Pro-Social Behavior In Rats Requires An Affective Motivation

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Running title: Rat helping requires affective motivation, Anxiolytic treatment blocks helping

Abstract: 43 words; Manuscript: 9000 words

Number of figures: 7; Supplementary material: 5

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Abstract

The motivations behind rodent helping behavior are unclear. We hypothesize that helping behavior is motivated by an affective state. To test this, we used a paradigm in which one rat can help another by releasing him from a restrainer. Rats were left untreated or received an injection of saline, midazolam (anxiolytic), or nadolol (peripherally acting sympatholytic). Midazolam but not nadolol or saline blocked helping. However, midazolam-treated rats opened the restrainer to access chocolate, showing that midazolam blocks helping through an effect on affect rather than through sedation or the like. To determine the role of HPA reactivity, we measured the corticosterone response to a trapped rat. Rats with the highest corticosterone responses evoked by a trapped rat did not develop consistent helping behavior. Together, these results demonstrate that rodent helping behavior is motivated by an affective state of anxiety and antagonized by physiological stress.

Keywords

emotional contagion, midazolam, empathy, helping, rodent, altruism

Introduction

Pro-social behavior refers to actions that help or benefit another individual or group of individuals (Cronin, 2012). Such behaviors have independently emerged in many species including rodents (Brosnan, 2013; Dugatkin, 1997; Sussman and Chapman, 2004), evidence for the profound evolutionary advantage gained by helping others (Hamilton, 1964). Rats exhibit several types of pro-social behavior such as sharing food (Marquez et al., 2015, Hernandez-Lallement et al., 2015), providing reciprocal aid to access food (Rutte and Taborsky, 2007), and releasing a trapped rat (Ben-Ami Bartal et al., 2011; Sato et al., 2015).

Despite the now numerous demonstrations of rodent pro-social behavior, the motivation driving rodent helping remains unclear. The trapped rat paradigm, in which one rat frees a rat trapped inside a Plexiglas tube, offers a chance to experimentally dissect the neurobiological motivations underpinning pro-social behavior. Some have suggested that helping in this paradigm may simply result from approach motivated by a positive cue (eg social contact) or avoidance of a negative cue (eg alarm calls) (Silberberg et al., 2014, Vasconcelos et al., 2012). Alternatively, rodent helping may be motivated by an affective state of distress that is caught by the helper from the trapped animal and resolved by the pro-social act, an idea that is consistent with the perception-action model of empathy (de Waal and Preston 2002). The present study is designed to test this latter hypothesis. Administering an anxiolytic to the potential helper rat tested the necessity of affect in motivating pro-social behavior. Finding that anxiolytic treatment blocked helping, we then asked whether the failure to show pro-social behavior by a minority of rats could stem from an elevated hypothalamo-pituitary-adrenal (HPA) reaction to a trapped conspecific. To this end, corticosterone (CORT) responses evoked by viewing a trapped rat were compared for rats that helped and for those that did not show consistent helping behavior.

In the helping behavior test used here, a "free" rat is given the opportunity to act pro-socially by releasing a trapped conspecific from a Plexiglas tube or restrainer (Ben-Ami Bartal et al., 2011; 2014). The restrainer, centrally located in a testing arena, can only be opened from the outside and thus only by the free rat. There is no explicit training nor is an external reward provided for opening the restrainer door. Across 12 daily sessions, rats typically learn to open the restrainer door within a few testing sessions, and thereafter continue to do so consistently at progressively shorter latencies.

We developed a method to isolate the day-to-day reinforcing effect of helping from other sources of learning, such as perceptual learning or mastery. Specifically, a simulation was created that tested whether helping (0 or 1) on days N and N+1 are independent. In the simulation, individual and day averages were used to calculate an expected probability of opening for each observation (each rat on each day). A random binary process was used to generate opening patterns for "null rats," rats where days N and N+1 are independent. Results from the simulated null rats were compared to the rats tested in this study. In this way, we were able to compare the number of sequential openings observed to the number that would be expected without reinforcement. This novel method provides a quantitative assessment of reinforcement.

Material and methods

Subjects. Two month-old Sprague-Dawley (SD) male rats (Charles River, Portage, MI) were used for all studies. Rats were housed in pairs with *ad libitum* access to chow and water in a 12:12 light-dark cycle, and allowed two weeks to acclimate to the housing environment and their cagemate. All rat pairs were cagemates.

Habituation. Two weeks after arriving at the animal facility, animals were habituated to the testing rooms, experimenters (who were kept constant for each cohort of rats), and testing arenas. Testing arenas were constructed of Plexiglas (50 x 50 cm, 32-60 cm high) and were kept constant for each pair or rats. On day 1 of habituation, rats were transported to the testing room and left undisturbed in their home cages. On day 2, rats were briefly handled. Starting with the second day of habituation, rats were weighed 3 times weekly for the duration of the experiment; no animal lost weight during the experiment. On days 3-6, rats were handled for 5 minutes by each experimenter and then placed together (in housing pairs) in the testing arenas for 30 minutes. After each habituation session, rats were returned to their home cages and to the housing room.

Free rats in one group of rats received no treatment whereas free rats in five groups received i.p. injections prior to every testing session. In order to habituate free rats to i.p. injections and minimize stress related to the injection itself, free rats in the four injection groups (high and low MDZ, nadolol, saline) received i.p. saline injections once daily for at least 5 days preceding testing. Rats in the uninjected group received no injections during habituation.

Open field testing. On the day following completion of habituation, rats were placed individually in an arena for 30 min and their activity recorded. Note that the arenas were the same as were used during habituation but that open field testing represented the first time each rat had been in the arena alone.

Protocol. On each testing day, rats were transported to the testing room and left undisturbed in their home cage for 15 minutes. Then rats were colored with markers to permit tracking the rats' individual movements. The free rat was colored red and the trapped rat colored blue. After coloring, rats in the uninjected group were placed into the arenas for testing.

Rats in the five injection groups were weighed after coloring and then injected with MDZ (2 mg/kg for the high dose conditions; 1.25 mg/kg for the low dose conditions, i.p.), nadolol (10 mg/kg) or saline (0.5 cc, i.p.). Rats were returned to their home cage after receiving an injection. After a waiting period (15 min for MDZ and saline; 30 min for nadolol), rats were placed in the arena and the helping behavior test began.

Trapped rat paradigm. The trapped rat was placed inside a restrainer and the restrainer was positioned in the arena center. Restrainers were Plexiglas tubes (25 X 8.75 X 7.5 cm; Harvard Apparatus, Holliston, MA) that had several slits, allowing for olfactory and tactile communication between rats. The free rat (the trapped rat's cagemate) was then placed in the arena and allowed to roam freely. The door to the restrainer could only be opened from the outside and therefore only by the free rat. If the free rat did not open the restrainer door within 40 min, the investigator opened the restrainer door halfway, to a 45° angle, greatly facilitating door-opening by either rat. Only door-openings that occurred prior to the halfway opening were counted as such.

Rat dyads always remained in the arena for a full hour. Hour-long testing sessions were repeated for 12 days and performed only once per day. All sessions were run during the rats' light cycle between 0800 and 1730. After each session, rats were returned to their home cages and the arena and restrainer were washed with 1% acetic acid followed by surface cleaner.

Blockers. Some trapped rats (n=30, 38%) succeeded in opening the door from inside the restrainer. When this happened, the trapped rat was placed immediately back in the restrainer, and a Plexiglas blocker was inserted, preventing his access to the door. If the free rat subsequently opened the door, the blocker was removed, allowing the trapped rat to exit the

restrainer. The blocker was then used for that trapped rat on all following test days. If the free rat failed to open the door by 40 min, the blocker was removed when the door was opened halfway.

Chocolate behavior test. Rats in the three chocolate conditions (high and low MDZ, saline), were introduced to chocolate chips for 3 weeks prior to the experimental sessions. After this exposure, they ate an average of 4.6 ± 0.4 chips at a time. On testing days, the restrainer was filled with 5 chocolate chips (Nestlé® Toll House, milk chocolate) and positioned in the arena center; chocolate was not available to rats outside of the testing sessions. The free rat was placed in the arena with the restrainer but without his cagemate; all other details of the experimental protocol were as described above. When rats opened the restrainer door, they always ate all 5 chips.

Door-opening analysis. Latency to door-opening was calculated as the minute when the restrainer door was opened minus the start time. For rats that never opened, a cutoff time of 40 min (the time of halfway opening) was assigned.

Behavioral testing for corticosterone measurements. Blood samples were collected via tail-nick from a cohort of 60 rats on the first and last days of testing in the helping behavior test (*trapped* condition, see above), run with slight modifications as detailed here. Rats were habituated to the arenas for 10 days. On day 1, restrainers were taped shut to ensure a common experience for all rats. On days 1 and 13, rats were removed from the arena after 40 minutes, and the door was not opened halfway. In the empty condition, procedures were identical, except that two cagemates were placed in an arena containing an empty restrainer.

Corticosterone measurements. Blood was collected at three time points of each session, on the first and last days of testing. On each day, the first sample (baseline) was collected an hour prior to placement in the arena. The second sample (test) was collected immediately after removal from the arena, and the third sample (post) was collected an hour after removal from the arena. Pilot experiments revealed that unstressed, male Sprague-Dawley rats show steady levels of CORT between 0830 and 1230. Therefore all samples were collected during this period.

Blood (200-500 µL) was collected via tail-nick, by experimenters who had handled the rats previously. Sampling was completed in less than three minutes (average 2:18). Samples were immediately centrifuged for 10 min at 5000 rpm at 4°C. Plasma was extracted and frozen at -20°C for further analysis via enzyme-linked immunoabsorbent assay (ELISA, IBL). The assay had a sensitivity of < 27.0 pg⁄ml. Two outliers were removed from analysis.

Statistical analysis. Opening latencies of each subject (16 per group) on each day (12 per subject) from each experimental group (5 trapped groups, 3 chocolate groups) were analyzed using a general linear model with the statistical software *R (R Foundation for Statistical Computing, Vienna, Austria, used under the General Public License)* and the R package "regress" (*David Clifford and Peter McCullagh, used under the General Public License).* The R code for the analysis as well as the original latency data (Supplement B) are included below.

The general linear model contains the following terms for sources of variance: *treatment* (fixed effect, 5 nominal levels), *day* (fixed effect, 12 ordinal levels), *rat* (fixed effect, 16 subjects per group * 12 days * 5 groups = 960 nominal levels), and interaction between *treatment* and *day*. The final source of variance considered was *Vrat* (see next section), which accounts for correlations between latencies on different days within the same subject. Note that there were two missing data points (one from day 1 of one rat injected with low MDZ and one from day 2 of one rat injected with high MDZ), bringing the total number of analyzed values to 958.

Rationale for and generation of Vrat. The data are correlated due to the fact that many measurements come from the same subject. However, because of a learning effect over days (i.e. opening latency decreases over time but does not increase), the correlation between latencies on two different days within the same subject decays as the interval between the days increases. Therefore, individual between-pair correlation coefficients (α) need to be generated for each interval in the data set. Vrat contains non-zero coefficients only for intervals within the same subject; for pairs containing opening latencies from two different subjects, there is no within-subject correlation and therefore the correlation coefficients are zero. Thus, *Vrat* is a 960*960 matrix with 12 non-zero values representing the correlation coefficients from the 12 possible day intervals that range from 0 to 11. The value of α for the day interval of 0 is 1.0 whereas α decays to 0.28 for the maximum interval of 11 days (see supplementary figure 1).

By generating *Vrat* with either an exponential decay or a linear decay and testing the log maximum likelihood estimate (LMLE) of the resulting models to compare their relative goodness of fit, we determined that an exponential decay is a better approximation. The α for each pair of opening latencies is therefore calculated as

$$\alpha = e^{-\gamma \cdot d},$$

where γ is a uniform correlation coefficient, constant across all pairs, that reflects the strength of correlation between latencies of two days within the same subject, and d is the absolute value of the difference between two days (e.g. latencies from day 1 and day 5 will have $d = 4$). We determined γ for each experiment by comparing models of different γ and selecting the value that resulted in the LMLE, as a larger LMLE indicates a better fit.

In a standard repeated-measures ANOVA, the correlation between days within the same subject is assumed to be constant across all intervals. However, since animals learn from their experiences, latencies on adjacent days are more correlated than are latencies separated by longer intervals. The matrix term *Vrat* offers the advantage of being able to accommodate smoothly changing correlation coefficients between pairs of days separated by increasing intervals. In sum, *Vrat* allowed us to account for experimental subjects' changing their behavior through learning.

Comparing hypotheses and testing significance of interactions. The alternative hypothesis is stated as follows.

$$latencies \sim (treatment + day + rat + treatment * day + Vrat)$$

The polynomial regression equation is the following:

$$Y_i = \beta_1 * treatment_i + \beta_2 * day_i + \beta_3 * treatment_i * day_i + \beta_4 * rat_i + \overrightarrow{\beta_5} * \boldsymbol{Vrat}_{i,j} + \epsilon_i \quad (i, j = 1, 2, 3, \cdots, 960)$$

Here β_n represents the respective regression coefficient calculated by regression analysis, i refers to the opening latency out of 960 latencies, and ϵ_i represents the unobserved random error.

The null hypothesis, which states that there is no interaction between *treatment* and *day*, can be stated as follows.

$$latencies \sim (treatment + day + rat + \boldsymbol{Vrat})$$

The polynomial regression equation for the null hypothesis is therefore the following.

$$Y_i = \beta_1 * treatment_i + \beta_2 * day_i + \beta_4 * rat_i + \beta_5 * \boldsymbol{Vrat}_{i,j} + \epsilon_i \quad (i, j = 1, 2, 3, \cdots, 960)$$

We then calculated the statistical significance of the difference between the goodness of fit of the null and alternative hypotheses. To do this, we looked up the probability of the following term on a χ^2 distribution with 4 degrees of freedom:

$$p = 1 - pchisq(2 * (LMLE_{alternative} - LMLE_{null}), \qquad df = 4$$

The results are reported as the calculated χ^2 value (see above), the degrees of freedom, and the corresponding probability.

The same analysis was performed on rats tested with a restrainer containing chocolate. However, since there were only three experimental groups (saline, high MDZ, low MDZ), the degree of freedom was two.

Differences in CORT levels were tested using MMA with "day" (1, 13) and "sampling point" (baseline, test, post) as the repeated measures. In addition, CORT *responses* (test value less baseline value) were analyzed with MMA with "day" as the repeated measure. Sidak tests were conducted for all post-hoc analysis. Pearson's R was used to calculate the correlation between the number of door-openings and CORT responses on day 1 of testing. All statistical comparisons were conducted using SPSS (PASW 18).

Testing for reinforcement. If a rat experiences opening the restrainer as rewarding, then door-opening behavior will be reinforced and the likelihood of opening on the *next day* will increase. Thus, reinforcement would be marked by a probability of opening on two sequential days (pSO) that is significantly higher than chance level. To test this, we compared the observed pSO to a distribution of chance pSO values generated with the following method.

To generate chance pSO values, a model was constructed that takes into account the effects of learning and also of individual differences. Rats may display non-associative learning over the course of testing sessions, putatively through processes that include habituation to the testing conditions, motor and perceptual learning, and conditioning. There are also individual differences between rats that might include motor, cognitive, and social differences. Together, these effects concentrate openings to later days in select rats, leading to high chance pSO levels. By incorporating the effects of learning and individual differences, the model produces realistic chance pSO values and allows a realistic estimate of the opening behavior on sequential days that would occur by chance, in the absence of reinforcement learning.

For each condition with N rats and 12 days, we created an observation matrix $\boldsymbol{M}$ with N rows and 12 columns. Opening observations from each condition were transformed into a binary distribution (1 for opening, 0 for not opening). The proportion of openings contributed by a rat (a measure of individual differences) was multiplied by the proportion of all openings that occurred on each day of testing among the group (a measure of learning). This product, after being normalized to the total number of openings in the group, is the probability that any given rat will open on any given day. Thus, we calculated an N by 12 matrix, $\boldsymbol{P}$, of probabilities for all instances of opening on day j by rat i. The matrix is given by:

$$\boldsymbol{P}_{i,j} = \frac{\sum_{k=1}^{N} \boldsymbol{M}_{k,j} \times \sum_{l=1}^{12} \boldsymbol{M}_{i,l}}{\sum_{k=1}^{N} \sum_{l=1}^{12} \boldsymbol{M}_{k,l}} \; (for\ i = 1,2,\dots,N; j = 1,2,\dots,12)$$

We then generated 10,000 binary matrices using the probabilities from $\boldsymbol{P}$ and calculated the pSO for each. From each distribution, we then estimated how extreme the observed pSO was compared to chance expectations by calculating a two-tailed p-value.

Results

Blocking distress in free rats tested with a trapped cagemate

Five groups of rats were studied. Rats that received no injection were compared to rats that received either vehicle (saline) or one of two doses (low 1.25 mg/kg; high 2.0 mg/kg) of the benzodiazepine anxiolytic, midazolam (MDZ). To distinguish between the direct anxiolytic effects and secondary sympatholytic effects of MDZ, a final group of rats received nadolol, a beta-adrenergic antagonist that does not cross the blood-brain barrier.

Overall, the opening latency decreased across days, reflecting learning (Fig. 1A). This decay in opening latency across days differed between treatment groups (general linear model as described in the Methods: $\chi^2(4)=12.0$; p=0.02; Fig. 1B; Table 1). Untreated rats as well as rats treated with saline or nadolol showed decreasing opening latencies across the days of testing (linear model analysis; uninjected: N(0,1)=-4.36, p<0.001; saline: N(0,1)=-3.56, p<0.001; nadolol: N(0,1)=-3.86, p<0.001). In contrast, there was no decay in latency across days for rats treated with either dose of MDZ (linear model analysis; low: N(0,1)=-1.67, p=0.09; high: N(0,1)=-0.19, p=0.85). Thus, rats treated with MDZ did not show evidence of learning across the test sessions. Interestingly, the average opening latency of rats treated with a high dose of MDZ started high and remained high throughout testing whereas the latency of rats treated with the low dose of MDZ tended to be low on the initial days of testing and relatively high on the final days of testing, giving rise to a shallow and non-significant downward trend in latency (p=0.09).

The most pronounced drops in latency occurred during the testing sessions on the middle 5-6 days (Fig. 1A). Therefore, to examine rats' stabilized performance, rather than the learning rate, the average latency recorded during days 10-12 was calculated; this was termed the *learned latency*. The learned latency was different between groups (Fig. 1C; one-way ANOVA;F(4, 75)=3.315, p=0.02). The learned latency of rats treated with a high dose of MDZ was significantly greater than that of uninjected rats (Tukey post hoc, p=0.01) or rats treated with nadolol (Tukey post hoc, p=0.04).

The group averages shown in Figure 1B-C fail to reveal an important within-group variation that resulted from two subpopulations of animals. In each condition, at least six rats, and as many as 10, never consistently opened the restrainer with some of these rats never opening the restrainer at all. Figure 2A reveals the two different subpopulations in each of the five conditions studied. Box plots show the downward trend in the median value as well as the shift of the latency distribution between days 1, 6 and 12 of testing (blue vertical histograms on right). In the high MDZ condition, no shift in opening latency distribution was observed.

Testing for reinforcement

As detailed in the methods, we constructed a model that estimates the probability of sequential day openings (pSO) that would occur in the absence of reinforcement. We then compared observed pSO values to chance values. The distribution of pSO values for all randomly generated matrices are illustrated in Figure 3 along with the two-tailed probability that the observed pSO (red dotted line) came from the null distribution of pSO values.

Several points are evident in comparing the observed and null distributions across groups. First, the probability of reinforced openings predicted by chance was highest (median=0.85) and least variable (10 to 90 percentile range = 0.09) for uninjected rats. Second, for rats injected with the high dose of MDZ, the probability of reinforced openings predicted by chance was relatively low

(median=0.38) and the distribution was broad (10 to 90 percentile range=0.30). Finally we calculated the probability that the probability of observed sequential opening occurred by chance (PpSO). Lower values of PpSO reflect a greater likelihood that the reinforcement did not occur by chance and is a measure of the strength of day-to-day reinforcement. We found that the observed number of reinforced openings was significantly greater than predicted by chance for rats that were either not injected or injected with saline, nadolol, or low MDZ (Fig. 3A-D; see figure for p values). Only in the case of rats injected with high MDZ was the observed pSO less than the median chance occurrence of reinforced opening (Fig. 3E). This was not a significant difference.

Opening streaks

Animals that opened the restrainer were a non-homogeneous group. Differences were observed in the opening patterns that the rats displayed. At one extreme, uninjected rats opened on the day immediately following 78 of 81 openings that occurred on days 1-11 (96%). Furthermore, whenever an uninjected rat opened for two days in a row, he opened on the next (third) day as well (69/69 opportunities). Because of this tendency to repeatedly open the restrainer door, uninjected rats opened for long streaks, including 2 animals that opened on all 12 days of testing (Fig. 4A). At the other extreme, rats treated with the high dose of MDZ opened the restrainer door on two sequential days on only 29% (4/14) of the opportunities and none opened for three days in a row (0/3 opportunities). Rats in the other groups opened for streaks of intermediate lengths (Fig. 4A).

The maximal possible length of an opening streak is greatest when rats open on the first day and declines thereafter (Fig. 4B, gray dotted line). We analyzed the longest streak for each rat and compared the streak length (1-12 days) to the first day of the streak (day 1-12). For uninjected rats, the median first opening occurred on day 3. The median length of the opening streak by uninjected rats was nearly the maximum value of 9 days. In contrast, the opening streaks of rats from all other groups were much shorter than the maximum possible. It is also notable that rats treated with MDZ started streaks earlier (day 1-5) than any other group but still had the shortest streak lengths (1-3 days). Thus the median streak length deviated from the maximal streak length by only 0.5 in the case of uninjected rats but by 9.5-10.5 days in MDZ-treated rats. The maximal streak length of nadolol- and saline-treated rats was less than the maximum possible by 3-5 days.

For rats that opened on at least two consecutive days on days 9-12 (uninjected, n=10; saline, n=7; nadolol, n=8; low MDZ, n=6; high MDZ, n=2), those treated with MDZ were more likely to take at least one break (red x-s, right axis of Fig. 4B) and also took longer breaks on average than rats from the other groups (black columns, left axis of Fig. 4C). This latter difference was significant between the 6 rats in the low MDZ group and 10 rats in the uninjected group that met the criteria for this analysis (one-way ANOVA; $F(4, 28)=3.81$, $p=0.01$).

Blocking distress in free rats tested with a chocolate-containing restrainer

To determine whether the reduction in door-opening observed in MDZ-treated rats could be due to a sedative effect of MDZ, rats were injected with a high (n=8) or low (n=8) dose of MDZ or saline (n=8) prior to testing with a restrainer containing chocolate, a non-social reward. As expected, the opening latency decreased across days (Fig. 5A). The decay in opening latency across days differed between treatment groups ($\chi^2(2)=13.2$; $p=0.001$). Rats treated with either dose of MDZ, but not those treated with saline, showed significantly decreasing opening latencies across the days of testing (saline: $N(0,1)=0.5$, $p=0.62$; low: $N(0,1)=-4.30$, $p<0.001$;

high: N(0,1)=-3.67, p<0.001). On the final 3 days of testing, when latencies had plateaued, the learned latency was significantly different between groups (Fig. 5C; one-way ANOVA;F(2, 21)=3.955, p=0.04). Tukey post hoc tests revealed that the average opening latency in saline-treated rats was greater than in rats injected with the low dose of MDZ (p=0.04). As with saline-injected rats tested with a trapped rat, MDZ-treated rats tested with chocolate showed a shift from longer to shorter opening latencies across the days of testing (Fig. 2B). In contrast, saline-injected rats tested with chocolate did not show a shift in latencies across the days of testing (Fig. 2B).

Comparison of reinforcement in chocolate and trapped conditions

The strength of reinforcement, as reflected by PpSOs, the probability that the probability of observed sequential opening occurred by chance, could not be directly compared between trapped conditions and chocolate conditions due to statistical power differences created by different number of rats studied (trapped conditions N=16; chocolate conditions N=8). To enable a valid comparison, we reduced the statistical power of the trapped models to the power level of chocolate models. This was accomplished with a power-matched bootstrapping of three trapped conditions that share pharmacological manipulations with chocolate conditions (high MDZ, low MDZ, saline). For each trapped condition, we created 100 bootstrapped samples, each containing 8 rats randomly chosen from the 16 rats. Almost a quarter of the samples from the high MDZ - trapped condition (n=23) were removed due to the absence of any openings. Bootstrapped samples were then analyzed with the null model, thereby generating bootstrapped PpSOs (low MDZ N=100; Saline N=100; high MDZ N=77) that represented the strength of reinforcement in the trapped conditions if they had been tested with the same statistical power that chocolate conditions had.

Since each bootstrapped PpSO has the same power as the PpSOs in chocolate conditions, we can compare strength of reinforcement between chocolate conditions and trapped conditions by comparing the PpSOs of a chocolate condition to the distribution of PpSOs of bootstrapped samples of the corresponding trapped condition (Fig. 6). Medians of the bootstrapped PpSOs (dashed line, saline: 0.08; low MDZ: 0.08; high MDZ: 0.85) were higher than the original PpSOs (cross, 0.01, 0.01, 0.40), reflecting reduced statistical power.

The PpSO of the high MDZ chocolate group (red marker, 0.26) is significantly lower than the bootstrapped PpSOs of high MDZ-trapped (77 out of 77 bootstrapped values are higher than 0.26, p<0.01), reflecting that high MDZ-treated rats were more reinforced when chocolate, rather than a trapped rat, was in the restrainer. The PpSO of the low MDZ-treated rats tested with chocolate (red marker, 0.10) is higher, but not significantly so, than the bootstrapped PpSOs of low MDZ rats tested with a trapped rat (43/100 of bootstrapped values were lower than 0.10, p=0.43). This reflects a roughly equal strength of reinforcement between chocolate and a trapped rat for free rats treated with low MDZ. Finally, the PpSO of saline-treated rats tested with chocolate (0.29) is significantly higher than bootstrapped PpSOs from saline-treated rats tested with a trapped rat (9/100 were higher than 0.29, p=0.09), reflecting greater reinforcement by a trapped rat than by chocolate.

In sum, high MDZ treatment renders chocolate more reinforcing than a trapped rat whereas saline treatment renders the trapped rat more reinforcing than chocolate.

Corticosterone responses to the helping behavior test

To further examine the biological mechanisms involved in helping behavior, corticosterone (CORT) levels were measured in rats exposed to a trapped cagemate. CORT is an index of hypohalamic-pituitary-adrenal (HPA) axis involvement. In this experiment, CORT levels were measured following the initial exposure to a trapped rat. CORT responses were calculated by subtracting a pre-session baseline from a measurement taken immediately after testing (see Methods). On this day only, the restrainer door was secured shut, ensuring that all free rats were exposed to the trapped rat for the full 40 minute duration. After the initial exposure to a trapped rat used to collect CORT, rats were tested in the standard paradigm described above for 12 days.

To test the relationship between CORT and helping behavior, a regression was performed between the average opening latency across the 12 days of standard testing and individual CORT responses. The CORT response of free rats was significantly correlated to the average opening latency across the 12 sessions (r^2=0.52; F(1, 15)=16.54, p<0.001; Fig 7A). In contrast, no significant correlation existed between the CORT response of the trapped rats and the average opening latency (r^2=-0.04; F(1,18)=0.69, p=0.42; Fig. 7B). Thus, a stronger HPA activation response is detrimental to successful helping and individuals with less HPA reactivity to a trapped rat are better helpers.

Discussion

This study demonstrates that rodent pro-social actions require affective processing that is blocked by the benzodiazepine anxiolytic MDZ. Although rats treated with MDZ did not open a restrainer to release their trapped cagemate, they did open a restrainer to access chocolate. Thus, the reduction in pro-social behavior produced in MDZ-treated rats was not due to a sedative, cognitive, or motor effect. Instead, the MDZ interfered specifically with the social affective processing that appears necessary to motivate the free rat to help a trapped rat. In humans, affective communication from one individual to another fuels an empathic understanding and pro-social actions. We hypothesize that the situation is similar in the simple helping situation presented to rats in the current experiments. Specifically the free rat needs to resonate with the trapped rat's affect in order to motivate his own pro-social actions.

MDZ modulates relative motivational value of helping trapped rat or accessing chocolate

Results from our power-matched bootstrapping analysis (see Results) show that MDZ treatment reduces the motivational value of helping a trapped cagemate. Yet, MDZ increased the reinforcement effect of chocolate. Rats treated with a high dose of MDZ opened the restrainer to access chocolate but not to liberate a trapped rat. Therefore, MDZ treatment diminishes the value of helping a trapped conspecific. Compared to MDZ, saline treatment had the opposite effect on the motivational values of helping and chocolate. Saline-treated rats were more likely to open for trapped rats than for chocolate. Saline-treated rats' lack of interest in chocolate is likely due to the stress induced by the injection procedure. Yet they were able to release a trapped cagemate. Social buffering decreases anxiety and rats' tendency for thigmotaxis (Kikusui et al., 2006). Therefore it is possible that saline-treated rats were less anxious when tested with a trapped rat, because of social buffering, than when placed alone in the arena with a restrainer containing chocolate. In light of the effect of social buffering on saline-treated rats, it is remarkable that no evidence of social buffering was observed in the behavior of MDZ-treated rats tested with a trapped cagemate. The ability of saline-treated rats to overcome their anxiety and help the

trapped rat demonstrates their degree of motivation, and puts into stark relief the complete lack of motivation observed in the non-stressed MDZ-treated rats to release their conspecifics.

Venturing into the arena center, a behavior rats typically prefer to avoid, crucially depends on a strong motivation to approach the restrainer. In MDZ-treated rats, chocolate provides such a motivation. However a trapped rat comprises a motivating force only for free rats treated with saline, but not for MDZ-treated ones. The most parsimonious explanation for these results is that the source of motivation in the trapped rat paradigm is the affect evoked in the free rat. And that motivating affect is sensitive to MDZ.

MDZ blocks helping through central actions

MDZ acts within the central nervous system to produce anxiolysis, which itself has a secondary, peripheral consequence of reducing sympathoexcitation. The peripherally acting beta-adrenergic blocker, nadolol, is capable of blocking sympathetic activation but does not cross the blood-brain barrier and therefore leaves central affective circuits unaltered. Nadolol treatment had no effect on helping behavior, resembling a saline injection in all respects. This result suggests that MDZ produces its effects through an antagonism of central affective circuits and not exclusively or primarily through a sympatholytic effect.

Social interaction is not the motivation for helping

The failure of MDZ-treated rats to release a trapped cagemate is further evidence that rats are motivated by negative affect rather than by a desire for social interaction as has been recently argued (Silberberg et al., 2014). Animals motivated primarily by a desire for social interaction would have opened a restrainer containing a trapped rat just as they opened a restrainer to access chocolate. However, this did not happen. Therefore it appears that rats open only for a rat in distress and only when they are capable of mounting an affective response. This idea is in line with previous results from our laboratory showing that rats repeatedly release cagemates even when subsequent social contact is prevented (Ben-Ami Bartal et al., 2011). Moreover rats that open a door to help a rat in distress do not do so to socially access a rat that is not in distress (Sato et al., 2015). Thus, a desire for social contact is neither necessary nor sufficient to motivate door-opening.

HPA reactivity is inversely correlated with helping behavior

We found that rats that responded to a trapped cagemate with a high CORT response, a measure of HPA reactivity or physiological stress, were less likely to develop consistent helping behavior than were rats that showed lower levels of physiological stress upon exposure to a trapped rat. It thus appears that HPA reactivity on the part of the free rat is detrimental to other-oriented actions. These results parallel findings in humans that personal distress has “egoistic” consequences, which oppose the expression of other-oriented empathy (Batson et al., 1987). Additional measures of HPA reactivity such as allogrooming are associated with a reduced propensity for pro-social behavior in chimps (Clay and de Waal, 2013). In humans, individuals with the short allele polymorphism of the serotonin transporter gene regulatory region (5-HTTLPR) have higher HPA reactivity (Gotlib et al., 2008) and lower pro-social tendencies (Stoltenberg et al., 2013). Physiological stress as measured by HPA reactivity therefore appears to antagonize helping, rendering individuals “afraid to help” in the words of Stoltenberg and colleagues (2013).

It may appear paradoxical that blocking anxiety through MDZ treatment prevents rats from helping whereas low HPA reactivity appears to allow or possibly promote helping. However HPA reactivity is a construct that is independent of the MDZ-sensitive affective state of anxiety (Lundberg and Frankenhaeuser 1980). As discussed above, the affective state of anxiety promotes helping and its blockade by MDZ precludes helping, regardless of the level of HPA activity. MDZ antagonizes the affective state of anxiety but does not directly antagonize HPA activity (Broadbear et al., 2004). Rats treated with MDZ do not develop helping because they are prevented from expressing the affect associated with anxiety. Regardless of their HPA reactivity, then, MDZ-treated rats would not help because they lack the required affect. In contrast, depressing HPA reactivity without affecting affective anxiety would be expected to promote helping. Indeed administration of a glucorticoid synthesis inhibitor extends empathic responses to strangers in mice and humans (Martin et al., 2015).

A novel method for evaluating reinforcement

The method introduced here to quantify day-to-day reinforcement can be adapted for use in many experimental conditions. The prerequisites are a binary choice and sequential testing. The advantage to this method is that it is able to test whether the outcome of a previous decision positively or negatively reinforces the decision while removing confounding effects created by non-associative learning that takes place across sessions. It therefore tests the strength of reinforcement against a parsimonious null hypothesis and quantitatively represents the strength of reinforcement.

Conclusion

In conclusion, this series of experiments clearly demonstrates the fundamental role of affect in motivating pro-social behavior in rodents. The helping behavior shown by rats in the present study is not a conditioned response motivated by either approach to a positive reward or avoidance of a negative cue. Instead, the motivation to help requires the helper to resonate with the affect of the victim. Pharmacological elimination of the affective state of anxiety in the potential helper prevented rats from helping. In addition, rats with high trait HPA reactivity did not develop a consistent pattern of helping. Thus, rodent helping appears to require both affective resonance and the ability to dampen HPA reactivity.

Conflict of interest statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author and contributors:

Trapped rat experiments: design (IB, PM, JD), data acquisition (IB, HS, NM, TM, PM); Chocolate experiments: design (IB, PM), data acquisition (IB, JW); Corticosterone experiments: design (IB, PM), data acquisition (IB, PM); Data analysis & statistics: (IB, HS, NM, TM, PM); Reinforcement model: (HS, PM); Drafting and revising manuscript: (IB, HS, NM, TM, JW, JD, PM).

Funding: Funds from the Pritzker Medical School and the Biological Sciences Division supported this work.

Acknowledgements:

The assistance of Miguel Barajas, Isabel Boni, Tony Logli, Maria Sol Bernardez-Sarria, Katie Ragsdale, David Rodgers, Yuri Sugano, and Jenny Wang, is gratefully acknowledged. We'd like to thank Dr. David White and Fanny Delebeque for their help with acquisition of corticosterone measurements. The authors are indebted to Peter McCullagh for patient and expert advice on crafting and coding the general linear models used.

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Table 1. Opening latencies declined across days. The decay of opening latency in each condition was compared to the decay observed for every other condition. P-values for these pair-wise comparisons are displayed (see Supplement for code).

	Uninjected	Saline	Nadolol	Low MDZ
Uninjected				
Saline	0.58			
Nadolol	0.73	0.83		
Low MDZ	0.06	0.18	0.12	
High MDZ	<0.01 *	0.02 *	<0.01 *	0.29

Figure 1

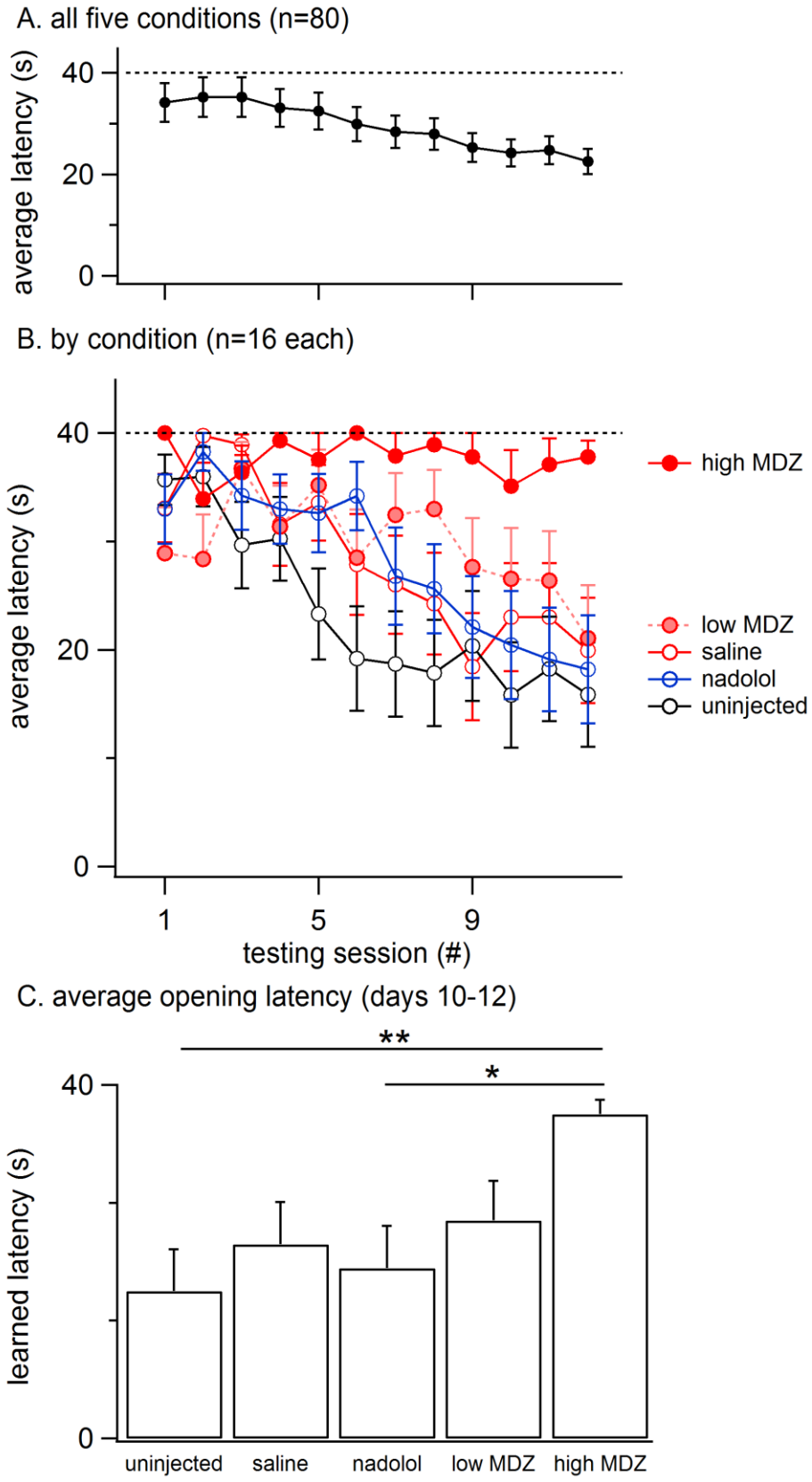

Figure 1. A: The mean (± SEM) latency to door-opening for all rats (n=80) decreased across the 12 days of testing, suggestive of learning. B: The decay in door-opening latency across testing sessions differed between the groups of rats tested (n=16 per group). C: The average opening latency during the final 3 days of testing, when latencies had plateaued, was significantly greater for rats treated with a high dose of MDZ than for rats that received no injection (**, p=0.01) or an injection of nadolol (*, p=0.04).

Figure 2

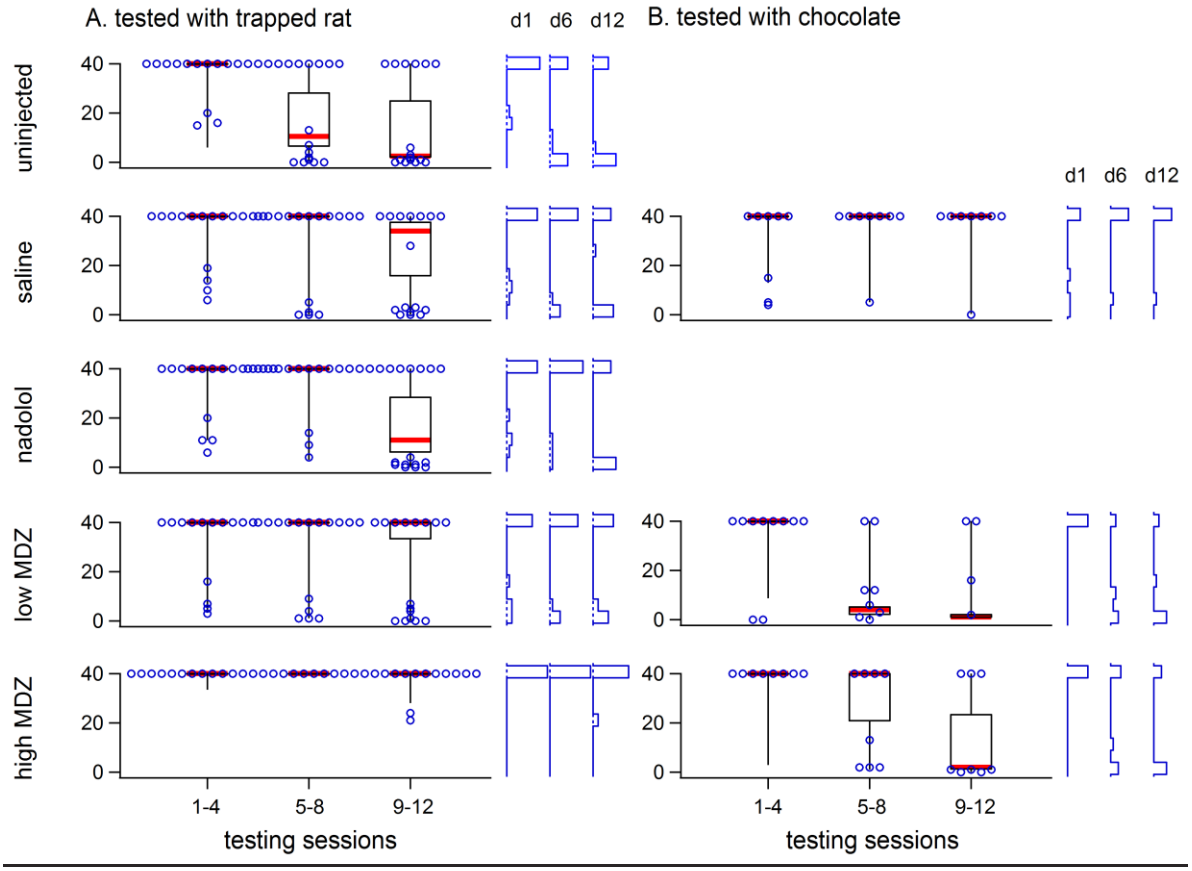

Figure 2. A: The variability of opening latency within groups is illustrated using box plots (40, 50, 60 percentile lines with the median marked in red, 10 and 90 percentile whiskers) showing latencies across the 12 days of testing. All individual latencies are ilustrated for days 1, 6, and 12 (hollow blue circles). Frequency histograms of latencies on those days are shown at the right for each group. In all groups except the high MDZ rats tested with a trapped rat and saline rats tested with chocolate, there was a shift from long to short latencies.

Figure 3

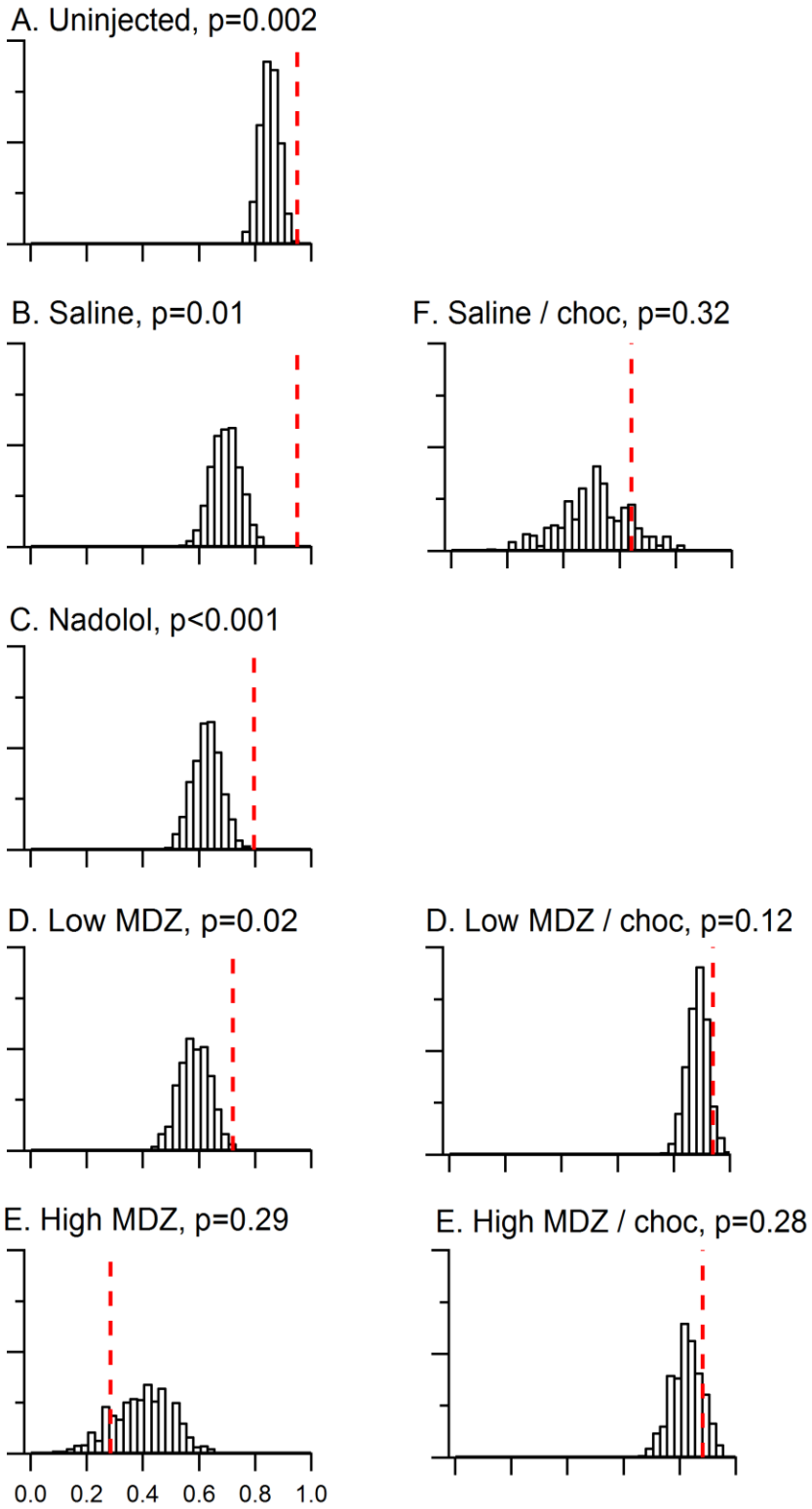

Figure 3. Day to day reinforcement occurred with a probability that was greater than chance for all groups except rats injected with the high dose of MDZ. A model that took into account the effects of learning and individual differences was constructed. The chance distribution of pSO (probability of sequential openings) values from 10,000 matrices is shown in histogram form for each group tested with a trapped rat. The observed pSO is marked by the red dotted line in each panel and the two-tailed probability of the observed pSO occuring by chance listed. For uninjected rats or rats injected with saline, nadolol or the low dose of MDZ, the observed pSO was significantly greater than would be expected by chance. However in the case of rats injected with the high dose of MDZ, the observed pSO was less than 85% of the chance pSO values.

Figure 4

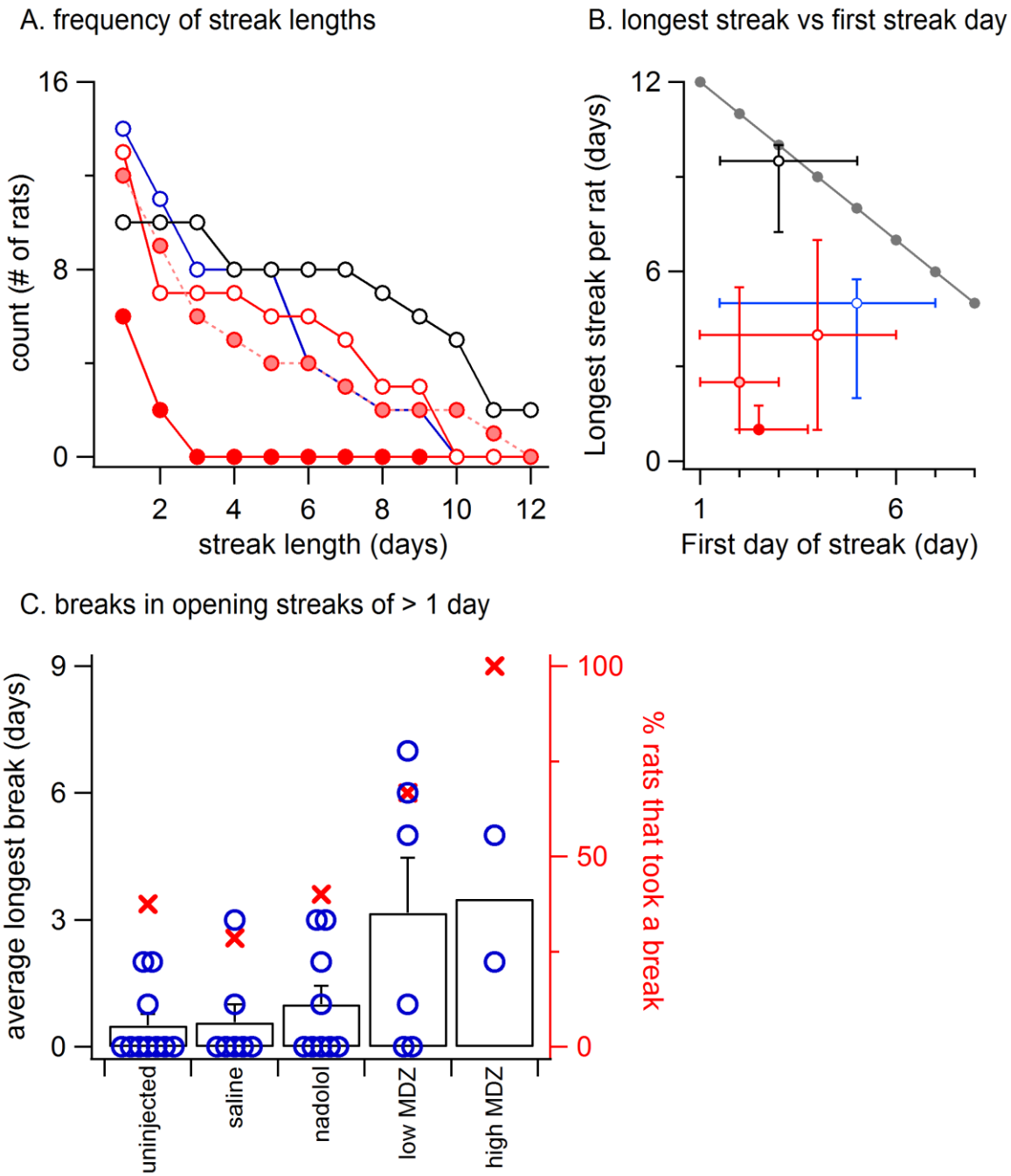

Figure 4. A: The frequency of opening streaks (consecutive day openings) of different lengths is illustrated for streaks of lengths from 2 to 12 days. At the left is the number of rats that opened at least once. B: The median length of the longest streak (± 25 and 75 percentiles) is graphed as a function of the median testing day (± 25 and 75 percentiles) on which the streak began. The gray line at the top shows the optimal possible performance (e.g. rats that began opening on day 1

could achieve a streak of 12 days). C: The failure of a rat to open for one or more days is termed a "break." An analysis of breaks for rats that opened on at least two consecutive days on days 9-12 (uninjected, n=10; saline, n=7; nadolol, n=8; low MDZ, n=6; high MDZ, n=2) shows that rats treated with MDZ were more likely to take at least one break (filled red circles, right axis). Rats treated with MDZ also took longer breaks on average than did rats from the other groups (black columns, left axis). The individual points for all rats considered in this analysis are illustrated by the hollow blue circles.

Figure 5

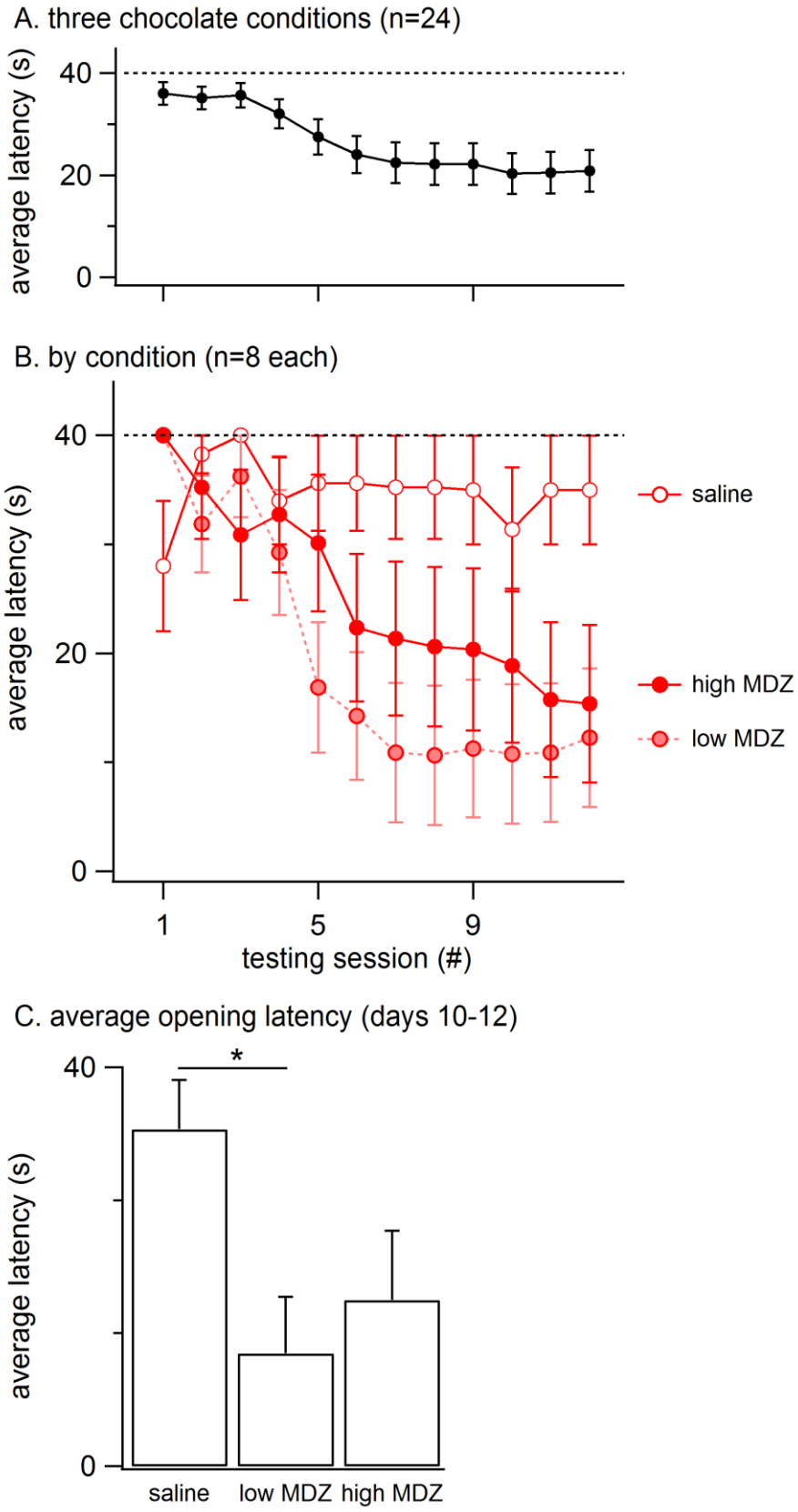

Figure 5. A: The mean (± SEM) latency to door-opening for all rats (n=24) decreased across the 12 days of testing, suggestive of learning. B: The decay in door-opening latency across testing sessions differed between the groups of rats tested (n=8 per group). C: The average opening latency during the final three days of testing, at a time when latencies had stabilized, was significantly less for rats treated with a high dose of MDZ than for rats that received saline (*, p=0.04).

Figure 6

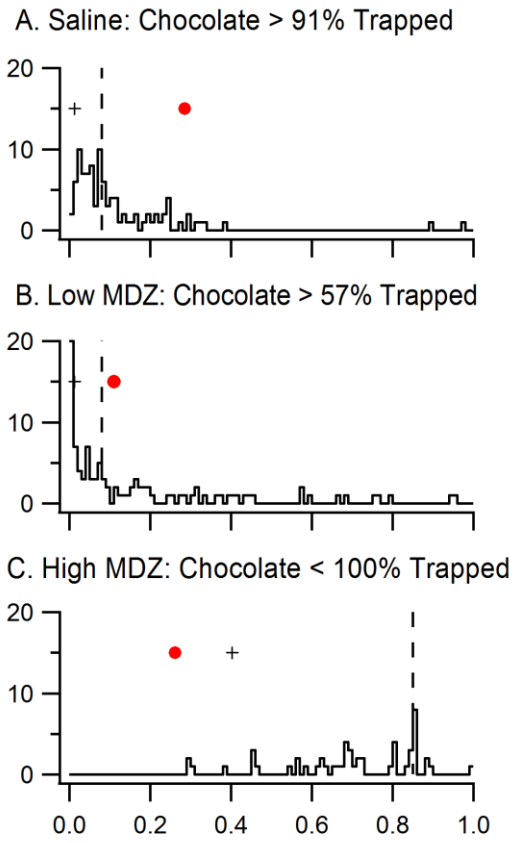

Figure 6. Data from rats tested with a trapped rat and injected with saline, low MDZ, or high MDZ was resampled to match the statistical power of chocolate conditions. The distributions of PpSOs from 100 bootstrapped samples from each trapped rat condition are shown in the histograms. The medians of these bootstrapped PpSOs (dashed line) are higher than the orginial PpSOs (crosses) from the complete data set of trapped rat conditions, showing reduction in statistical power. In this way the PpSO of rats tested with chocolate (red dots) can be compared to that of rats tested with a trapped rat.

Figure 7

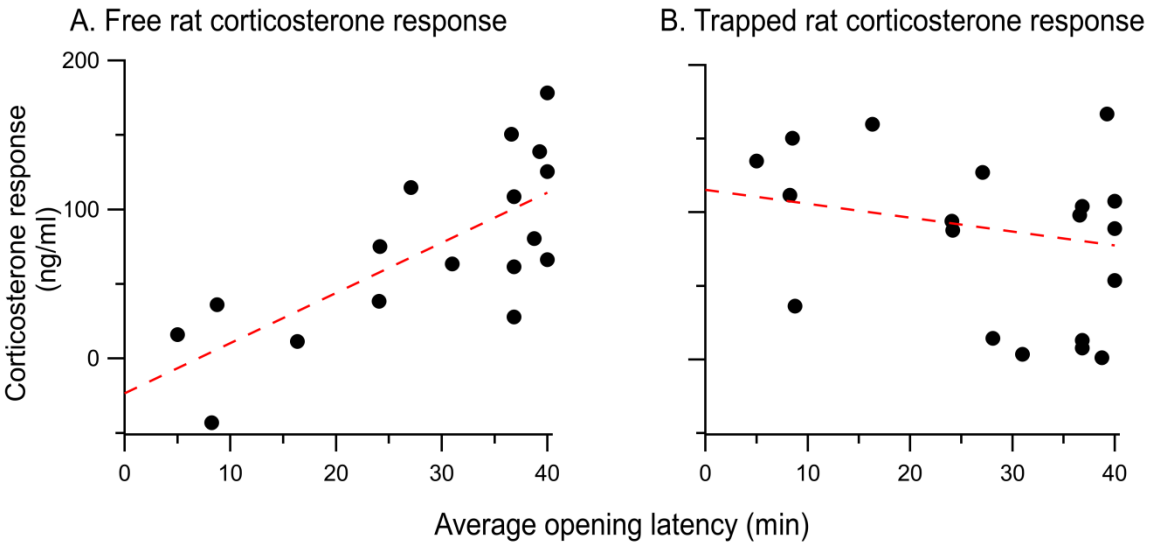

Figure 7. A: The corticosterone response of each free rat evoked by a trapped rat is positively correlated with the average opening latency over the 12 days of testing. behavior. B: There was no correlation between the the trapped rat's corticosterone response and the ensuing opening behavior of the free rat.

Supplementary material

A. R code for general linear model for the 5 groups tested with a trapped rat
B. The original latency data for all rats tested with a trapped rat in comma separated value format
C. Matlab code for the null model testing for day-to-day reinforcement
D. R code for general linear model for the 3 groups tested with a chocolate-containing restrainer
E. The original latency data for all rats tested with a chocolate-containing restrainer in comma separated value format

Supplement A: R code for general linear model

Numbers in text are given for trapped rat condition. Numbers for chocolate condition are provided in the comments.

```
data <- read.csv("data.csv", header=TRUE) #read the data file
time <- c(data$D1, data$D2, data$D3, data$D4, data$D5, data$D6, data$D7, data$D8, data$D9,
      data$D10, data$D11, data$D12) #latencies from day1(D1) to day12(D12) are read
w <- !is.na(time);  ndays <- 12;  nrats <- 80 #two data points are missing, for chocolate groups,
      nrats=24
y <- time[w]
drug <- relevel(as.factor(rep(data[,1], ndays))[w], ref="uninjected") #treatments are read as
      factors (i.e. nominal)
rat <- as.factor(rep(data[,2], ndays))[w] #individual rat IDs are read as factors (i.e. nominal)
day <- rep(1:ndays, rep(nrats, ndays))[w] #days are read ordinally
d <- abs(outer(day, day, "-")) #the intervals between each pair of days are calculated
c(length(y), length(drug), length(rat), length(day), length(fday), dim(d)) #check the lengths of all
      the vectors
gammahat <- 0.116   #The uniform correlation coefficient. It is estimated by codes below under
      "Gamma evaluation" gammahat for chocolate groups is 0.13
Vrat <- outer(rat, rat, "==")  * exp(-gammahat *d) #The matrix Vrat is produced with an
      exponential function

fit <- regress(y~day*drug, ~rat+Vrat, start=c(1,1,1), pos=c(1,1,1)) #data is fitted to the general
      linear model.
summary(fit) #result of fitting is displayed
ndlevs <- length(levels(drug)) #number of levels in treatment is read

K <- model.matrix(~day+drug)
fit0 <- regress(y~day+drug, ~rat+Vrat, start=c(1,0.1,1), pos=c(1,1,1))   # null model with no
      interaction
fit1 <- regress(y~day*drug, ~rat+Vrat, start=c(1,0.1,1), pos=c(1,1,1), kernel=K)  # essentially the
      same as fit, but with kernel K
X2 <- 2*(fit1$llik - fit0$llik) #difference between the two models is plugged into a chi-square
      distribution
1 - pchisq(X2, df=ndlevs-1) #calculate p-value for interaction

###### Gamma evaluation
gamma <- seq(0.15, 0.08, -0.01) #gamma should be between 0 and 1. Previous testing showed
      that it should fall within 0.08-0.15.
llik <- matrix(0, length(gamma), 4) #the same model with different gamma values is reiterated
      and the likelihood of different models are compared
for(i in 1:length(gamma)){
  Vrat <- outer(rat, rat, "==") * exp(-gamma[i]*d)
  fit1 <- regress(y~fday*drug, ~rat+Vrat, start=c(1,1,1), pos=c(1,1,1))
  llik[i,] <- c(fit1$llik, fit1$sigma)
}
```

```
plot(gamma, llik[,1], cex=0.5) #maximum likelihood of the model at different values of gamma
        are plotted, and the gamma value that results in the largest maximum likelihood is chosen
```

Supplement B. The original latency data for all rats tested with a trapped rat in comma separated value format

drug,rat,D1,D2,D3,D4,D5,D6,D7,D8,D9,D10,D11,D12
saline,R1,40,40,40,40,40,40,40,40,40,40,40,40
saline,R2,40,40,25,12,2,0,0,1,0,0,0,0
saline,R3,19,40,40,40,40,0,4,0,0,0,0,2
saline,R4,10,40,40,40,40,40,40,40,40,40,40,40
saline,R5,6,40,40,40,40,40,40,40,4,40,40,3
saline,R6,14,36,38,2,4,0,0,0,0,0,0,0
saline,R7,40,40,40,40,40,40,40,40,4,40,40,40
saline,R8,40,40,40,40,40,40,35,40,0,4,4,3
saline,R9,40,40,40,40,40,40,6,11,7,2,0,1
saline,R10,40,40,40,6,40,5,10,1,0,2,4,2
saline,R11,40,40,40,40,40,40,40,40,40,40,40,40
saline,R12,40,40,40,40,40,40,40,40,40,40,40,28
saline,R13,40,40,40,5,11,1,1,1,0,0,0,0
saline,R14,40,40,40,40,40,40,40,14,40,40,40,40
saline,R15,40,40,40,40,40,40,40,40,40,40,40,40
saline,R16,40,40,40,40,40,40,40,40,40,40,40,40
uninjected,R17,40,40,40,40,40,40,40,40,40,40,40,40
uninjected,R18,40,40,40,40,6,0,4,0,2,2,0,0
uninjected,R19,40,40,40,40,40,40,40,40,40,40,40,40
uninjected,R20,40,40,40,40,15,40,40,4,40,2,40,6
uninjected,R21,15,12,3,1,1,0,0,1,1,1,0,1
uninjected,R22,40,40,40,40,40,7,5,2,1,0,2,3
uninjected,R23,40,40,15,5,5,0,1,0,0,0,0,1
uninjected,R24,40,40,40,40,40,40,40,40,40,40,40,40
uninjected,R25,40,40,7,25,33,2,2,0,0,0,0,0
uninjected,R26,16,40,40,36,17,0,1,35,2,1,0,0
uninjected,R27,40,40,40,40,4,13,3,3,40,5,33,1
uninjected,R28,40,40,8,1,8,4,0,1,0,2,3,2
uninjected,R29,40,40,40,40,40,40,40,40,40,40,40,40
uninjected,R30,40,40,40,40,40,40,40,40,40,40,40,40
uninjected,R31,20,4,2,16,4,1,3,0,0,0,1,0
uninjected,R32,40,40,40,40,40,40,40,40,40,40,40,40
lowMDZ,R33,40,40,39,18,40,1,1,1,0,1,2,4
lowMDZ,R34,40,40,40,40,40,4,40,40,40,40,40,40
lowMDZ,R35,3,40,40,40,40,40,40,40,29,40,40,7
lowMDZ,R36,3,22,40,6,40,40,40,40,40,40,40,40
lowMDZ,R37,7,5,40,40,40,40,40,40,40,40,40,40
lowMDZ,R38,16,40,40,40,40,40,40,34,2,2,1,0
lowMDZ,R39,40,40,40,40,40,40,40,40,40,40,40,40
lowMDZ,R40,,40,40,40,40,40,40,40,40,40,40,40
lowMDZ,R41,40,40,40,40,40,40,40,40,40,40,40,40
lowMDZ,R42,5,4,40,40,40,40,40,40,40,18,26,1
lowMDZ,R43,40,40,39,3,3,1,1,13,0,0,0,0

lowMDZ,R44,40,14,28,40,40,40,40,40,9,0,1,0
lowMDZ,R45,40,40,40,40,40,40,40,40,40,40,40,40
lowMDZ,R46,40,2,2,0,0,1,2,0,0,1,0,0
lowMDZ,R47,40,40,40,40,40,9,40,40,40,40,40,5
lowMDZ,R48,40,5,40,35,40,40,35,40,40,40,32,40
highMDZ,R49,40,40,40,40,40,40,40,40,40,40,40,40
highMDZ,R50,40,,40,40,40,40,40,40,40,40,40,40
highMDZ,R51,40,6,40,40,40,40,40,40,40,40,40,40
highMDZ,R52,40,40,40,40,40,40,40,40,40,40,40,40
highMDZ,R53,40,40,40,40,40,40,40,40,40,40,40,40
highMDZ,R54,40,40,40,40,40,40,6,40,40,40,40,24
highMDZ,R55,40,40,40,40,40,40,40,40,40,40,40,40
highMDZ,R56,40,19,15,40,1,40,40,23,5,40,2,21
highMDZ,R57,40,40,40,40,40,40,40,40,40,40,40,40
highMDZ,R58,40,40,40,40,40,40,40,40,40,40,40,40
highMDZ,R59,40,4,40,40,40,40,40,40,40,2,40,40
highMDZ,R60,40,40,7,40,40,40,40,40,40,40,40,40
highMDZ,R61,40,40,40,40,40,40,40,40,40,40,40,40
highMDZ,R62,40,40,40,29,40,40,40,40,40,0,32,40
highMDZ,R63,40,40,40,40,40,40,40,40,40,40,40,40
highMDZ,R64,40,40,40,40,40,40,40,40,40,40,40,40
nadalol,R81,40,40,40,40,40,40,40,30,15,5,6,2
nadalol,R82,40,40,40,40,40,40,40,8,1,3,3,0
nadalol,R83,20,40,18,8,40,40,40,40,40,37,40,40
nadalol,R84,40,40,40,40,40,40,40,10,3,0,0,1
nadalol,R85,40,40,40,40,40,9,4,5,3,1,1,0
nadalol,R86,40,40,40,40,30,40,7,10,1,0,1,4
nadalol,R87,11,12,40,40,40,40,40,40,40,40,40,40
nadalol,R88,40,40,40,40,2,40,40,40,40,40,40,40
nadalol,R89,40,40,4,40,40,40,40,40,40,40,40,40
nadalol,R90,40,40,40,40,40,40,15,40,40,40,11,0
nadalol,R91,40,40,40,40,40,40,40,40,40,40,40,40
nadalol,R92,6,40,6,12,40,40,40,23,11,0,3,1
nadalol,R93,40,40,40,7,9,4,0,0,0,1,1,2
nadalol,R94,11,40,40,21,1,14,2,4,0,0,0,1
nadalol,R95,40,40,40,40,40,40,40,40,40,40,40,40
nadalol,R96,40,40,40,40,40,40,1,40,40,40,40,40

Supplement C. Matlab code for the null model testing for day-to-day reinforcement

```
ratnumber=16; %Define size of data matrices
iteration=10000; %Define number of iterations of null simulations
distpN11=zeros(1,iteration); %Create storage vector for probability of sequential opening from
        each simulation

indv=zeros(1,ratnumber); %Create storage vector for individual differences coefficients
daye=zeros(1,12); %Create storage vector for day-to-day differences coefficients

%Calculate differences coefficients for the data
for m=1:12
   daye(m)=sum(data(:,m))/sum(sum(data));
end
for n=1:ratnumber
   indv(n)=sum(data(n,:))/sum(sum(data));
end

%Iterations
for i=1:iteration
   null=zeros(ratnumber,12); %Generate a null matrix that matches the data matrix
   c11=0; c1deno=0; %Reset counters of sequential openings and openings
   for m=1:12 %Generation of binary simulated observations
      for n=1:ratnumber
         np=rand;
         if np>=daye(m)*indv(n)*sum(sum(data))
            null(n,m)=0;
         else null(n,m)=1;
         end
      end
   end

   for m=1:11 %Count sequential openings and total openings
      for n=1:ratnumber
         if null(n,m)==1
            c1deno=c1deno+1;
            if null(n,m+1)==1,
               c11=c11+1;
            end
         end
      end
   end

   pN11=c11/c1deno; %Calculate probability of sequential opening
   distpN11(i)=pN11; %Store probability of sequential opening from this iteration
end
```

```
%%
cd11=0;c1ddeno=0; %Reset sequential opening and total opening counters
for m=1:11 %Calculate probability of sequential opening for the data
   for n=1:ratnumber
      if data(n,m)==1
         c1ddeno=c1ddeno+1;
         if data(n,m+1)==1,
            cd11=cd11+1;
         end
      end
   end
end

pD11=cd11/c1ddeno; %Calculate probability of sequential opening
%%
display(pD11)
display(mean(distpN11))
ExN11=0; %Reset extreme simulation counter

for i=1:iteration %Calculate number of simulations that are as extreme as the observation (two-
         tailed)
   if abs(distpN11(i)-mean(distpN11))>=abs(pD11-mean(distpN11))
      ExN11=ExN11+1;
   end
end

pvalue11=ExN11/iteration; %Calculate p-value
display(pvalue11)
```

Supplement D: Matlab code for power-matching bootstrapping analysis

```
rawtestiteration=100; %number of bootstrapped samples
pvalue=zeros(1,rawtestiteration); %Storage for ppSOs from bootstrapped analysis
vec=1:1:16;

data=zeros(8,12); % Analogous as Supplement C
for a=1:rawtestiteration
   index=datasample(vec,8);
   for b=1:8
      data(b,:)=raw(index(b),:);
   end
   ratnumber=8;
   iteration=5000;
   distpN11=zeros(1,iteration);

   indv=zeros(1,ratnumber);
   daye=zeros(1,12);

   for i=1:iteration
      null=zeros(ratnumber,12);
      c11=0; c1deno=0; c0deno=0;
      cd11=0;c1ddeno=0; c0ddeno=0;
      for m=1:12 % Individual Differences
         daye(m)=sum(data(:,m))/sum(sum(data));
         for n=1:ratnumber
            indv(n)=sum(data(n,:))/sum(sum(data));
            np=rand;
            if np>=daye(m)*indv(n)*sum(sum(data))
               null(n,m)=0;
            else null(n,m)=1;
            end
         end
      end
      for m=1:11
         for n=1:ratnumber
            if null(n,m)==1
               c1deno=c1deno+1;
               if null(n,m+1)==1,
                  c11=c11+1;
               end
            end
         end
      end
      pN11=c11/c1deno;
      distpN11(i)=pN11;
```

```
    end


    %%
    for m=1:11
        for n=1:ratnumber
            if data(n,m)==1
                c1ddeno=c1ddeno+1;
                if data(n,m+1)==1,
                    cd11=cd11+1;
                end
        end
        end
    end
    pD11=cd11/c1ddeno;

    %%
    ExN11=0;
    for i=1:iteration
        if abs(distpN11(i)-mean(distpN11))>=abs(pD11-mean(distpN11))
            ExN11=ExN11+1;
        end
    end
    pvalue(a)=ExN11/iteration;
end
```

Supplement E. The original latency data for all rats tested with a chocolate-containing restrainer in comma separated value format

drug,rat,D1,D2,D3,D4,D5,D6,D7,D8,D9,D10,D11,D12
LowMDZ,R1,40,18,10,1,1,1,0,1,5,3,0,0
LowMDZ,R2,40,40,40,40,40,40,40,40,40,40,40,16
LowMDZ,R3,40,40,40,6,6,3,0,1,5,0,1,0
LowMDZ,R4,40,29,40,40,5,12,0,0,0,0,0,0
LowMDZ,R5,40,40,40,40,30,12,5,1,0,1,1,40
LowMDZ,R6,40,40,40,40,40,40,40,40,40,40,40,40
LowMDZ,R7,40,8,40,36,11,6,1,1,0,1,4,0
LowMDZ,R8,40,40,40,31,2,0,1,1,0,1,1,2
HighMDZ,R9,40,40,40,40,40,40,40,40,40,40,40,40
HighMDZ,R10,40,40,40,40,38,2,1,1,1,0,1,0
HighMDZ,R11,40,40,40,40,40,40,40,40,40,40,40,40
HighMDZ,R12,40,40,40,40,40,40,40,40,40,29,2,1
HighMDZ,R13,40,40,40,0,40,40,40,40,40,40,40,40
HighMDZ,R14,40,2,2,40,0,2,1,1,0,0,0,0
HighMDZ,R15,40,40,5,42,3,2,2,1,2,1,2,1
HighMDZ,R16,40,40,40,20,40,13,7,2,0,1,1,1
Saline,R17,5,40,40,40,40,40,40,40,40,40,40,40
Saline,R18,4,40,40,40,40,40,40,40,40,40,40,40
Saline,R19,40,40,40,40,40,40,40,40,40,40,40,40
Saline,R20,40,40,40,40,40,40,40,40,40,40,40,40
Saline,R21,40,40,40,40,40,40,40,40,40,40,40,40
Saline,R22,40,40,40,40,40,40,40,40,40,40,40,40
Saline,R23,40,40,40,12,40,40,40,40,40,10,40,40
Saline,R24,15,26,40,20,5,5,2,2,0,1,0,0