Behavioral Neuroscience
2013, Vol. 127, No. 4, 515–523

0735-7044/13/$12.00 DOI: 10.1037/a0032962

Social Defeat Stress Switches the Neural System Mediating Benzodiazepine Conditioned Motivation

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Benzodiazepines have been demonstrated to have a high abuse liability in persons suffering from anxiety but have demonstrated mixed abuse liability findings in preclinical models. We hypothesized that by modeling anxiety in a male C57BL/6 mouse model it would be possible to reveal a preference for benzodiazepines within this subpopulation through negative reinforcement. Using the Tube Test of Social Dominance and the Resident/Intruder Paradigm we investigated whether animals identified as dominant or submissive/defeated would differentially display a preference for midazolam (a short acting benzodiazepine) in a conditioned place preference paradigm. Consistent with our hypotheses, benzodiazepine conditioned motivation was mediated by negative reinforcement as submissive but not dominant mice displayed a preference for midazolam. Furthermore, different neural systems mediated midazolam conditioned motivation depending on the stress status of the animal (single vs. repeated stress—as induced by the Resident/Intruder Paradigm). Singly stressed animals showed midazolam place preferences through a dopamine-independent pathway, whereas the place preferences of repeatedly stressed animals were mediated through a dopamine-dependent pathway. This demonstrates that stress is sufficient for switching the neural system mediating midazolam conditioned motivation. Finally, midazolam reinforcement in the conditioned place preference paradigm was shown to be predictive for dominance/submission status.

Keywords: conditioned place preference, animals models of anxiety, resident/intruder paradigm, benzodiazepine motivation, male C57B6 mice

Benzodiazepines are a regularly prescribed class of drugs for the treatment of insomnia, anxiety, and depression; however, they have been demonstrated to have high abuse liability in poly drug users and persons with comorbid anxiety and depression (Licata & Rowlett, 2008; Zawertailo, Busto, Kaplan, Greenblatt, & Sellers, 2003). Despite this demonstrated abuse liability in humans, preclinical animal studies investigating benzodiazepine preferences have yielded mixed results depending on the apparatus used (self-administration vs. conditioned place preference, CPP) and previous drug exposure of the animals (di Scala, Oberling, Rocha, & Sander, 1992; Gray, Allison, & Pratt, 1999; Leri & Franklin, 2000; Pettit, Batsell, & Mueller, 1989; Spyraki, Kazandjian, & Varonos, 1985).

A review by Ator and Griffiths (1987) examined studies of benzodiazepine self-administration and discovered half of the studies reviewed failed to reveal benzodiazepine self-administration. Further examination of the particular studies revealed that studies which demonstrated benzodiazepine self-administration used animals that were previously exposed to sedatives. Furthermore, using a biased CPP paradigm, Spyraki et al. (1985) and Gray, Allison, and Pratt (1999) demonstrated a CPP for diazepam, whereas, using an unbiased CPP, benzodiazepine preference was not observed (di Scala et al., 1992; Leri & Franklin, 2000; Pettit et al., 1989). The aforementioned studies suggest that for biased CPP procedures, benzodiazepines overcome the aversion associated with the initially least preferred side, but unbiased CPPs are unable to replicate this. Conversely, when benzodiazepine preference is examined in the context of poly drug use, a consistent and synergistic preference is revealed (Licata & Rowlett, 2008; Walker & Ettenberg, 2003; Walker & Ettenberg, 2005). These inconsistencies in benzodiazepine CPP necessitate further investigation into the relevant conditions associated with benzodiazepine preference.

Given that abuse liability studies identify poly drug users to be at risk for using benzodiazepines, it is possible that, to investigate benzodiazepine preferences in an animal model, clinically relevant conditions must be considered. As previous studies have investigated benzodiazepines in the context of poly drug exposure, this study considers another clinically relevant condition for benzodiazepine abuse by exploring benzodiazepines in the context of an animal model of anxiety. Furthermore, this work assesses the neural mechanism promoting benzodiazepine preference (positive

This article was published Online First June 3, 2013.

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This work was funded by the Canadian Institutes of Health Research (CIHR). The authors would like to acknowledge Wiplove Lamba for contributions to the social defeat conditioned place preference experiment. The authors would also like to acknowledge Dr. David Hampson and his lab for introducing us to the Tube Test and allowing us to borrow it. Derek van der Kooy and Lilian Riad-Allen designed research and wrote the article. Lilian Riad-Allen performed research and analyzed data.

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vs. negative reinforcement) and considers the impact of single versus repeated stress on this preference.

Previous studies additionally demonstrated that, depending on the drug status of an animal (previously drug naive vs. drug dependent), a neurobiological switch in the systems mediating drug preference is observed. Previously opiate naive animals experience opiate preference through a tegmental pedunculopontine nucleus (TPP) mediated system and opiate dependent animals experience opiate preference through a mesolimbic dopamine (DA) mediated system (Bechara, Harrington, Nader, & van der Kooy, 1992; Nader & van der Kooy, 1997). Perhaps most interestingly, withdrawal is crucial to this neurobiological switch as the absence of withdrawal will maintain animals in a previously drug naive-like state (Bechara et al., 1992). Therefore, it is possible that repeated stress caused by withdrawal is involved in facilitating this neural switch. To explore this possibility, the neural systems mediating midazolam preferences were compared between singly stressed animals and repeatedly stressed animals.

Materials and Methods

Animals

All animals used in these experiments were adult male (25g–35g) C57BL/6 mice. Dopamine receptor subtype 1 (D1) and dopamine receptor subtype 2 (D2) knockout mice (KO) were backcrossed to a C57BL/6 background for more than 20 generations. With the exception of knockout mice, all animals were ordered from Charles River (Montreal, Canada) and allowed to habituate to their new environment for one week prior to any conditioning. D2 receptor knockout mice (D2KO; generated as previously described by Kelly et al., 1998) were birthed from heterozygotic breeding pairs that were mated at the University of Toronto and subsequently genotyped by polymerase chain reaction (PCR). D1 knockout mice (D1KO) were a generous gift from Dr. Susan George (University of Toronto) and were generated as previously described by Drago et al. (1994). There were no observable differences between wild-type (WT) and knockout animals in terms of locomotion or weight, and previous reports on the same animals suggest that there are no postnatal differences in basic motor skills between wild-type and D2KO animals (Ting-A-Kee, Dockstader, Heinmiller, Grieder, & van der Kooy, 2009). Additionally, El-Ghundi et al. (1999) have reported that D1KO do not display any locomotor impairments compared to wildtype animals.

With the exception of the group housed animals used in the prediction experiment, all animals were singly housed in clear Plexiglas mouse cages in a sound attenuated, temperature controlled room. Animals were on a 12-hour light cycle with lights on from 7:00 am until 7:00 p.m. Access to food and water was available ad libitum except during behavioral conditioning and testing. All experiments were approved by the University of Toronto's Animal Care Committee, in accordance with the Canadian Council on Animal Care Guidelines.

Conditioned Place Preference Apparatus

The conditioning apparatus (Place Conditioning Chamber, Med Associates Inc.) consists of a series of eight conditioning chambers each with overall dimensions of 46.5 cm × 12.7 cm × 12.7 cm separated into two distinct compartments separated by a blocked off gray zone which is 7.2 cm long. The two compartments, each 16.8 cm long, are distinct from one another with one being all black with a stainless steel rod floor and the other, all white with a stainless steel mesh floor. Ceiling lights on the lid of both compartments ensured adequate lighting and light intensity was manipulated in order to ensure that no baseline preference existed for either environment.

Conditioned Place Preference Procedure

An unbiased conditioned place preference paradigm was utilized to assess the conditioned motivational state of the animals. Animals were conditioned for five minutes in the two distinctly different compartments described earlier, and all sessions were fully counterbalanced such that half of the animals received drug on the white side and the other half received drug on the black side. Within those groups, half of the animals received midazolam on the first day and the other half received vehicle. Conditioning sessions lasted for 6 days (once daily; three pairings with each environment). Animals were conditioned and tested at the same time each day. Following conditioning, animals were undisturbed for 3 days and tested on the fourth day. Testing consisted of removing the partition between the black and white compartments allowing the mice to explore both areas for 10 minutes. Due to observed state dependent memory effects associated with midazolam, (preliminary studies of midazolam CPP revealed that animals tested in a drug-free state demonstrated no preference for the midazolam conditioned side and spent an equal amount of time exploring both compartments) all animals were tested under the influence of midazolam. Following testing, time spent in the saline paired side was subtracted from time spent in the drug paired side. Therefore, a positive score indicated a preference for the drug and a negative score denoted an aversion. All sessions were checked to ensure that no black/white preference existed and in the uncommon event of a compartment preference, those experiments were excluded.

Drugs

Midazolam 5 mg/ml (Sandoz, Montreal, Canada) was dissolved in 0.9% saline solution and injected intraperitoneally at a concentration of 0.25 mg/kg. This dose was selected based on the results of a dose response curve that tested preference for midazolam at 0.025 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg, and 2.5 mg/kg (see Figure 1). Because midazolam is known to induce state-dependent memories (File, Goodall, Mabbutt, Harris, & Skelley, 1993), animals were conditioned and tested under the influence of 0.25 mg/kg midazolam.

Alpha-flupenthixol (Sigma Aldrich), a broad spectrum dopamine antagonist was dissolved in 0.9% saline solution and injected intraperitoneally 1 hour prior to conditioning at a concentration of 0.5 mg/kg. This drug and dose was selected as it has been demonstrated not to produce any motivational effects (preferences or aversions Grieder et al., 2010; Laviolette & van der Kooy, 2003) and antagonizes D1 and D2 receptors equally (Andersen, 1988; Creese, Burt, & Snyder, 1976). All alpha-flupenthixol pretreated groups were administered alpha-flupenthixol on both drug and vehicle conditioning days to counterbalance any independent motivational effects of this drug.

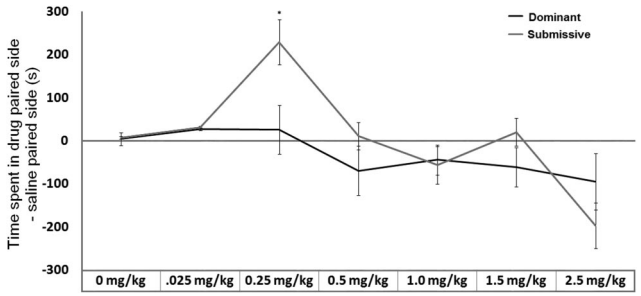

Figure 1. Dose Response Curve for Midazolam. To explore the differential conditioned motivational effects of dose on dominant versus submissive animals, a dose response curve between 0.0 mg/kg and 2.5 mg/kg was conducted (each data point contains a minimum of six animals). A 2-way ANOVA (Dose × Dominance status) revealed a significant interaction, $F(6, 90) = 2.84$, $p = .01$. To better understand this interaction, simple main effects post hoc analysis were conducted, revealing that at the doses tested, submissive animals revealed a significantly greater preference over dominant animals at a dose of .25 mg/kg ($p = .001$). At all other doses tested, dominant and submissive animals had nonsignificantly different place preference scores from one another (0 mg/kg, $p = .96$; .025 mg/kg, $p = .94$; .5 mg/kg, $p = .22$; 1 mg/kg, $p = .83$; 1.5 mg/kg, $p = .17$; and 2.5 mg/kg, $p = .08$). Submissive animals revealed a significant place preference at a dose of 0.25 mg/kg. At all other doses, neither dominant nor submissive animals display any conditioned motivational effects of midazolam. Data represent means +/− SEM. Asterisks represents a significant difference between dominant and submissive animals over dose.

Tube Test of Social Dominance: Identifying Dominant and Submissive Mouse Pairs

A modified Tube Test of Social Dominance, described earlier by Lindzey, Winston, and Manosevitz (1961) was used to identify pairs of dominant and submissive animals. This test was performed by introducing weight and age matched mice into opposite ends of a clear Plexiglas tube (2.5 cm inner diameter, 30.5 cm length). Mice were simultaneously released and advanced into the tube until being met by the other animal. In the event that one animal was dominant, he proceeded into the tube while the submissive animal retreated. A submissive animal was identified once all four paws reversed out of the tube. In the event that there was no difference in the dominant/submissive hierarchy, it was observed that neither animal would retreat, or both animals would retreat simultaneously. In order to ensure reliable tube test results, trials were timed and any trial exceeding two minutes was excluded. Additionally, trials where mice did not meet directly in the middle of the tube were excluded. Last, the side to which the submissive animal retreated was recorded in order to ensure that no side bias existed. The tube was cleaned with Virox (Virox Technologies, Inc.) and rinsed and dried between each trial.

In order to confirm the submissive status identified by the tube test, mice were introduced into a neutral cage and allowed to interact until signs of dominance or submission were revealed. In the rare event that the mouse pair behaved in a way not predicted by the tube test, that is, the submissive mouse displayed dominant behaviors (sniffing of the other mouse, instigating a fight, tail shaking, etc.) or a dominant mouse displayed submissive behaviors (avoidance of the other mouse, digging, squealing, standing in an upright defensive position), that mouse pair was excluded. Following this interaction, animals were left to recover for a minimum of 24 hours prior to any conditioning sessions.

Inducing Repeatedly Stressed Animals

Mice were repeatedly stressed using the resident/intruder paradigm. Submissive animals, as identified by the tube test, were introduced to the home cage of the dominant animal each day prior to conditioning (prior to drug and vehicle conditioning resulting in a total of six physical encounters). The mice were left to interact for up to 5 minutes or until an attack was instigated. At the end of this interaction session, animals (WT, D1KO, and D2KO) were given an intraperitoneal injection of either 0.25 mg/kg midazolam or vehicle and placed directly into the appropriate conditioning chamber. Alpha-flupenthixol pretreated animals were injected with 0.5 mg/kg of alpha-flupenthixol 1 hour prior to this interaction and subsequent conditioning (alpha-flupenthixol pretreatment was administered prior to vehicle and midazolam conditioning to counterbalance any motivational effects of this drug).

Inducing Singly Stressed Animals

Singly stressed mice were identified as being either dominant or submissive using the tube test. Once dominance status was identified, animals were placed into a neutral cage and allowed to interact in order to confirm their submission status. Conversely to the repeatedly stressed mice, the singly stressed mice only experience a single physical encounter with the dominant animal, which was performed in a neutral cage. However, singly stressed mice were conditioned in a compartment adjacent to the dominant animal, such that, singly stressed submissive mice were directly exposed to the scent of their paired dominant mouse at the time of conditioning without allowing any physical interaction between the animals. Conditioning sessions were performed by injecting each animal with either an intraperitoneal injection of 0.25 mg/kg of midazolam or vehicle and directly placing them into the appropriate conditioning chamber. Submissive and dominant mice were conditioned in adjacent boxes so that the scent of the animals could be detected but no physical interaction was possible. Alpha-flupenthixol pretreated animals were injected with 0.5 mg/kg of alpha-flupenthixol 1 hour prior to conditioning.

Examining the Differential Response to Social Defeat in Dominant and Submissive Animals

In order to identify the response of dominant and submissive animals to this social interaction, animals were identified as being either dominant or submissive using the protocol described earlier and assessed in a conditioned place preference paradigm. This was performed by inducing stress in the submissive animal by placing them in the dominant animal's home cage as in the repeated stressed procedure. Following this exposure, animals were placed in one of the compartments of the conditioned place preference apparatus. The following day, the animals were not exposed to one another and conditioning occurred without any interaction. This process was repeated for a 6-day conditioning cycle, whereby each animal was exposed to the defeat interaction three times and the noninteraction three times. Irrespective of the conditioning interaction, submissive mice were conditioned in a compartment adja-

cent to the dominant animal. Therefore, submissive mice were directly exposed to the scent of their paired dominant mouse at the time of conditioning without allowing any physical interaction between the animals. Following a 1-day rest period, the mice were tested for their compartment preference. Time spent in the interaction paired side was subtracted from time spent in the noninteraction paired side, such that, a positive score indicates a preference for defeat interaction and a negative score indicates an aversion to the defeat interaction.

Statistical Analyses

All statistical analyses were performed using SPSS 17.0 (IBM). For all ANOVAs, tests of normality and equal variances were performed and followed up by Bonferroni multiple comparison post hocs or simple main effect post hocs, where appropriate. For any *t* tests, two-tailed independent samples *t* tests were used. In all cases, data is presented as Mean +/− Standard Error and significance was determined at an alpha of less than 0.05.

Results

Negative Reinforcement Promotes Midazolam Conditioned Motivation

The absence of benzodiazepine abuse in the general population suggests that benzodiazepines are not positively reinforcing; consequently, their abuse in anxious subpopulations suggests that their appetitive properties may be mediated by negative reinforcement (the removal of an aversive stimulus) rather than positive reinforcement (the addition of an appetitive stimulus). In order to investigate which type of reinforcement drives benzodiazepine abuse, a dose-response curve comparing repeatedly stressed submissive and dominant animals' CPP to midazolam was performed. This dose-response curve revealed a significant interaction between dose (0 mg/kg–2.5 mg/kg) and status (dominant vs. submissive), $F(6, 90) = 2.84$, $p = .01$, such that, submissive animals displayed a significantly greater preference for a compartment paired with 0.25 mg/kg midazolam, and dominant animals do not display rewarding conditioned motivational effects at any dose tested (see Figure 1). The differential response by repeatedly stressed submissive and dominant animals treated with 0.25 mg/kg of midazolam supports our hypothesis that midazolam conditioned motivation is mediated by negative reinforcement as midazolam preference is only demonstrated by submissive animals. Additionally, this dose response curve demonstrates that response to midazolam in dominant animals is not shifted to the left or the right as a preference is not observed at any dose tested. Importantly, at the highest dose, both dominant and submissive animals trend toward aversions. To examine whether different neural systems mediate midazolam preference dependent on the level of stress, we next examined preference to midazolam in singly and repeatedly stressed mice.

Stress Status (Repeated vs. Single) Determines the Neural System Mediating Midazolam Preference From Dopamine-Independent to Dopamine-Dependent

To test whether repeated stress is capable of switching the neural system mediating midazolam preference, CPPs for midazolam were compared between singly stressed mice (submissive animals that were identified by the tube test but not subsequently physically interacted with a dominant animal) and repeatedly stressed mice (submissive animals that physically interacted with and were socially defeated by a dominant animal on six separate occasions) under saline pretreatment and alpha-flupenthixol pretreatment conditions.

In order to assess whether midazolam preference is mediated by the dopaminergic system in repeatedly stressed mice, we antagonized D1/D2 receptors with alpha-flupenthixol pretreatment and predicted that if midazolam preference is dopamine dependent, submissive animals would no longer display a preference for midazolam. Saline pretreated repeatedly stressed submissive and dominant mice were conditioned to 0.25 mg/kg midazolam and compared with alpha-flupenthixol pretreated repeatedly stressed and dominant mice. The analysis of this 2-way ANOVA revealed a significant interaction between status (dominant vs. submissive) and pretreatment (saline vs. alpha-flupenthixol), $F(1, 95) = 14.40$, $p = .001$. Saline pretreated repeatedly stressed submissive mice were the only group to demonstrate a midazolam preference (Figure 2a), Furthermore, this preference was reversed when repeatedly stressed submissive mice were pretreated with the D1/D2 antagonist alpha-flupenthixol, suggesting that midazolam preference is dopamine mediated in these mice (Figure 2a).

In order to explore potential explanations for this reversal, we examined the response of dominant and submissive animals to repeated defeat. Animals were conditioned to a compartment paired with social interaction (by dominant and submissive mice) or a compartment paired with no interaction. This test revealed a nonsignificant trend $t(29) = 2.04$, $p = .051$, such that, dominant mice demonstrated a preference for the compartment paired with interaction and submissive mice demonstrated an aversion to the compartment paired with interaction (Figure 2b). These results suggest that submissive mice may experience social defeat to be aversive and, therefore, by blocking the effects of midazolam with alpha-flupenthixol, this aversion is revealed.

The previous analysis of repeatedly stressed submissive mice revealed that midazolam preference is dopamine mediated in these animals (Figure 2a). To assess whether stress is capable of switching the neural system mediating midazolam preference, saline and alpha-flupenthixol pretreated singly stressed mice were conditioned to 0.25 mg/kg and tested in a 2-way ANOVA. If stress is capable of switching the neural system mediating midazolam preference, it would be expected that unlike repeatedly stressed mice, singly stressed mice experience midazolam preference through a dopamine independent pathway. In support of our hypothesis, there was only a significant main effect of dominance status, $F(1, 70) = 10.62$, $p = .002$, such that the only significant difference between alpha-flupenthixol singly stressed mice and saline pretreated singly stressed mice was whether they were dominant or submissive (see Figure 3). Both singly stressed saline and alpha-flupenthixol pretreated submissive mice maintained their preference for midazolam, demonstrating that unlike repeatedly stressed submissive mice, singly stressed submissive mice demonstrate midazolam preferences through dopamine independent pathways.

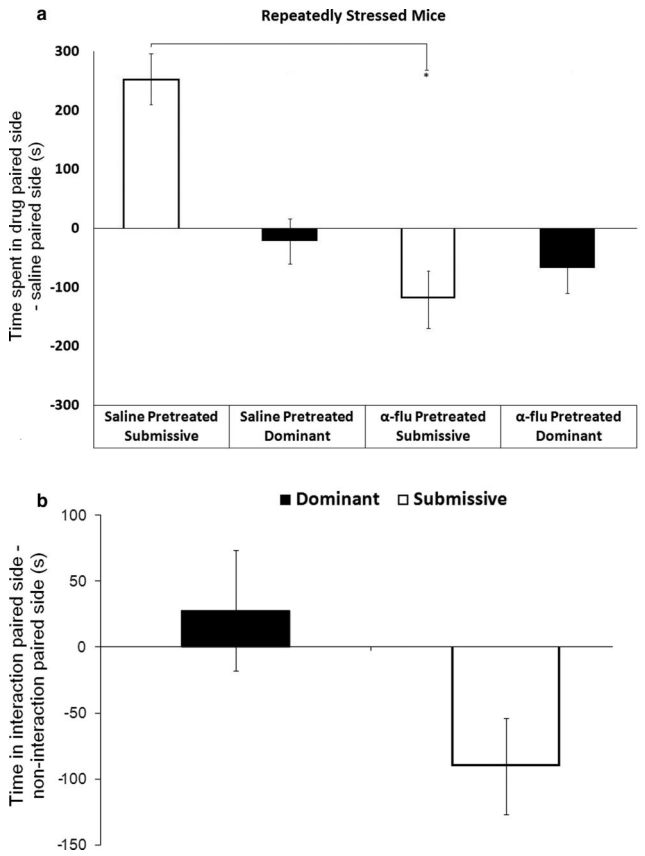

Figure 2. Midazolam CPP in Repeatedly Stressed Mice. 2a. To explore the role of dopamine in repeatedly stressed submissive and dominant mice, a 2-way ANOVA revealed a significant interaction $F(1, 95) = 14.40$, $p = .001$ between dominance status (dominant vs. submissive) and pretreatment (alpha-flupenthixol vs. saline). Simple main effects post hocs revealed that saline pretreated repeatedly stressed submissive mice ($n = 24$) displayed a significantly greater preference than their alpha-flupenthixol pretreated counterparts ($n = 19$), $p = .001$. No significant difference exists between saline pretreated dominant mice ($n = 31$) and alpha-flupenthixol pretreated dominant mice ($n = 25$), $p = .43$. The robust preference for midazolam by saline pretreated repeatedly stressed submissive animals but not dominant animals demonstrates that midazolam preferences are only observed when an aversive stimulus is present (negatively reinforcing). Furthermore, the reversal of the observed preference in alpha-flupenthixol pretreated repeatedly stressed submissive mice implicates the dopaminergic system in midazolam preference of repeatedly stressed submissive mice as blocking the dopaminergic system reverses the previously observed preference. (Asterisks represent a significant post hoc difference between saline and alpha-flupenthixol pretreated repeatedly stressed submissive mice). 2b. Dominant mice ($n = 15$) displayed a trending preference for a compartment previously paired with interaction. Conversely, submissive mice ($n = 16$) displayed a trend toward an aversion to the compartment paired with interaction. Data represent means +/− SEM.

Blockade of D1 or D2 Receptors Attenuates Midazolam Preference in Repeatedly Stressed Mice

Because repeatedly socially defeated submissive mice display a significantly attenuated midazolam preference when pretreated with the D1/D2 receptor antagonist alpha-flupenthixol, it was then investigated whether D1 or D2 receptor blockade alone could attenuate midazolam preference. To investigate this, D1 and D2 knockout mice were repeatedly socially defeated and then compared with a wild-type control group and an alpha-flupenthixol treated wild-type group. For submissive animals there was a significant one-way ANOVA, $F(3, 54) = 12.23$, $p = .001$, such that, wild-type submissive mice were the only group to demonstrate a preference for midazolam (Figure 4a). This preference was significantly different from the aversions demonstrated by D1KO, ($p = .001$), D2KO, ($p = .01$), and alpha-flupenthixol pretreated mice, ($p = .001$). Both submissive D1 and submissive D2 knock out animals were not significantly different from the submissive alpha-flupenthixol treated group, ($p > .05$), suggesting that antagonism of either D1 or D2 receptors is sufficient to block midazolam preference in repeatedly socially defeated animals. Dominant D1 and dominant D2 knock-outs were not significantly different from dominant wild-type and dominant alpha-flupenthixol pretreated animals in a one-way ANOVA, $F(3, 51) = .102$, $p = .96$ (Figure 4b) suggesting that there are no conditioned motivational effects of midazolam on nonstressed animals.

Midazolam Preference Predicts Dominance/Submission Status

Based on our previous finding that midazolam preference is mediated by negative reinforcement (see Figure 1), we hypothesized that we could predict which animals were submissive based on their CPP for midazolam. To test this, group housed mice (three or four male mice in one cage) were conditioned with 0.25 mg/kg

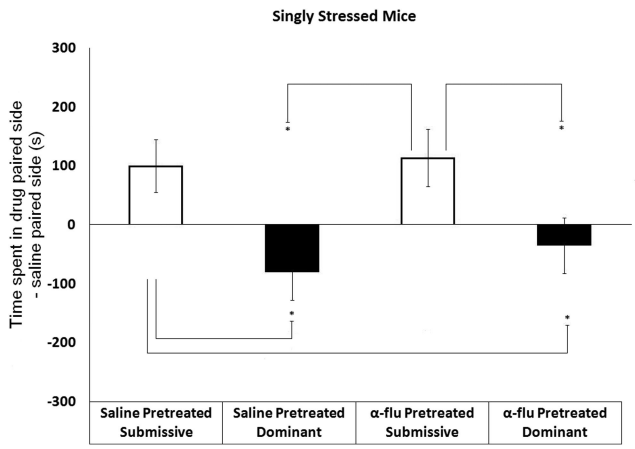

Figure 3. Midazolam CPP in Singly Stressed Mice. To compare singly stressed dominant and submissive animals under saline and alpha-flupenthixol pretreatment, a 2-way ANOVA revealed only a significant main effect of dominance status, $F(1, 70) = 10.62$, $p = .002$. Suggesting that, singly stressed alpha-flupenthixol pretreated submissive mice ($n = 18$) maintain their preference for midazolam at a similar level to their singly stressed saline pretreated counterparts ($n = 20$); therefore, for singly stressed submissive mice, midazolam preference is dopamine independent. Saline pretreated dominant mice ($n = 18$) and alpha-flupenthixol pretreated dominant mice ($n = 18$) do not demonstrate a preference for midazolam. Data represent means +/− SEM. Asterisks represent a significant main effect of dominance status; such that the only significant difference exists between dominant and submissive animals.

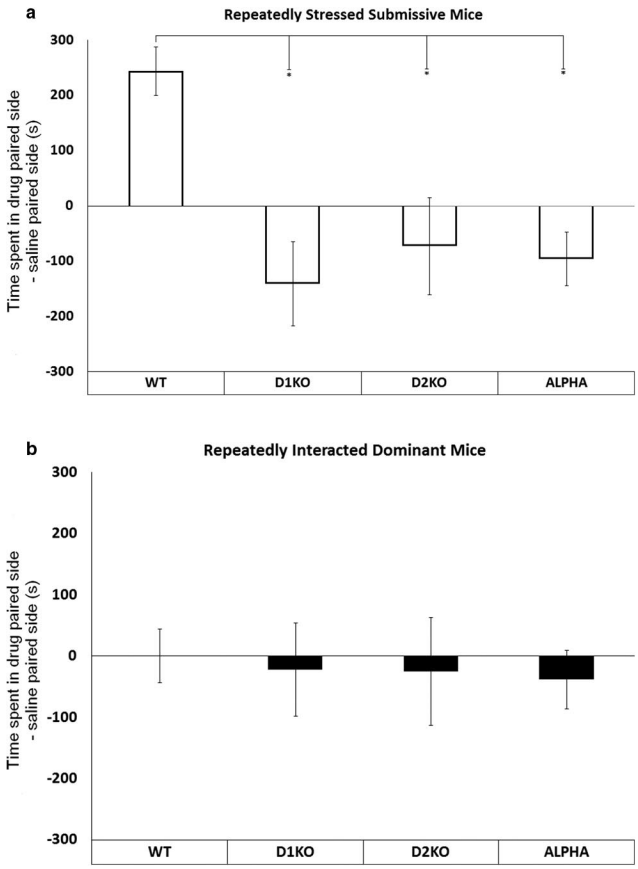

Figure 4. Dopamine Specificity in Repeatedly Stressed Dominant and Submissive Mice. 4a. Time spent in the midazolam paired side (vs. saline paired side) revealed that wildtype repeatedly stressed submissive mice ($n = 22$) were the only repeatedly stressed submissive animals to display a preference for 0.25 mg/kg midazolam in a one-way ANOVA. Compared with wildtype repeatedly stressed submissive mice, D1KO ($n = 8$), $p = .001$; D2KO ($n = 8$), $p = .01$; and alpha-flupenthixol pretreated mice ($n = 20$), $p = .001$ do not display a preference for midazolam, indicating that blockade of either D1 or D2 like receptors is sufficient for blocking midazolam preference in submissive mice. Asterisks represent a significant main effect of genotype such that wildtype animals are the only group tested to display a significant preference for midazolam. 4b. In order to explore whether D1, D2, or alpha-flupenthixol pretreated dominant mice respond differently to midazolam than dominant wildtype mice, response to 0.25 mg/kg midazolam was examined between these groups using a one-way ANOVA. No dominant mouse group displayed a preference for midazolam. Additionally, there are no significant differences between wildtype dominant mice ($n = 21$) and D1KO dominant mice ($n = 8$), $p = .99$; D2KO dominant mice ($n = 8$), $p = .99$; and alpha-flupenthixol pretreated dominant mice ($n = 18$), $p = .99$ in their response to midazolam. Data represent means +/− SEM.

midazolam and their place preferences assessed. Following testing, and with the experimenter blinded to the conditioning results, animals were tube tested in order to determine their dominance/submission status. Following identification of their status, a two-tailed, independent samples t test was performed comparing the CPP of dominant and submissive animals. This analysis revealed a significant t test, $t(13) = 2.38$, $p = .04$, such that, mice that displayed a midazolam preference were more likely to be submissive in the tube test. Conversely, mice that did not show a preference for midazolam were more likely to be dominant. These results suggest that social status can be accurately predicted based on midazolam preference (see Figure 5). Furthermore, these results suggest that social status can be accurately predicted based on midazolam preference and provides more evidence in support of the negative reinforcement hypothesis of midazolam conditioned motivation, as animals that display a midazolam preference are significantly more likely be submissive.

Discussion

Benzodiazepine Conditioned Motivation is Mediated by Negative Reinforcement

Unlike opiates, which are thought to be positively reinforcing in dependent animals (Bechara & van der Kooy, 1989; Bozarth & Wise, 1981; van der Kooy, Mucha, & O'Shaughnessy, & Bucenieks, 1982) and, therefore, pose a high abuse liability for the general population, benzodiazepines are thought to have a low abuse liability for the general population but a high abuse liability for poly drug users and persons with comorbid anxiety and depression (Barnas, Rossmann, Roessler, Riemer, & Fleischhacker, 1992; Brunette, Noordsy, Xie, & Drake, 2003; Ciraulo, Sands, & Shader, 1988; Griffiths & Weerts, 1997; Licata & Rowlett, 2008; Martinez-Cano, Gauna, Vela-Bueno, & Wittchen, 1999; Rooney, Kelly, Bamford, Sloan, & O'Connor, 1999; Zawertailo et al., 2003). We hypothesized that benzodiazepine preference was mediated by negative reinforcement and tested this empirically by using animals identified as being either dominant or submissive using the tube test of social dominance. As expected from the clinical portrait of anxious/depressed persons abusing benzodiazepines, submissive animals but not dominant animals displayed a preference for benzodiazepines. These data suggest that benzodi-

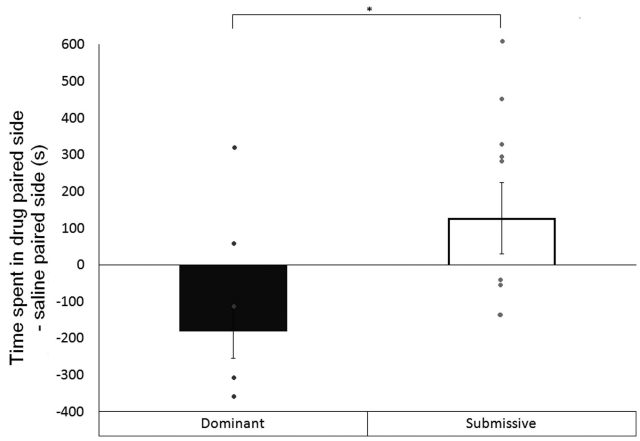

Figure 5. Midazolam CPP Predicts Dominance/Submission Status. A two-tailed independent samples t test revealed that dominance/submission status could be predicted from midazolam preference, such that submissive animals ($n = 9$) are more likely to display a preference for midazolam whereas dominant animals ($n = 6$) are more likely to display an aversion. Dots indicate the scores of individual mice. Bars represent means +/− SEM. Asterisks signify a significant difference between dominant and submissive animals.

azepines on their own are not positively reinforcing; that is, they do not possess appetitive properties and preferences are only revealed when in the presence of an aversive stimulus, in this case, the anxiety associated with social defeat or submissive social status. Additionally, the larger preference in repeatedly stressed (vs. singly stressed mice) is consistent with our hypothesis that benzodiazepines are negatively reinforcing. Furthermore, these results suggest that it is conditioned motivation (conditioned motivation here is defined as a guiding force that promotes approach or avoidance behavior following conditioning), rather than associative learning that promotes midazolam place preferences as submissive but not dominant animals display a place preference to midazolam. Additionally, animals did not display any significant differences in locomotor activity on any of the conditioning or test days and all environments were fully counterbalanced to ensure that the observed results were not caused by initial preferences for one environment over another. Finally, as expected by the negative reinforcement hypothesis, these effects are larger in the repeatedly stressed group than the singly stressed group.

The differentiation between the positive reinforcing effects of opiates/stimulants and the negative reinforcing effects of benzodiazepines may explain why dominant/nonstressed animals do not experience a preference for benzodiazepines but can still display a drug preference for opiates, as there is evidence to suggest that opiates are intrinsically rewarding (Bechara & van der Kooy, 1989; Stewart, de Wit, & Eikelboom, 1984). Moreover, this provides a potential explanation as to why benzodiazepines are largely nonproblematic in the general population, yet pose a high abuse liability in anxious/depressed subpopulations.

Stress Status (Repeated or Single) Switches the Neural System Mediating Midazolam Preference From Dopamine-Independent to Dopamine-Dependent

Previous evidence suggests that different neurobiological systems mediate the rewarding properties of drugs of abuse, depending on the motivational state of an animal (deprived vs. nondeprived; Bechara et al., 1992; Bechara & van der Kooy, 1992; Bechara, Nader, & van der Kooy, 1998). However, this switching hypothesis was not previously explored for benzodiazepines. Given the involvement of negative reinforcement on benzodiazepine preference, it appeared relevant to examine varying levels of stress and its ability to switch the neurobiological systems involved in benzodiazepine preference.

We show that submissive singly stressed mice (mice that were physically defeated by a dominant mouse on one occasion) and submissive repeatedly stressed mice (mice that were physically defeated by a dominant mouse on six occasions) both exhibit a preference for midazolam. However, repeatedly stressed mice, but not singly stressed mice demonstrate an attenuated midazolam preference when pretreated with the D1/D2 receptor antagonist alpha-flupenthixol. This finding suggests a neurobiological switch dependent on the stress status of submissive mice (single vs. repeated). Interestingly, this neurobiological switch perfectly complements the one observed for opiates as previous evidence suggests that opiate dependent and withdrawn animals, but not, nondeprived animals have an attenuated preference for opiates when pretreated with the D1/D2 receptor antagonist alpha-flupenthixol (Bechara et al., 1998; Bechara & van der Kooy, 1992). An important distinction to be made between these groups (opiate and midazolam treated animals) is that for opiate dependent and withdrawn animals, animals were made drug dependent prior to conditioning and were subsequently conditioned at the peak of withdrawal—in the deprived state. Interestingly, opiate dependent but not withdrawn animals still experienced opiate reward through a dopamine independent pathway (TPP), similarly to nonphysically dependent/nondeprived animals (Bechara & van der Kooy, 1992). Therefore, it is possible that the stress experienced by an animal in opiate withdrawal is necessary for switching the neural system mediating opiate reward from the TPP to the mesolimbic dopamine system. Additionally, studies have demonstrated that by injecting BDNF in the ventral-tegmental area of naive animals, the neural system mediating their opiate preference switched to a dependent-like system (dopamine dependent; Vargas-Perez et al., 2009) suggesting that stress and/or changes in BDNF levels are capable of mediating this neurobiological switch.

Conversely, in the midazolam treated group, all animals were conditioned in the nondeprived state with the only difference being the level of interaction between the dominant and submissive animals (either reexposed prior to each conditioning session as in the repeated stressed group or never reexposed following the status test as in the single stressed group). This switch, observed in midazolam treated submissive mice suggests that repeated stress is capable of modifying the neurobiological system mediating midazolam preference from a non-deprived-like state (dopamine independent) to a deprived-like state (dopamine dependent).

Given what it known about sensitization and dopamine, as described by the incentive-sensitization model (Robinson & Berridge, 1993; Robinson & Berridge, 2001), another interpretation of this data may be that with repeated stress and drug exposures, the dopaminergic system becomes sensitized and, therefore, involved in the conditioned motivation for benzodiazepines. For the present data, this interpretation is unlikely as we were unable to reveal a preference for benzodiazepines in both single and repeatedly stressed animals without negative reinforcement. Further to that, if these findings demonstrated a sensitization to dopamine with repeated exposures, an increase in locomotion may be expected (Robinson & Berridge, 1993); however, in these experiments no significant increases in locomotion was observed with repeated conditioning exposures (data not shown). Lastly, if these findings were truly a product of dopamine sensitization as a function of repeated exposures, it may be hypothesized that both repeatedly stressed dominant and submissive animals are responsive to dopamine antagonism/deletion, rather than submissive animals alone. Our data demonstrate that only submissive animals display changes in conditioned motivation when dopamine is antagonized/depleted; therefore, it is unlikely that incentive-sensitization theory is able to explain these findings.

D1 and D2 Receptor Blockade Attenuates Midazolam Preference in Repeatedly Stressed Mice

Following the identification that repeatedly stressed mice experience midazolam preference through a dopamine-dependent pathway, we sought to identify whether we could elucidate the specific dopamine subunit mediating this preference. Using receptor knockout mice we identified that both D1 and D2 receptor knockout mice experienced an attenuated midazolam preference, sug-

gesting that blockade of either D1 or D2 is sufficient for blocking midazolam preference in repeatedly stressed submissive mice. This unexpected finding suggests the involvement of both D1-like and D2-like receptors in midazolam preference and further distinguishes benzodiazepines from other drugs of abuse which appear to be mediated by only one receptor subtype (Ting-A-Kee et al., 2009). This finding further demonstrates the unique nature of benzodiazepines and offers a starting point by which drug targets aimed at treating benzodiazepine dependence may build upon.

One limitation of the aforementioned studies is that the neurobiology of benzodiazepine preference was examined using systemic injections of midazolam and, thus, it is not possible to elucidate where in the brain this potential switch is occurring. Future attempts to localize where this neurobiological switch occurs could be conducted by intracranially injecting midazolam into the VTA of dominant and submissive mice, as this neural region has been previously implicated in the neurobiological switch for opiates (Laviolette & van der Kooy, 2004; Nader & van der Kooy, 1997). By identifying where exactly in the brain midazolam preference is processed, depending on the stress status of an animal, a clearer understanding of the neurobiology of benzodiazepine conditioned motivation may be established.

Midazolam Preference Predicts Dominance/Submission Status

As expected by previous work from Cao et al. (2010), which states that 60% to 70% of mice in their study were determined to be susceptible to repeated social defeat stress (submissive), preliminary testing suggested that six of 15 animals were determined to be dominant (40%) and nine out of 15 were determined to be submissive (60%). Furthermore, we demonstrate that group housed mice that were determined to be submissive had a significantly greater preference for the compartment paired with midazolam compared with animals that were determined to be dominant. Reinforcing that, midazolam conditioned motivation is negatively reinforcing. However, one limitation of this protocol is the inability to determine the strength of dominance and submission between animal pairs. Attempts to correlate latency to attack, number of attack attempts, circling, and other dominant behaviors with midazolam preference in submissive animals were nonsignificant. One explanation for the lack of significance in these correlation experiments are that submissive animals are known to increase social avoidance following defeat stress (Blanchard, McKittrick, & Blanchard, 2001) and so it is possible that by effectively avoiding a strongly dominant mouse, a defeated submissive animal may receive the same score (if measuring the number of attacks, circling behaviors, etc.) as a weakly defeated submissive animal.

Other questions raised by this work focus on what factors determine submission status. It is possible that early postnatal encounters with littermates contribute to the early determination of status, causing long-term submission in adult life. Likewise, it is also possible that the embryonic environment influences the status of these animals. Mice used in these experiments were birthed and reared in a group housed environment prior to being delivered to our facility, similarly, all KO mice were group housed prior to the start of experiments; therefore, we are unable to identify which of the explanations listed above determine submission status. However, it is likely that no one explanation can entirely explain the factors determining dominance/submission but rather a complex interaction between the embryonic environment and early interactions with littermates. Furthermore, in naturalistic interactions, it is possible that genetic variability also contributes to the differences observed in dominance and submission status; this may be observed by comparing different mouse strains using the Tube Test and/or the Resident Intruder Paradigm. Understanding the neurochemical adaptations that occur following social defeat and potentially other repeated forms of stressors may be crucial to understanding benzodiazepine preferences.

In summary, this work demonstrates that it is possible to demonstrate midazolam conditioned motivation in an animal model as submissive but not dominant animals display a preference for midazolam. This conditioned motivation is mediated by separate neural systems depending on the stress status of the animal: singly stressed submissive animals demonstrate midazolam preference through a dopamine independent system, whereas repeatedly stressed submissive animals demonstrate midazolam preference through a dopamine dependent system. Within repeatedly stressed mice, antagonism of the D1 or D2 receptor is sufficient for blocking midazolam conditioned motivation and last, within group housed animals, midazolam preference predicts social defeat status.

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Received February 7, 2013
Revision received April 1, 2013
Accepted April 2, 2013 ■