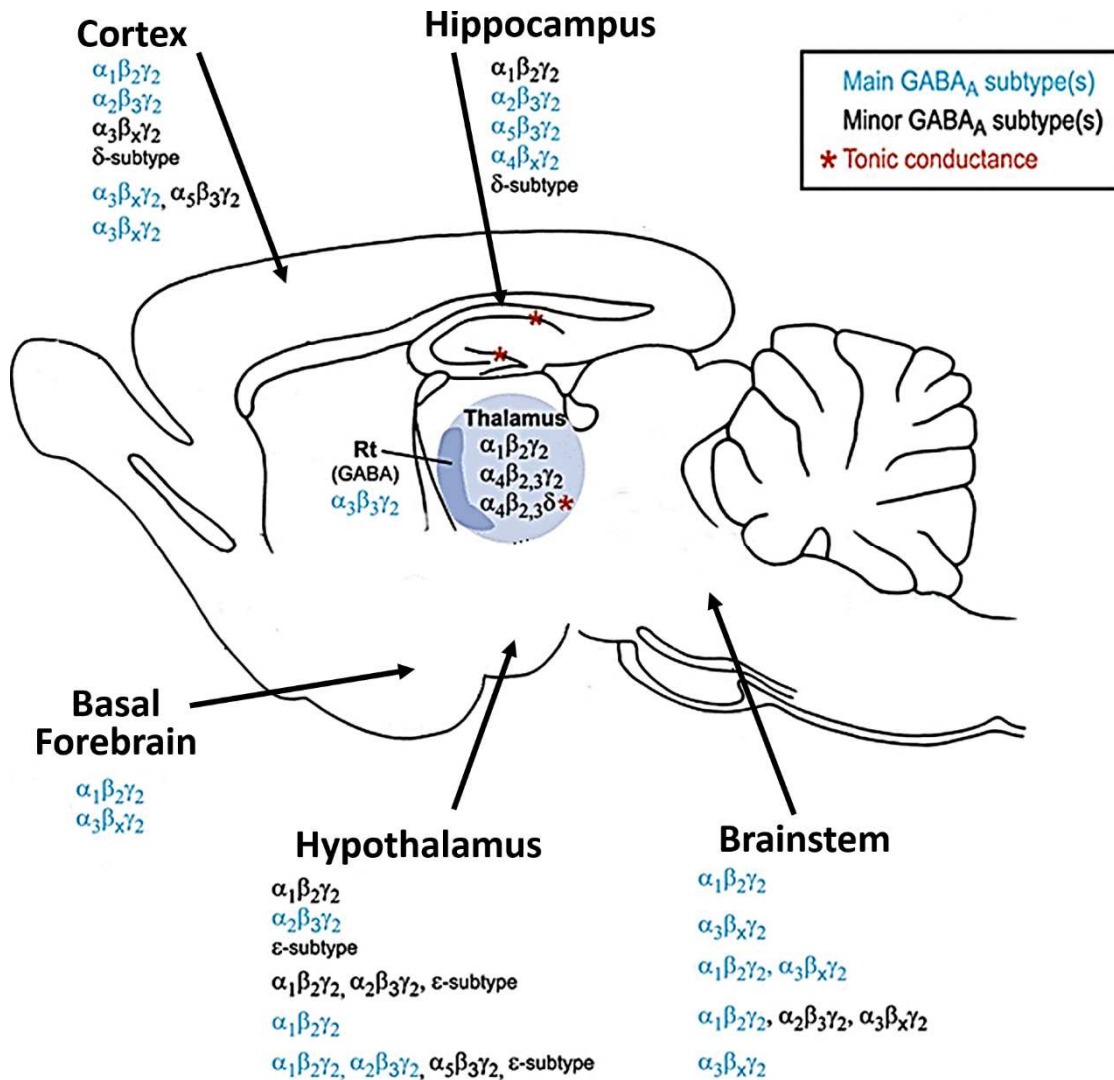
Figure 2-5.   GABA$_A$Rs modulated in the arousal system

GABA$_A$Rs are expressed in many regions that regulate arousal and sleep.

ACh, acetylcholine; ADO, adenosine; DA, dopamine; DpMe, deep mesencephalic reticular nucleus; DR, dorsal raphe nucleus; Glu, glutamate; Hcrt, hypocretins ∕ orexins; His, histamine; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; MCH, melanin concentrating hormone; NA, noradrenaline; n.d., not determined; nRt, reticular nucleus of the thalamus; PeF, perifornical nucleus; PH, posterior hypothalamus area; PPTg, pedunculopontine tegmental nucleus; SubC, subcoeruleus nucleus; TM, tuberomammillary nuclei; VLPAG, ventrolateral periacqueductal gray; VLPO, ventrolateral preoptic area; 5-HT, serotonin. Reprinted with permission.Winsky-Sommerer, R., *Role of GABAA receptors in the physiology and pharmacology of sleep.* Eur J Neurosci, 2009. **29**(9): p. 1779-94 [215].

[98, 214]. Due to the effects found in point mutation mice it appears that modulation of α1-GABA$_A$R induces sedation while modulation of α2-GABA$_A$R induces sleep.

Motor Inhibition

Sedation in mice is usually measured as a decrease in motor activity. Movement is in general there is a balancing act between cortical excitation and inhibition from the basal ganglia. Excitation and inhibition of locomotor activity is controlled by the MLR and pedunculopontine nucleus (PPN) respectively within the mesopontine tegmentum. The MLR activates muscle tone through the locus caeruleus (LC), raphe nuclei and excitatory reticulospinal system and motion through CPGs. MLR activation is caused by activation of cholinergic and glutamatergic receptors. PPN decreases muscle tone hyperpolarizing the motoneurons and possibly suppressing CPGs possibly decreasing locomotion[220]. The basal ganglia specifically the substantia nigra pars reticulate (SNr) GABAergic neurons project to the MLR and PPN providing tonic inhibition and modulate locomotion and muscle tone. The basal ganglia receives innervation from the cerebral cortex and as stated before sends projections to the spinal cord through the mesopontine tegmentum.

The cortical-basal ganglia circuit is a major component of volitional movement. Glutamate and GABA control movement through direct and indirect circuits. This direct circuit is a loop in that the glutamatergic neocortical neurons projects to the striatum which also receives dopaminergic projections onto the D1 receptors both projections exciting the striatum. The striatum sends GABA to the internal pallidum inhibiting its GABAergic neurons which would send GABA to the thalamus, since the thalamus is no longer inhibited by the internal pallidum it then sends glutamate back to the neocortex thus initiating movement. Inhibition of movement occurs when the D2 receptors on the striatum of activated by dopamine leading to GABA release from the striatum onto the internal and external pallidum. The external pallidum would send inhibitory projections onto the subthalamic nucleus but due to the inhibition from the striatum does not and this allows the subthalamic nucleus to send glutamatergic projections to the internal pallidum causing a net excitation. With the internal pallidum activated it sends GABA to the thalamus restricting glutamatergic release onto the neocortex [221].

Project Purpose

The purpose of this project was to examine tolerance, cross-tolerance, withdrawal, and molecular alterations in zolpidem and diazepam-treated C57/BL6J mice after different treatment durations. Elucidating the mechanisms behind zolpidem tolerance is necessary due to the ongoing use and development of subunit specific GABA$_A$R PAMs and the overall understanding of GABA$_A$R mechanisms. We hypothesized that 3 days of repeated zolpidem or diazepam would result in no behavioral and molecular alterations. We hypothesized that 7 days of zolpidem and diazepam would result in tolerance, cross-tolerance, withdrawal, and alterations in GABAergic and glutamatergic mRNA and

protein levels. We hypothesized that 30 days of zolpidem and diazepam would result in tolerance and cross-tolerance to zolpidem's effects and alter GABAergic mRNA levels.

CHAPTER 3.   EXPERIMENTAL METHODS[*]

The purpose of Study 1 was to determine the effects of 3 days of zolpidem and diazepam on the sedative effects of zolpidem treatment and mRNA levels of $GABA_AR$ subunits and $GABA_AR$ associated proteins. The purpose of Study 2 was to determine the effects of 7 days of zolpidem and diazepam on the sedative effects of zolpidem treatment, spontaneous withdrawal, flumazenil-induced withdrawal, and mRNA levels of $GABA_AR$ subunits, $GABA_AR$ associated proteins, AMPAR subunits, and NMDA subunits. Study 2 also examined the effect of treatments on total and cell surface expression of $GABA_AR$ receptor subunits proteins. The purpose of Study 3 was to determine the effects of 30 days of chronic zolpidem and diazepam on the sedative effects of zolpidem treatment and mRNA levels of $GABA_AR$ subunits and $GABA_AR$ associated proteins.

Studies 1-3 examined sedative tolerance by measuring the locomotor activity of mice during open field testing. Locomotor activity was defined as the horizontal distance traveled (in cm) during a 60-min open field test. In rodents, decreases in open field locomotor activity is a standard behavioral test for assessing the sedative effects of drugs [66, 69, 222-226]. Tolerance to drug sedation is commonly observed as the diminished effectiveness to reduce locomotor activity [25, 105].

Withdrawal-like symptoms were measured by locomotor activity during open field testing and anxiety-like behavior in the elevated plus maze (EPM). In rodents and humans, abrupt discontinuation of a sedative can cause hyperactivity and anxiety-like effects [53, 121, 227]. Hyperactivity was characterized as increased distance traveled in the open field. The Open-field anxiety-like behaviors were identified by the decreases in the percent of time and entries into the center zone and a decrease in frequency with which the mice stood on their hind legs in the maze (frequency of vertical activity or rearing) [17]. Anxiety-like behaviors in the EPM were identified as decreases in the percent of entries or time in the open arms [228-231].Tolerance and withdrawal from BZs and zolpidem are also associated with alterations in mRNA expression. Messenger RNA was measured by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). We examined mRNA expression in 4 brain regions: cortex, hippocampus, amygdala, and PFC. The cortex and PFC have roles in mediating locomotor activity and would possibly be affected by repeated diazepam and zolpidem [87]. It is thought that the hippocampus and amygdala have roles in mediating withdrawal induced anxiety and possibly tolerance from BZs [63, 232, 233].The cortex and prefrontal cortex have been found to have changes in mRNA expression due to BZs and zolpidem [107, 109, 111, 113, 115, 117, 234]. Other studies have found changes in mRNA expression in the hippocampus as well [102, 104, 118, 119, 202, 203]. Tolerance and withdrawal from BZs

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and zolpidem can also alter total and surface expression of proteins in the cortex and hippocampus as measured by western blot [107, 117].

Materials

Mice

Male C57BL/6J mice about 3-6 months old and weighing about 18-30 g (Jackson Laboratories, Maine) were used in these studies. Mice were housed in standard group cages (n = 5), allowed full access to food and water, and maintained on a 12 hour light/dark cycle (light on from 7:00 a.m.). Once in the vivarium, mice were allowed to acclimate there for at least a week before being used in experiments. Experiments were performed during the light cycle and were approved by the UTHSC Institutional Review Board. All experiments followed the principles and standards of animal care outlined by the NIH publication "Guide for the Care and Use of Laboratory Animals" [235].

In Study 1, two cohorts of mice were used for the experiments. Cohort 1 included mice treated with vehicle (n = 11), diazepam (n = 11), and zolpidem (n = 12). Cohort 2 of Study 1 included mice treated with vehicle (n = 9), diazepam (n = 10), and zolpidem (n = 9). In Study 2, five cohorts of mice were used for experiments. Cohort 1 included vehicle (n = 13), diazepam (n = 13), and zolpidem (n = 13) treated mice. Cohort 2 of Study 2 included mice treated with vehicle (n = 9), diazepam (n = 9), and zolpidem (n = 8) mice. Cohort 3 of Study 2 included mice treated with vehicle (n = 4), diazepam (n = 4), and zolpidem (n = 5) mice. Cohort 4 of Study 2 included mice treated with vehicle (n = 6), diazepam (n = 8), and zolpidem (n = 9) mice. Cohort 5 of Study 2 included mice treated with vehicle (n = 6), diazepam (n = 6), and zolpidem (n = 6) mice. In Study 3, two cohorts of mice were used. Cohort 1 was composed of vehicle (n = 6), diazepam (n = 6), and zolpidem (n = 7) treated mice and Cohort 2 included mice treated with vehicle (n = 12), diazepam (n = 10), and zolpidem (n = 19) mice.

Drugs and Antibodies

Diazepam, zolpidem (catalog numbers D0899 and Z-103, respectively; Sigma, St Louis, MO) and flumazenil (catalog number 1328; Tocris, Bristol, UK) were suspended in a vehicle which was composed of saline with 0.05 % Tween-80. Animal weights were recorded before all drug injections. Daily injections and behavioral testing were conducted in distinct environments to prevent context-specific tolerance [236]. Drugs were administered intraperitoneally (i.p.) at a volume of 10 ml/kg. In Studies 1-3 mice were treated twice a day (8:00 am and 5:00 pm) with 5 mg/kg of zolpidem, 10 mg/kg of diazepam, or vehicle. Treatment doses were based on previous experiments in rats and mice designed to study the development of sedative tolerance to diazepam or zolpidem [25, 28, 112, 123, 128, 129]. The test dose of zolpidem was selected from dose-response curves indicating the 50 % effective dose (ED50) for the locomotor-impairing effects of zolpidem in rats and mice ranging from 1.0 to 2.5 mg/kg [72, 224]. The test dose of the

GABA$_A$R antagonist flumazenil was used based on prior experiments showing flumazenil, at a dose of 10 mg/kg, induces mild withdrawal symptoms like increased locomotor activity and anxiety-like behaviors in mice previously given chronic BZ treatments [38, 121, 230, 237].

Primary GABA$_A$R subunit antibodies used for cross-linking/western blot analyses were as follows: α1 subunit (1:1000; Millipore, Billerica, MA, USA), α2 subunit (1:1000; Alomone Labs, Jerusalem, Israel), α3 subunit (1:500; Abcam, Cambridge, UK), and γ2 subunit (1:1000; Affinity Bioreagents, Waltham, MA). The primary NMDAR subunits antibodies used were NR1 subunit (1:1000; Abcam, Cambridge, UK) and NR2A subunit (1:400; Millipore, Billerica, MA). Peroxidase labeled anti-rabbit IgG (1:5000) and peroxidase labeled anti-mouse IgG (1:5000) were used as secondary antibodies (both from Vector Laboratories, Burlingame, CA). An antibody for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:3000; Fitzgerald, Acton, MA) was used as a control protein and to normalize results. The GAPDH was a monoclonal mouse antibody and the other primary antibodies were polyclonal rabbit antibodies.

Justification for Why We Selected 3, 7, and 30 Days as Our Experimental Time Points

Most drug studies investigate the behavioral and molecular changes that occur after different treatment durations of drugs. Most studies that have looked at zolpidem tolerance have examined development after 7-14 days, however there are few that look at the effects of short-term or long-term zolpidem treatment. Studies investigating BZ tolerance examine tolerance after 1-3 days (short-term), 7-14 days, and > 28 days (long-term) [122, 127, 130, 131, 151].

Justification for Two Injections for Methodology

For this project we decided to give mice two injections to better mimic the high dose levels that can occur when humans abuse zolpidem by self-administering multiple high doses, sometimes throughout the day [139, 147, 148, 174]. In addition, since mice metabolize zolpidem faster than humans, two doses increase the probability of mimicking high circulating concentrations that occur in humans abusing zolpidem [31, 33, 37, 39, 131, 179]. The 50% effective dose (ED50) for zolpidem in rodents needed to impair locomotor activity is 1.0-2.5 mg/kg and the ED50 for diazepam is 1.21 mg/kg [72, 224, 238]. The doses are 10x (zolpidem) or 20x (diazepam) greater than needed to induce sedation in mice. Likewise, humans who abuse zolpidem go beyond the clinical dose (5-10 mg) and can use 10-20 times the clinical dose (100-1000 mg), defined as a supratherapeutic dose.

<center>**Methods**</center>

Figure 3-1 is a simple flow-chart of the experimental designs of Studies 1-3. In all studies, mice received treatment injections of vehicle, diazepam, and zolpidem on consecutive days (i.e. 3, 7, or 30). All behavioral tests and molecular analyses were started 16-20 hours after the last treatment injection, unless otherwise stated. In Study 1, two cohorts of mice received vehicle, diazepam, or zolpidem for 3 days. Both cohorts were tested for sedative tolerance measured by locomotor activity in the open field. Immediately after testing Cohort 2 was given one injection of their assigned drug, then sacrificed 16-20 hours later for analysis of mRNA expression. The mRNA expression of GABA$_A$R α1-5, β1-3, γ1-2 and δ subunits, GABA$_A$ clustering protein gephyrin, and GABA transporter 1 (GAT-1) were measured by qRT-PCR.

In Study 2, five cohorts of mice received vehicle, diazepam, or zolpidem for 7 days. Cohort 1 was tested for sedative tolerance measured by locomotor activity in the open field. Cohort 2 was tested for spontaneous withdrawal measured by locomotor activity and anxiety-like behaviors measured in the open field. Cohort 3 was tested for flumazenil-induced withdrawal measured by anxiety-like measures in the EPM and open field as well as locomotor activity in the open field. Cohort 4 was used for analysis of mRNA expression of GABA$_A$R α1-5, β1-3, γ1-2 and δ subunits, gephyrin, GAT-1, NMDA subunits (GluN2A, GluN2B, and GluN1), and AMPA subunits (GluR1 and GluR2) measured by qRT-PCR. Cohort 5 was used to analyze total protein expression of GABA$_A$R subunits α1, α2, α3, and γ2, and the surface expression of GABA$_A$R subunit α1. Protein expression was measured by western blot in the cortex and hippocampus.

In Study 3, two cohorts of mice received vehicle, diazepam, or zolpidem for 30 days. Both cohorts were tested for sedative tolerance measured by locomotor activity in the open field. Immediately after testing Cohort 2 was given their assigned drug regimen, then sacrificed 16-20 hours later for analysis of mRNA expression of GABA$_A$R α1-5, β1-3, γ1-2 and δ subunits, gephyrin, and GAT-1 measured by qRT-PCR. A separate cohort of mice were for injected for 1 day (acute), to measure the effect of acute diazepam, zolpidem, and vehicle on mRNA expression measured by qRT-PCR.

Tolerance and Spontaneous Withdrawal: Open Field

Tolerance and spontaneous withdrawal were measured by examining locomotor activity during a 60-min open field test session [226]. Mice were given either 2 mg/kg of zolpidem (tolerance) or vehicle (spontaneous withdrawal) and placed back in the home cage for 2 min prior to testing. A 2-min latency between test drug injection and testing onset has been successfully used to identify tolerance to the locomotor-impairing effect of the BZ midazolam, which, like zolpidem, is relatively short acting and rapidly eliminated [239, 240]. After 2 min, mice were individually placed in open field chambers (44.45 cm long, 45.09 cm wide, and 29.21 cm high) constructed from a clear polycarbonate and equipped with four 24-beam infrared arrays across the base of each chamber wall (MED Associates, Model ENV-520). All measures in the open field were

<center>30</center>
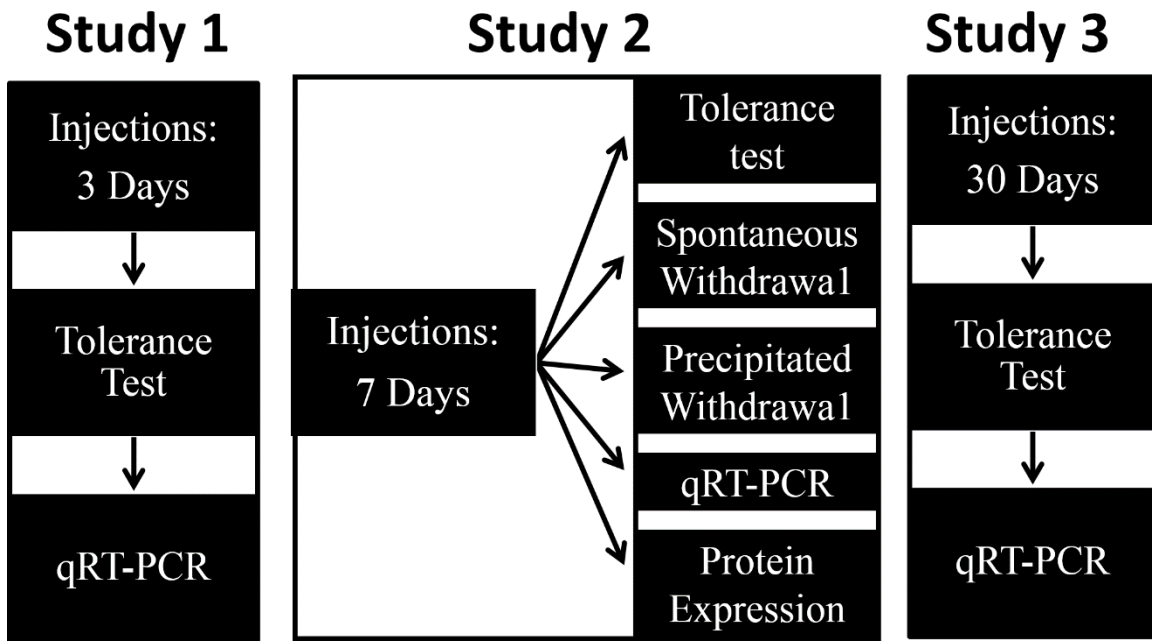
Figure 3-1.   Overview flow chart of experimental studies
The studies were conducted to evaluate the effect of treatment durations on locomotor activity, mRNA levels, and protein expression. The mice used in these studies were male C57BL/6Js. In Study 1, two cohorts of mice were used, both were used for tolerance and cohort 2 was used for qRT-PCR. In Study 2, five cohorts of mice were used, each cohort was used in only one experiment. In Study 3, two cohorts of mice were used, both were used for tolerance and cohort 2 was used for qRT-PCR

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automatically recorded and collected by the MED Associates' Activity Monitor Data Analysis software installed on an IBM-based computer. For tolerance experiments, the horizontal distance (in cm) was recorded and used as an index of motor sedation. For withdrawal experiments, horizontal distance, vertical counts (frequency of rearing), time spent in the center, and number of center entries were recorded and used as an indices of withdrawal. The central zone was defined as the compartment of the floor centrally located 8 cm from the perimeter of the chamber walls. Testing was conducted under standard test room lighting (100 lux), and each animal was tested once.

Flumazenil-Induced Withdrawal: EPM and Open Field

Cohort 3 was used to measure flumazenil-induced withdrawal. After a 7 day treatment with diazepam, zolpidem, or vehicle, mice were tested for flumazenil-induced withdrawal on two consecutive days. On Day 1, mice were given a 10 mg/kg injection of flumazenil 20 min before a 5 min EPM test session. After EPM testing, mice were immediately placed in the open field for a 60-min test session. On Day 2 mice were given a 10 mg/kg injection of flumazenil 20 min before a 60-min open field test session. Mice were then individually placed in open field chambers.

The EPM was constructed of wood, painted white, and consisted of 4 arms that were 30.48 cm long and 6.99 cm wide and elevated by a wooden base to a height of 76.20 cm. Two of the EPM arms were without walls and 2 arms were enclosed by 17.78cm high walls. Animal movements were recorded overhead with the use of a camera (SENTECH). Recordings were analyzed with video-tracking software (ANY-maze©, Stoelting CO, USA). The anxiety-like behaviors tracked in the EPM were percent time spent in the open arms and the number of entries into the open arms. The testing in the open field was conducted as stated above in Tolerance and Withdrawal: Open Field. For flumazenil-induced withdrawal the dependent variables were the same as for spontaneous withdrawal.

qRT-PCR

About a day after their last treatment injection, mice were sacrificed by cervical dislocation. Immediately afterwards, whole mouse brains were rapidly isolated and immediately frozen on crushed dry ice. Tissue isolation was performed by sectioning brains (300-μmthick) on a Leica cryostat, followed by dissection of the PFC, cortex, amygdala, and the hippocampus using a stereomicroscope and a scalpel blade under RNAse-free conditions. After the collection, samples were stored in buffer RLT Lysis buffer (QUIAGEN) at −20 °C.

Samples were then homogenized by sonication and RNA was isolated with the RNeasy protect mini kit (QIAGEN). RNA concentration and purity were determined using a microplate reader in conjunction with a Take3™ Micro-Volume plate (BioTek Instruments, Inc., Winooski, VT). The ratio of absorbance at 260 and 280 nm of samples

were commonly in the range of 1.8–2.2. Samples with A260/A280 of less than 1.8 were considered to be contaminated by protein and excluded from further analyses. RNA integrity was determined using the Agilent Bioanalyzer 2100 (Agilent Technologies, UK) to generate an electropherogram and an RNA integrity number (RIN). RIN scores ranged between 5.3 and 9.4.

RNA was transcribed to cDNA using Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN, USA) following the manufacturer's instructions. One reaction consisted of 2 µL Anchored oligo(dT) primer (2.5 µM), 4 µL random hexamer primer (60 µM), 8 µL reaction buffer 5× (8 mM), 1 µL protector TNase inhibitor (20 U), 4 µL deoxynucleotide mix (1 mM), and 1 µL reverse transcriptase (10 U). Varying volumes of RNA (amounting to 100 ng) and PCR-grade water were added for a 20-µL reaction mixture. Complementary DNA (cDNA) was transcribed using an Eppendorf Mastercycler thermal cycler (Eppendorf) gradient PCR machine. Reverse transcription incubation conditions were 25 °C for 10 min, 55 °C for 30 min, and 85 °C for 5 min.

The relative expression levels of α1-5, β1-3, γ1-2 and δ subunits, gephyrin, and GAT-1 were measured in Studies 1-3. In Study 2 the relative expression levels of GluN2A, GluN2B, GluN1, GluR1 and GluR2 were also measured. Measurements for mice given acute administration were limited to α1-3, α5, β2, γ2, and GAT-1. Primer and probe combinations were designed using ProbeFinder software (version 2.45; Roche Diagnostics, Indianapolis, IN, USA). The oligonucleotide sequences used for the primers (Integrated DNA Technologies) and gene transcript IDs are shown in **Table 3-1[16]**.

To determine PCR efficiencies, we generated standard curves for each target and reference genes (β-tubulin, β-actin, cyclin D, hypoxanthine phosphoribosyltransferase 1, ribosomal protein S19, and TATA-binding protein) by plotting the threshold value (Ct) versus the log of the amount of serial dilutions of cDNA (2 µg–0.2 ng). Separate standard curves were generated for each brain region analyzed. The amplification efficiencies of target and reference genes were within an acceptable slope range of −3.6 to −3.1, corresponding to efficiencies close to 100%. TATA-binding protein was used as the endogenous reference gene and amplified in parallel with target genes. This reference gene showed stable expression across samples and between brain regions. PCR efficiencies between the target and reference genes were relatively equal. Real-time PCR was performed using the TaqMan detection method and LightCycler® 480 System (Roche Diagnostics, Indianapolis, IN, USA) [241]. Each reaction was performed in triplicate and consisted of 0.1 µL of UPL probe (10 µM), 0.1 µL of forward primer (20 µM), 0.1 µL of reverse primer (20 µM), 5 µL of LC 480 2X master mix (Roche Diagnostics, Indianapolis, IN, USA), 2.7 µL of PCR grade water, and 2 µL of cDNA (10 ng). The PCR reaction consisted of initial incubations at 95 °C for 5 min followed by 50 amplification cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 10s

Table 3-1.   Gene transcripts and primer sequences

Gene ID	Forward Primer Sequence	Reverse Primer Sequence	Ensembl Transcript ID
Gabra1	5'-CTT TTC TCC CGG GTC TGG-3'	5'-TCT TCA TCA CGG GCT TGC-3'	ENSMUST00000169305
Gabra2	5'-ACA AAA AGA GGA TGG GCT TG-3'	5'-TCA TGA CGG AGC CTT TCT CT-3'	ENSMUST00000000572
Gabra3	5'-CTT GGG AAG GCA AGA AGGTA-3'	5'-GGA GCT GGT GTT TTC TT-3'	ENSMUST00000137517
Gabra4	5'-AAA GCC TCC CCC AGA AGT T-3'	5'- CAT GTT CAA ATT GGC ATG TGT-3'	ENSMUST00000031121
Gabra5	5'-GAC GGA CTC TTG GAT GGC TA-3'	5'-ACC TGC GTG ATT CGC TCT-3'	ENSMUST00000068456
Gabrb1	5'-CCC TCT GGA TGA GCA AAA CT-3'	5'-AAT TCG ATG TCA TCC GTG GTA-3'	ENSMUST00000031122
Gabrb2	5'-CGA TGG CAC TGT CCT GTA TG-3'	5'-ATA CCG CCT TAG GTC CAT CA-3'	ENSMUST00000169077
Gabrb3	5'-CGT GGG TGT CCT TCT GGA T-3'	5'-ATG GTG AGC ACG GTG GTA AT-3'	ENSMUST00000085240
Gabrd	5'-CGGAGCTGATGAACTTCAAAT-3'	5'-ATGTAGACGCCCCGGTTC-3'	ENSMUSG00000029054
Gabrg1	5'-AGG CAG GAA GCT GAA AAA CA-3'	5'-TTC ATG GGA ATG AGA GTG GA-3'	ENSMUST00000101143
Gabrg2	5'-ACA GAA AAT GAC GCT GTG GA-3'	5'-CAT CTG ACT TTT GGC TTG TGA A-3'	ENSMUST00000070725
GEPHYRIN	5'-TGA TCT TCA TGC TCA GAT CCA-3'	5'-GCA AAT GTT GTT GGC AAG C-3'	ENSMUST00000052472
GAT-1	5'-GCC TGG TCA ATA CCA CCA AC-3'	5'-CCA TCT GTC ATC TGG TGC AT-3'	ENSMUST00000032454
Grin2a	5'-ATT CAA CCA GAG GGG CGT A-3'	5'-TTC AAG ACA GCT GCG TCA TAG-3'	ENSMUSG00000059003
Grin2b	5'-GTGAGAGGAAATCTCGGGGC-3'	5'- GGATGCCGGGGATAGAAAGG-3'	ENSMUSG00000030209
Grin1	5'-GTCCTCCAAAGACACGAGCA-3'	5'-AGCTCTCCCTATGACGGGAA-3'	ENSMUSG00000026959
Gria1	5'-AGGGATCGACATCCAGAGAG-3'	5'-TGCACATTTCCTGTCAAACC-3'	ENSMUSG00000020524
Gria2	5'-CCAATGGGATAAGTTCGCATA-3'	5'-GCACAGCTTGCAGTGTTGA-3'	ENSMUSG00000033981

The gene transcripts and sequences used in the qRT-PCR experiments. Gabra1-α1 subunit, Gabra2-α2 subunit, Gabra3-α3 subunit, Gabra4-α4 subunit, Gabra5-α5 subunit, Gabrb1-β1 subunit, Gabrb2-β2 subunit, Gabrb3-β3 subunit, Gabrd-δ subunit, Gabrg1-γ1 subunit, Gabrg2-γ2 subunit, Grin2a-GluN2A, Grin2b-GluN2B, Grin1-GluN1, Gria1-GluR1, and Gria-GluR2. Modified with permission. Wright, B.T., C.F. Gluszek, and S.A. Heldt, *The effects of repeated ZOLP treatment on tolerance, withdrawal-like symptoms, and GABAA receptor mRNAs profile expression in mice: comparison with DZP.* Psychopharmacology (Berl), 2014. **231**(15): p. 2967-79 [16].

Bissulfosuccinimidyl Suberate (BS3) Cross-Linking/Western Blot

The water soluble protein BS3 reacts with cell-surface proteins to selectively form amine bonds on the cell surface. These cross-linked surface proteins can be isolated, visualized, and quantified by western blot analysis because they appear at a higher molecular weight compared to non-cross-linked intracellular protein [242].

About a day after mice were given their last treatment injection in Study 2, they were sacrificed by cervical dislocation. The whole mouse brains were rapidly isolated and placed on ice. BS3 cross-linking assays were performed following a protocol modified from Boudreau [242]. Tissue isolation was performed by sectioning brains (1mm thick) with an acrylic matrix (Brain Tree©), followed by dissection of the left and right cortex and hippocampus with a scalpel blade. After dissection, right cortex and hippocampus tissue samples were placed in tubes containing artificial cerebrospinal fluid (ACSF) and stored on ice. Left cortex and hippocampus samples were placed in tubes containing a 52mM BS3/ACSF solution and incubated for 30 min in a 4^0C refrigerator with gentle mixing. After BS3 incubation, the tissue was quenched in 100mM glycine solution and placed back into the 4^0C refrigerator for a 10 min gentle mixing period. After quenching was completed, BS3 treated and non-treated tissue samples were spun down in for 2 min and the supernatant was discarded. Cortical and hippocampal BS3 treated and untreated tissue samples were placed in lysis buffer containing protease and phosphatase inhibitors (Complete ULTRA tablets, Roche Diagnostics, Indianapolis, IN, USA) and sonicated to lyse cells. Samples were again spun down and supernatant was collected. The protein concentration was quantified by the Pierce^{TM} BCA protein assay kit (ThermoFisher Scientific, Waltham, MA).

Equal amounts of protein (20 mg) were separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad). After transfer, the membrane was blocked with tris-buffered saline with 0.05% Tween 20 (TBST) containing 5% milk for 1 hr at room temperature. After the membrane was blocked, it was incubated with primary antibody (α1, α2, α3, γ2, GluN1, GluN2B) diluted in TBST, overnight at 4^0C. The membrane was washed three times in TBST, and incubated with horseradish peroxidase-conjugated second antibody diluted in TBST at room temperature for 1 hr. After incubation, the membrane was washed three times in TBST. Proteins were visualized by an enhanced chemiluminescence detection system (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific GermanyLtd. & Co.KG) and images were taken with a gel imaging system (Alpha Innotech,Thermo Fisher Scientific GermanyLtd. & Co.KG). The GAPDH antibody (Fitzgerald) diluted in TBST was used as a control protein. The results of western blotting were analyzed by the AlphaView software system. All results were normalized with GAPDH.

Data Reduction and Statistical Analyses

   Tolerance, Spontaneous, and Flumazenil-Induced Withdrawal. In Studies 1-3, group differences in tolerance were assessed by analyses on distance traveled during each 10-min block using a two-way ANOVA with treatment (VEH, DZP, and ZOLP) as the between-subject factors and time (Blocks 1–6) as a within-subject factor. Follow-up analyses were done with one-way ANOVAs and Dunnett's t-tests where appropriate.

   For spontaneous withdrawal, two-way ANOVAs were conducted on distance traveled in the center, periphery, and entire apparatus. These ANOVA were performed to assess whether distance traveled across the test session time (Blocks 1–6) influenced measures of open-field anxiety. Two-way ANOVAs were conducted on each of the following anxiogenic measures; frequency of rearing, number of center zone entries, and percent of center zone time during each 10-min block. The between-subject factor was treatment (VEH, DZP, and ZOLP) and the within-subject factor was time (Blocks 1–6). Follow-up analyses were done with one-way ANOVAs and Dunnett's t-tests where appropriate on each measure.

   For flumazenil-induced withdrawal, analyses were conducted on percent time open arm time and entries into open arms EPM using a one-way ANOVA for each measure. Distance traveled, frequency of rearing, number of center zone entries, and percent of center zone time during each 10-min block on each day were analyzed using two-way ANOVAs for with treatment (VEH, DZP, and ZOLP) as between-subject factors and time (blocks 1–6) as a within-subject factor for each measure. Follow-up analyses were done with one-way ANOVAs and Dunnett's t-tests where appropriate for each open field measure.

   mRNA and Protein Expression. Calculations of standard curves, CT values, and quantification of mRNA expression levels were performed by Gene Scanning software (version 1.5.0) provided with the LightCycler® 480 Instrument (Roche Applied Science). The comparative CT method ($\Delta\Delta CT = \Delta CT$ reference-ΔCT target) was used to compare expression levels of mRNAs between the control (VEH) and treatment (ZOLP and DZP) groups. For ease of presentation, data in tables and figures are expressed as relative fold change by applying the 2-$\Delta\Delta CT$ equation. Statistical comparisons were carried out for each analyzed region (cortex hippocampus, amygdala, and prefrontal cortex) and treatment protocol independently. Statistical analyses for mRNA and protein expression in the cortex and hippocampus were performed using one-way ANOVAs with Dunnett's t-tests for pairwise comparisons against a single control (VEH) to maintain a 0.05 error rate for each analysis. In vehicle-treated animals, the relative abundance of mRNA was as follows: cortex, α1 > γ2 > β2 > α3 > α4 > β3 > δ > α5 > β1 > α2 > γ1; hippocampus, γ2 > α5 > β3 > α1 > β2 > α4 > β1 > α2 > α3 > δ > γ1; amygdala, γ2 > α3 > β2 > α1 > β3 > α4 > γ1 > β1 > α5 > α2 > δ; and PFC, α1 > α3 > γ2 > β2 > β3 > α2 > β1 > α5 > δ > α4 > γ1.

CHAPTER 4.   RESULTS*

Study 1: The Effect of 3-Day Zolpidem and Diazepam Treatment on Behavioral Sedation and mRNA Expression

Tolerance to the Sedative Effects of Zolpidem

The purpose of Study 1 was to examine if a 3-day treatment regimen of zolpidem or diazepam induces significant changes to the sedative effects of zolpidem, as measured by open field activity, and significant changes in mRNA expression, as measured by qRT-PCR. Alterations to the sedative effects were examined in Cohort 1 and 2 mice previously treated with vehicle, diazepam, or zolpidem **(Figure 4-1)**. A two-way ANOVA found significant effects of time (Blocks), $F(5, 290) = 44.94$, $p < 0.01$, treatment (VEH, DZP, ZOLP), $F(2, 58) = 4.08$, $p < 0.05$, and a significant interaction of Time x Treatment, $F(10, 290) = 3.79$, $p < 0.01$. The main effect of time indicated that activity between Blocks differ from each other, generally increasing over time. The main effect of treatment indicated significant differences between treatment groups in overall activity, independent of time. One-way ANOVAs performed at each level of time revealed significant differences at Blocks 2 and 3, $F(2, 58) = 10.84$, $p < 0.01$ and $F(2, 58) = 6.20$, $p < 0.01$, respectively. There were no differences at Blocks 1 and 4-6, $Fs(2, 58) < 6.20$, $ps > 0.05$. Follow-up Dunnett's t-tests with VEH mice as controls showed the DZP mice displayed more activity at Blocks 2 and 3 than the VEH mice, $ps < 0.05$. These analyses indicated that after 3 days of diazepam, but not zolpidem, tolerance to zolpidem's sedative effects occur.

Changes in mRNA Expression Measured by qRT-PCR

Cohort 2 mice were used to examine changes in mRNA expression. One-way ANOVAs were used to compare group levels of each mRNA in the cortex, hippocampus, amygdala, and PFC. These ANOVAs revealed no significant differences in levels of any GABA$_A$R subunit mRNA or GABA$_A$R-related mRNA in the cortex, $Fs(2, 24) < 1.3$, $ps > 0.05$, amygdala, $Fs(2, 19) < 0.94$, $ps > 0.05$, or PFC, $Fs(2, 25) < 1.9$, $ps > 0.05$ **(Figure 4-2)**.

In the hippocampus, there was a significant treatment effect on the expression of α5-GABA$_A$R mRNA, $F(2, 24) = 3.45$, $p < 0.05$. A Dunnett's t-test showed the ZOLP group had reduced α5-subunit expression compared to the VEH group, $p < 0.05$. There were no significant treatment differences in the levels of other mRNA, $Fs(2, 24) < 3.45$, $ps > 0.05$ **(Figure 4-3)**.

Figure 4-1.   Decreased locomotor impairing effect of zolpidem after 3-day administration of zolpidem, diazepam, or vehicle
Distance traveled in open field after test administration of 2 mg/kg of ZOLP in mice previously given subchronic treatment of ZOLP, DZP, or VEH. Analyses of each 10-min block showed that differences between groups were most evident in the first half of the 60-min test session. Data are presented as mean + sem. Number sign denotes VEH < DZP groups ($p < 0.05$).
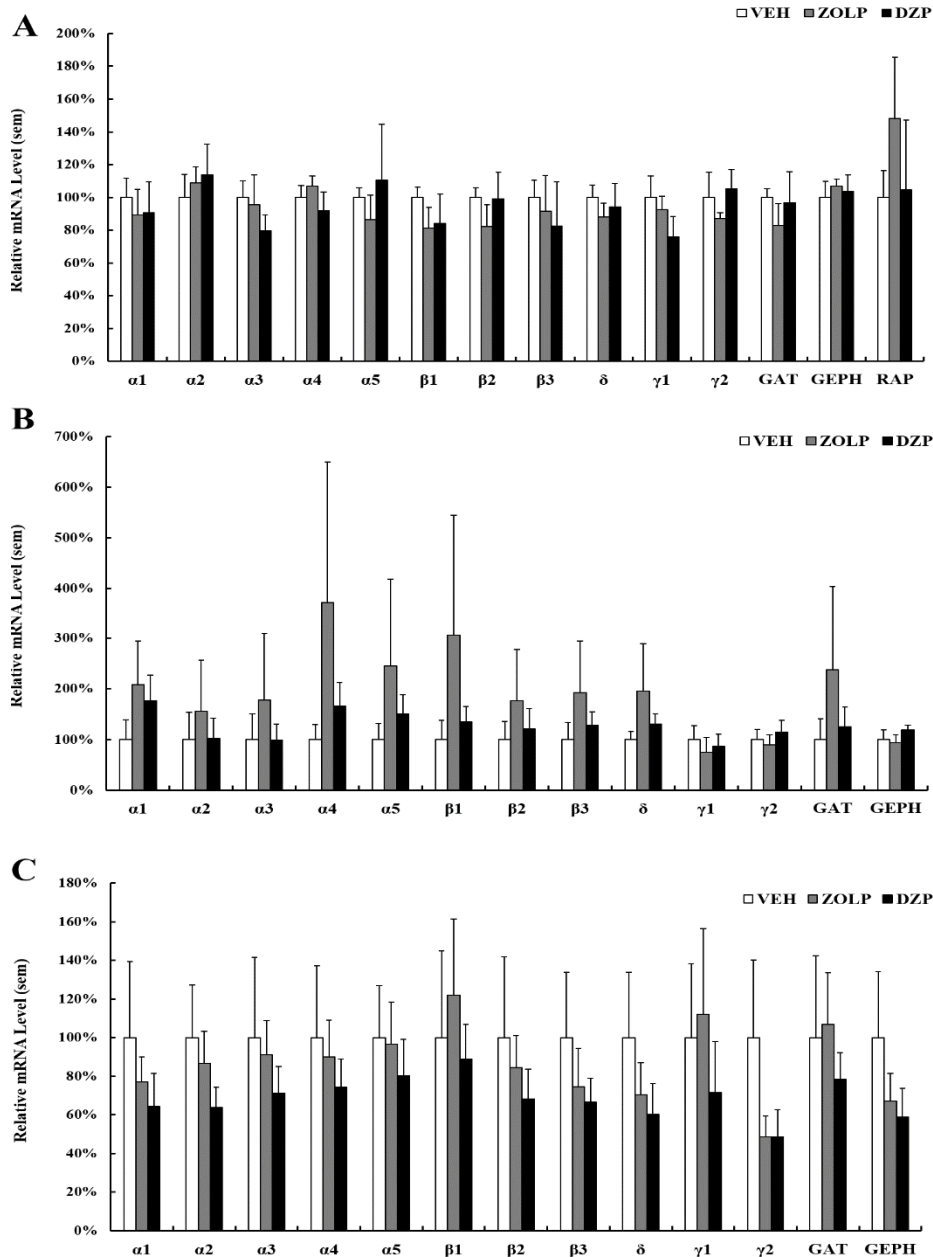
Figure 4-2.   Relative levels of mRNA in the cortex, amygdala, and pfc following 3-day administration of zolpidem, diazepam, or vehicle

Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's *t*-test for pairwise comparisons against a VEH for (A) cortex, (B) amygdala, and (C) pfc. Data are presented as mean + sem. There was no significance among these regions, $p > 0.05$.
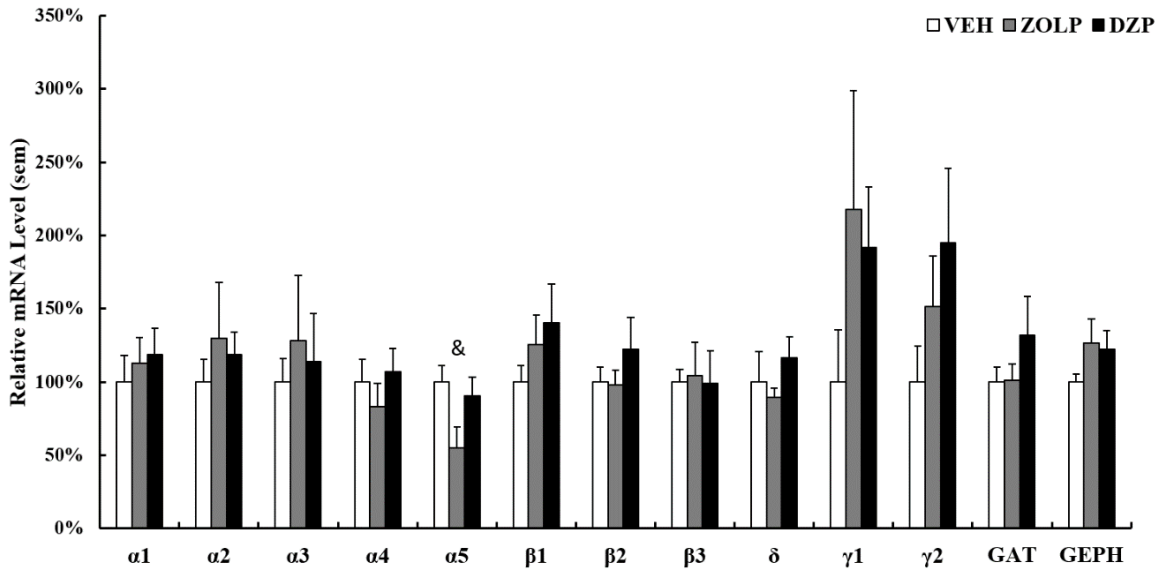
Figure 4-3.   Relative levels of mRNA in the hippocampus following 3-day administration of zolpidem, diazepam, or vehicle

Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100 %). Statistical analyses were performed by ANOVA with Dunnett's *t*-test for pairwise comparisons against a VEH. Data are presented as mean + sem. Ampersand denotes VEH > ZOLP treatments ($p < 0.05$.).

In summary, results from Study 1 indicated that the sedative effects of zolpidem was significantly reduced in mice given diazepam for 3 days. The decreased sedative efficacy of zolpidem in mice given prior exposure to diazepam is indicative of cross-tolerance. In contrast, the efficacy of zolpidem was unaffected in mice previously given zolpidem, suggesting no tolerance developed in these mice. There were no significant changes in the expression of $GABA_AR$ subunit or associated mRNAs in the cortex, hippocampus, amygdala, or PFC after 3 days of diazepam. However, the expression of $\alpha5$-$GABA_AR$ mRNA was reduced in the hippocampus after 3 days of zolpidem treatment.

Study 2: The Effect of 7-Day Zolpidem and Diazepam Treatment on Behavioral Sedation, Withdrawal, mRNA, and Protein Expression

The purpose of Study 2 was to examine if a 7-day treatment regimen of zolpidem or diazepam causes 1) changes in the sedative effect of zolpidem, 2) spontaneous withdrawal after discontinuation of treatment, 3) flumazenil-*induced* withdrawal, 4) changes in mRNA expression measured by qRT-PCR, and/or 5) changes in total and cell surface expression of selective receptor subunits, as measured by western blotting.

Tolerance to the Sedative Effects of Zolpidem

Changes to the sedative effects of zolpidem were examined in Cohort 1 mice that were previously treated with vehicle, diazepam, or zolpidem. A two-way ANOVA yielded significant main effects of treatment, $F(2, 46) = 3.40$, $p < 0.05$, time, $F(5, 230) = 118.80$, $p < 0.01$, and a reliable Treatment $\times$ Time interaction, $F(10, 230) = 8.00$, $p < 0.01$, signifying performance over time was differentially affected by drug treatment. To assess the interaction, one-way ANOVAs at each level of time were performed. These tests revealed group differences at Blocks 1, 2 and 3, $F(2,46) = 8.16$, $p < 0.01$; $F(2,46) = 16.34$, $p < 0.01$; and $F(2,46) = 6.61$ $p < 0.01$, respectively. Follow-up Dunnett's *t*-tests revealed that the activity in ZOLP and DZP groups was significantly greater than in the VEH group at Blocks 1–3, $ps < 0.05$ **(Figure 4-4).** There were no group differences at Blocks 4-6, $Fs(2,36) < 3.05$, $ps > 0.05$.

Spontaneous Withdrawal

The presence of spontaneous withdrawal after discontinued zolpidem, diazepam, or vehicle treatments was assessed in Cohort 2. We looked at overall activity to determine if mice had the differences in the rate of habituation over time, which might confound our interpretation of results. A 2-way ANOVA found no significant main effect of time, $F(5,105) = 0.01$, $p > 0.05$, no main effect of treatment, $F(2,21) = 0.02$, $p > 0.05$, and no significant interaction of Time x Duration, $F(10,105) = 0.11$, $p > 0.01$. The analysis for rate of habituation show that all groups show a similar rate of habituation.
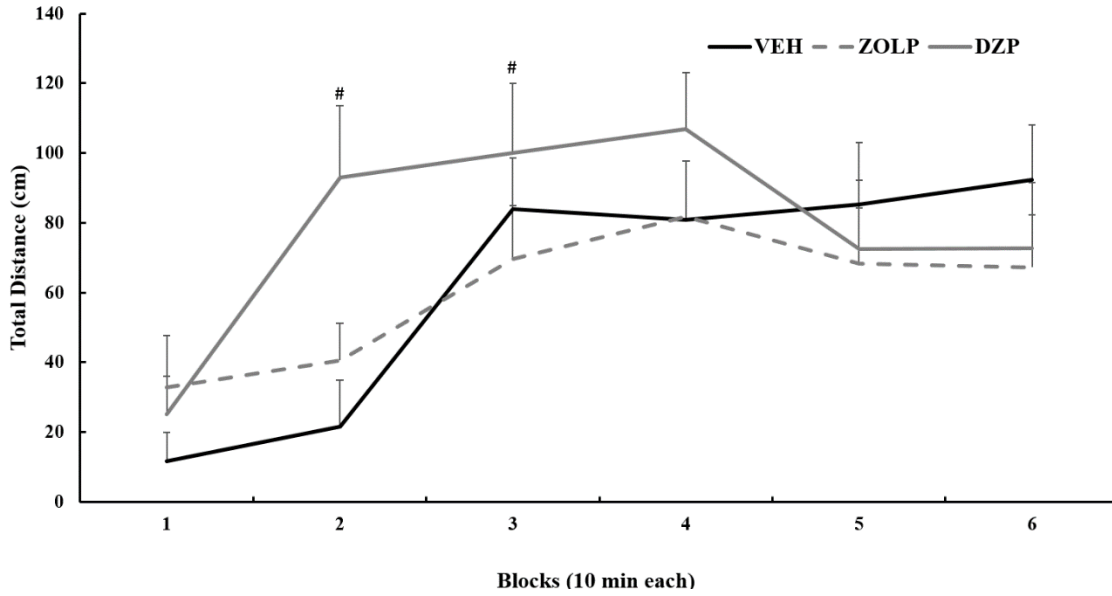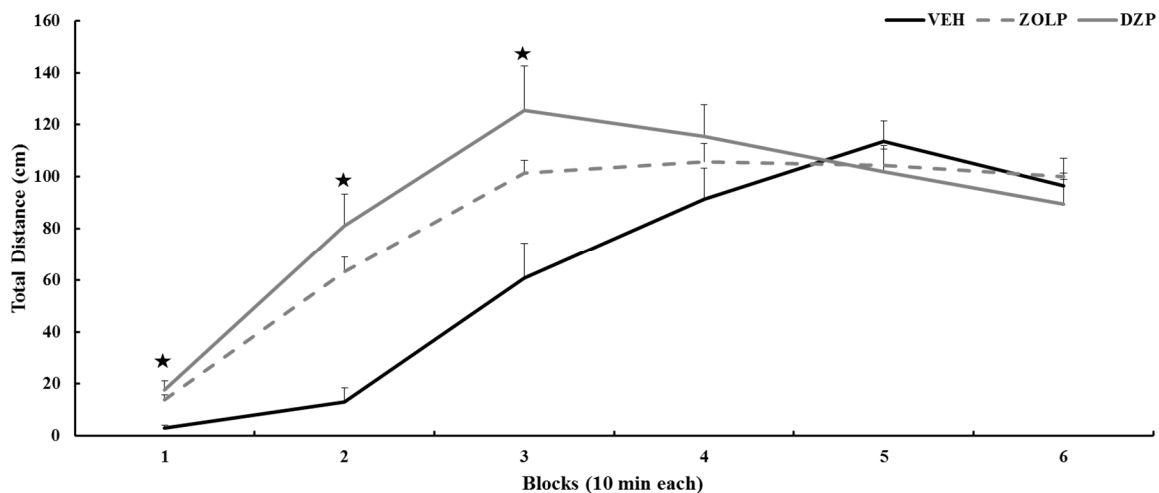
Figure 4-4.   Decreased locomotor impairing effect of zolpidem after 7-day administration of zolpidem, diazepam, or vehicle

Distance traveled in open field after test administration of 2 mg/kg of ZOLP in mice previously given subchronic treatment of ZOLP, DZP, or VEH. Analyses of each 10-min block showed that differences between groups were most evident in the first half of the 60-min test session. Data are presented as mean + sem. Black star denotes VEH-ZOLP < ZOLP-ZOLP and DZP-ZOLP groups ($p < 0.05$). Reprinted with permission. Wright, B.T., C.F. Gluszek, and S.A. Heldt, *The effects of repeated ZOLP treatment on tolerance, withdrawal-like symptoms, and GABAA receptor mRNAs profile expression in mice: comparison with DZP.* Psychopharmacology (Berl), 2014. **231**(15): p. 2967-79 [16].

For distance traveled in the entire open field, a two-way ANOVA found that there was a main effect of time, $F(5,105) = 34.78$, $p < 0.01$, a main effect of treatment, $F(2,21) = 8.08$, $p < 0.01$, and a significant interaction of Time x Duration, $F(10,105) = 2.97$, $p < 0.01$. To assess the interaction, a one-way ANOVAs at each level of time were performed. There were group differences at Block 1, $F(2,21) = 16.82$, $p < 0.01$, Block 2, $F(2,21) = 8.25$, $p < 0.01$, Block 3, $F(2,21) = 4.29$, $p < 0.05$, and Block 5, $F(2,21) = 4.70$, $p < 0.05$. Follow-up Dunnett's t-tests revealed that the activity of ZOLP and DZP groups was significantly lower than the VEH groups at Blocks 1 and 2, $p < 0.01$ and the activity of DZP groups was significantly lower than the VEH groups at Blocks 3 and 5, $p < 0.05$. There was no significantly differences at Blocks 4 and 6, Fs $(2,21) > 3.11$, $p > 0.05$ **(Figure 4-5)**.

For percent of center zone time, a two-way ANOVA yielded main effects of treatment, $F(2, 23) = 5.27$; $p < 0.02$, time, $F(5, 115) = 8.36$; $p < 0.01$, and a significant Treatment × Time interaction, $F(10, 115) = 2.49$; $p = 0.01$. One-way ANOVAs followed by Dunnett's t-tests indicated significant differences during Blocks 4-6, $F(2,23) = 7.82$, $p < 0.01$; $F(2,23) = 4.25$, $p < 0.05$; and $F(2,23) = 5.95$, $p < 0.01$. There were no significant differences between groups at Blocks 1-3, $F(2,23) < 3.3$, $p > 0.05$. Overall, ZOLP and DZP groups spent significantly less percent of time in the center zone in Blocks 4-6 compared to VEH mice, ps < 0.01 **(Figure 4-6)**.

For the number of center zone entries, a two-way ANOVA yielded, main effects of treatment, $F(2, 23) = 8.20$; $p < 0.01$, and time, $F(5, 115) = 41.67$; $p < 0.01$, were significant. The Treatment × Time interaction, $F(10, 115) = 2.67$; $p < 0.01$ was also significant. ANOVAs followed by t-tests indicated that ZOLP and DZP groups had fewer center zone entries during Blocks 1 and 2 **(Figure 4-6)**. Analyses of frequency of rearing showed reliable effects of time, $F(5, 115) = 6.28$; $p < 0.01$, and treatment, $F(2, 23) = 41.67$; $p < 0.01$. Overall, the frequency of rearing was lower in both ZOLP and DZP groups in comparison to the VEH group **(Figure 4-6)**.

Flumazenil-Precipitated Withdrawal

Cohort 3 mice were used to assess the presence of flumazenil-precipitated withdrawal symptoms on two consecutive days (Day1 and Day 2) after vehicle, diazepam, or zolpidem treatments. On Day 1, mice were given flumazenil (10 mg/kg/i.p) 20 min before EPM and subsequent open field testing. One-way ANOVA analyses of EPM measures revealed no significant differences between groups for percent time in the open arms or entries into the open arms, Fs$(2,10) < 1.38$, ps > 0.05 **(Figure 4-7)** Group differences in Day 1 open field dependent measures were assessed using two-way ANOVAs. For distance traveled, this analysis indicated a significant effect of time, $F(5,45) = 52.21$, $p < 0.01$, and a significant interaction of Time x Treatment, $F(10,45) = 2.18$, $p < 0.05$. There was no significant effect of treatment, $F(2,9) = 1.13$, $p > 0.05$. A one-way ANOVA found significant group differences at Block 6, with ZOLP and DZP groups displaying less activity than the VEH group, ps < 0.05 **(Figure 4-8)**. For percent time spent in the open field center zone, analysis indicated a significant effect of time,
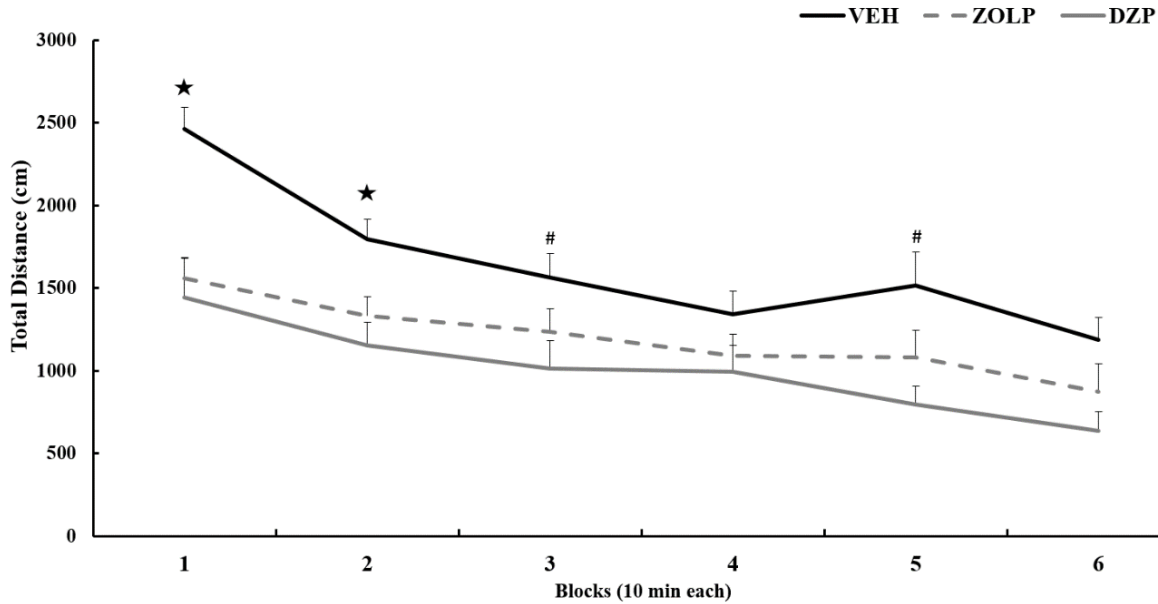
Figure 4-5. Decreased locomotor activity in zolpidem and diazepam groups after cessation of 7 days of zolpidem and diazepam
Distance traveled in open field after test administration of VEH in mice previously given subchronic treatment of ZOLP, DZP, or VEH. Analyses of each 10-min block showed that differences between groups were most evident in the first half of the 60- min test session. Data are presented as mean + sem. Black star denotes VEH> ZOLP and DZP groups ($p < 0.05$). Number sign denotes VEH > DZP group ($p < 0.05$). Reprinted with permission. Wright, B.T., C.F. Gluszek, and S.A. Heldt, *The effects of repeated ZOLP treatment on tolerance, withdrawal-like symptoms, and GABAA receptor mRNAs profile expression in mice: comparison with DZP*. Psychopharmacology (Berl), 2014. 231(15): p. 2967-79 [16].
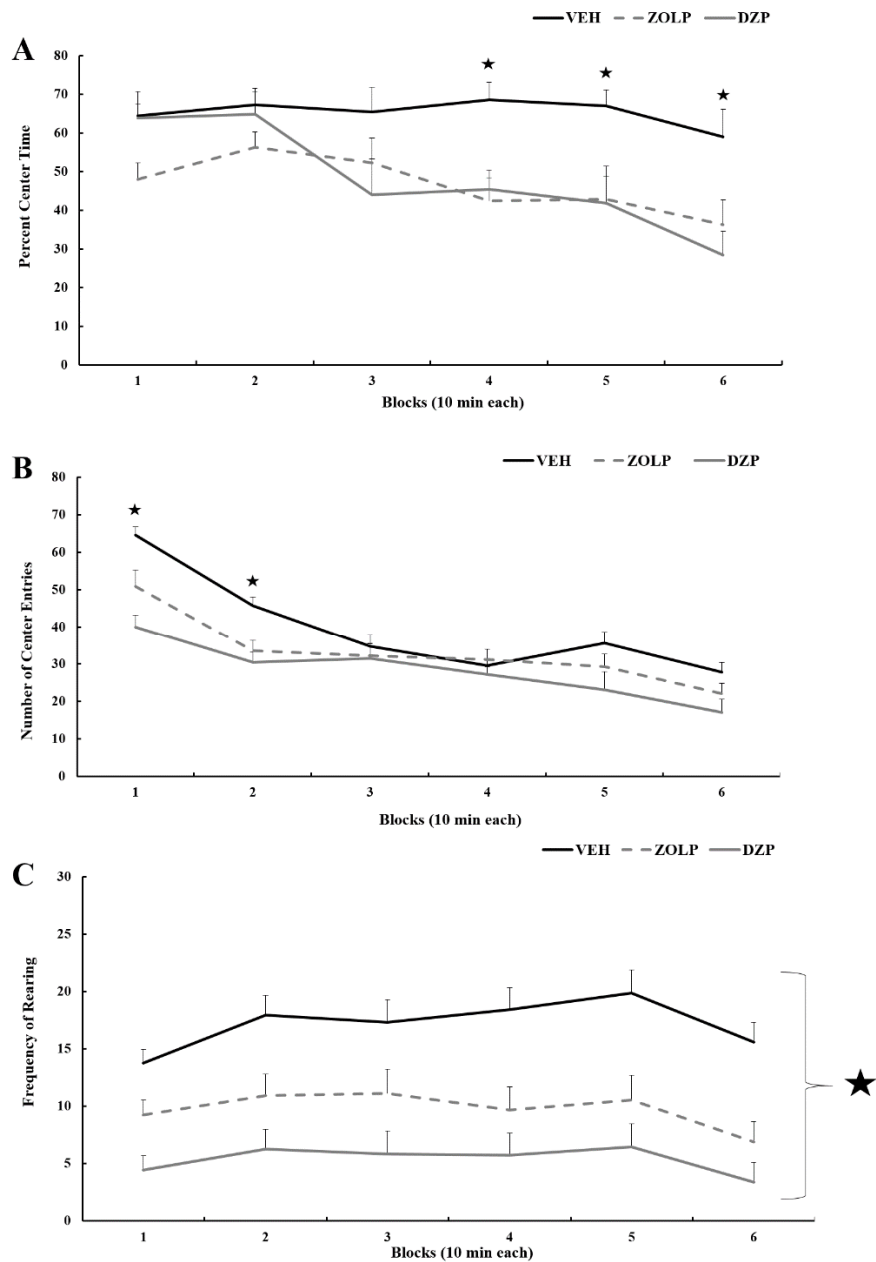
Figure 4-6.   Decreased percent time in center, center entries, and rearing frequency after cessation of 7 days of zolpidem and diazepam
Multiple exploratory behaviors were decreased following cessation of drug. (A) Percent of center time, (B) Number of center entries, and (C) Rearing frequency. Analyses were conducted on means of each 10-min block. Data are presented as mean + sem. Black star denotes VEH significantly different than ZOLP and DZP groups ($p < 0.05$). Reprinted with permission. Wright, B.T., C.F. Gluszek, and S.A. Heldt, *The effects of repeated ZOLP treatment on tolerance, withdrawal-like symptoms, and GABAA receptor mRNAs profile expression in mice: comparison with DZP*. Psychopharmacology (Berl), 2014. 231(15): p. 2967-79 [16].
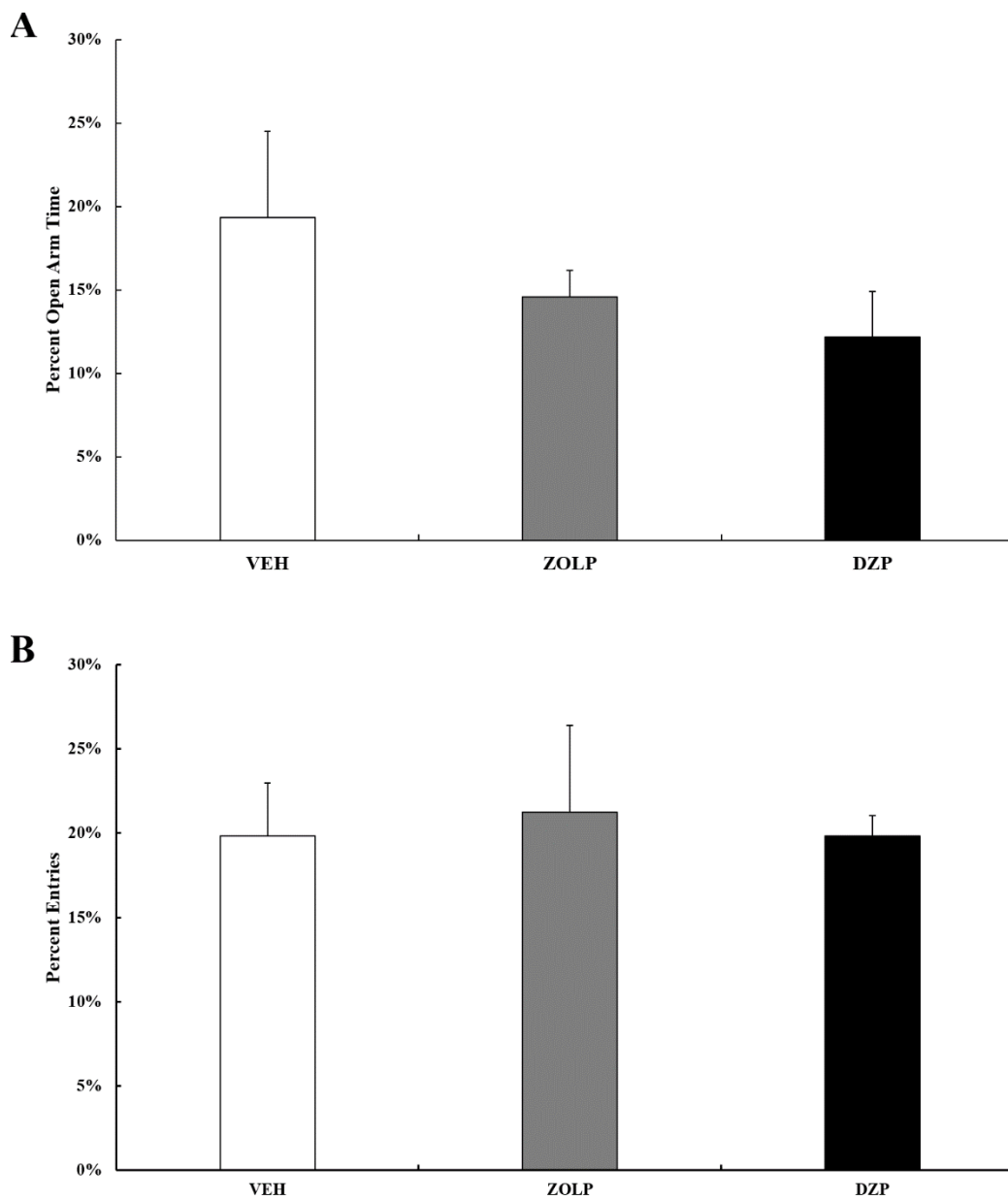
Figure 4-7.   Percent time spent and percent entries into the open arms of the EPM after acute flumazenil administration after 7-day administration of zolpidem, diazepam, or vehicle

Histogram comparing (A) percent of time spent in the open arms and (B) percent entries into the EPM. Statistical analyses were performed by ANOVA with Dunnett's *t*-test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were no significant differences between the groups ($p > 0.05$).
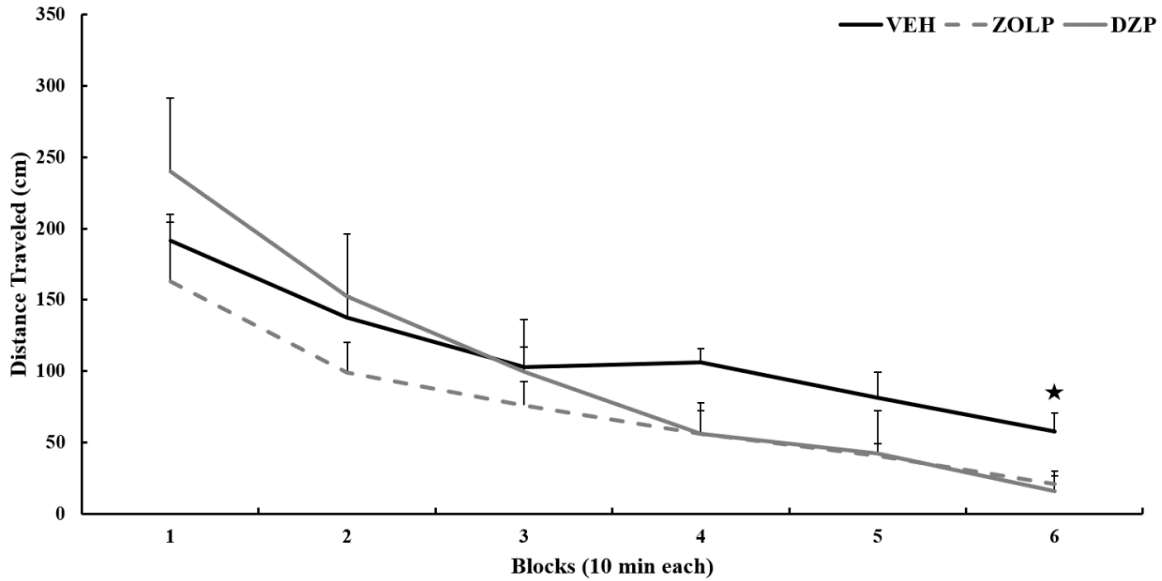
Figure 4-8.   Locomotor effect of flumazenil on test day 1 after 7-days administration of zolpidem, diazepam, or vehicle

Distance traveled in open field after test administration of FLU in mice previously given subchronic treatment of ZOLP, DZP, or VEH on test day 1. Analyses were conducted on means of each 10- min block. There was a significant decrease in activity at Block 6 on day 1, but no significant differences between groups on day 2. Data are presented as mean + sem. Black star denotes VEH significantly different than ZOLP and DZP groups ($p < 0.05$).

$F(5,45) = 45.41$, $p < 0.01$, but no significant effect of treatment, $F(2,9) = 0.83$, $p > 0.05$, or a significant interaction of Time x Treatment, $F(10,45) = 1.31$, $p > 0.05$ (**Figure 4-9**). For center entries, the analysis indicated a significant effect of time, $F(5,45) = 22.70$, $p < 0.01$, but no significant effect of treatment, $F(2,9) = 2.83$, $p > 0.05$, or a significant interaction of Time x Treatment, $F(10,45) = 0.38$, $p > 0.05$ (**Figure 4-9**). For frequency of rearing on Day 1, analysis indicated a significant effect of time, $F(5,45) = 23.01$, $p < 0.01$, but no significant effect of treatment, $F(2,9) = 1.54$, $p > 0.05$, or a significant interaction of Time x Treatment, $F(10,45) = 0.40$, $p > 0.05$ (**Figure 4-9**). 0.05). Data are presented as mean + sem.

On Day 2, mice were given of flumazenil (10 mg/kg/i.p) 20 min before open field testing, and group differences in dependent measures were assessed using two-way ANOVAs. The analysis of group difference in distance traveled showed a significant effect of time, $F(5,50) = 18.76$, $p < 0.01$, but no significant effect of treatment, F(2, 10) = 0.627, $p > 0.05$ or a significant interaction of Time x Treatment, $F(10,50) = 0.933$, $p > 0.05$ (**Figure 4-10**). For percent time spent in center on Day 2, analysis indicated a significant effect of time, $F(5,50) = 4.41$, $p < 0.01$ but no significant effect of treatment, $F(2,10) = 0.791$, $p > 0.05$ or significant interaction of Time x Treatment, $F(10,50) = 0.21$, $p > 0.05$ (**Figure 4-11**). For center entries, analysis indicated a significant effect of time, $F(5,50) = 20.03$, $p < 0.01$, and a significant interaction of Time x Treatment, $F(10,50) = 3.33$, $p > 0.01$, but no significant effect of treatment, $F(2,10) = 0.61$, $p > 0.05$. A one-way ANOVA found significant group differences at Block 1, $F(2,10) = 4.38$, $p < 0.05$, with the ZOLP group having less center entries than the VEH groups (**Figure 4-11**). There were no significant group differences at Blocks 2-6, $F(2,10) < 4.32$, $p > 0.05$. For frequency of rearing, t analysis indicated a significant effect of time, $F(5,50) = 5.46$, $p < 0.01$ but no significant effect of treatment $F(2,10) = 0.45$, $p > 0.05$ or a significant interaction of Time x Treatment, $F(10,50) = 2.01$, $p > 0.05$ (**Figure 4-11**).

Changes in mRNA Expression Measured by qRT-PCR

Cohort 4 was used to measure changes in mRNA expression of GABA$_A$R subunits and related proteins in the cortex, hippocampus, amygdala, and PFC. In the cortex (**Figure 4-12**), a one-way ANOVA identified group differences for the subunits α1, $F(2, 17) = 12.97$, $p < 0.01$; α3, $F(2, 17) = 6.92$, $p < 0.01$; β2, $F(2, 17) = 5.80$, $p < 0.05$, and δ, $F(2, 17) = 5.93$, $p < 0.05$. Follow-up Dunnett's t tests revealed decreased expression levels of α1, α3, β2, and δ subunits in ZOLP and DZP groups compared to VEH (ps < 0.05) and DZP groups but not ZOLP groups, induced significant decreases in expression of α4, α5, β1, and γ2 subunits after chronic treatment (ps < 0.05). In addition to subunits, group differences were identified for gephyrin, $F(2, 17) = 4.95$, $p < 0.05$, and GAT-1, $F(2, 17) = 5.80$, $p < 0.05$. There were no significant differences for the other subunits, Fs$(2, 17) < 4.39$, ps > 0.05.

In the hippocampus (**Figure 4-13**), a one-way ANOVA identified group differences for the α5 subunit, $F(2, 18) = 3.82$, $p < 0.05$ and γ2 subunit, $F(2, 17) = 5.22$, $p < 0.05$. Follow-up Dunnett's t tests revealed decreased expression levels of α5 and γ2
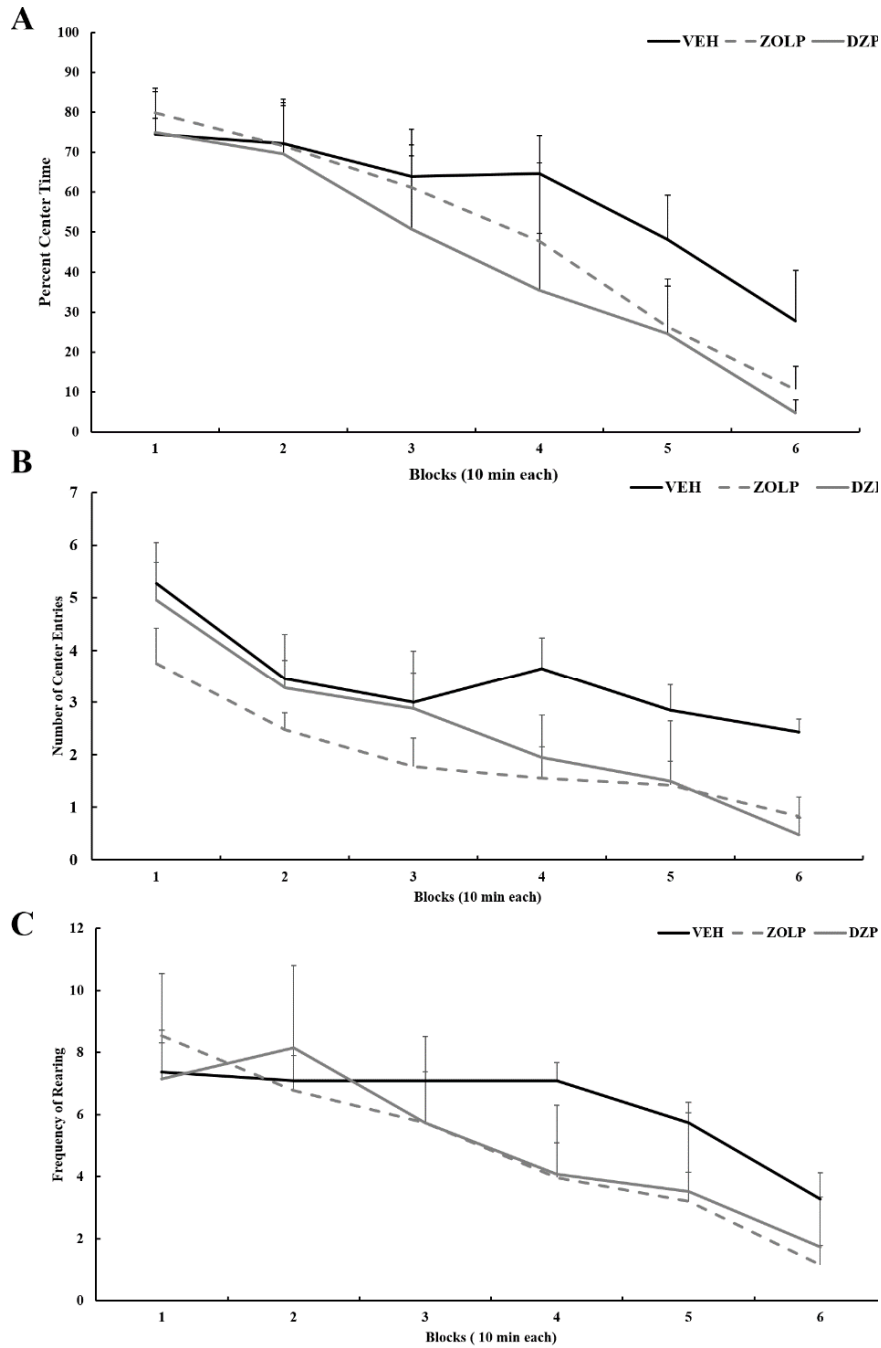
Figure 4-9.   Effect of flumazenil on percent time in center, center entries, and rearing frequency on test day 1 after 7-days administration of zolpidem, diazepam, or vehicle

Percent time in center, center entries, and rearing frequency in open field after test administration of FLU in mice previously given subchronic treatment of ZOLP, DZP, or VEH on test day 1. Analyses were conducted on means of each 10- min block. There were no significant differences between the groups on day 1 ($p > 0.05$). Data are presented as mean + sem.
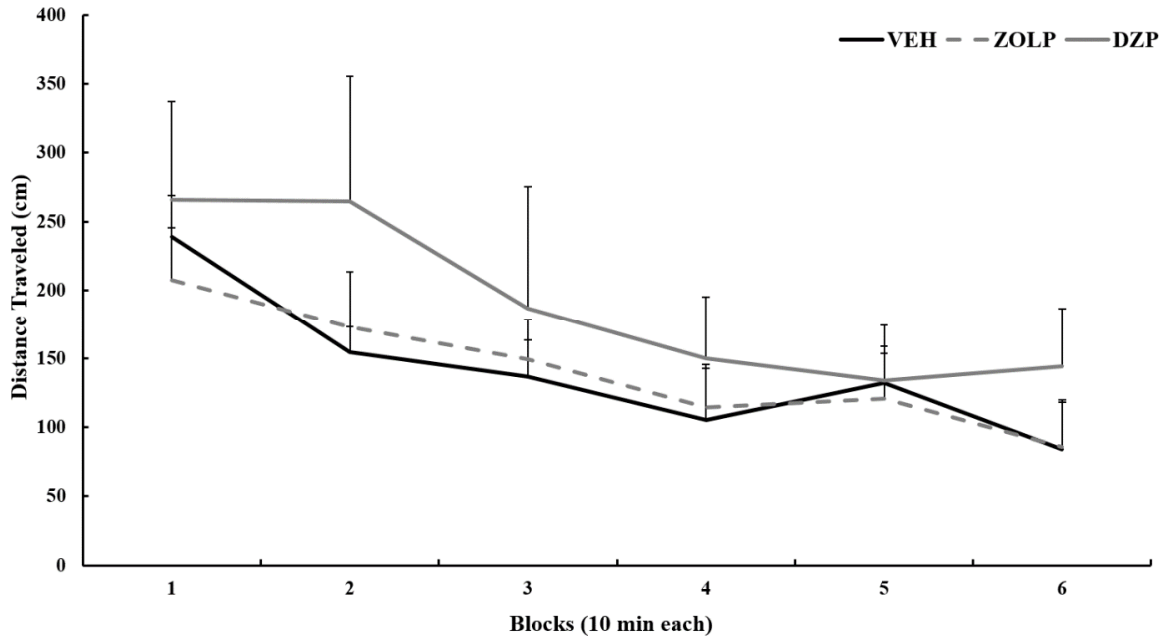
Figure 4-10.   Locomotor effect of flumazenil test day 2 after 7-day administration of zolpidem, diazepam, or vehicle
Distance traveled in open field after test administration of FLU in mice previously given subchronic treatment of ZOLP, DZP, or VEH day 2. Analyses were conducted on means of each 10- min block. There were no significant differences between groups, $p > 0.05$. Data are presented as mean + sem.
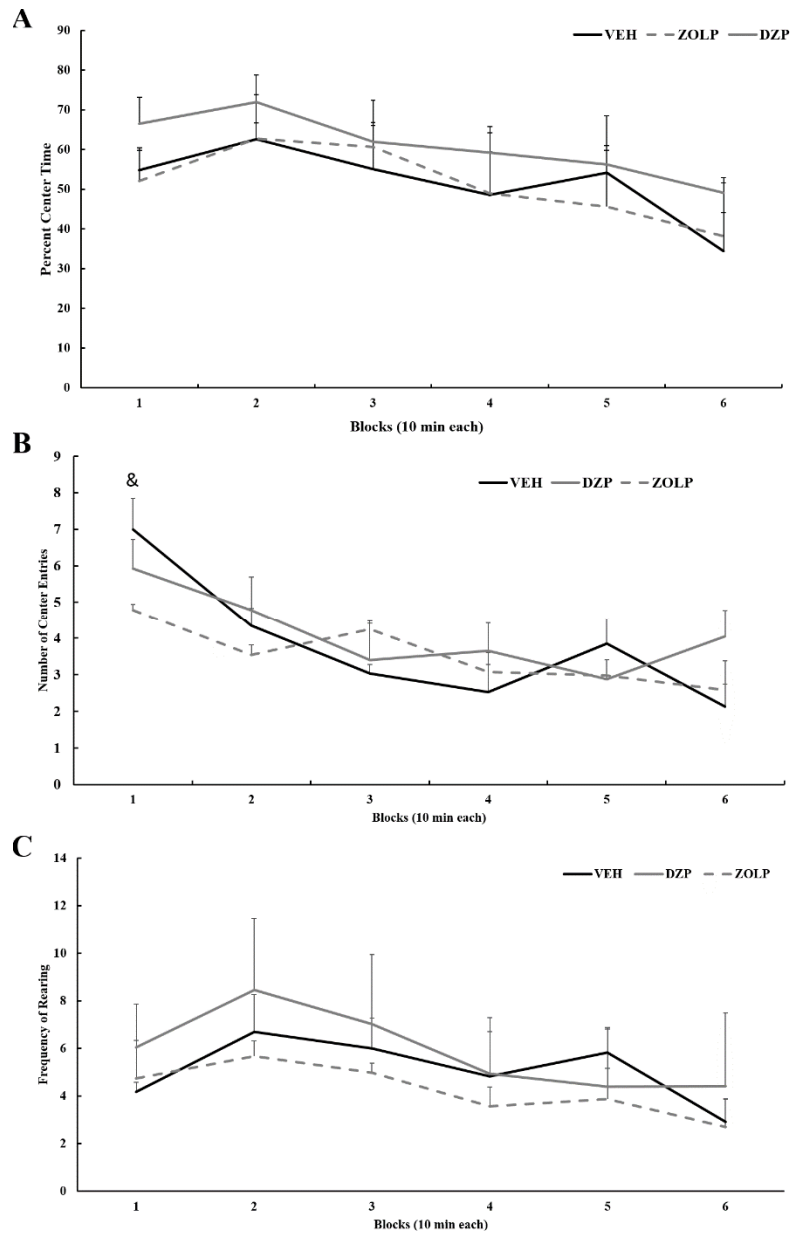
Figure 4-11. Effect of flumazenil on percent time in center, center entries, and rearing frequency on test day 2 after 7-days administration of zolpidem, diazepam, or vehicle
Percent time in center, center entries, and rearing frequency in open field after test administration of FLU in mice previously given subchronic treatment of ZOLP, DZP, or VEH on test day 2. Analyses were conducted on means of each 10- min block. There were significant group differences on the number of center entries at Block 1. ZOLP groups had less center entries than VEH groups. There were no significant group differences at other measures, $p > 0.05$. Data are presented as mean + sem. Ampersnad denotes VEH significantly different than ZOLP and DZP groups ($p < 0.05$).

Figure 4-12. Relative levels of mRNA in the cortex following 7-day administration of zolpidem, diazepam, or vehicle

Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. Black star denotes VEH significantly different than ZOLP and DZP treatments ($p < 0.05$); Number sign denotes VEH significantly different than DZP treatment ($p < 0.05$). Reprinted with permission. Wright, B.T., C.F. Gluszek, and S.A. Heldt, *The effects of repeated ZOLP treatment on tolerance, withdrawal-like symptoms, and GABAA receptor mRNAs profile expression in mice: comparison with DZP.* Psychopharmacology (Berl), 2014. **231**(15): p. 2967-79 [16].

Figure 4-13.   Relative levels of mRNA in the hippocampus following 7-day administration of zolpidem, diazepam, or vehicle
Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100 %). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. Black star denotes VEH significantly different than ZOLP and DZP treatments ($p < 0.05$); Number sign denotes VEH significantly different than DZP treatment ($p < 0.05$). Reprinted with permission. Wright, B.T., C.F. Gluszek, and S.A. Heldt, *The effects of repeated ZOLP treatment on tolerance, withdrawal-like symptoms, and GABAA receptor mRNAs profile expression in mice: comparison with DZP.* Psychopharmacology (Berl), 2014. **231**(15): p. 2967-79 [16].

subunits in DZP groups compared to VEH groups ($ps < 0.05$). There were no significant differences for the other subunits, $Fs(2, 18) < 5.22$, $ps > 0.05$. There were no significant group differences in the amygdala, $Fs(2, 20) < 1.90$, $ps > 0.05$, or the PFC, $Fs(2, 20) < 0.95$, $ps > 0.05$ for the other subunits (**Figure 4-14**).

The mRNA expression of AMPAR and NMDAR subunits in the cortex, hippocampus, amygdala, and PFC was also measured in Cohort 4. In the hippocampus, a one-way ANOVA showed significant differences between groups for GluN2A, $F(2,16) = 4.650$, $p < 0.05$. Follow-up Dunnett's *t*-test found that there was a decrease in GluN2A in the DZP group compared to the VEH group, $p < 0.05$ (**Figure 4-15**). There were no significant group differences for other AMPA or NMDA subunits in the hippocampus. Likewise, there were no group differences in the cortex, $Fs(2, 12) < 0.625$, $ps > 0.05$; amygdala, $Fs(2, 16) < 0.76$, $ps > 0.05$; or the PFC, $Fs(2, 20) < 1.73$, $ps > 0.05$ for the other subunits (**Figure 4-16**).

Changes in Total and Surface Subunit Protein Expression

Cohort 5 mice who were given previous zolpidem, diazepam, and vehicle treatment were used to measure differences in cortical and hippocampal protein expression. In the cortex, one-way ANOVAs found significant differences between groups for total α2 subunit, $F(2,15) = 4.95$, $p < 0.05$, and γ2, $F(2, 6) = 18.16$, $p < 0.01$. In the cortex, Dunnett's *t*-tests found that total α2 subunit expression is decreased in ZOLP mice and total γ2 subunit expression is decreased in DZP mice (**Figures 4-17** and **4-18**). ANOVAs revealed no groups differences in total α1, $F(2,15) = 2.47$, $p > 0.05$; total α3, $F(2,6) = 1.30$, $p > 0.05$; total GluN1, $F(2,5) = 0.235$, $p > 0.05$; and total GluN2B, $F(2, 5) = 2.50$, $p > 0.05$ (**Figures 4-19** through **4-22**). A one-way ANOVA found significant differences between groups for intracellular α1, $F(2,6) = 5.49$, $p < 0.05$ and intracellular α1 was decreased in DZP groups, $p < 0.05$. A one-way ANOVA found no group differences with surface α1 subunits $F(2,6) = 0.176$, $p > 0.05$, (**Figure 4-23**). In the hippocampus one-way ANOVAs found no significant differences between groups for total α1, $F(2,5) = 2.72$, $p > 0.05$ or total α2, $F(2,5) = 3.37$, $p > 0.05$ (**Figures 4-24** and **4-25)**.

In addition to the parametric tests, non-parametric tests were run for the data sets for total α1 in the cortex and total α2 in the hippocampus did not meet the assumptions needed for conducting parametric tests, therefore non-parametric tests were used for analysis. Non-parametric tests using Kruskal-Wallis H test showed that there were no significant differences in protein expression between the treatment groups for total α1, $\chi^2(2) = 4.82$, $p > 0.05$, and for total α2, $\chi^2(2) = 5.14$, $p > 0.05$.

In summary, mice previously given zolpidem or diazepam treatment for 7 days were less sensitive to the sedative effects of zolpidem treatment when compared to vehicle mice, indicating the presence of tolerance and cross-tolerance, respectively. Zolpidem and diazepam treated mice given vehicle injections prior to testing showed evidence of spontaneous withdrawal. Specifically, these mice displayed anxiety-like
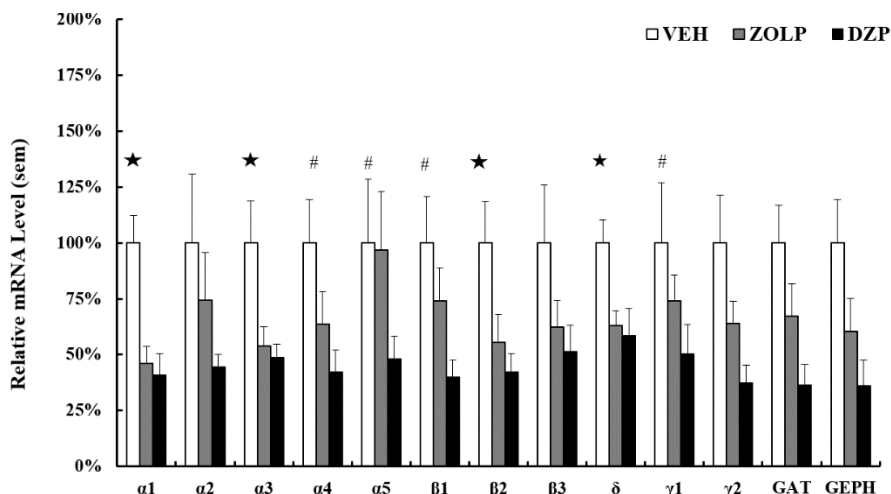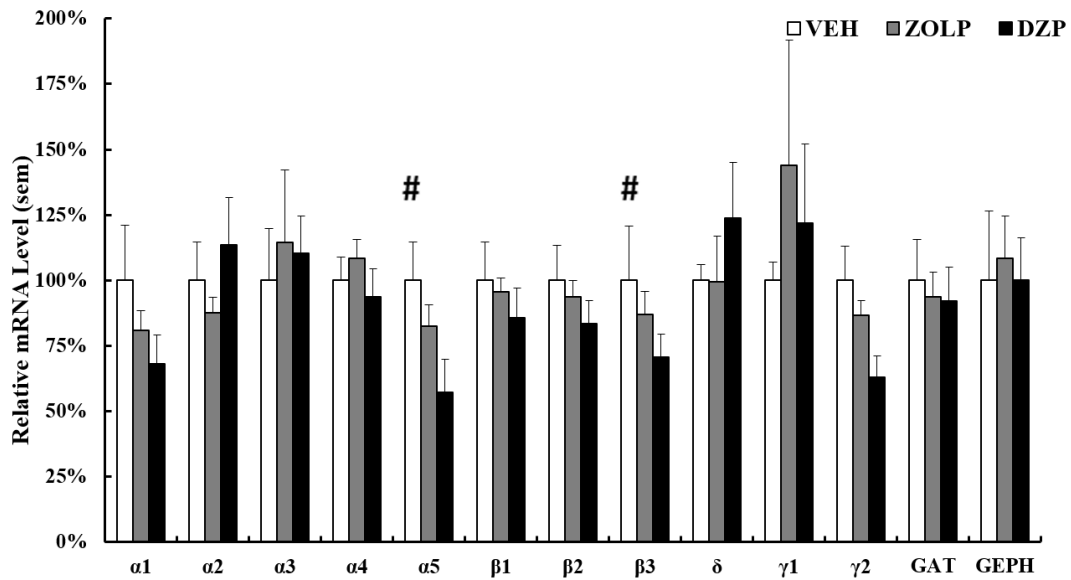
A

B
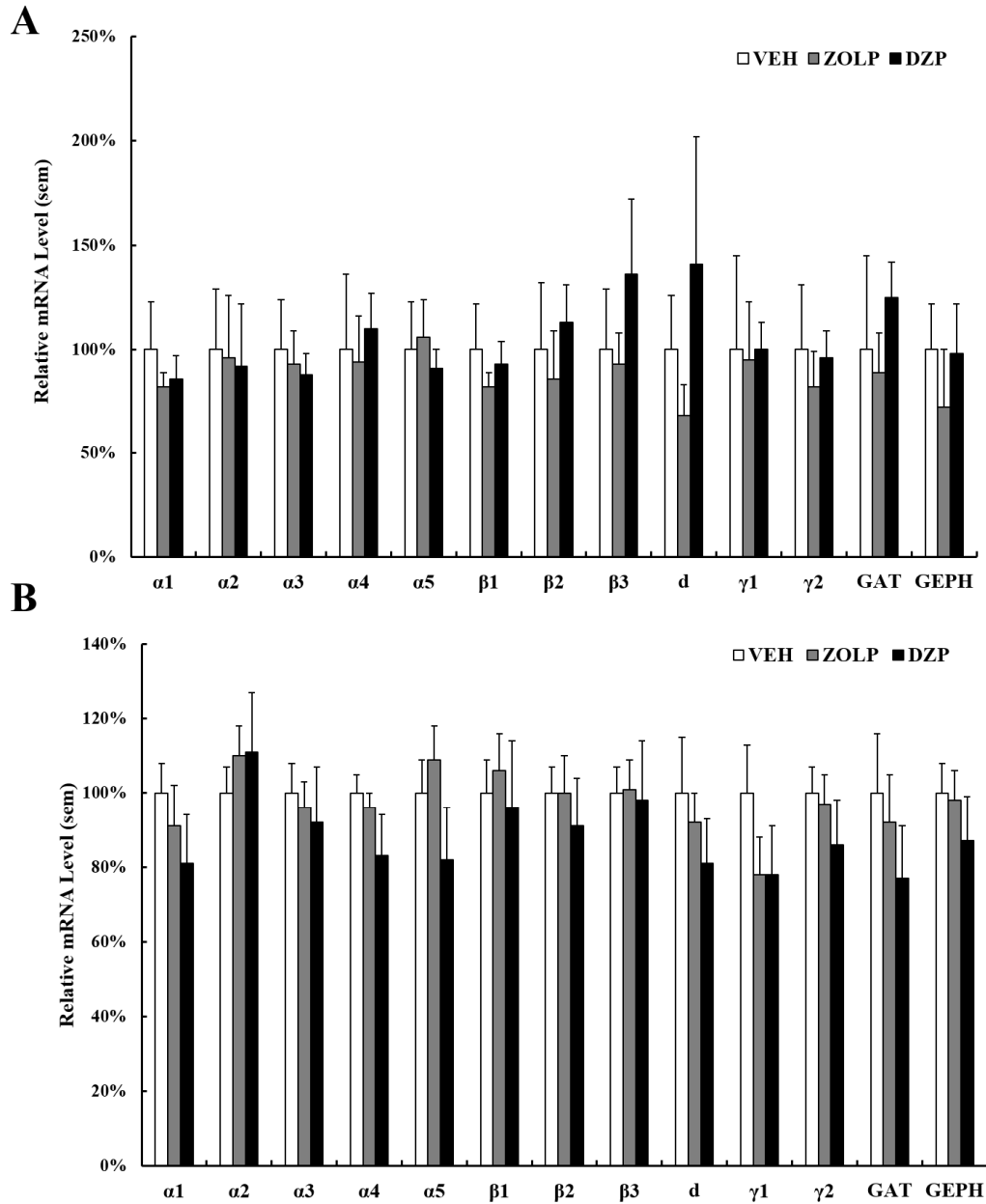
Figure 4-14.   Relative levels of mRNA in the amygdala and pfc following 7-day administration of zolpidem, diazepam, or vehicle
Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100 %). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH in the (A) amygdala and (B) pfc. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05. Reprinted with permission. Wright, B.T., C.F. Gluszek, and S.A. Heldt, *The effects of repeated ZOLP treatment on tolerance, withdrawal-like symptoms, and GABAA receptor mRNAs profile expression in mice: comparison with DZP.* Psychopharmacology (Berl), 2014. **231**(15): p. 2967-79 [16].
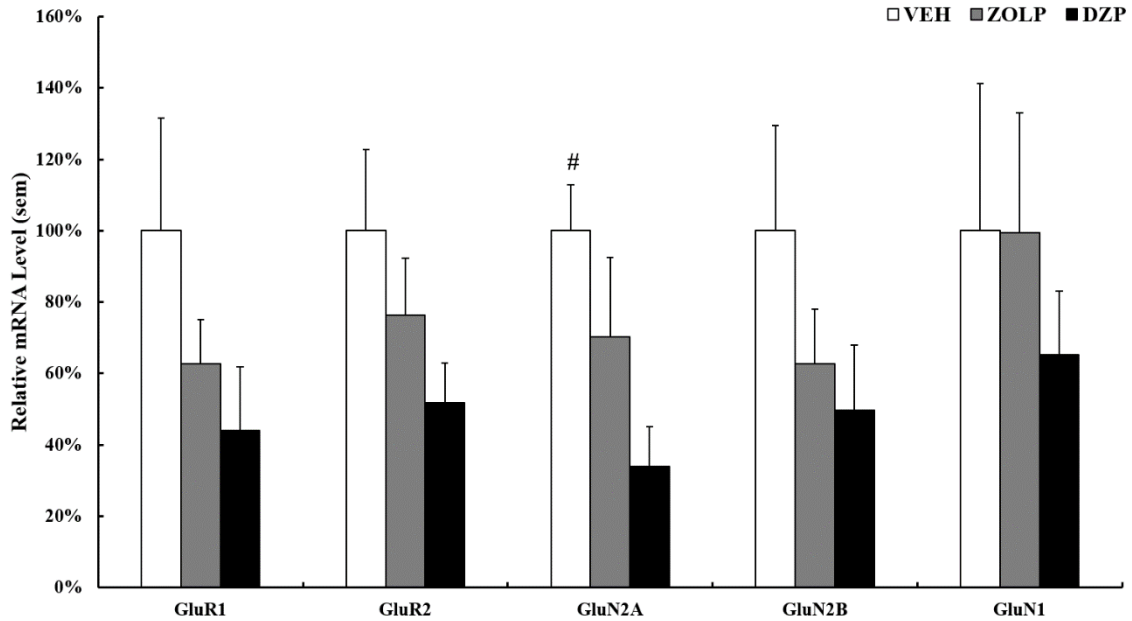
Figure 4-15. Relative levels of mRNA in the hippocampus following 7-day administration of zolpidem, diazepam, or vehicle

Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100 %). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. Number sign denotes VEH significantly different than DZP treatment ($p < 0.05$).
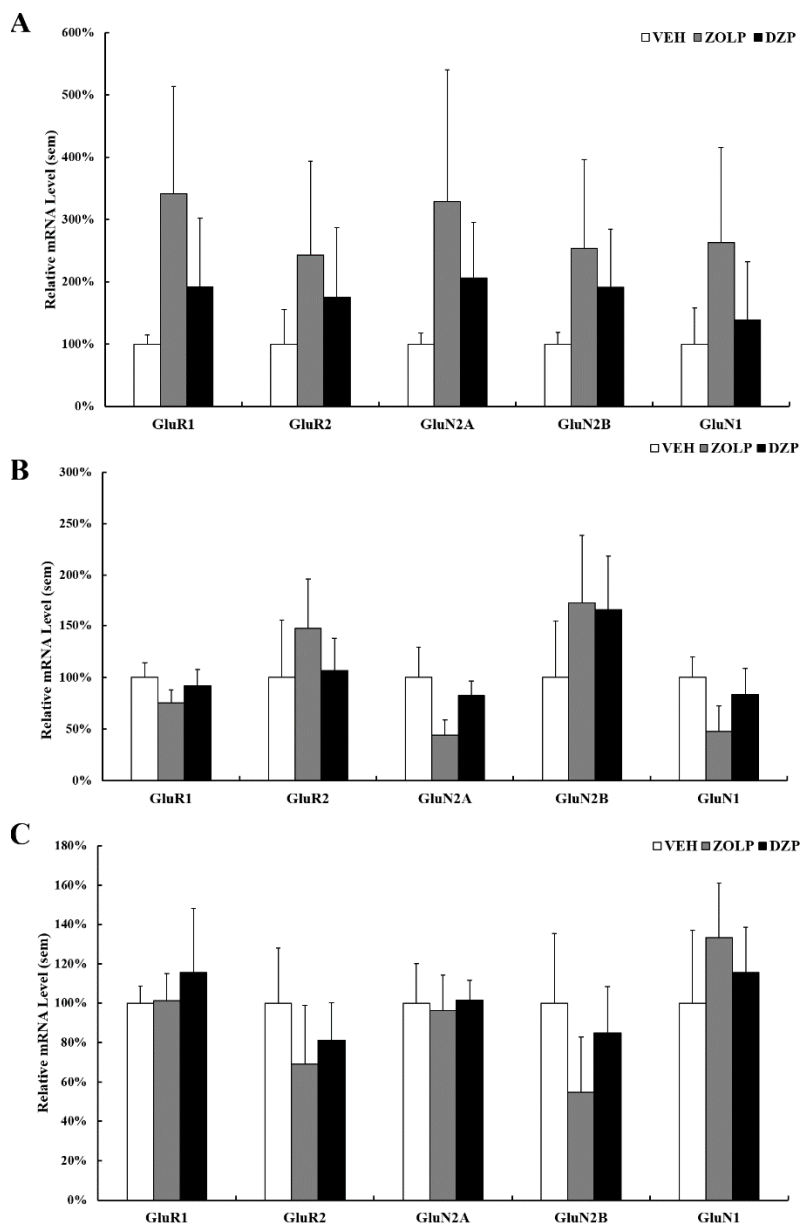
Figure 4-16.   Relative levels of mRNA in the cortex, amygdala, and pfc following 7-day administration of zolpidem, diazepam, or vehicle

Relative expression of protein from each group.Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100 %). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH in the (A) cortex, (B) amygdala, and (C) pfc. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05.
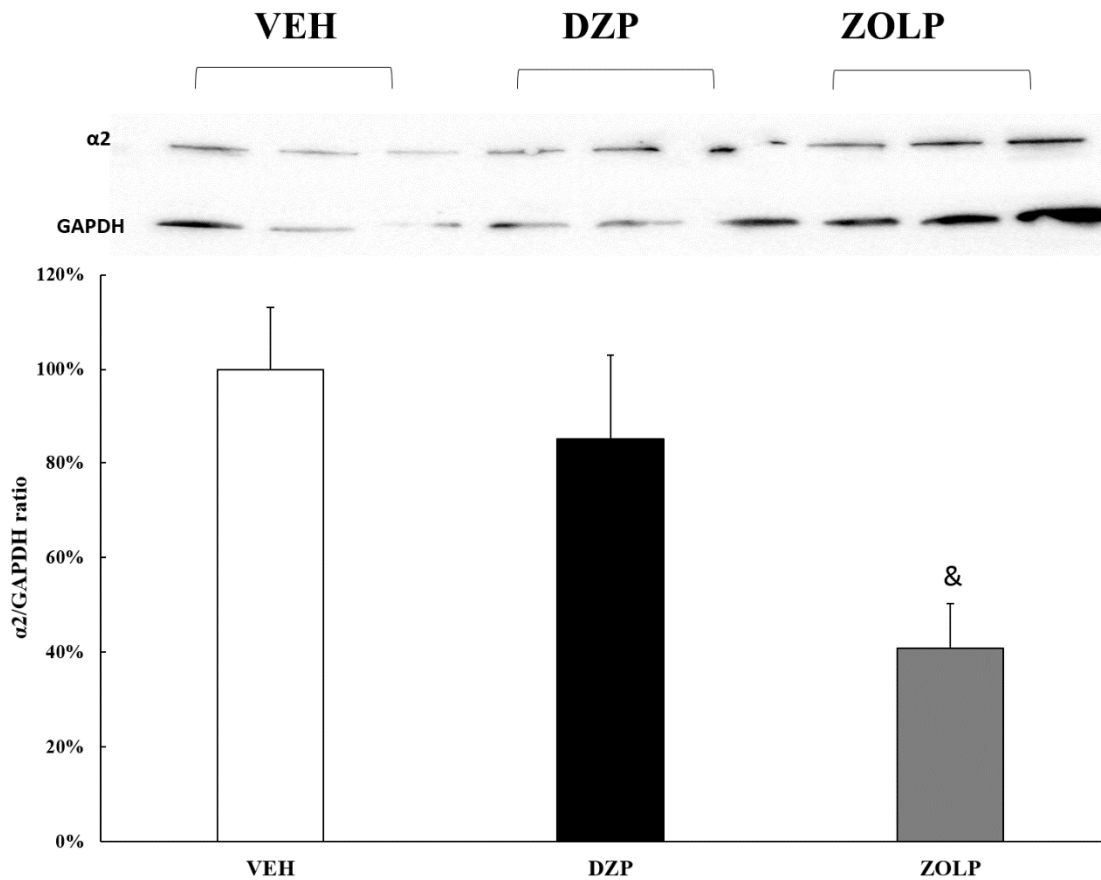
Figure 4-17.   Relative α2 subunit expression in the cortex following 7-day administration of zolpidem, diazepam, or vehicle
Relative expression of protein from each group. Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were significant differences between groups, ps < 0.05. The ZOLP group had less α2 protein expression the VEH group, p < 0.05.
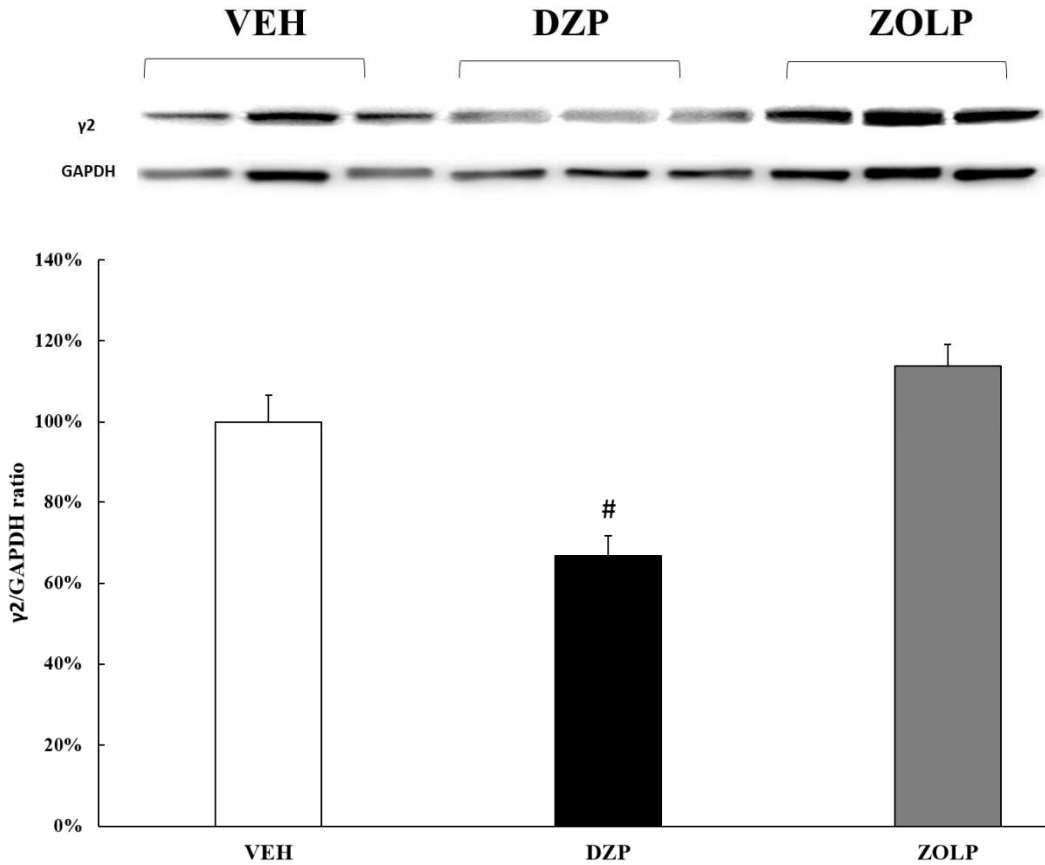
Figure 4-18.   Relative γ2 subunit expression in the cortex following 7-day administration of zolpidem, diazepam, or vehicle

Relative expression of protein from each group. Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were significant differences between groups, ps < 0.05. The DZP group had less γ2 protein expression the VEH group, p < 0.05.
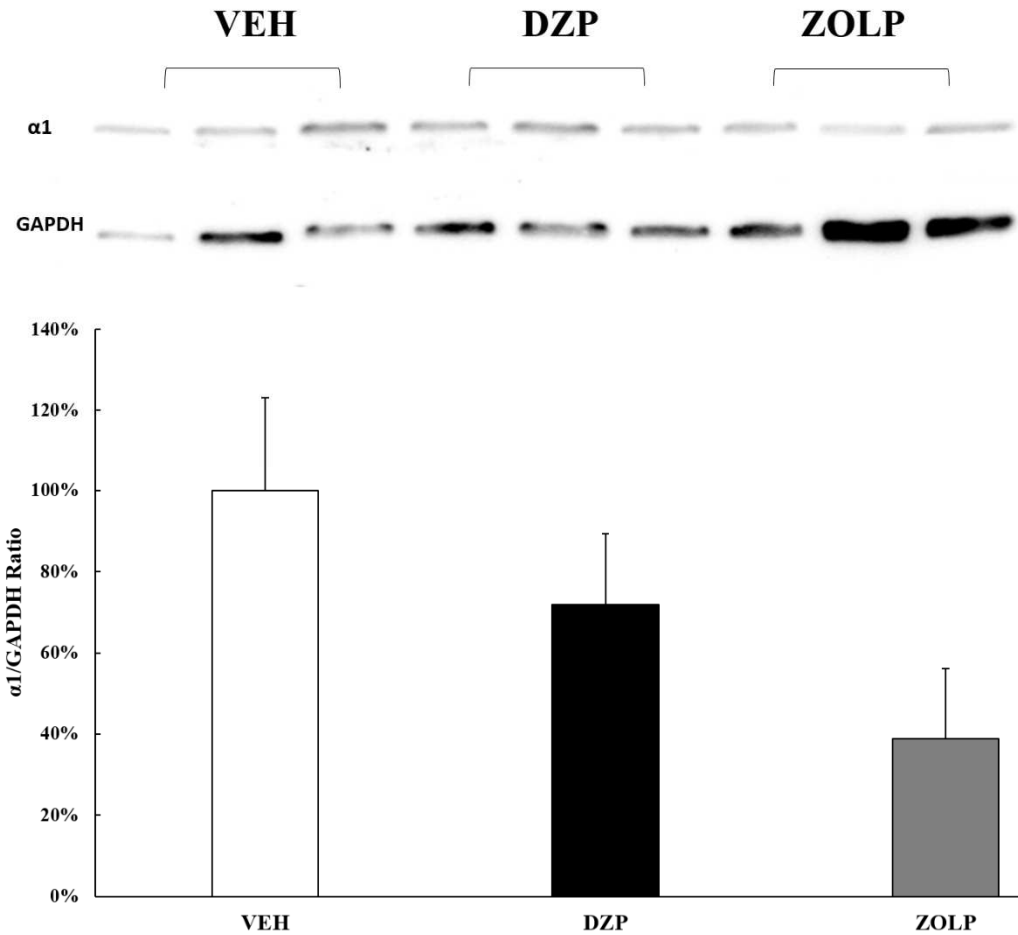
Figure 4-19.   Relative α1 subunit expression in the cortex following 7-day administration of zolpidem, diazepam, or vehicle
Relative expression of protein from each group. Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05.
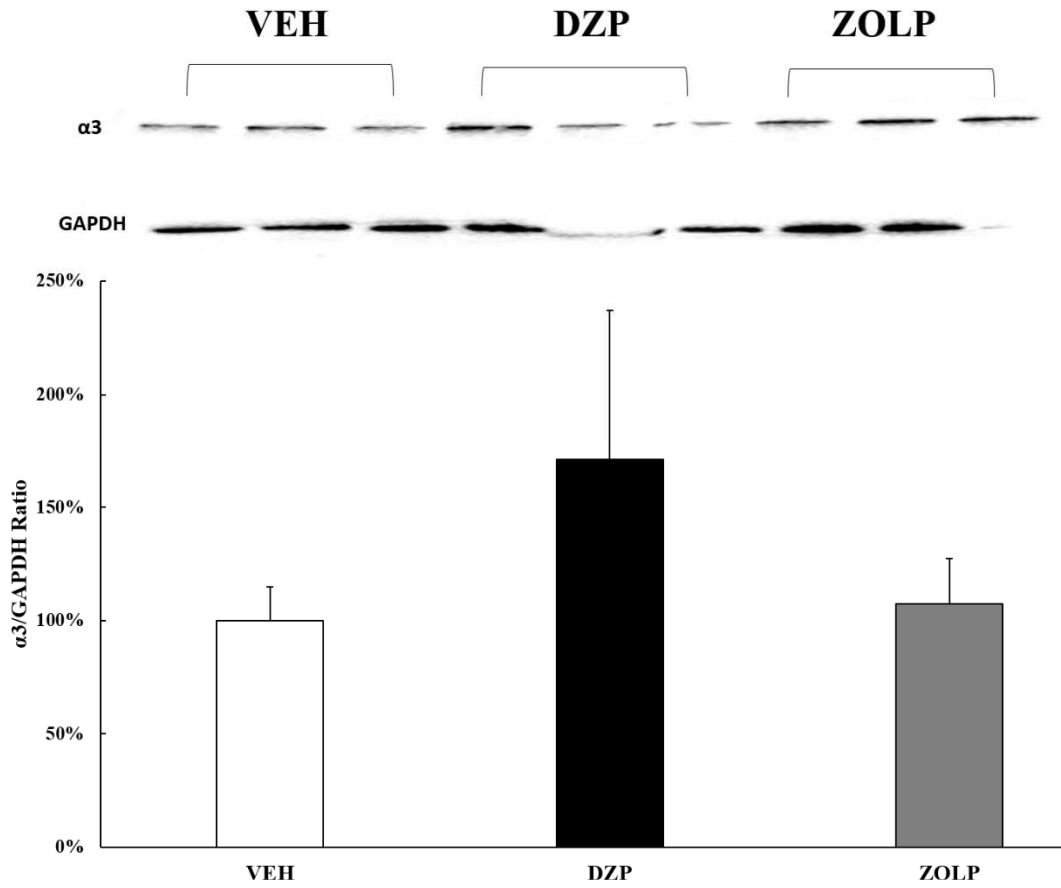
Figure 4-20.   Relative α3 subunit expression in the cortex following 7-day administration of zolpidem, diazepam, or vehicle
Relative expression of protein from each group. Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05.
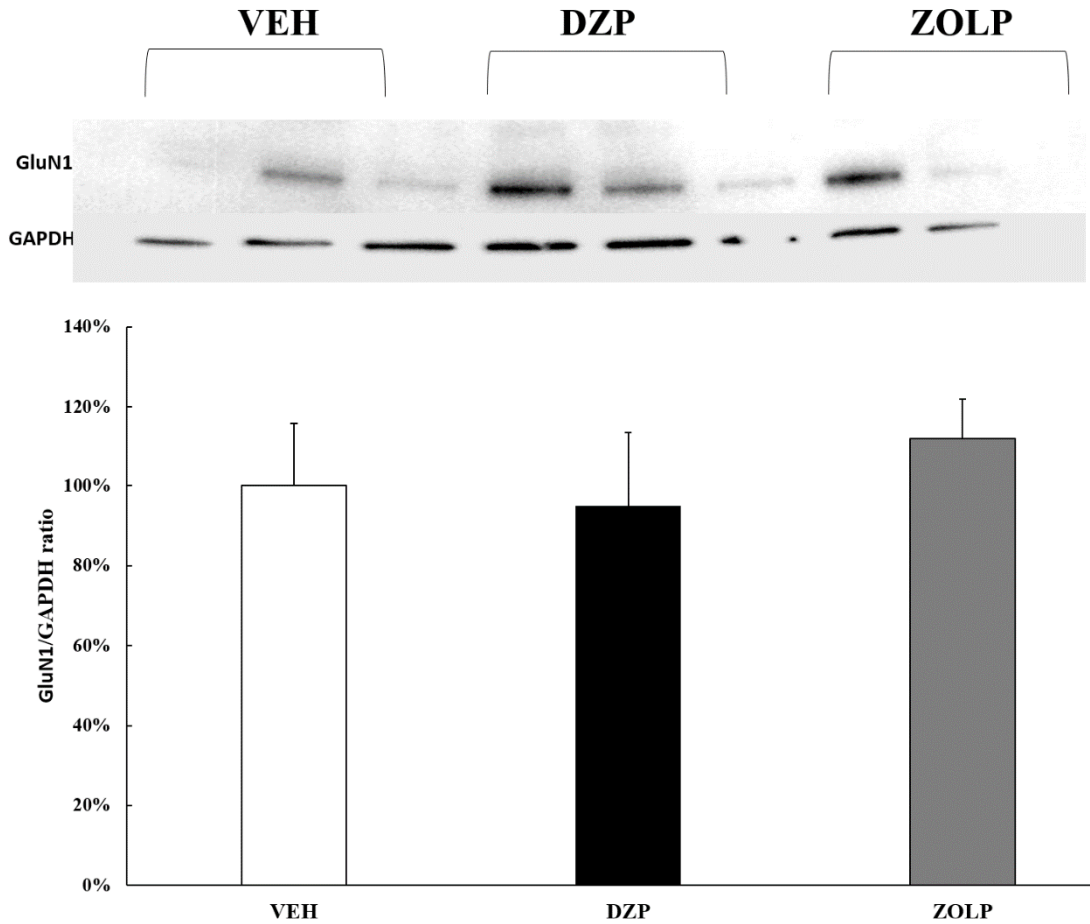
Figure 4-21.   Relative GluN1 subunit expression in the cortex following 7-day administration of zolpidem, diazepam, or vehicle
Relative expression of protein from each group. Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05.
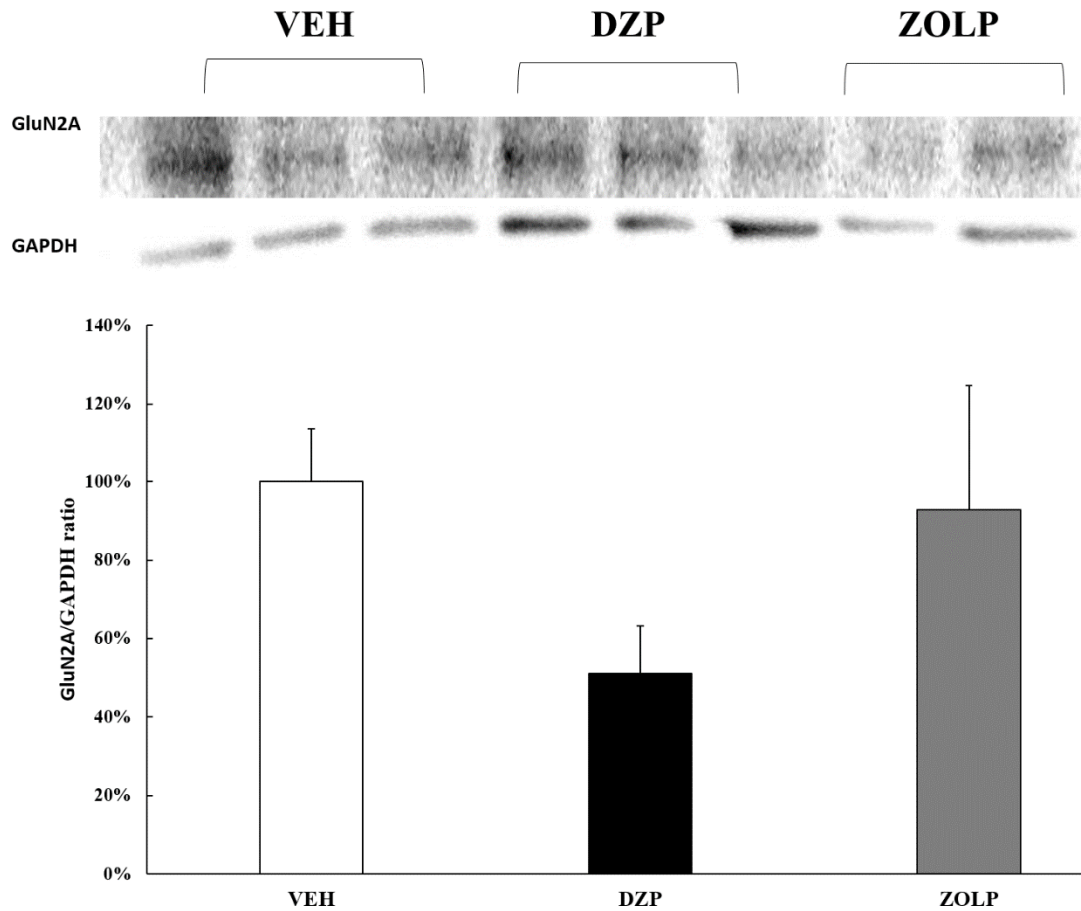
Figure 4-22. Relative GluN2A subunit expression in the cortex following 7-day administration of zolpidem, diazepam, or vehicle
Relative expression of protein from each group. Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05.
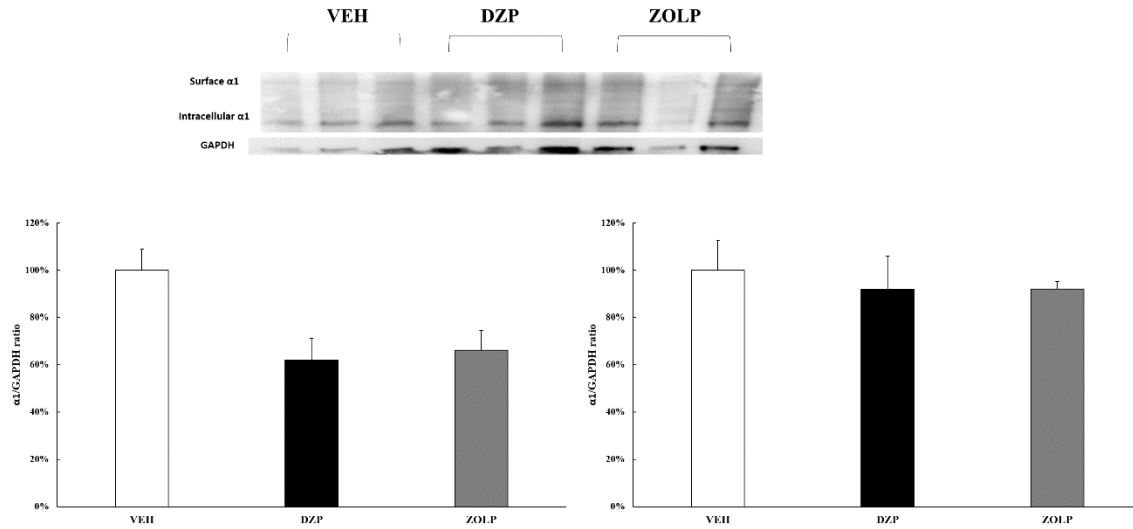
Figure 4-23. BS3 treated samples, surface and intracellular α1 protein expression levels in the cortex following 7-day administration of zolpidem, diazepam, or vehicle Relative expression of protein from each group. Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05.
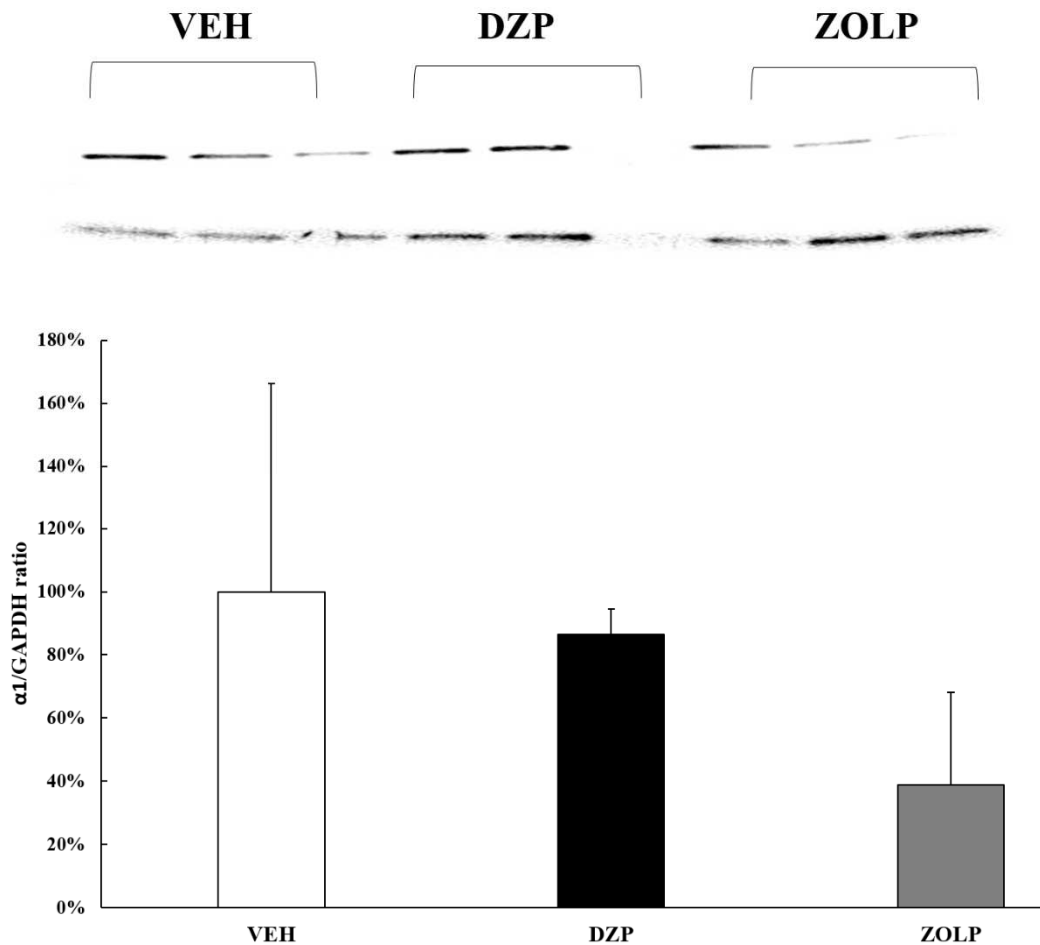
Figure 4-24. Relative α1 protein expression levels in the hippocampus following 7-day administration of zolpidem, diazepam, or vehicle

Relative expression of protein from each group. Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05.
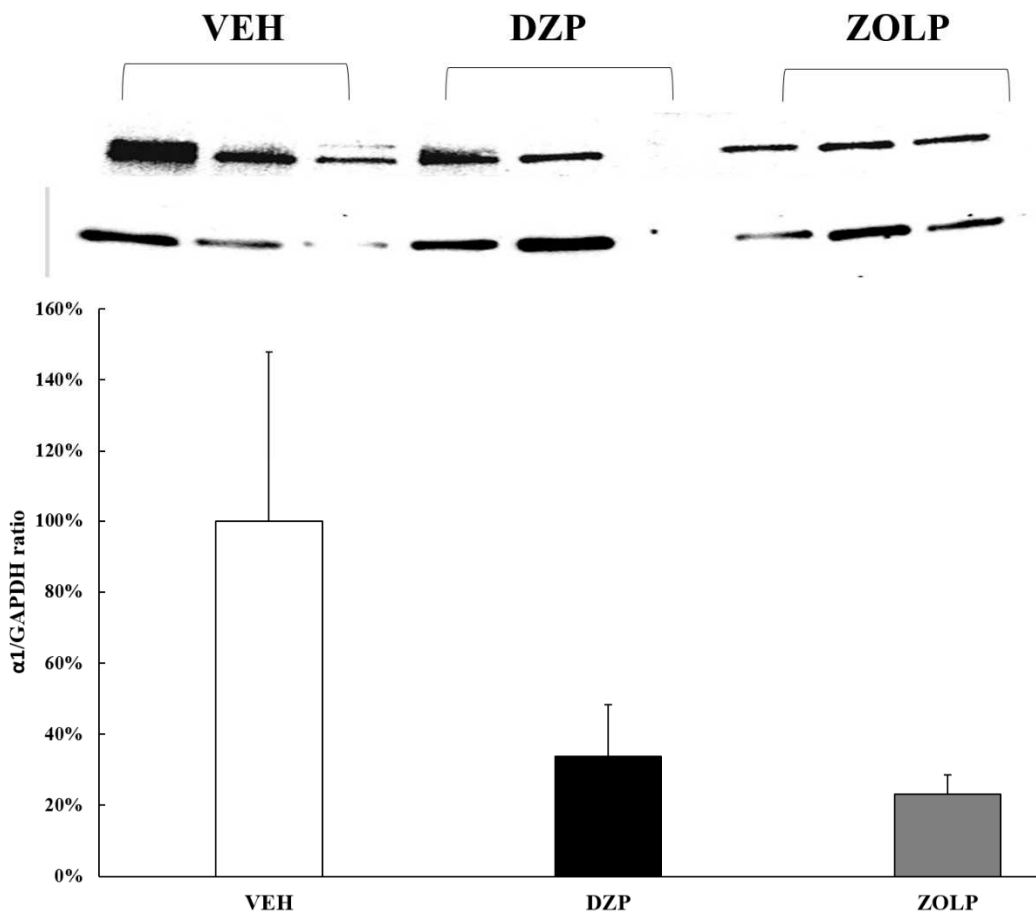
Figure 4-25. Relative α2 protein expression levels in the hippocampus following 7-day administration of zolpidem, diazepam, or vehicle

Relative expression of protein from each group. Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05.

behaviors, including decreases in activity, frequency of rearing, and center zone entries. These mice also showed a progressive decrease percent of time in center zone, suggesting a gradual increase in anxiety during testing. In contrast, zolpidem and diazepam treated mice showed minimal withdrawal symptoms after flumazenil treatment, as indicated by the lack of anxiety-like behaviors when flumazenil was given prior to testing 1 and 2 days after drug termination.

In addition to changes in behaviors, mice given zolpidem or diazepam treatment for 7 days also showed different levels of mRNA expression in the cortex when compared to vehicle mice. Both drugs caused decreases in the expression of $\alpha 1$-, $\alpha 3$-, $\beta 2$-, and $\delta$-GABA$_A$R subunits in the cortex. Diazepam treatment also caused decreases in the expression $\alpha 4$-, $\alpha 5$-, $\beta 1$-, and $\gamma 2$-GABA$_A$R subunits in the cortex. Mice given diazepam treatment also showed different levels of mRNA expression in the hippocampus. Specifically, these mice displayed lower mRNA expression levels of $\alpha 5$- and $\gamma 2$-GABA$_A$R subunits and the NMDA GluN2A subunit.

Study 2 also identified differences in local protein expression. Protein expression of total $\alpha 2$ subunit was decreased after zolpidem treatment and protein expression of total $\gamma 2$ subunit was decreased after diazepam treatment in the cortex. The intracellular $\alpha 1$ subunit protein was decreased after zolpidem treatment, while surface $\alpha 1$ subunit protein was unaffected in the cortex. Protein expression of total $\alpha 1$ and $\alpha 2$ subunits in the hippocampus were not affected.

Study 3: The Effect of 30-Day Zolpidem and Diazepam Treatment on Behavioral Sedation and mRNA Expression

Tolerance to the Sedative Effects of Zolpidem

The purpose of Study 1 was to examine if a 30-day treatment regimen of zolpidem or diazepam induces significant changes to the sedative effects of zolpidem, as measured by open field activity, and significant changes in mRNA expression, as measured by qRT-PCR. Changes to the sedative effects were examined in Cohort 1 and 2 mice previously treated with vehicle, diazepam, and zolpidem. A two-way ANOVA on found a significant main effect of time, $F_{(5,175)} = 196.10$, $p < 0.01$, a significant main effect of treatment, $F_{(2,35)} = 8.95$, $p < 0.01$, and a significant interaction of Time x Treatment, $F_{(10,175)} = 2.57$, $p < 0.01$ (**Figure 4-26**). To examine the interaction, one-way ANOVAs were performed at each level of time. There were significant group differences at Block 1, $F_{(2,54)} = 4.15$, $p < 0.05$, and Block 2, $F_{(2, 54)} = 6.57$, $p < 0.01$. There were no significant differences at Blocks 3-6, $F_{(2,54)} < 4.14$, $p > 0.05$. Follow-up Dunnett's t-tests found that the DZP group displayed more activity than the VEH group at Block 1, $p < 0.05$. At Block 2, both DZP and ZOLP groups exhibited more activity than the VEH group, ps < 0.05.
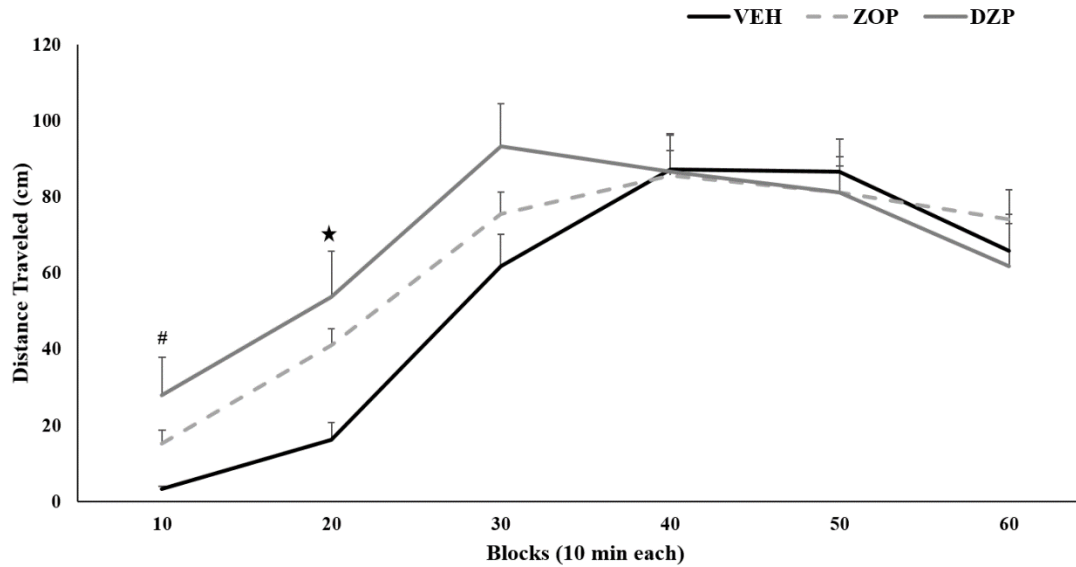
Figure 4-26. Decreased locomotor impairing effect of zolpidem after 30-day administration of zolpidem, diazepam, or vehicle
Distance traveled in open field after test administration of 2 mg/kg of ZOLP in mice previously given subchronic treatment of ZOLP, DZP, or VEH. Analyses of each 10-min block showed that differences between groups were most evident in the first half of the 60-min test session. Data are presented as mean + sem. Black star denotes VEH< ZOLP and DZP groups and Number sign denotes VEH < DZP group ($p < 0.05$).

Changes in mRNA Expression Measured by qRT-PCR

Cohort 2 mice were used to examine changes in mRNA expression. One-way ANOVAs were performed to compare group levels of each mRNA in the cortex, hippocampus, PFC, and amygdala. In the cortex, one-way ANOVAs revealed significant group differences in mRNA levels of α1 subunits, $F(2,35) = 5.18$, $p < 0.05$, and GAT-1, $F(2,35) = 3.30$, $p < 0.05$. In the hippocampus, ANOVAs revealed significant group differences in levels of α4 subunits, $F(2,31) = 5.89$, $p < 0.01$, γ2 subunits, $F(2,31) = 3.60$, $p < 0.05$, and gephyrin, $F(2,31) = 4.39$, $p < 0.05$. In the amygdala, a one-way ANOVAs revealed significant group differences in mRNA levels of γ2 subunits, $F(2,18) = 6.84$, $p < 0.01$. Follow-up Dunnett's *t*-tests performed on all group differences above show that DZP or ZOLP groups were not significantly different from the VEH groups, *p*s > 0.05 **(Figure 4-27)**.

In the PFC, one-way ANOVAs found group differences in mRNA levels of subunits α1, $F(2,30) = 6.67$, $p < 0.01$; α2, $F(2,27) = 6.70$, $p < 0.01$; α3, $F(2,31) = 5.39$, $p < 0.05$; β1, $F(2,32) = 4.23$, $p < 0.05$; β2, $F(2,32) = 3.75$, $p < 0.05$; β3, $F(2,32) = 5.33$, $p < 0.05$; δ, $F(2,32) = 4.16$, $p < 0.05$; γ1, $F(2,32) = 5.90$, $p < 0.01$; and γ2, $F(2,32) = 4.07$, *p*s < 0.05. Follow-up Dunnett's *t*-test found significant differences between the VEH and ZOLP groups for subunits α1, α2, α3, β1, β2, β3, δ, γ1, and γ2, *p*s < 0.05. (**Figure 4-28**).

In summary, this study indicated that mice given a 30-day treatment of diazepam were cross-tolerant to the sedative effects of zolpidem. This study also indicated that mice given zolpidem showed some evidence of zolpidem tolerance; however, this effect was relatively weak and only observed at time Block 2. There were no significant changes in mRNA levels after 30 days of diazepam. However, mice given a 30-day zolpidem treatment did significantly decrease the levels of mRNA for $GABA_A R$ subunits α1, α2, α3, β1, β2, β3, γ1, and γ2 in the PFC.

Comparisons of Zolpidem and Diazepam Sedative Effects after 3-, 7-, and 30-Day Treatment Durations

Statistical analyses of Studies 1-3 revealed more prominent deficits to the sedative effects of zolpidem in mice given zolpidem and diazepam for 7 days in comparison to 3 days and 30 days. To further inspect these findings, we compared differences in treatment duration by drug treatment. To complete this, we first examined whether differences in treatment duration (3-, 7-, and 30-days) influenced the distance traveled across the test session time (Blocks 1–6) in mice given vehicle treatments (VEH-ZOLP). This comparison indicated no significant of time, $F(5,240) = 98.09$, $p < 0.01$; duration, $F(10,240) = 1.35$, $p > 0.05$; or a significant Time x Duration interaction, $F(2, 48) = 0.90$, $p > 0.05$. These results suggest that the number of injections prior to testing did not impact locomotor activity during testing and was not likely a confounding factor influencing differences in the sedative effects of zolpidem among studies **(Figure 4-29)**.
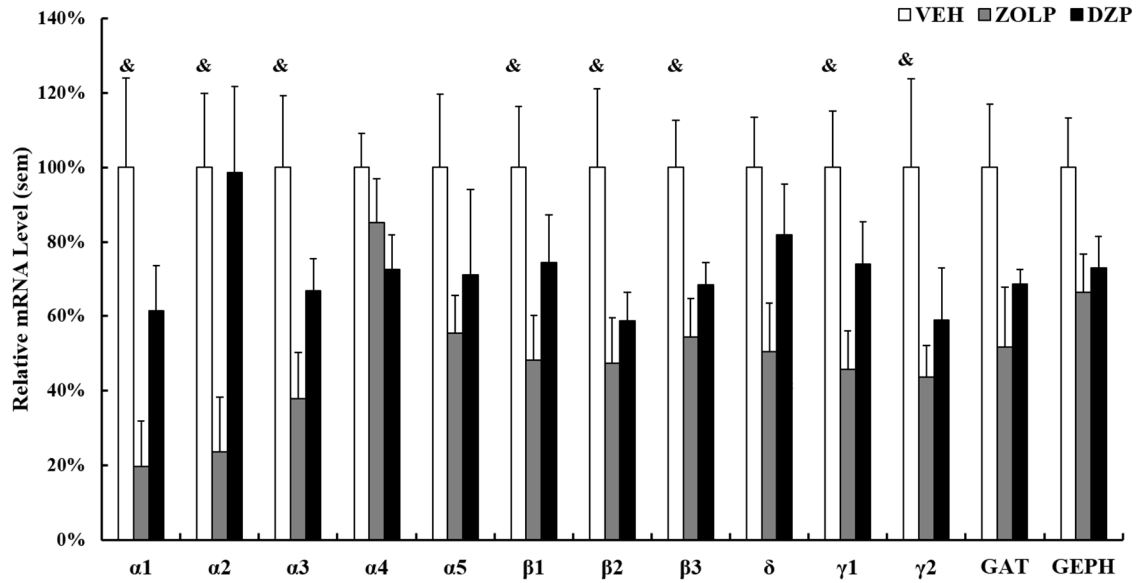
Figure 4-27. Relative levels of mRNA in the pfc following 30-day administration of zolpidem, diazepam, or vehicle

Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. Ampersand sign VEH > ZOLP treatment ($p < 0.05$).
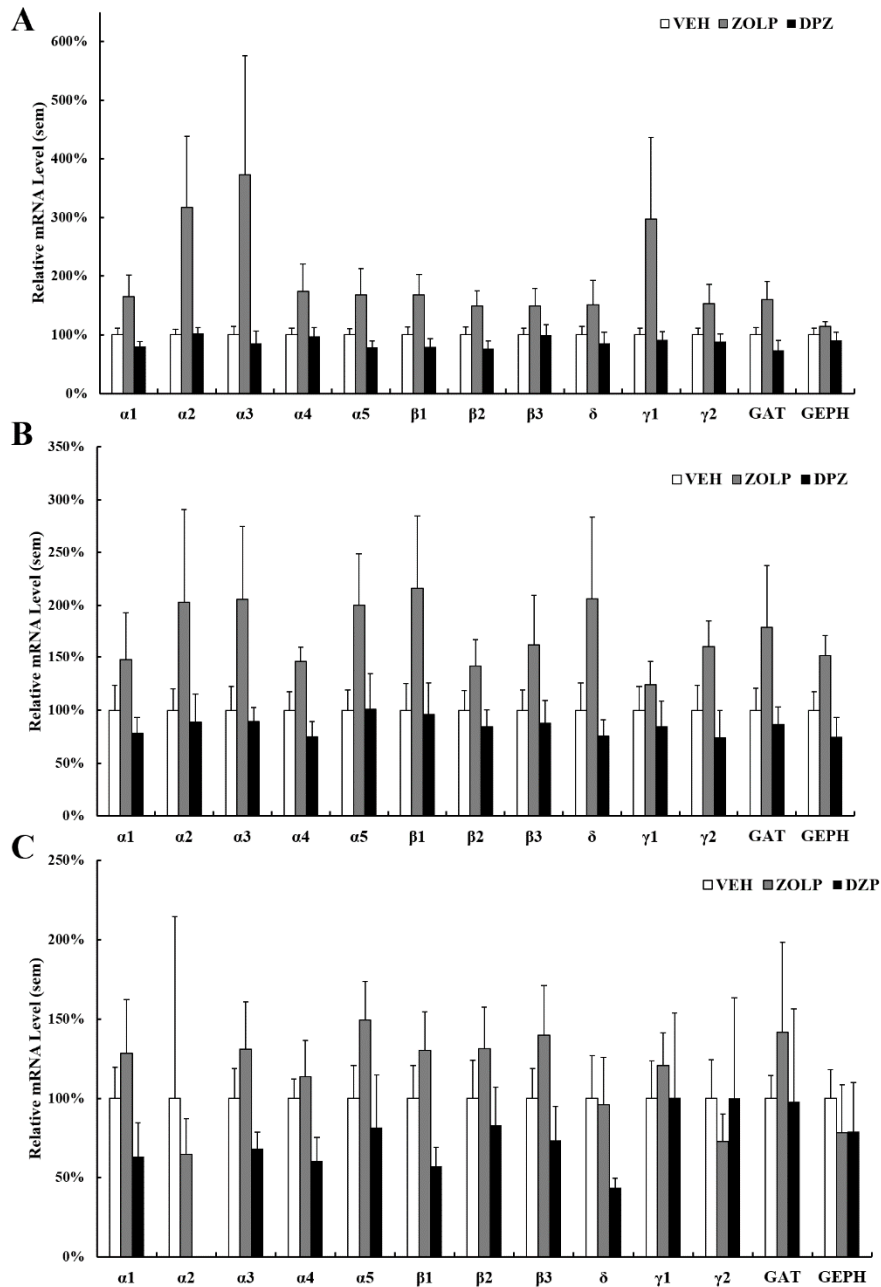
Figure 4-28. Relative levels of mRNA in the cortex, hippocampus, and amygdala following 7-day administration of zolpidem, diazepam, or vehicle
Histograms comparing levels of transcripts are expressed as mean percentage of VEH control (100%). Statistical analyses were performed by ANOVA with Dunnett's t test for pairwise comparisons against a VEH. Data are presented as mean + sem. There were no significant differences between groups, ps > 0.05.
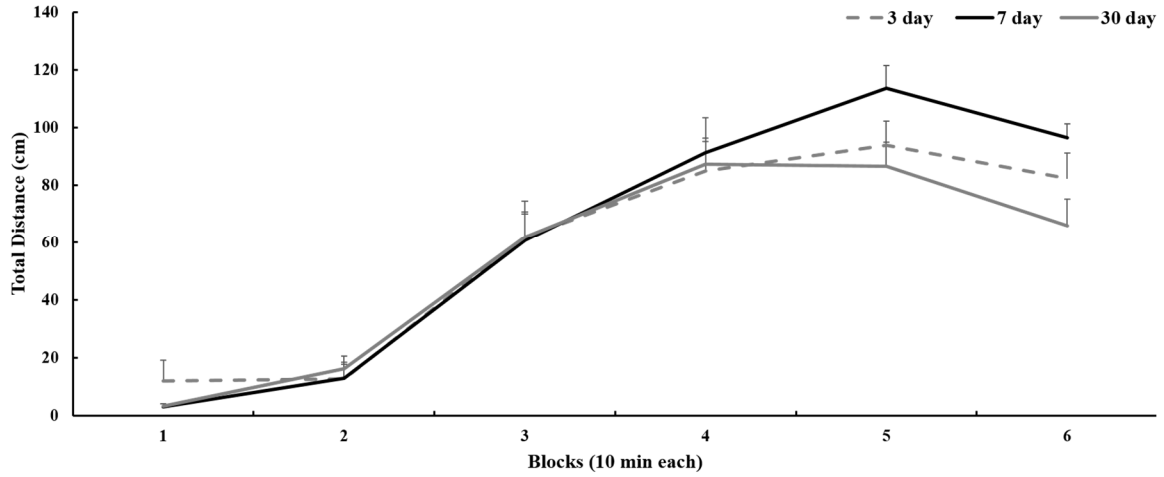
Figure 4-29. No significant differences between vehicle groups from each study on motor activity
Distance traveled in open field after test administration of 2 mg/kg of ZOLP in mice previously given subchronic treatment of VEH for 3, 7, or 30 days. Analyses of each 10-min block showed no differences between. Data are presented as mean + sem.

Next, we used the same approach in an attempt to affirm the more prominent 7-day deficits by separately examining differences in treatment durations of zolpidem and diazepam. In mice given diazepam treatments (DZP-ZOLP), the two-way ANOVA revealed a main effect of time, $F(5,240) = 52.20$, $p < 0.01$. There was no effect of duration, $F(2,48) = 1.60$, $p > 0.05$ or a significant interaction of Time x Duration, $F(10, 240) = 1.43$, $p > 0.05$ **(Figure 4-30)**.

In mice given zolpidem treatments (ZOLP-ZOLP), the two-way ANOVA found a main effect of time, $F(5,295) = 105.08$, $p < 0.01$, duration, $F(2,59) = 6.72$, $p < 0.05$, and a significant interaction of Time x Duration, $F(10,295) = 1.92$, $p < 0.05$. To evaluate the interaction, one-way ANOVAs were conducted on each block of time. There was a significant difference between groups at Block 2, $F(2,59) = 3.98$, $p < 0.05$, Block 3, $F(2,59) = 9.18$, $p < 0.05$, and Block 6, $F(2,59) = 3.85$, $p < 0.05$. Mice who received zolpidem for 7 days displayed greater activity at Blocks 2, 3, and 6 than mice who received zolpidem for 3 or 30 days, $p < 0.05$. There were no significant differences between groups at Blocks 1, 4 and 5, $Fs(2,59) < 3.10$, $ps > 0.05$ **(Figure 4-31)**.

Comparison of Center, and Periphery Distance Traveled: Spontaneous Withdrawal

To assess whether differences in the distance traveled across the test session time (Blocks 1–6) influenced measures of open-field anxiety, two-way ANOVAs were performed on distance traveled in the center and periphery of the open field. If both regions show decreases in activity, then activity would affect interpretation of anxiety-like measures.

For distance traveled in the center, a two-way ANOVA found that there was a main effect of time, $F(5,105) = 31.01$, $p < 0.01$, a main effect of treatment, $F(2,21) = 7.21$, $p < 0.01$, and a significant interaction of Time x Duration, $F(10,105) = 3.24$, $p < 0.01$. To assess the interaction, a one-way ANOVAs at each level of time were performed. There were group differences at Block 1, $F(2,21) = 14.82$, $p < 0.01$, Block 2, $F(2,21) = 9.09$, $p < 0.01$, Block 3, $F(2,21) = 4.05$, $p < 0.05$, and Block 5, $F(2,21) = 3.51$, $p < 0.05$. Follow-up Dunett's t-tests revealed that the activity of ZOLP and DZP groups was significantly lower than the VEH groups at Blocks 1 and 2, $p < 0.01$ and the activity of DZP groups was significantly lower than the VEH groups at Blocks 3 and 5, $p < 0.05$. There was no significantly differences at Blocks 4 and 6, $Fs(2,21) > 3.17$, $p > 0.05$ **(Figure 4-32).**

For distance traveled in the periphery, a two-way ANOVA found that there was a main effect of time, $F(5,105) = 11.36$, $p < 0.05$, no main effect of treatment, $F(2,21) = 2.03$, $p > 0.05$, and no significant interaction of Time x Duration, $F(10,105) = 0.81$, $p > 0.05$ **(Figure 4-33)**.
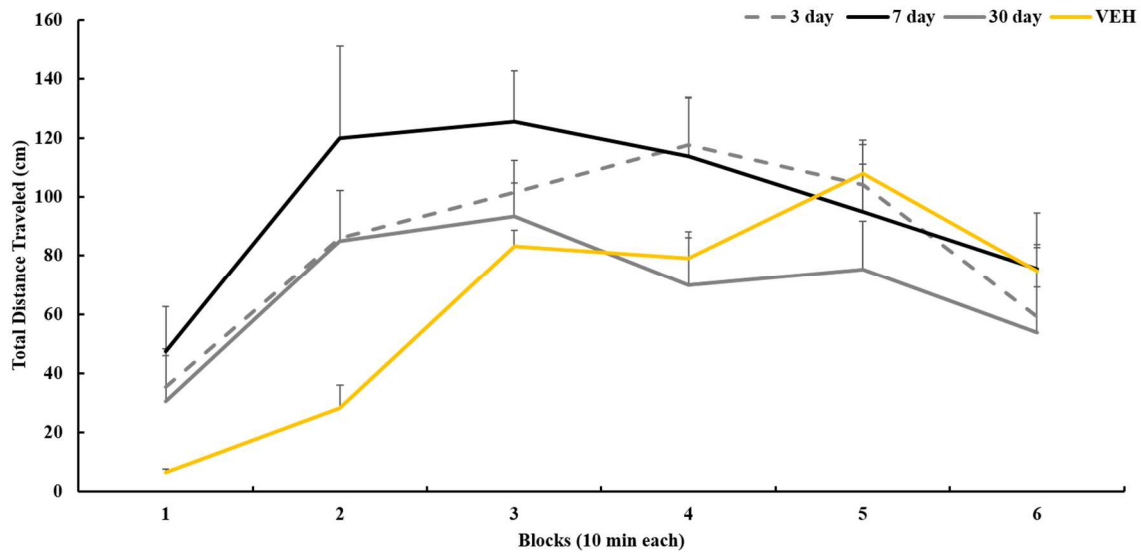
Figure 4-30.   No significant differences between diazepam groups from each study on motor activity.

Distance traveled in open field after test administration of 2 mg/kg of ZOLP in mice previously given subchronic treatment of DZP for 3, 7, or 30 days. Analyses of each 10-min block showed no differences between. Data are presented as mean + sem.
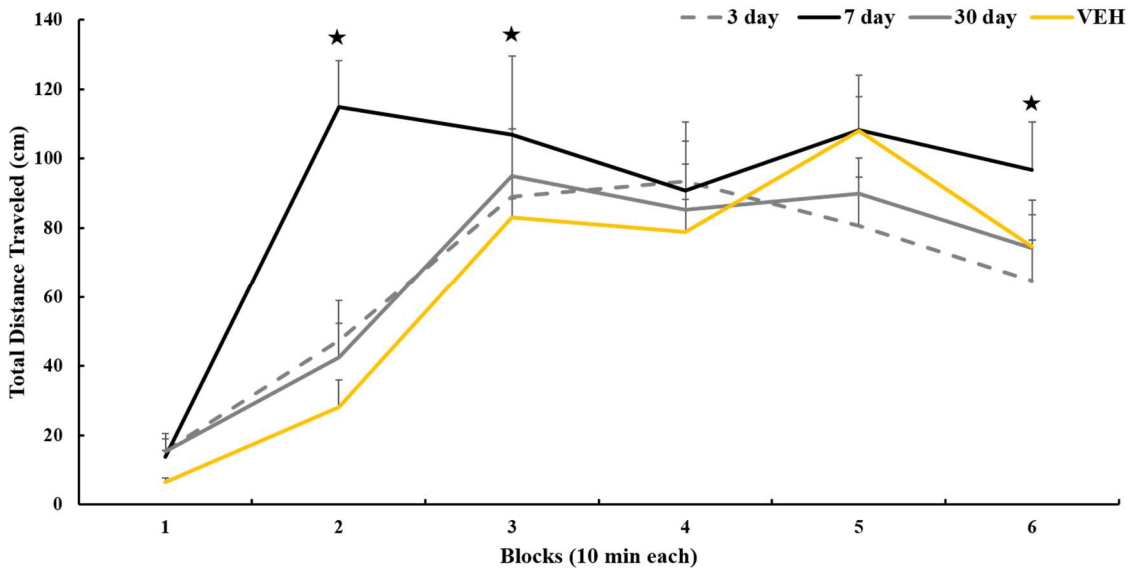
Figure 4-31.   Increased motor activity due to 7 days of ZOLP compared to 3 or 30 days of ZOLP

Distance traveled in open field after test administration of 2 mg/kg of ZOLP in mice previously given subchronic treatment of ZOLP for 3, 7, or 30 days. Analyses of each 10-min block showed differences between groups. The 7 day ZOLP group showed greater activity than the 3 or 30-day group. Data are presented as mean + sem.
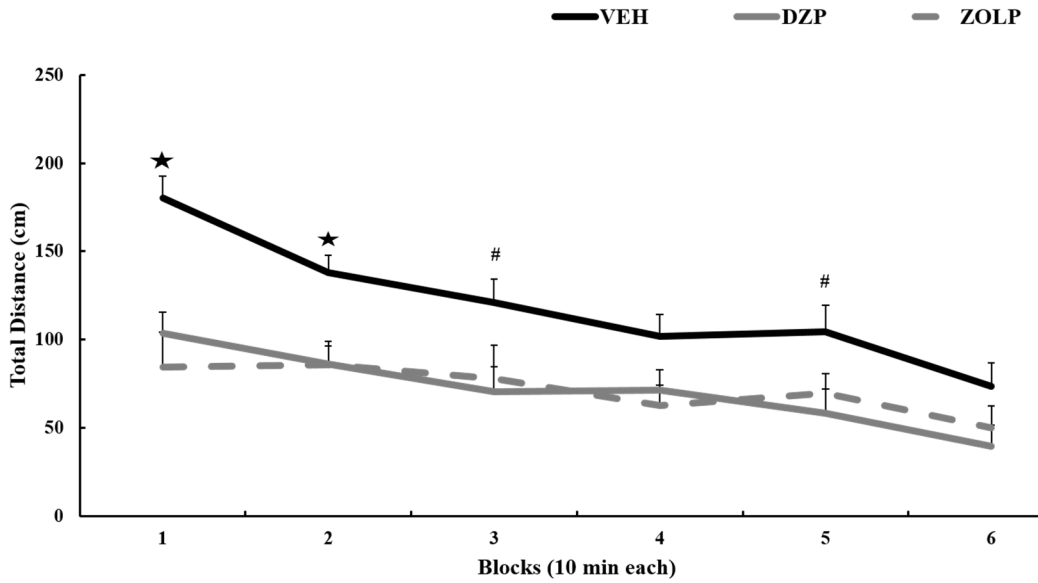
Figure 4-32. Decreased motor activity in the center region of the open field apparatus

Distance traveled in open field after administration of VEH in mice previously given subchronic treatment of ZOLP, DZP, or VEH. Analyses of each 10-min block showed that differences between groups, with the VEH group showing greater activity than the DZP or ZOLP groups. Data are presented as mean + sem. Black star denotes VEH< ZOLP and DZP groups and Number sign denotes VEH < DZP group ($p < 0.05$).
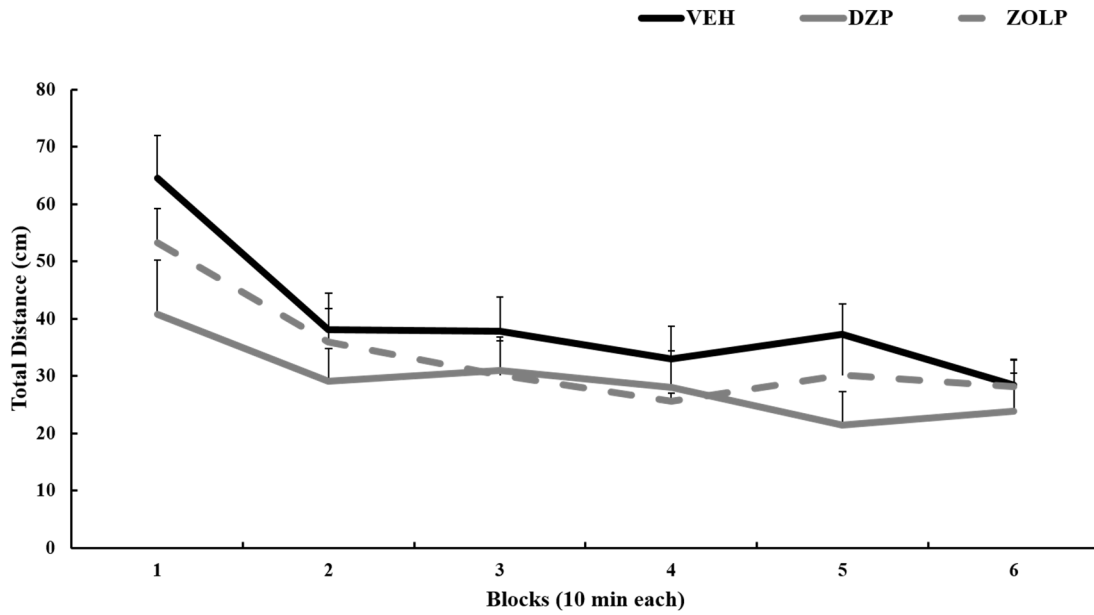
Figure 4-33.   No significant differences between groups in the peripheral region of the open field apparatus on motor activity
Distance traveled in open field after administration of VEH in mice previously given subchronic treatment of ZOLP, DZP, or VEH. Analyses of each 10-min block showed no differences between groups, Data are presented as mean + sem.

CHAPTER 5.   DISCUSSION*

The development of tolerance to the sedative-hypnotic effects of zolpidem and symptoms of withdrawal are becoming a growing concern as usage increases in the general population [22, 23, 136]. Using mouse models, researchers are discerning what factors influence tolerance and withdrawal and the neuromechanisms associated with these phenomena [226]. The purpose of our studies were to examine whether 3-, 7-, and/or 30-day treatment regimens of zolpidem or diazepam diminished the sedative effects of zolpidem in mice. In addition, these studies examined whether treatment regimens changed the expression of mRNA and proteins believed to be associated with tolerance and withdrawal.

Behavioral Tolerance and Cross-Tolerance

Numerous studies have indicated that tolerance to the sedative effects of BZs can develop following repeated treatments in rodents; however, there is conflicting evidence concerning the development of tolerance to zolpidem [123, 243, 244]. Our finding of tolerance after 7 days of zolpidem is in agreement with past studies that show mice develop sedative tolerance after 10 days of treatment [25, 105, 123]. However, in Studies 1 and 3, mice given zolpidem for 3 or 30 days prior to testing show minimal evidence of reduced sedation. Past studies examining the development of tolerance to zolpidem's varying effects have primarily examined changes in efficacy or potency after 7-14 days of treatment. For example, tolerance to zolpidem's ataxic, hypothermic, and anticonvulsant effects have been observed in rodents and primates during these time periods [25, 29, 38, 105, 138]. Other studies have reported no tolerance after zolpidem administration in rodents [28, 109]. The detection of zolpidem tolerance in past studies is likely influenced by numerous factors, like the time between test injection and behavioral testing. One major difference between our and previous studies was the latency between our test-drug injection and the onset of tolerance testing. In most studies, the time between injections and the start of testing is 15-30 min. Because zolpidem is relatively short acting and quickly metabolized, 15-30 min delay between drug injection and testing may hamper detection of tolerance. In our studies, the time between the test injection and testing was only 2 min. As seen in **Figure 4-3**, the sedative effects of zolpidem are clearly evident during the first 30 min following this test-drug injection and less evident during the latter half of the test session.

In contrast to 7 days, 3 and 30 days of zolpidem treatment produced little evidence of sedative tolerance in mice. The lack of tolerance after 3 days may be due to the fact that sedative tolerance gradually develops and reaches detectable levels only at later time points (e.g. 7 days). It is possible that higher treatment dose levels, different dose regimens, or higher testing dose levels may result in more apparent detection of sedative tolerance. Several experiments can answer this question by increasing the dose to > 5 mg/kg or incrementally increasing the dose across time [211]. It is also possible that a difference in treatment regime (i.e. subcutaneous or oral treatments) may cause tolerance detection at earlier or later time points [123]. This experiment could possibly lead to different behavioral manifestations of tolerance. It is unclear if the rates of tolerance to zolpidem's effects develop at differently.

In Studies 1-3, we found repeated diazepam treatments diminished the sedative effects of zolpidem, a phenomena termed cross-tolerance. In the case of tolerance, the sedative effects of BZs develop before the other effects (e.g. anxiolytic or anticonvulsant) [127, 132, 246]. In a recent study, Vinkers et al. showed that mice continually administered diazepam for 28 days displayed tolerance to diazepam's sedative effects as well as its hypothermic and anxiolytic effects [112]. While numerous studies have documented cross-tolerance between different BZs in rodents, relatively few have examined cross-tolerance between zolpidem and BZs [24, 105, 247-249]. The Vinkers study also showed that mice given continual zolpidem treatment for 28 days displayed cross-tolerance diazepam's hypothermic and anxiolytic-like effects but not to diazepam's sedative effects [112].

Possible evidence for different mechanisms mediating BZ and zolpidem tolerance have been found with experiments using point-mutation mice. These mice have a single point mutation in an α-subunit protein, allowing for the receptor to be BZ- and zolpidem-insensitive. One study found that tolerance to the sedative effects of diazepam in mice lacking BZ sensitive α5-GABA$_A$Rs are greatly reduced [129]. This suggests continual activation of α5-GABA$_A$Rs is an important contributing factor to the development of tolerance to diazepam. Zolpidem has very little affinity and efficacy at α5-GABA$_A$Rs; thus, it is unlikely that tolerance to the sedative effects of zolpidem is due to activation of α5-GABA$_A$Rs. This may be indicative of different mechanisms mediating the development of sedative tolerance to BZs versus zolpidem

Together with our results, these findings suggest that chronic BZ treatments can cause cross-tolerance to zolpidem's sedative effects, whereas chronic zolpidem treatments are more limited in inducing cross-tolerance to the effects of BZs. In addition, this implies that deficits in zolpidem-induced sedative effects are mediated by continued allosteric modulation of α1-GABA$_A$Rs. However, at the doses used in our studies it is likely that α2- or α3-GABA$_A$Rs are activated; thus we cannot exclude the possibility of these GABA$_A$Rs mediating tolerance. Whereas deficits in BZ-induced sedative effects may be due to the repeated allosteric activation of other BZ sensitive GABA$_A$R subtypes combined with the α1-GABA$_A$R subtype. This may indicate that different GABA$_A$R subtypes are associated with BZ and zolpidem sedative tolerance and sedative effects.

In addition to tolerance, is it possible that the sedative effects of BZs and zolpidem are mediated by different or a combination of different GABA$_A$Rs? Another question is if tolerance and/or cross-tolerance persists after BZ treatment ends. It has been found in human case studies that patients who abuse zolpidem had previous benzodiazepine abuse as well [26]. This may be indicative of permanent changes in GABA$_A$Rs functioning and it would be valuable to see if rodents exhibit sedative tolerance after drug cessation.

Behavioral Withdrawal

In humans, the abrupt cessation of prolonged BZ treatments can be followed by unpleasant withdrawal symptoms, which are believed to play a role in BZ abuse and dependence [4, 250]. The abrupt cessation of supratherapeutic doses of zolpidem can also lead to a BZ-like withdrawal syndrome [139, 147, 251-254]. In nonhuman primates and rodents, repeated BZ administration also produces physical dependence, detected by spontaneous and flumazenil-induced withdrawal symptoms [6, 255-258]. In nonhuman primates, these withdrawal effects are also observed after repeated treatment with zolpidem [133, 150]. Withdrawal-like symptoms are less likely observed in rodents after zolpidem discontinuation in comparison to diazepam [24, 28, 38, 152, 259-261]. The distinctive pharmacological profile observed in rodents and primates may be explained by differences in the distribution of α1-GABA$_A$R subtypes and/or the binding characteristics of zolpidem. *In vitro* autoradiography experiments reveal substantial differences in [3H]zolpidem binding in the brains of rats and humans [76]. These differences in the binding characteristics of specific brain regions may likely contribute to the distinctive pharmacological effects of zolpidem in rodents and primates.

In our study zolpidem and diazepam treated mice given vehicle prior to testing showed spontaneous withdrawal. These mice displayed anxiety-like with decreases in activity, frequency of rearing, and center entries. There were also progressive decreases in percent time spent in the center zone, indicative of increasing anxiety during testing. The display of anxiety-like behavior from spontaneous withdrawal in our study are in line with rodent studies that have identified anxiety-like behavior in zolpidem treated mice after spontaneous withdrawal [28, 259, 260]. Our results are also consistent with previous studies showing anxiety-like behaviors in the elevated plus maze, open field, and social interaction test when assessed 1-3 days after prolonged diazepam treatments [101, 227, 228, 246, 255, 262]. Though both groups of mice displayed anxiety-like behavior in the open field, they also showed reduced locomotion. Unlike our findings, hyper-locomotion, a typical symptom of spontaneous withdrawal, is detected after abrupt cessation of diazepam [125]. Possible that testing could produce anxiety-like behaviors concomitant with hyper-locomotion at later time points.

While it is possible that the decreases in activity were associated with the residual drug effects during testing, evidence from previous studies suggest this is not the case. In rats, the $t_{1/2}$ from plasma is approximately 30 min for zolpidem and 70 min for diazepam and its active main metabolite desmethyldiazepam [33, 34, 37, 263-265]. In the case of

zolpidem, daily injections do not change $t_{1/2}$ values or result in accumulation in plasma or the brain [35]. Repeated injection of zolpidem for up to 28 days is virtually undetectable 3–4 h after the last injection, and the degradation of zolpidem does not lead to the formation of metabolites that can accumulate and sustain drug action [31, 35, 266]. Daily diazepam injections do not change $t_{1/2}$ values and most studies but not all reveal no plasma accumulation of diazepam or its metabolite after long-term treatment [124, 233, 265, 267, 268]. These data argue against dramatic changes in the pharmacokinetics as the basis for the detection of withdrawal.

In contrast to spontaneous withdrawal, Study 2 showed that zolpidem and diazepam treated mice given flumazenil after treatments displayed minimal flumazenil-induced withdrawal, as measured by anxiety-like behavior. The lack of flumazenil-induced withdrawal in diazepam treated mice is contrary to studies that have found that flumazenil treatment results in anxiety-like behavior and hyper-locomotion [51-53]. Our study is in line with another study that did not find anxiety-like behavior in zolpidem treated mice given flumazenil [38].

Study 2 found that spontaneous withdrawal results in anxiety-like behavior and hypo-locomotion. Both spontaneous and flumazenil induced withdrawal can induce other withdrawal symptoms like handling induced convulsions that could be detected. A possible experiment would be to see if cessation of drug can cause handling induced convulsions could be detected after 3, 7, or 30 days of zolpidem or diazepam, as noted by past studies [269]. In addition, examining spontaneous withdrawal at time points beyond one day may provide information about the duration and severity of withdrawal symptoms. While withdrawal can be observed hours after drug discontinuation, some studies have found that BZ withdrawal may not occur until up to 3 days after drug treatment has stopped. If the spontaneous withdrawal experiment conducted had tested withdrawal days later, there may have been hyper-locomotion. Study 2 focused on anxiety-like behavior and locomotor activity due to flumazenil-induced withdrawal but are there other withdrawal symptoms or tests that could occur like convulsions and since flumazenil resulted in minimal effects is it possible that other drugs like PTZ can better induce withdrawal symptoms [25, 200, 269].

It is hypothesized that zolpidem withdrawal and tolerance are due to long-term supratherapeutic dose activation of the α2- and α3-GABA$_A$Rs along with α1-GABA$_A$Rs [175]. While zolpidem binds to α1-subunits with an affinity that is five- to ten-fold greater than α2- and α3-GABA$_A$Rs, *in vitro* studies show the relatively high dose of zolpidem used in this study might be sufficient to activate these lower-affinity GABA$_A$Rs *in vivo* and contribute to deficits in the locomotor-impairing effects of zolpidem and anxiety-like behaviors [17, 270].

mRNA and Protein Expression

GABA$_A$R Subunits mRNA and Protein

Repeated BZ administration produces numerous neuroadaptive changes in specific brain regions; however, it is unknown which neuroadaptive factors predict whether a certain BZ possesses the potential to produce tolerance. In our studies, we chose to examine mRNA changes in the cortex, hippocampus, amygdala, and PFC due to their involvement in the control of locomotor activity, sedation, and withdrawal symptoms as well as documented mRNA alterations that occur due to BZs and zolpidem [111, 113, 187, 234]. Many studies suggest changes in GABA$_A$R subunit mRNA contribute to tolerance and withdrawal after long-term BZ treatment regimens [92, 106, 108, 111, 113, 271, 272].

In agreement with Study 2, most data suggest repeated diazepam treatments alter α1-subunit mRNA expression in the cerebral cortex, although this is not observed in all studies [105-113]. Other studies have shown that diazepam treatment decreases α1-subunit mRNA in the hippocampus and prefrontal cortex [105, 107]. Also diazepam treated mice showed a decrease in α4, β1, and γ2 subunits in the cortex and α5-, γ2-GABA$_A$R subunits, and GluN2A subunits in the hippocampus. This latter finding is consistent with some reports that find decreases in mRNA expression of α5 subunits [107, 113, 116]. Some studies find that repeated diazepam treatment (> 7 days) induced significant increases in α3, α4, α5, β1, and γ3 subunits as well as decreases in β2 and γ2 subunits [107, 273]. Others find long term diazepam caused an increase in the α1-subunit mRNA expression in the parietal cortex and ventral pallidum but no change in α1 or γ2 in the other areas [273]. Study 2 observed cortical decreases in α3- and δ-subunit mRNA after both treatments and α4-subunit mRNA after diazepam treatment. It has been found that long-term treatment with diazepam increased α3 and α4 mRNA expression in the cerebral cortex, whereas other studies have found no such effects [107, 111, 115, 116].

In mice treated with zolpidem for 3 days, we observed a decrease in the mRNA expression of the α5 subunit in the hippocampus and in mice treated with zolpidem for 30 days showed a decrease in the mRNA expression of α1, α2, α3, β1, β2, β3, γ1, and γ2 subunits in the PFC. We observed that zolpidem treated mice showed a decrease in α1, α3, β2, and δ subunits in the cortex. We also found that an acute dose of either drug did not affect mRNA levels (Appendix). One of the most consistent finding is that long-term zolpidem treatment decreases α1 mRNA in cerebral cortex after 14 days of treatment in rats [234]. An upregulation of α4 mRNA in the cortex has also been identified after long-term treatment with zolpidem [234]. No previous studies have noted changes in δ mRNA after long-term zolpidem exposure *in vivo* [107, 234].

It must be noted that many studies have shown inconsistent findings that limit the essential or key changes underlying tolerance and withdrawal. Also of note is that rats have been historically used to investigate mRNA changes associated with tolerance and withdrawal after BZ treatments, while mice have been used in only a few studies [271,

272]. Neuroanatomical and behavioral differences between these two species may contribute to published discrepancies [274-276]. Other inconsistencies are due to differences between treatment regimen and subanatomical regions. For example some studies used infusions pumps or s.c. injections [115]. Others look at specific regions like the CA1 region of the hippocampus, frontoparietal motor cortex, or frontoparietal somatosensory cortex [107, 112].

Both α4- and δ-GABA$_A$Rs are usually found in perisynaptic and extrasynaptic regions of the brain, including the cortex [277, 278]. These receptors are insensitive to BZs and zolpidem, indicating that changes in GABA$_A$R mRNA levels are not necessarily dependent on direct drug-receptor interaction. In cultured cells, withdrawal from repeated treatment of diazepam and zolpidem has been shown to increase α4-subunit mRNA and decrease α1-subunit mRNA, suggesting that the molecular events that underlie withdrawal may involve a shift from BZ-sensitive to BZ-insensitive GABA$_A$R subtypes [188, 189]. While zolpidem preferentially binds to α1-GABA$_A$Rs, it also possesses some agonistic action on α3-GABA$_A$Rs at high doses which may mediate the decrease in α3 mRNA after zolpidem treatment [17, 279]. It is noteworthy that the anxiolytic effects of BZs are putatively mediated by α2- and α3-GABA$_A$Rs [99, 280, 281]. While no mRNA changes were detected in the amygdala, which is known to be involved in fear and anxiety, changes in the function of these receptors in the cortex may play a role in the processing of sensory information that mediate anxiety-like behavior seen in diazepam and zolpidem groups treated with vehicle in Study 2 [232].

Due to the development of cross-tolerance in diazepam treated mice to zolpidem and the lack of tolerance in zolpidem treated mice in Study 1 and 3, it was expected that the diazepam treated mice would show changes in mRNA expression. Vinkers 2012, found that 28 days of diazepam resulted in an increase in the α2 subunit in the hippocampus (CA1 region) [112]. Though this study showed mRNA changes in the α2 subunit in the hippocampus, like our study it did not show any changes in the cortex or in the hippocampus with α1 or α5 subunit. It has been shown that the mRNA expression of the α4 subunit increases during tolerance and during withdrawal from diazepam and zolpidem [115, 234]. In the present study we did not see a similar increase in either group.

One would expect since BZs like diazepam have similar affinity and efficacy to multiple GABA$_A$Rs, that changes in mRNA transcripts would be more pronounced and frequent than zolpidem. This may be the reason we saw more pronounced alterations due to diazepam versus zolpidem in Study 2. An expectation is that zolpidem would only alter α1 subunits because it primarily binds to α1-GABAARs. As our study and other studies have found repeated zolpidem does have more broad effects on other GABAergic transcripts. Studies 1-3 focused on measuring the mRNA expression of GABA$_A$Rs subunits. Is it possible that other non-GABAergic changes occurred after 3 or 30 days of zolpidem or diazepam? Future experiments should examine if changes in non-GABAergic (eg. NMDAR or AMPAR subunit mRNA) occur after these time points in zolpidem or diazepam groups. Another unexamined question is the temporal duration of

identified mRNA changes in this study. Experiments looking at mRNA changes greater or less than 1 day after drug cessation might identify acute or more long term changes.

Examination of selective GABA subunits at the protein level indicated mice given zolpidem had decreased expression of total α2 protein in the cortex and mice given diazepam had decreased expression of total γ2 protein and decreased intracellular α1 protein in the cortex. Mice given prior zolpidem or diazepam did not show any changes in the cortex of total α1, α3, NR1, NR2A, or surface α1 protein expression. There were also no changes in the hippocampus of mice given prior zolpidem or diazepam of total α1 or α2 protein expression. Most *in vivo* studies of repeated BZ treatment show a decrease in α1 and increases in α5, β2, and γ2 protein [107, 109]. We found a decrease in γ2 protein but no changes in α1 protein expression. *In vitro* studies of long-term diazepam treatment on rat cerebellar neurons found diazepam treatment decreased α1 and γ2 protein expression during treatment and after withdrawal increased α4 protein expression [189, 254].

Some studies observed decreased number of BZ binding sites after long-term BZ treatment [244]. Receptor uncoupling, internalization, and degradation of GABA$_A$R subunits have been proposed as mechanisms underlying BZ tolerance [151, 197, 282, 283]. While these mechanisms are not fully understood, posttranslational processes, such as phosphorylation, are likely to play key roles [284, 285]. Other studies have demonstrated that altered mRNA levels induced by chronic BZ exposure can correlate to their respective protein levels *in vivo* [107, 109]. Thus, it is conceivable that mRNA changes seen in this study coincide with changes in the expression of their respective proteins. However, the relationship between changes in mRNA levels and corresponding protein level is not well characterized. While mRNA serves as an intermediate between DNA and protein, they are subject to posttranscriptional processing by RNA-binding proteins that bind to multiple sites on numerous RNAs to function in diverse time-dependent processes [286].

It has been hypothesized that due to zolpidem binding preferentially to α1-GABA$_A$Rs that tolerance would be associated with decreases in α1 and γ2 protein expression [105]. We also expected to see a decrease in surface expression of α1 subunit for the same reason. It is possible that the high variability and the failure to see statistical significance with our mRNA and protein experiments were due to small sample sizes. Some of the experiments only had 3-6 mice per group, leading to high variability which may have masked significant results. Future experiments with increased sample size may reduce variability and identify significant differences.

Our study only looked at protein expression after 7 days of drug treatment, however it is possible that changes in protein occur earlier or later. Experiments should be conducted at 3 or 30 days of drug treatment. The study focused on the changes of select GABA$_A$R and NMDAR subunits and it may be possible that changes occur at other signaling transcripts or proteins like KCC2, mGlu5, or PKC [204, 211]. Since there was a decrease in α2 subunit expression after zolpidem treatment, the use of the α2 point mutation mice might show if α2-GABA$_A$Rs affect zolpidem sedative tolerance. A single

high dose of diazepam (30 mg/kg) given to wild-type mice can also produce brain-region-dependent reduction in the expression level of mRNA transcripts involved in the regulation of synaptic plasticity such as c-Fos, CaMKII, and BDNF [287]. Interestingly, many of the transcript changes induced by diazepam in wild-type mice are not altered in mice with α1-GABA$_A$Rs that have been rendered diazepam-insensitive by point mutations, indicating that these transcript changes are specifically mediated by α1-containing GABA$_A$Rs.

NMDA

Repeated BZ treatment also affects ionotropic glutamate receptor subunit gene expression. As reviewed elsewhere, alterations in other neurotransmitter systems have been implicated as neuroadaptive mechanisms underlying the development of tolerance and withdrawal to BZ-like drugs, including an upregulation of the glutamatergic system [151, 288]. In Study 2 it was found that only diazepam treated mice showed a decrease of the mRNA expression of GluR2A subunit mRNA in the hippocampus. There were no other group differences in the hippocampus of GluR1, GluR2B, GluN1 or GluN2 mRNA levels. There were also no group differences for any of the NMDA or AMPA subunit mRNA levels in the cortex, amygdala, or PFC. Our results differ from other studies that find increases in GluR1 and GluR2B, but no increase in GluR2A mRNA levels in the hippocampus after diazepam treatment [118, 119]. Increases in the expression of AMPA receptor subunit mRNAs and AMPA receptor ligand binding have been reported in a number of brain areas after BZ withdrawal [53, 289]. We expected that Study 2 would identify increases in GluR1 and GluR2B mRNA, as found in other studies looking at repeated diazepam in mice [118, 119]. One difference between our study and others is the time point of sacrifice after drug termination. Our study waited 16-20 hours after last injection whereas others have waited as long as 96 hours after drug termination. Another difference is the method of mRNA analysis. The duration of treatment between Study 2 and past examinations differed. In our study treatment lasted 7 days as opposed to 3 or 14 days [117, 119]. We used qRT-PCR as opposed to *in situ* hybridization.

Because other studies have noted changes in NMDAR and AMPAR subunits at varying time points, it is possible that changes in these subunit mRNA may have occurred at 3 or 30 days of treatment. Also is it possible that these changes would only occur during withdrawal. This study focused on ionotropic glutamate receptors but is it possible that our repeated zolpidem and diazepam could result in changes in metabotropic glutamate receptors. One study has found that repeated zolpidem treatment can increase mGluR5 protein [204]. There is evidence that mGlur5 interacts with α1-GABA$_A$Rs [205].

At the protein level, zolpidem- and diazepam-treated mice did not show any changes in the cortical GluR1 and GluR2 subunits. This is in contrast to other studies that found increases in GluR1 but not GluR2A protein expression [117]. Electrophysiological data indicate that a single *in vivo* dose of zolpidem or diazepam can produce an increase in the AMPA/NMDA current ratio and activity of mesolimbic dopamine neurons [167]. Gene-targeted mice lacking the GluR1 subunit of AMPARs show less tolerance to a

number of the acute behavioral effects of flurazepam (e.g., loss of righting reflex, walking beam impairments) after high-dose treatments [121, 290]. In contrast, greater flumazenil-induced withdrawal-like symptoms are seen after treatment termination. Together, these findings suggesting alterations in AMPA receptors are involved in mediating the processes involved in BZ tolerance and withdrawal.

A variable to consider is the small sample sizes of Studies 1 and 3 which may have resulted in high variation in our data sets. The small sample sizes of our data sets may have masked a possible significant result. Therefore future experiments should have a greater number of mice per group to address this problem. This project focused on mRNA alterations of GABA$_A$R subunits and associated proteins in Studies 1-3 and Study 2 also focused on alterations of NMDA and AMPA receptor subunits. As mentioned above, the focus of this study may have missed other mRNA and protein alterations like those of mGluR, KCC2, PKA, and PKC, that have been found to be associated with BZ and zolpidem tolerance and are known to interact with GABA$_A$Rs [204, 205, 209, 211].

Connection Between Sedation, Withdrawal, and Observed Alterations in the mRNA and Protein Levels in the Cortex, PFC, Hippocampus, and Amygdala

Our results, similar to past studies, found tolerance and cross-tolerance to zolpidem after 7 days of repeated zolpidem and diazepam treatments. We also found that an abrupt end of treatment caused withdrawal anxiety and a reduction in motor activity, a measure of sedation. The most significant changes in mRNA and protein expression occurred during this treatment duration. The cortex and the hippocampus were the only regions that had decreased expression of GABA$_A$R subunits and NMDAR subunits. Both zolpidem and diazepam groups had decreased mRNA levels of α1-, α3-, β2-, and δ-GABA$_A$R subunits in the cortex. The decreases in α1, α3, and β2 are in line with other studies, but the decrease in cortical δ mRNA levels has not been previously documented in the cortex or other brain regions. Past studies hypothesize decreases of α1, α3, and β2 may be indicative of a decrease in GABA$_A$Rs in the cortex. A decrease in these subunits may result in a decrease in efficacy of diazepam and zolpidem in the cortex and contribute to our measure of behavioral tolerance observed in the study. The decrease of the δ-subunit mRNA has not been seen in other studies. This finding is surprising given that neither zolpidem nor diazepam bind to GABA$_A$Rs that contain the δ subunit. Because the location of δ-subunits in the cortex is not firmly established, it is unclear how this affects the development of tolerance. However δ-subunits are known to be incorporated in extrasynaptic GABA$_A$Rs that mediate tonic inhibition, thus reduction may cause an overall decrease in baseline tonic inhibition resulting in an increase in excitatory tone.

The diazepam group had decreased mRNA levels of α4-, β1-, γ2-subunits, GAT, and gephyrin in the cortex. In addition this group showed significant decreases of α5- and β3-GABA$_A$R subunits and GluN2A subunit in the hippocampus. A decrease in the mRNA of the γ2-subunit and gephyrin in the cortex due to repeated diazepam may indicate decrease in BZ-sensitive GABA$_A$Rs, as the γ2-subunit is necessary for BZ binding and gephyrin plays a role in maintaining GABA$_A$Rs on the cell surface. It is

unclear how a decrease in GAT-1 influences tolerance, because a decrease in GAT-1 should lead to a greater concentration of GABA in the synaptic cleft and increased inhibition. It is also unclear why there was a decrease in $\alpha 4$ or $\beta 1$ mRNA levels because diazepam nor zolpidem bind to GABA$_A$Rs with these subunits, although a decrease in $\alpha 4$-subunit could result in a decrease in GABA$_A$Rs responsible for tonic inhibition. Other studies tend to see an increase in $\alpha 4$ mRNA levels, which could result in an increase in BZ- and zolpidem-insensitive GABA$_A$Rs.

At the protein level our findings did not completely correspond with changes in mRNA levels. Future studies with more refined and sophisticated experimental designs may help resolve discrepancies between mRNA and protein levels seen in our studies. Although some of our results are unresolved, they generally confirm that modulation of neocortical circuits by changes in GABA$_A$Rs play a role in the motor impairing effects of PAMs and eventual development of tolerance to zolpidem and diazepam [103].

The diazepam groups had a decrease of $\alpha 5$- and $\beta 3$-GABA$_A$R subunits, and the GluN2A subunit in the hippocampus. The decrease of $\alpha 5$ is in line with other studies that examine the effects of repeated diazepam in the hippocampus as diazepam binds to GABA$_A$Rs with this subunit. It is unlikely that a decrease in either of these GABA$_A$R subunits influence sedative cross-tolerance to zolpidem, because $\alpha 5$-GABA$_A$Rs do not participate in the sedative effect of zolpidem. Alterations in $\alpha 5$-GABA$_A$Rs could, however, alter memory functions, because hippocampal $\alpha 5$-GABA$_A$Rs are known to be involved in memory and learning. The decrease in GluN2A mRNA levels are novel, though it is unclear how it affects sedative tolerance. Past studies have reported that changes in GluN1 and GluN2B but not GluN2A mRNA and protein levels are associated with the contextual dependent features of PAM tolerance [118, 119, 249]. Because GluN2A is involved in learning in memory, these changes may be associated with contextual dependent features.

Three days of repeated zolpidem did not produce sedative tolerance but did result in a decrease in $\alpha 5$ subunit mRNA levels in the hippocampus. These results only partially supported our hypothesis. As stated above, it is known that changes in hippocampal $\alpha 5$-GABA$_A$Rs do not influence the sedative effects of zolpidem. It is possible that the decrease in $\alpha 5$ subunits is a compensatory change or associated with neuroadaptive changes unrelated to sedation. Although this project found cross-tolerance to zolpidem occurs after 3 days, there were no corresponding changes in mRNA levels. Past studies have found that there are alterations in GluN1 and GluN2B after 4 days [118, 119]. Thus mechanisms associated sedative cross-tolerance after short-term durations may not be associated with measurable GABAergic alterations.

Thirty days of repeated zolpidem did not produce sedative tolerance but did result in molecular alterations. These results only partially supported our hypothesis. Although 30 days of zolpidem did not result in sedative tolerance, our findings do not rule out the development of withdrawal symptoms afterwards. Thirty days of repeated zolpidem did result in the decrease of $\alpha 1$-, $\alpha 2$-, $\alpha 3$-, $\beta 1$-, $\beta 2$-, $\beta 3$-, $\gamma 1$-, and $\gamma 2$-subunit mRNA levels in the PFC. PFC glutamatergic projections to the amygdala strongly inhibit the amygdala;

thus, the loss of prefrontal inhibition may increase the glutamatergic drive to the amygdala which can increase anxiety associated with withdrawal symptoms [100]. Although this project found cross-tolerance to zolpidem occurs after 30 days, there were no corresponding changes in GABAergic mRNA levels. Vinkers et al. (2012), have shown that 28 days of diazepam treatment resulted in sedative tolerance to diazepam but no detectable cortical GABAergic mRNA changes and few hippocampal changes [112]. Thus, the presence of sedative tolerance after long term PAM treatments are not always dependent on observable GABAergic alterations.

Limitations

Our studies provide insight into possible mechanisms behind zolpidem tolerance, although there are limitations to our interpretations. Protein expression was not looked at in all studies. Though GABA$_A$R mRNA and protein expression have been shown to correlate with each other that may not be the case here [107, 109]. We found in Study 1 and 3 that changes in mRNA expression were not associated with the development of cross-tolerance. The diazepam groups in both studies showed cross-tolerance but no changes in mRNA expression, and the zolpidem group in both studies showed no tolerance and minimal changes in mRNA expression. Studies 1 and 3 measured tolerance and mRNA expression in the same mouse whereas Study 2 did not. This would have allowed for correlations on distance and mRNA expression. If prominent changes behavioral and mRNA changes were identified in the same animal in Study 2 correlations may have identified a more definitive relationship between behavioral and neuroadaptive changes. In addition to GABAergic alterations, changes in other neurotransmitter systems and proteins could be involved in zolpidem and diazepam tolerance, but were not investigated in our studies which focused on GABA$_A$R subunits and to a lesser extent AMPAR and NMDAR subunits.

Conclusions

In summary, this project found that 3 and 30-day zolpidem use did not result in tolerance to zolpidem's sedative effects whereas diazepam treatment at all treatment durations produced cross-tolerance. Three and 30 day treatments of zolpidem but not diazepam resulted in minimal GABAergic mRNA changes. Though there were minor changes in mRNA expression in the PFC with 30-day zolpidem and minor changes in the hippocampus after 3 day zolpidem, these changes are likely independent from sedative tolerance. Seven-day zolpidem and diazepam administration can produce acute deficits in the locomotor-impairing effects of zolpidem and anxiety-like behaviors upon drug termination (spontaneous withdrawal) but not precipitated withdrawal. Our results demonstrated that zolpidem and diazepam treatments have differing effects on GABA$_A$R subunit mRNA expression, mainly in the cortex, which may partially contribute to mechanisms underlying the behavioral phenomena. In general, repeated zolpidem produced fewer changes in the efficacy in of zolpidem in comparison to repeated

diazepam. However, continued research into tolerance and withdrawal to zolpidem are necessary due to the growing abuse of zolpidem.

Clinical Significance and Implications of Research

This project showed that 7 days of repeated zolpidem and diazepam resulted in the emergence of sedative tolerance and withdrawal. Along with sedative tolerance, there were significant changes, primarily in the cortex, of GABAergic mRNA and protein levels associated with BZ and zolpidem functioning. Our research indicates the cortical alterations may play a major role in the development of tolerance to zolpidem, similar to what other studies have found. Cortical GABA$_A$Rs appear to be mediate tolerance and the development of future sedative-hypnotics should focus on creating drugs that are either GABA$_A$R subtype selective or drugs that work directly on the arousal system. Subtype selective PAMs that only bind to α1-GABA$_A$Rs with little or no affinity or efficacy to other GABA$_A$R subtypes may prevent the development of sedative tolerance and withdrawal. Recently developed orexin receptor antagonists (Suvorexant) that target arousal regions like the locus coeruleus, TMN, and raphe nucleus have been developed and marketed as alternatives to currently used PAMs. Early results suggests orexin antagonists show reduced propensity of typical BZ and zolpidem side effects, like dependence, suggesting they are safer than hypnotic PAMs. Comparing zolpidem to orexin antagonists or other α1-GABA$_A$R selective PAMs may help us better understand the relationship between molecular alterations and the development of tolerance and withdrawal in humans and better aid in the development of treatment options for the treatment of insomnia.

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APPENDIX. MRNA EXPRESSION AFTER 1 DAY OF VEH, DZP, AND ZOLP

Region	Vehicle		Zolpidem		Diazepam	
Cortex	Mean	SEM	Mean	SEM	Mean	SEM
α1	1.00	0.04	1.01	0.06	0.99	0.05
α2	1.00	0.08	1.02	0.09	1.08	0.07
α3	1.00	0.10	1.02	0.08	1.02	0.09
α5	1.00	0.07	1.00	0.09	1.02	0.08
β2	1.00	0.09	0.92	0.06	0.87	0.08
γ2	1.00	0.09	1.01	0.03	0.95	0.05
GAT-1	1.00	0.06	0.97	0.07	0.94	0.08
Hippocampus	Mean	SEM	Mean	SEM	Mean	SEM
α1	1.00	0.19	1.31	0.22	1.40	0.17
α2	1.00	0.12	0.94	0.15	1.08	0.11
α3	1.00	0.30	0.80	0.07	0.84	0.22
α5	1.00	0.27	1.41	0.17	1.49	0.28
β2	1.00	0.16	1.22	0.14	1.15	0.18
γ2	1.00	0.10	1.01	0.13	1.01	0.05
GAT-1	1.00	0.22	0.65	0.10	0.76	0.05
Amygdala	Mean	SEM	Mean	SEM	Mean	SEM
α1	1.00	0.08	1.12	0.10	1.21	0.05
α2	1.00	0.14	1.23	0.21	1.34	0.10
α3	1.00	0.21	1.43	0.15	1.41	0.09
α5	1.00	0.34	0.75	0.09	0.94	0.13
β2	1.00	0.11	1.01	0.12	1.07	0.06
γ2	1.00	0.06	1.05	0.11	1.10	0.03
GAT-1	1.00	0.08	0.90	0.09	1.16	0.06
Amygdala	Mean	SEM	Mean	SEM	Mean	SEM
α1	1.00	0.10	0.85	0.23	0.77	0.02
α2	1.00	0.07	0.94	0.27	0.83	0.06
α3	1.00	0.14	0.70	0.36	0.32	0.14
α5	1.00	0.17	1.28	0.10	1.79	0.28
β2	1.00	0.08	0.84	0.29	0.62	0.06
γ2	1.00	0.07	0.90	0.19	0.78	0.02
GAT-1	1.00	0.11	0.85	0.25	0.64	0.09

There were no differences in mRNA expression after 1 day of zolpidem or diazepam. mRNA levels expressed as proportion of VEH controls. Modified with permission. Wright, B.T., C.F. Gluszek, and S.A. Heldt, *The effects of repeated zolpidem treatment on tolerance, withdrawal-like symptoms, and GABAA receptor mRNAs profile expression in mice: comparison with diazepam.* Psychopharmacology (Berl), 2014. **231**(15): p. 2967-79.

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