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Pilot Studies on the Novel Hypothesis that TSPO (Peripheral Benzodiazepine Receptor) Is Involved in Benzodiazepine/"Z-Drug" Physical Dependence and/or Withdrawal

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Abstract

Benzodiazepines and other benzodiazepine receptor agonists, such as the "Z" drugs, are widely prescribed medications mainly used for treating anxiety and seizures, and for inducing sedation. Unfortunately, despite their popularity, benzodiazepine prescribing often exceeds recommendations and the consequences can be severe. On September 23, 2020, the United States FDA announced a new requirement for a Boxed Warning for benzodiazepines prescribing. Along with this announcement, the FDA stated that relevant information regarding the initiation, continuation, and discontinuation of benzodiazepines is lacking. Here, we describe initial pilot studies intended to investigate the questions 1) can animal models be developed that demonstrate benzodiazepine physical dependence and/or withdrawal symptoms, and 2) determine whether translocator protein (TSPO) plays a role in benzodiazepine dependence and/or withdrawal processes. The former was demonstrated, methodological limitations prevented the latter.

Keywords

Benzodiazepine, TSPO, Rats, *In Vivo* Tests, Western-Blot Analysis

1. Introduction

Benzodiazepines (BZDs) are some of the most widely-prescribed medications [1]

[2] [3]. They are commonly used for alleviating anxiety (anxiolytic action), preventing or stopping certain seizures, and inducing sedation [4]-[8]. Since the development of the first chlordiazepoxide in the 1960s, BZDs have been largely and widely promoted and accepted as having a favorable safety profile and low abuse potential with little or no withdrawal syndrome [9]. In fact, BZDs were superior in safety to existing drug classes such as barbiturates, but despite their wide popularity and efficacy, benzodiazepine dependence/withdrawal is not only more common than first thought, but can be very severe and prolonged [4] [6] [10] [11]. As pointed out in the FDA warning, prescribing often exceeds medical evidence (in either initiation or duration of use).

Drugs associated with potential abuse act in part by activating reward centers of the brain [12]. In this context, reward refers to a positive, or pleasant, affective experience ("liking") [13]. An animal model that is commonly used for the assessment of rewarding stimuli is Conditioned Place Preference (CPP) [14]. In CPP, an animal learns to associate one of a pair of environmental cues (locale) with the rewarding stimulus. During an evaluation, an animal will spend a significantly greater amount of time in the locale (a "paired" chamber in this case) in which it was administered the rewarding drug than it will in a locale in which it was not administered the drug ("neutral" chamber). "Disliking" can also be measured: conditioned place-aversion (CPA) is demonstrated when the animal spends less time in the "paired" chamber. Previous studies have demonstrated that the most popular BZD, alprazolam, induces positive place-preference in male rats. In the present study, we show that this positive-place preference can be attenuated by flumazenil, a direct antagonist of the $GABA_A$ receptor, which benzodiazepines allosterically modulate.

Abrupt withdrawal from BZDs can be associated with serious short- and long-term consequences. In addition, patients undergoing dependence management via dose-tapering often fail to complete it due to intense withdrawal symptoms and the extended process required to weaning patients off. Thus, there is a pressing need to better understand the physiology of benzodiazepine dependence and withdrawal.

Guidelines exist with recommendations of only short-term use of BZDs; however, long-term use has become increasingly popular [15]. Even therapeutic doses for as few as 3 - 6 weeks have been linked to dependence. 44% of chronic users experience symptoms of anxiety and depression and 40% - 50% of those who use for >6 months experience severe withdrawals [11] [15] [16].

Unfortunately, the physiology of benzodiazepine withdrawal is virtually unknown. There is some evidence of down-regulation of benzodiazepine binding sites, increased calcium flux, and serotonin (5-HT) activity during withdrawal in animal models. Nonetheless, numerous studies support the presence of both psychological and physical withdrawal symptoms. Furthermore, roughly 10% - 25% of chronic users suffer extended periods of withdrawal, up to 12 months and even longer [11] [17] [18].

Benzodiazepines are known to modulate $GABA_A$ chloride channels within the CNS, increasing Cl^- influx, resulting in hyperpolarization and an overall decrease in neuronal function. These channels are well understood and not thought to be involved in withdrawal symptoms. However, BZDs have also been shown to interact with a translocator protein called TSPO, found on mitochondria widely distributed throughout the body and on immune cells. TSPO plays many important roles in the body, most notably in steroid production and stress regulation [19] [20]. In the context of BZDs, no studies have yet linked the expression of TSPO to the consequences of withdrawal from BZDs.

2. Methods

Male and female Sprague Dawley rats (250 - 350 g) were purchased from Envigo Laboratories (Indianapolis, IN) and maintained in a climate-controlled room on a 12-h light/dark cycle with food and water available *ad libitum*. All experiments were performed in accordance with policies and recommendations of the International Association for the Study of Pain (IASP) and the National Institutes of Health (NIH) guidelines for the handling and use of laboratory animals. The protocol and testing procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona. All efforts were made to avoid or minimize animal suffering and to reduce the number of animals used.

2.1. Conditioned Place Preference (CPP)

The CPP protocol was conducted over a 14-day period using a test system (San Diego Instruments) consisting of two "pairing" chambers and a smaller, well-lit "middle" chamber. The pairing chambers were dimly lit, and consisted of solid-colored walls (grey) with a textured floor, and the second chamber was markedly different: it had striped walls (black and white) and a smooth floor. The smaller middle chamber was used to introduce the animal to the system, and had no artificial odor associated with it. On the first day, a rat was individually placed in the middle chamber, and had free access to all chambers over a period of 15 minutes. Automated infrared beams were used to quantify the time spent in each of the chambers (San Diego Instruments; San Diego, CA). During this period, the time spent in each chamber was recorded to ensure that there were no inherent biases for one chamber over another. If an animal showed an innate bias towards one chamber (defined as spending more than 80% of the time there), it was removed from the study without further evaluation. On the second day of testing, the animals entered the conditioning protocol. Based on previous studies, they were injected with flumazenil (2 mg/kg, s.c.; N = 20 male rats) and alprazolam (3 mg/kg, s.c.; N = 20) or flumazenil (2 mg/kg, s.c.; N = 19 male rats) and vehicle (10% DMSO, 10% Tween-80, and 80% saline, 1 mL/kg; N = 19 male rats) and were immediately placed into one of the two pairing chambers for 30 min, without access to the other chambers. This became the chamber in which

the animals would receive drug injection, so it was designated the "paired chamber"; the chamber not associated with drug injection was designated the "neutral chamber". The pairing for each animal was balanced, *i.e.*, half of the rats received drug (or vehicle) treatment in the solid-colored chamber, and the other half received drug (or vehicle) in the striped chamber. This pairing was repeated for a total of five drug exposures.

Testing was performed on the following day (day 5 of the protocol) by placing the animals individually in the middle chamber and allowing it access to roam freely across all chambers. This day did not include any injections. The time spent in each chamber was recorded, and a difference score was derived by subtracting the time spent in the neutral chamber from the time spent in the paired chamber. In this paradigm a significant positive difference score (a >50-s increase over baseline) is indicative of preference for the drug-pairing (CPP); a significant negative difference score (a >50-s decrease from baseline) is indicative of aversion to the drug-pairing CPA.

The mean time that the rats spent in the paired-chamber and the neutral-chamber was calculated for each group (±SEM). Student's t-test was used to evaluate differences between the mean times for statistical significance ($P < 0.05$).

2.2. Elevated plus Maze (EPM)

EPM baseline behavior was measured prior to animals receiving any compound. Compounds were administered twice daily for 14 days in two treatment groups: one group received vehicle (10:10:80 mixture of Tween80, DMSO, and saline; N = 16 females; N = 28 males); the other received alprazolam (3 mg/kg, s.c., twice a day for 14 days; N = 16 females; N = 28 males). Beginning on Day 15, behaviors were evaluated daily over a 5-day period.

The EPM consists of four elevated arms (50 cm long and 10 cm wide) with two opposing arms containing 30 cm high opaque walls. EPM testing occurred in a quiet testing room with ambient lighting at ~500 lux. On day of testing, rats were allowed to acclimate to the testing room for 20 minutes. Each rat was placed individually in the center of the maze, facing the enter platform and cage mates started in the same closed arm. Each rat was allowed 5 minutes to explore the EPM and then returned to its home cage. Between animals the EPM was cleaned thoroughly with Versa-Clean (Fisher Scientific). EPM performance was recorded using an overhead video camera (MHD Sport 2.0 WiFi Action Camera, Walmart.com) for later quantification. Open- and closed-arm entries were defined as the rat's head entering the arm, and open-arm time began the moment the head entered the open arm and ended upon exit. The duration of the test was recorded using the ANY-maze (Stoelting Co) software and the amount of time the animal spent in each set of arms was determined.

2.3. Tactile Thresholds

Baseline behavior was measured prior to the animals receiving any compound.

Compounds were administered twice daily for 14 days in two treatment groups: one group received vehicle (10:10:80 mixture of Tween80, DMSO, and saline; N = 16 female rats; N = 28 male rats) and the other received alprazolam (3 mg/kg, s.c., twice a day for 14 days; N = 16 female rats; N = 28 male rats). Beginning on Day 15, behaviors were evaluated daily over a 5-day period.

Assessment of tactile sensory thresholds was determined by measuring the withdrawal response to probing the hind paw with a series of calibrated fine (von Frey) filaments. Each filament was applied perpendicularly to the plantar surface of the paw of the rats held in suspended wire mesh cages. The withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength ("up and down" method), and data were analyzed using the nonparametric method of Dixon, as described by Chaplan *et al.* and expressed as the mean withdrawal threshold [21].

2.4. Open Field

Baseline behavior was measured prior to the animals receiving any compound. Compounds were administered twice daily for 14 days in two treatment groups: one group received vehicle (10:10:80 mixture of Tween80, DMSO, and saline; N = 12 female rats) and the other received alprazolam (3 mg/kg, s.c., twice a day for 14 days; N = 12 female rats). Beginning on Day 15, behaviors were evaluated daily over a 5-day period.

The open-field arena (33 cm × 28 cm × 33 cm) was a translucent box with an open top and a black floor. A rectangle (16.5 cm ×14 cm) was marked in the center of the field. Sessions began by placing the rat in the center rectangle and ended after a preset 5 minutes. The entire session was recorded by a video camera mounted 1.5 m above the floor. The trial was recorded and analyzed using ANY-maze software to determine the amount of time the animal spent in the center of the box *vs* the perimeter of the box.

2.5. Western Blot Analysis

Whole-cell lysates were analyzed for the expression of translocator protein TSPO. Cell protein samples (20 - 30 μg) were resolved on 10% SDS-polyacrylamide gels (TGX Criterion XT; Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane. Protein transfer was verified by Ponceau-S staining, and the polyvinylidene difluoride membrane was incubated in 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (v/v; TBST). Membranes were incubated with TSPO antibody, appropriate secondary antibodies, and developed using Clarity ECL Substrate (Bio-Rad). Bands were quantified and corrected for background using ImageJ software (Wayne Rasband, Research Services Branch, NIMH, Bethesda, MD).

3. Results

The male rats that received vehicle (10:10:80 of DMSO:Tween80:Saline) and in-

jection of flumazenil (2 mg/kg, s.c.) had a non-significant ($P > 0.05$) mean difference score between chambers, indicating a lack of pre-testing preference for a chamber.Conversely, the male rats that received alprazolam (3 mg/kg, s.c.) and antagonist flumazenil (2 mg/kg, s.c.) demonstrated statistically significant ($P \leq 0.01$) decrease in time spent in the drug-paired chamber compared to baseline (Figure 1).

To differentiate the possible anxiety-induced behavior from abrupt withdrawal of alprazolam, we performed elevated plus maze (EPM) experiments in both male and female rats. The male rats that received 14 days of alprazolam (3 mg/kg, twice daily), displayed only a significant difference in the amount of time spent in the open arms when compared to the control group (vehicle, twice daily for 14 days) on day 3 of withdrawal (Figure 2) whereas the animals who received chronic dosing of alprazolam spent significantly less time in the open arms on that day. Conversely, there were no significant differences ($P > 0.05$) between time spent in the open arms when comparing the female rats in the vehicle *vs* alprazolam groups on days 1 - 5 of withdrawal (Figure 3).

Mechanical allodynia can be assessed with tactile sensitivity testing using von

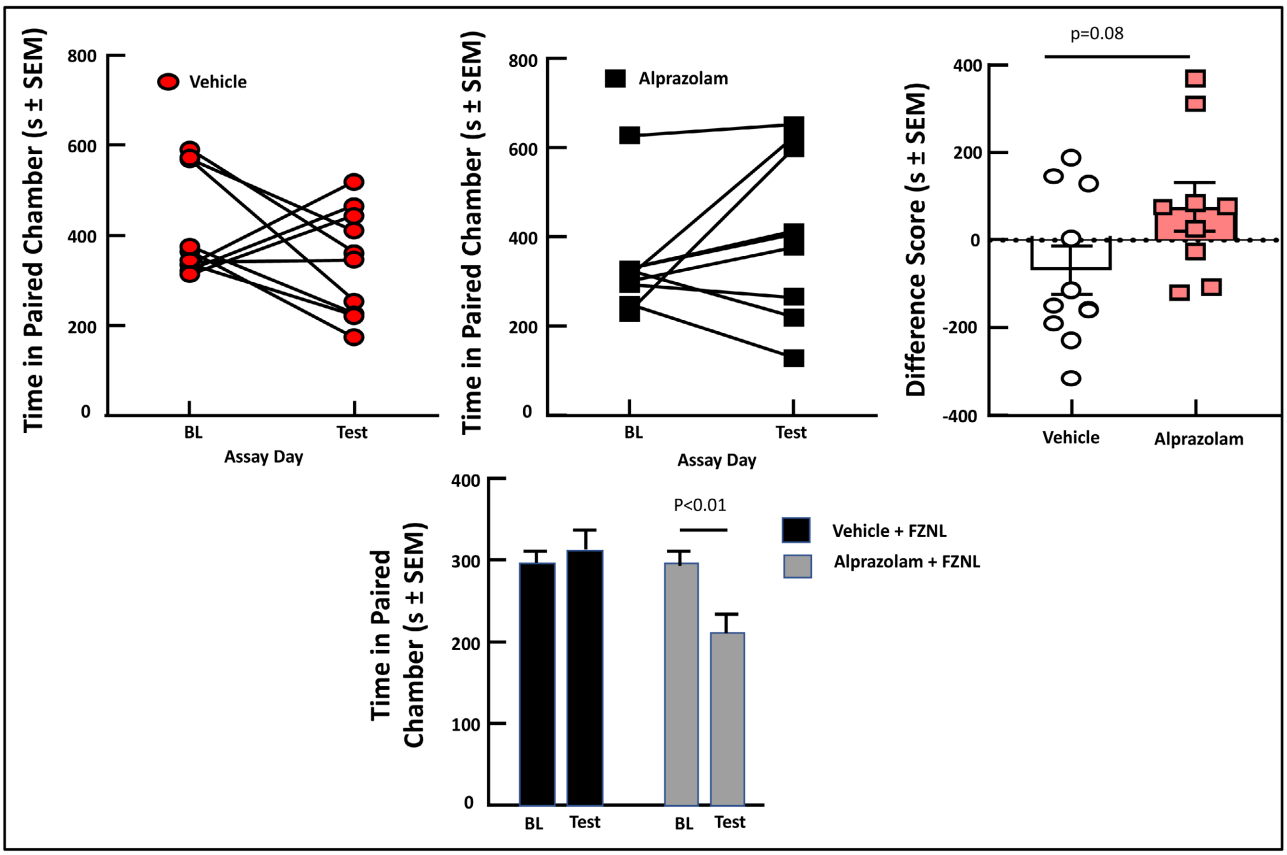


Figure 1. The mean (±SEM) time, in seconds, spent in the paired chamber at baseline and on test day for rats receiving vehicle [upper left] or alprazolam (3 mg/kg, s.c.) [upper middle] is shown. The difference scores of vehicle and alprazolam are shown [upper right]. There was no significant difference in the time spent in the paired chamber between baseline and test day for animals injected with the vehicle and antagonist flumazenil (2 mg/kg, s.c.; FZNL). In contrast, alprazolam and flumazenil injection resulted in a significant ($P \leq 0.01$) decrease in time spent in the paired chamber after treatment.

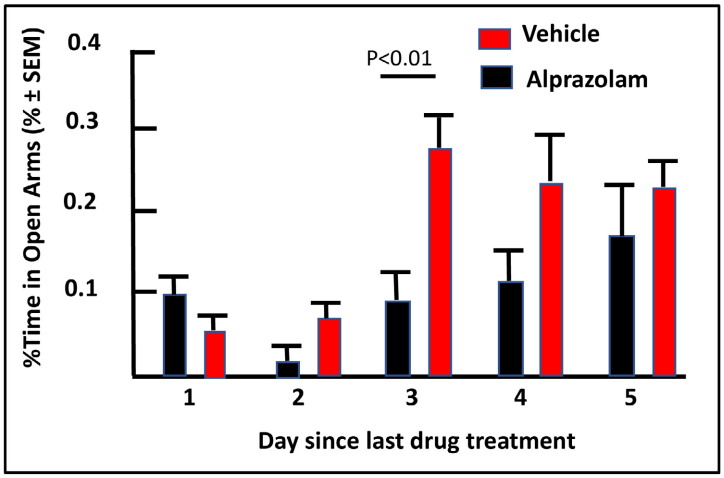


Figure 2. The % time spent in the open arms of the elevated plus maze is plotted as a function of days of withdrawal after male rats (N = 28) were dosed with 14 days of alprazolam (twice daily, 3 mg/kg) *vs* those who received vehicle twice daily. Less time in the open arms suggests anxiogenic behavior. A significant difference (P < 0.01) in time spent in the open arms was demonstrated on day 3 of withdrawal, where animals who received chronic alprazolam spent significantly less time in the open arms. ** = P < 0.01.

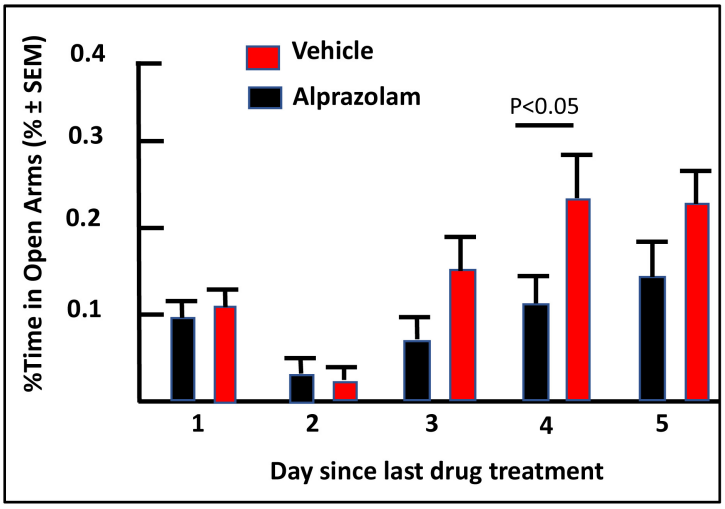


Figure 3. The % time spent in the open arms of the elevated plus maze is plotted as a function of days of withdrawal after female rats (N = 16) were dosed with 14 days of alprazolam (twice daily, 3 mg/kg) *vs* those who received vehicle twice daily. Less time in the open arms suggests anxiogenic behavior. There was no significant difference (P > 0.05) in time spent in the open arms demonstrated on days 1 - 5 of withdrawal.

Frey filaments, a normally non-noxious stimulus. Increased sensitivity to tactile stimulation can be demonstrated as an anxious behavior in rodents. There was no significant difference in paw withdrawal threshold (P > 0.05) between male rats chronically dosed with alprazolam (**Figure 4**) or female rats chronically dosed with alprazolam (**Figure 5**) *vs* those treated with vehicle on days 1 - 5 of

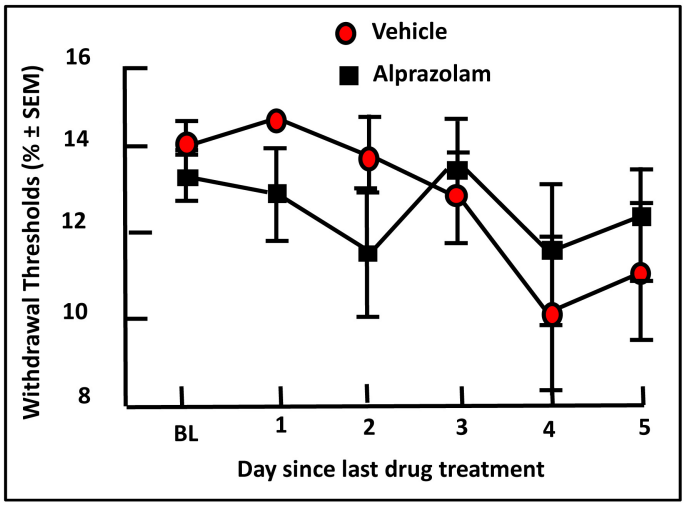


Figure 4. The paw withdrawal threshold (in grams, von Frey filaments) in male rats (N = 28) after chronic dosing of alprazolam (3 mg/kg twice daily for 14 days) *vs* those treated with vehicle.

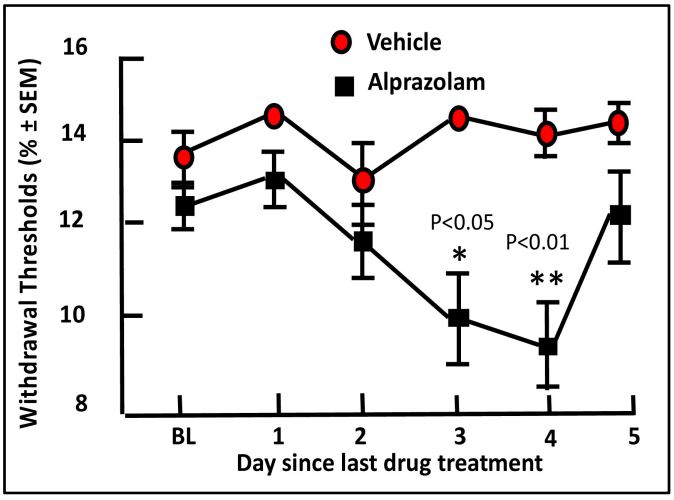


Figure 5. Paw withdrawal threshold (in grams, von Frey filaments) in female rats (N = 16) after chronic dosing of alprazolam (3 mg/kg twice daily for 14 days) *vs* vehicle.

withdrawal.

The open-field experiment was meant to also address possible anxiogenic behavior in rats withdrawing from chronic alprazolam (3 mg/kg, twice daily for 14 days). More time in the center of the open field as opposed to the edges of the test field is indicative of anxiogenic behavior. There was no significant difference between time spent in the center of the chamber in rats chronically dosed with alprazolam *vs* those who received the vehicle on days 1 - 5 of withdrawal (**Figure 6**).

Naïve tissue samples from liver, kidney, adrenal glands, brainstem, spinal cord, spleen, and cortex were analyzed for expression of TSPO. Unfortunately, Western blot analysis was unsuccessful in demonstrating a baseline level of TSPO in these samples, despite multiple attempts (**Figure 7** and **Figure 8**).

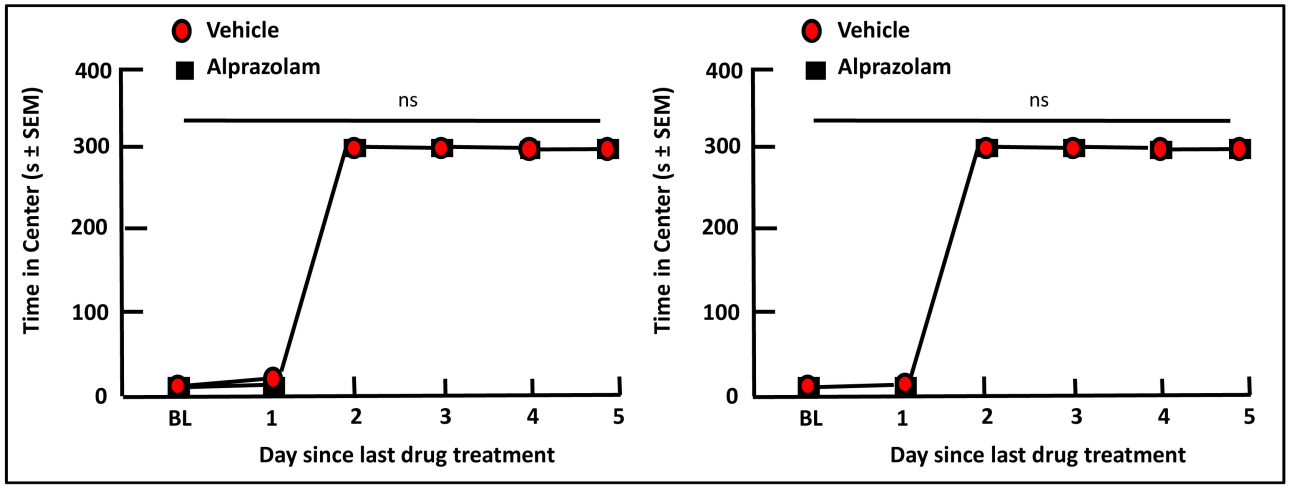
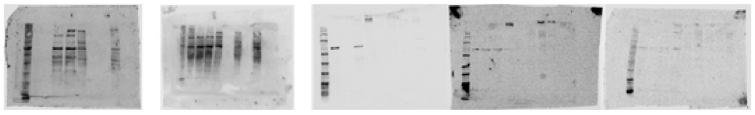
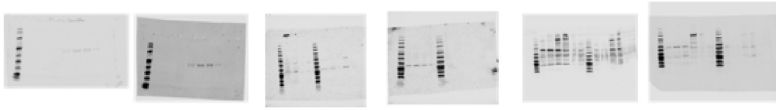


Figure 6. Open field testing in female rats (N = 12), comparing those dosed chronically with alprazolam (3 mg/kg, twice daily for 14 days) *vs* vehicle. There was no significant difference (P > 0.05) in time spent in the center of the open field in the two groups on days 1 - 5 of withdrawal.



Date	6.13.19	6.19.19	6.20.19	6.22.19	6.25.19
Primary antibody	1:1,000, BSA	1:1,000, BSA	1:1,000, milk	1:1,000, milk	1:1,000, milk
Secondary antibody	1:20,000, milk	1:20,000, milk	1:10,000, milk	1:10,000, milk	1:10,000, milk
Miscellaneous changes	30ug protein	30ug protein, 1:1 dilution of tissue	20ug protein, centrifuge	20ug protein, centrifuge	Re-diluted samples (1:1 RIPPA/inhibitor), stopped running gel early
Result	Missing wells	Wells still missing	Wells missing	Wells missing	Wells missing, bands very faint, band appearing at the wrong size, protein not migrating

Figure 7. Western blot analysis intended to analyze TSPO receptor levels in naïve male rodent tissues using various protocols.



Date	7.10.19	7.11.19	8.1.19	8.2.19	8.7.19	8.8.19
Primary antibody	1:1,000, milk	1:1,000, milk	1:1,000, BSA TSPO: 1:4,000	1:1,000, BSA TSPO: 1:4,000	1:1,000, BSA TSPO: 1:4,000	1:1,000, BSA TSPO: 1:4,000
Secondary antibody	1:20,000, milk	1:20,000, milk	1:20,000, milk	1:20,000, milk	1:10,000, milk	1:10,000, milk
Miscellaneous changes	New primary antibody, new DTT, 30ug protein	Stripped blot from 7.10	Increased voltage during transfer to 200V		Rehomgenized adrenal tissue	Stripped blot from 8.7
Result	Gel ripped on transfer, primary was left unagitated all night, wells missing and few faint bands	Tublin bands only showed up for half of samples	Protein in each well, bands very thin	Good bands, all benzo wells missing	Some solid bands, still wells missing	Lost a lot of bands and not all wells showed up

Figure 8. Western blot analysis intended to analyze TSPO receptor levels in naïve male rodent tissues using various protocols.

4. Discussion

The first goal of the current study was achieved. We were successful in demonstrating attenuation of conditioned place preference, a model of drug "liking", in male rats that were treated with alprazolam and the benzodiazepine receptor antagonist, flumazenil. This supports the idea that 1) alprazolam induces rewarding-like behavior in rats [22] [23], and 2) that alprazolam, and possibly other benzodiazepine receptor agonists, have the potential to result in addiction since blocking the benzodiazepine receptor with flumazenil attenuated the conditioned place preference.

Unfortunately, we were unable to demonstrate significant withdrawal behaviors in either male or female rats using an elevated-plus maze apparatus, von Frey tactile stimulation methodology, or open-field experiments. However, male rats did exhibit a significant anxiogenic behavior using open-field testing on day 3 of withdrawal from chronic alprazolam. Additional studies are required to investigate withdrawal models in order to understand the nuances regarding the differences in models used to probe the physiology of benzodiazepine withdrawal.

In addition, due to the methodological shortcomings of the antibody probes, we were unable to optimize Western blot analysis of TSPO receptor expression in naïve male rodent tissues. Additional studies are required to analyze the effects of the TSPO receptor in benzodiazepine reward and withdrawal for possible clinical implications.

A mechanistic explanation for the perplexing prolonged withdrawal symptoms that haunt a subset of patients following cessation of benzodiazepine use thus remains elusive [24] [25].

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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