Punishment Risk Task: Monitoring anxiogenic states during goal directed actions in mice

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Summary/Abstract: Canonical preclinical studies of anxiety-related behavioral states have been critical to developing mechanistic insights and therapeutics for neuropsychiatric disorders. Most of these assays use exploration of novel spaces to test approach-avoidance conflicts such as the open field test, elevated plus maze, and light-dark box. However, these assays cannot evaluate complicated behaviors in which competing states of motivation result in anxiogenic behaviors. Furthermore, these assays can only test the approach-avoidance conflict once due a reliance on spatial novelty. Here we demonstrate the punishment risk task in male and female, group- and singly-housed mice, a model initially described in singly-housed male rats by Park and Moghaddam (2017). The task tests how probabilistic punishment affects reward-seeking behavior. In particular, it measures the delay to pursue a reward while the likelihood of punishment actively impinges reward-associated actions. Elucidating how these competitive behavioral states are integral to adaptive behavior and change over time and experience to coordinate anxiogenesis should greatly benefit anxiety disorder research. Specifically, implementing this assay in mice will enable cell-type selective interrogation of these processes and further our understanding of the neural basis of anxiogenesis.

Introduction: Animal models are key to understanding the etiology and neurobiology of neuropsychiatric disorders such as anxiety and depression. Unfortunately, the heterogeneity of anxiety disorders in humans combined with their complex diagnostic criteria produces obstacles for modeling of clinical conditions in animals[1,2]. In an effort to model human pathological anxiety in rodents, an extensive range of behavioral models have been established[3–10]. Many of these behavioral models use simplistic approaches designed to evaluate animals' natural responses to aversive stimuli or contrast their propensity to approach or avoid exploratory behavior or social interaction against the aversive properties of novel open, brightly lit, or elevated spaces (i.e., open-field, light-dark box, and elevated zero/plus mazes). These novelty-based physical exploration assays that exploit this approach-avoidance conflict limit assessment of more complex behavioral states in which competing motivations can impede normal functioning. Parsing how these more explicitly competing behavioral states are integral to adaptive behavior, predict susceptibility to stressors, and coordinate anxiogenesis will greatly benefit anxiety disorder research.

Recently, a rodent behavioral model initially described by Park and Moghaddam (2017)[11] assessed reward-guided actions in the absence or presence of probabilistic punishment. The task was designed so that an instrumental action (nosepoke) always produces a reward (sugar pellet), but the same action probabilistically leads to punishment (electrical foot shock) with varying degrees of contingency in a block-wise manner. In the task, different blocks have increasing probability of punishment associated with the action while the action-reward contingency remains constant (i.e., fixed-ratio 1). In this manner, animals display increased latency to perform the rewarded actions as the probability of punishment increases. This exacerbated punishment-induced latency suggests a transient state of hesitancy that dramatically affects performance during the session, particularly within the final block which has the highest probability of punishment. The authors confirmed that diazepam, a well-established anxiolytic, significantly diminished this latency in within-subject trials compared to saline-treated sessions, demonstrating the predictive validity of the task as a model to induce and examine anxiogenic behavior. Furthermore, this assay imposes two competing motivations onto one explicit instrumental action: active punishment avoidance and reward pursuit/procurement. Despite static reward contingency, a relatively low probability of punishment drives a distinct behavioral pattern in which animals hesitate before pursuing instrumental actions. For example, in some cases animals approach the nosepoke port, but do not complete the actions. Park and Moghaddam (2017)[11] suggest that these punishment-based behavioral changes are due to both learning the punishment contingency as well as the state-dependent effects of punishment. Importantly, the animals show

distinctly slower instrument reaction times, but not reward reaction times, as entries to procure the food are left unaffected. The implication is that punishment likely does not induce anhedonia or avolition but generates a behavioral state in which animals are hesitant to pursue known reward-related actions explicitly due to a miniscule probability of punishment. We hypothesize that this behavioral state is anxiogenic. If so, this model could be used to not only to evoke an anxiogenic state, but also to predict susceptibility to stressors or determine the efficacy of potential therapeutic treatments.

Many experimental procedures evaluate anxiety states following exposure to unfamiliar aversive environments that can be elicited or exacerbated by a range of threatening stimuli such as predator exposure or pain[3,10,12]. Although baseline behaviors are presumably appropriate and adaptive for the present conditions in rodents, anxiety disorders in humans constitute maladaptive or pathological responses to the existing environment[1], suggesting that the ideal rodent behavioral assay could be tested repeatedly in the same animals. The need to differentiate distinct anxiety states has given rise to concepts “state” and “trait” anxiety[13,14]. State anxiety refers to the anxiety a subject experiences at a particular moment of time that can be worsened by the presence of an anxiogenic stimulus. On the other hand, trait anxiety encompasses anxiety that has minimal moment-to-moment variation and is considered to be an “enduring feature of an individual”[3]. However, only recently have these concepts been implemented to separate these potentially anxiety-related behaviors in rodents[15,16]. Establishing behavioral paradigms that exclusively evaluate these distinct states requires an intuitive behavioral approach. The model used by Park and Moghaddam (2017)[11] examines a particular intersection of behavior in which established reward environments subsequently gain relatively low probability punishments. Initially, we could expect the environment to induce very low levels of anxiety, while the introduction of low-risk probability would shift this state to an ever-increasing anxiogenic state. This enables testing of “state” anxiety through the introduction of the shock punishment and the maintenance of delayed reward responding within subjects, while also presenting an opportunity to evaluate “trait” anxiety through examination of inter-subject variability, experimental treatment groups, genetic mouse models, and strain differences.

Animal models of neuropsychiatric disorders should have face, construct, and predictive validity. These require the model to demonstrate similar outcomes to the clinical condition (i.e., similar symptoms, underlying biology, and effectiveness of clinically-relevant treatment[3–5,17–19]). Indeed, reproducibility, reliability, and predictive validity are vital for valid and useful animal models. While rats are powerful models and tremendously useful for neuropsychiatric behavioral research[15,20–25], technical advances in mouse genetics and neurophysiological tools have given

rise to circuit and cell-selective manipulations that have made mice incredibly advantageous in neuroscience research[26–30]. This has driven adaptation of many well-established and validated behavioral models of anxiety in rats to mice. To broaden these efforts, we have extensively adapted and tested the punishment risk task that Park and Moghaddam (2017) introduced in rats to demonstrate its validity in mice. The work we present here should provide a foundation for more comprehensive interrogation of the neural circuitry underlying behaviors related to anxiety. Careful replication and extension of this important behavioral model from rats to mice will enable important studies with genetic tools to alter gene expression and enable *in vivo* imaging and neuronal manipulation during behavior. Because of the task-based nature of this repeatable behavioral model, important temporal dynamics of the underlying circuit function can be probed with this approach as demonstrated by Park and Moghaddam (2017). When fully-implemented across species, this assay could provide important insights to inform the development of novel treatment strategies for anxiety disorders.

Results: To adapt the punishment risk task (PRT) from rats to mice, we first sought to replicate the task as closely as possibly to the rat protocol. However, we needed to accommodate size differences between the species. This required reducing the total number rewards to from 150 to 45 pellets. To accommodate this change in reward number we also modified the contingencies used for the probabilistic punishment for Block 2 (0.06 to 0.066) and Block 3 (0.10 to 0.133). We initially kept the shock amplitude (0.3 mA) and duration (300 ms) the same[11]. To test if these changes would be sufficient to port the behavior to mice, we transferred animals from our breeding facility to our laboratory facility and allowed them to acclimate for one week. We next food restricted the animals to 90% of their initial body weight to increase motivation to seek food rewards during the light cycle. Next, animals were trained to self-administer sweetened food pellets on a fixed-ratio 1 schedule for 10 days (or until animals collected 45 pellets on 2 consecutive days) (**Figure 1a, Supplementary Figure 1a-e)**. Following fixed-ratio 1 training with no punishment contingency, animals were tested with the adapted probabilistic punishment schemes (**Figure 1b**). On this first test session animals increased latency to respond to the cue (main effect of block: one-way ANOVA $F(1.221, 10.99)=11.01$, $p = 0.005$, Tukey's comparison (Block 1 vs. Block 3, $p < 0.05$) as previously described[11] (**Figure 1c,** left). However, four out of the fourteen animals we tested did not complete all 45 trials and were thus excluded the analysis (**Supplementary Figure 1f**). To determine whether this increased nosepoke response latency would carry over to future tests, we tested the animals again three days later. Encouragingly, on Test Day 2, there was no retained increased latency in Block 1 and the increased latency in Block

3 was recapitulated following the probabilistic shock exposure (main effect of block: one-way ANOVA F(1.008, 4.031) = 10.14, p = 0.033, Tukey's comparison Block 1 vs. Block 3, *ns*) (**Figure 1c,** right). Unfortunately, however, far fewer mice completed the second test day (n=5/14; **Supplementary Figure 1f**) and these multiple comparisons did not reveal differences between groups. Considering almost all of the animals completed all 45 trials when there was no probabilistic punishment, we interpreted these results to mean the shock amplitude was likely too great for the assay to be effective and repeatable in mice at 0.3 mA.

To directly test the effect of shock amplitude on PRT performance, we used a second cohort of naïve animals. We transferred this cohort from our breeding facility and acclimated them to our laboratory facility. Because future use of PRT would likely employ neurophysiological approaches that may require single housing, we also randomly assigned each mouse to be singly-housed or kept in their group housing (**Figure 2a**). To determine whether this adult social isolation alters baselined approach-avoidance behaviors, animals were tested in canonical exploration-based activity measures to examine approach-avoidance behaviors prior to PRT testing (**Supplementary Figure 2a**). In the open field test, we did not observe any differences in exploration of the center zone between group- and single-housed animals (unpaired t test, t (39) = 0.1066, p = 0.9156) or between sexes within or between these housing conditions (two-way ANOVA (housing x sex), no effect of housing f(1,37) = 0.0093, p = 0.9233 or effect of sex F(1, 37) = 3.913, p = 0.0554) **(Supplementary Figure 2a-b**). However, a two-way ANOVA (housing x sex) analyzing distance traveled revealed a main effect of group (F(1, 37) = 7.912, p = 0.0078), but no effect of sex (F(1,37) = 2.701, p = 0.1087). Tukey's multiple comparisons revealed single-housed female mice had moved significantly greater distance during testing as compared to group-housed female mice (p = 0.0289) and male mice (p = 0.0152). Though trending, these isolated mice were not significantly different from single-housed male mice (p = 0.09)**(Supplementary Figure 2c-d)**. Further testing in the elevated plus maze revealed no difference in the time spent in the open arms for group- and single-housed animals (unpaired t test, t (39) = 0.1446, p = 0.1561 **(Supplementary Figure 2e, h**). Additionally, further analysis (two-way ANOVA) did not reveal any differences in open arm exploration between sexes (F(1, 37)= 0.229, p = 0.6351) or these housing conditions (F(1, 37) = 1.86, p = 0.1809) . Housing conditions affected distance traveled (F(1, 37)= 7.543, p = 0.0093), but sex did not have any effect on distance traveled in the EPM **(Supplementary Figure 2g).** Tukey's multiple comparisons did not reveal any differences between any groups. Although social isolation (i.e. single housing) can impact approach-avoidance behaviors, we show here that this did not occur when the isolation is performed in adulthood as described previously[31].

Following OFT and EPM testing, animals were then trained to self-administer sweetened food pellets on a fixed-ratio 1 schedule for 10 days (or until animals collected 45 pellets on 2 consecutive days) (**Supplementary Figure 3b-d, h,i)**. Following fixed-ratio 1 training with no punishment contingency (**Figure 2b**), animals were assigned to specific shock amplitudes in a counterbalanced manner and tested in the punishment risk task on separate days, with four days in between test days. Briefly, the punishment risk task (PRT) as adapted for mice requires mice learning to nosepoke in response to a cue to receive a sucrose pellet under a fixed ratio one schedule of reinforcement (FR1) for a total of 45 trials. During PRT this conditioning behavior is separated into 3 blocks (15 trials each), with each having increasing probability of nosepoke-contingent foot shock (0, 0.066, and 0.133). (**Figure 2c**). This design yields two discrete outcomes: 1) nosepoke latency, which can be considered the primary independent variable of PRT, quantifies the delay in instrumental action following cue onset; and 2) reward latency, a secondary independent PRT variable that quantifies the time an animal takes to retrieve the reward after it is delivered (**Figure 2c**). Each test required the animals complete all three blocks to be included in behavioral data analysis **(Supplemental Figure 3a**). Prior to shock introduction, animals tested with no punishment probability in blocks 2 and 3 had similar nosepoke latency across blocks and between groups (**Supplementary Figure 3e).** Despite no difference in the motivation to respond to the cue, these groups were shown have an increase in reward latency as they progressed through the test ($p < 0.0001$) (**Supplementary Figure 3f**) and did not consume all pellets received (**Supplementary Figure 3g**), indicating a potential effect of time or satiety on the motivation to retrieve a reward following successful operant conditioning behavior without a probabilistic punishment.

To establish an effective punishment without producing fear conditioned behavior we tested two different 300 ms shock amplitudes (0.1 mA and 0.3 mA). A repeated measures 3-way ANOVA examining nosepoke latency by 3 factors (housing, shock amplitude, and test block) revealed a significant effect of block ($F(1.631, 97.84) = 35.87$, $p < 0.0001$) and housing ($F(1, 60) = 4.036$, $p = 0.049$), but no significant effect of shock amplitude ($F(1, 60) = 0.2835$, $p = 0.5964$) (**Figure 2d**). This indicates that the animals were slower to respond as the probability of punishment increases in each block. There was no significant interaction between any of the factors. Tukey's multiple comparisons test revealed significant differences between average nosepoke latency in *Block 1* and *Block 3* for 0.1 mA shock in isolated animals ($p = 0.0075$) and grouped animals ($p = 0.0243$). Additionally, average nosepoke latency was significantly increased from *Block 2* to *Block 3* for 0.1 mA shock in isolated animals ($p = 0.0436$), but not group-housed animals ($p = 0.3797$). Multiple comparisons did not reveal any relevant significant differences

comparing blocks between isolated and grouped animals for 0.3 mA shock. Further, a repeated measures 3-way ANOVA examining reward retrieval latency by 3 factors (housing, shock amplitude, and test block) revealed a significant effect of block ($F(1.603,96.18) = 26.37$, $p < 0.0001$) and housing ($F(1, 60) = 10.68$, $p = 0.0018$), but no significant effect of shock amplitude ($F(1, 60) = 1.547$, $p = 0.2184$) (**Figure 2e**). There was no significant interaction between any of the factors. Tukey's multiple comparisons test revealed significant differences between average reward retrieval latency in *Block 1* and *Block 3* for 0.3 mA shock in isolated animals ($p = 0.0009$) and grouped animals ($p = 0.0367$). Additionally, average reward retrieval latency was increased between *Block 1* and *Block 2* for 0.3 mA shock in isolated animals ($p = 0.0063$), but not group-housed animals ($p = 0.1499$). Multiple comparisons did not reveal any relevant significant differences between isolated and grouped housed animals for 0.3 mA shock or any significant differences in any block or housing condition for 0.1mA. Together, these results suggest that higher intensity shock may be more likely to increase reward retrieval latency alongside latency to respond to cue presentation. As described in Park and Moghaddam (2017)[11], this increased reward retrieval latency confounds clear interpretation of the hesitancy to nosepoke for reward following the cue. Given these data, further examination of PRT with drug treatments only used a 0.1 mA shock in the procedure.

Given that sex had a minimal effect on canonical activity measures, we examined whether sex and housing had any influence on nosepoke latency within the PRT test. A 3-way, repeated measures ANOVA (block x shock x housing) examining average nosepoke latency among male animals revealed a significant effect of block ($F(2, 38) = 15.13$, $p = 0.0001$), housing ($F(1, 32) = 5.42$, $p = 0.026$), and an interaction of block, housing, and shock ($F(2, 32) = 8.348$, $p = 0.0012$) (**Supplementary Figure 4a**). Tukey's multiple comparisons test revealed significantly increased average nosepoke latency in *Block 3* compared to *Block 1* for 0.1mA shock in group-housed mice ($p < 0.0001$), but not in single-housed mice ($p = 0.8842$). Further, comparisons revealed significantly increased average nosepoke latency in *Block 3* compared to *Block 1* for 0.3mA shock in single-housed mice ($p < 0.01$), but not in group-housed mice ($p = 0.6922$). A 3-way, repeated measures ANOVA (block x shock x housing) examining average nosepoke latency among female mice revealed a significant effect of block ($F(2, 24) = 13.84$, $p = 0.0001$), but no effect of housing ($F(1, 17) = 0.0522$, $p = 0.8221$), or shock intensity ($F(1, 12) = 0.0075$, $p = 0.9324$) (**Supplementary Figure 4b)**. There was no interaction of these factors within female mice.

Following Park and Moghaddam (2017) we then used known benzodiazepine anxiolytic, diazepam to test whether the increased nosepoke latency in *Block 3* can be reversed similar to anxiety states observed in humans. A repeated measures 2-way ANOVA (block x drug treatment)

examining average nosepoke latency among group-housed animals showed a significant effect of block ($F(2, 22) = 11.14$, $p = 0.0004$), drug treatment ($F(1, 11) = 15.03$, $p = 0.0026$), and an interaction of drug treatment and block ($F(2, 22) = 9.263$, $p = 0.0012$) (**Figure 3a**). Tukey's multiple comparisons test revealed significantly increased average nosepoke latency in *Block 3* compared to *Block 1* following saline treatment ($p < 0.0001$), but not following diazepam treatment ($p = 0.9361$). Additionally, average nosepoke latency in *Block 3* was significantly lower following diazepam compared to saline treatment ($p < 0.0001$). A repeated measures 2-way ANOVA (block x drug treatment) examining average nosepoke latency among single-housed animals revealed a significant effect of block ($F(2, 24) = 11.62$, $p = 0.0003$), drug treatment ($F(1, 12) = 14.78$, $p = 0.0023$), and an interaction of drug treatment and block ($F(2, 24) = 8.606$, $p = 0.0015$) (**Figure 3b**). Average nosepoke latency significantly increased in *Block 3 compared to Block 1* following saline treatment (Tukey's multiple comparisons test, $p < 0.0001$), but not following diazepam treatment ($p = 0.7933$). Additionally, average nosepoke latency in *Block 3* was significantly lower following diazepam compared to saline treatment ($p < 0.0001$). A repeated measures 2-way ANOVA (block x drug treatment) examining average reward retrieval latency among group-housed animals revealed a significant effect of block ($F(2, 24) = 9.255$, $p = 0.001$), drug treatment ($F(1, 12) = 6.38$, $p = 0.0266$), and an interaction of drug treatment and block ($F(2, 22) = 11.63$, $p = 0.0003$) (**Figure 3c**). Average reward retrieval latency significantly increased in *Block 3* compared to *Block 1* following saline treatment (Tukey's multiple comparisons test, $p < 0.0001$), but not following diazepam treatment ($p = 0.9992$). Additionally, average nosepoke latency in *Block 3* was significantly lower following diazepam compared to saline treatment ($p < 0.0001$). A repeated measures 2-way ANOVA (block x drug treatment) examining average reward retrieval latency among single-housed animals revealed a significant effect of block ($F(2, 24) = 15.32$, $p = 0.0003$), drug treatment ($F(1, 12) = 6.956$, $p = 0.0217$), and an interaction of drug treatment and block ($F(2, 24) = 13.38$, $p = 0.0001$) (**Figure 3d**). Tukey's multiple comparisons test revealed significant increase in average nosepoke latency in *Block 3* compared to *Block 1* following saline treatment ($p < 0.0001$), but not following diazepam treatment ($p > 0.9999$). Additionally, average reward retrieval latency in *Block 3* was significantly lower following diazepam compared to saline treatment ($p < 0.0001$). These data suggest that anxiolytic diazepam prevents the increased latency to respond to cue presentations during sessions with higher punishment probability. These experiments also demonstrated that saline injection has an effect on reward retrieval latency which was not observed in previous 0.1 mA shock PRT testing. Despite repeated handing prior to drug treatment experiments, this may be due to added stress that injection procedures may have on animals that were not used in previous testing. Further, our data demonstrated that

higher intensity shock drives increased nose poking during *Block 3* for group and single-housed animals (**Supplementary Figure 5a**). A repeated measures 3-way ANOVA revealed an effect of block ($F(2, 52) = 9.834$, $p = 0.0002$), shock intensity ($F(1, 26) = 33.53$, $p < 0.0001$), and an interaction of block and shock intensity, but no effect of housing ($F(1, 26) = 0.1259$, $p = 0.7256$). Post hoc comparisons showed that greater intensity shock (0.3 mA) increases the average number of total nosepokes in *Block 3* for both group animals (Group - Block 1 vs. Group - Block 3, **** $p < 0.0001$; Group - Block 2 vs. Group - Block 3, **** $p < 0.0001$; Group (0.1 mA) - Block 3 vs. Group (0.3 mA) - Block 3, #### $p < 0.0001$; and single-housed animals (Isolation (0.1 mA) - Block 3 vs. Isolation (0.3 mA) - Block 3, #### $p < 0.001$). Additionally, our data demonstrated that probability of punishment drives increased hopper entries throughout the test, regardless of housing or shock intensity (**Supplementary Figure 5b**). A repeated measures 3-way ANOVA revealed an effect of block ($F(2,52) = 4.320$, $p = 0.0184$), no effect of shock intensity ($F(1, 26) = .4633$, $p = 0.5021$), and no effect of housing ($F(1, 26) = 0.4117$, $p = 0.5267$). However, post hoc comparisons did not reveal differences between groups.

Following drug treatment testing, our data demonstrated that diazepam (2 mg/kg) treatment differentially affects the average number of nosepokes per block for single-housed animals (**Supplementary Figure 5c**). A repeated measures 3-way ANOVA revealed an effect of block ($F(2, 48) = 37.48$, $p < 0.0001$), drug treatment ($F(1, 24) = 9.042$, $p = 0.0061$), and housing ($F(1, 24) = 14.67$, $p = 0.0008$). It also revealed an interaction of block and treatment ($F(2, 39) = 6.964$, $p = 0.0026$ and block and housing ($F(2, 48) = 16.05$, $p < 0.0001$), but no interaction of treatment and housing ($F(1, 24) = 0.0153$, $p = 0.9026$), nor an interaction of all three factors ($F(2, 39) = 0.9689$, $p = 0.3885$). Post hoc comparisons showed that diazepam treatment increases average nosepokes for single-housed animals in *Block 1* (Saline - Block 1 vs Diazepam - Block 1, # $p < 0.05$; as compared to saline treatment and as compared to group-housed animals (Diazepam (group) - Block 1 vs Diazepam (isolation) - Block 1, ### $p < 0.001$. Single-housed animals have greater average nosepokes in *Block 1* following saline and diazepam treatment (Saline - Block 1 vs. Saline - Block 3, ** $p < 0.01$; Diazepam - Block 1 vs Diazepam - Block 3, **** $p < 0.0001$).

Additionally, these data demonstrate that diazepam (2 mg/kg) differentially affects the average number of entries per block for group-housed animals (**Supplementary Figure 5d**). A repeated measures 3-way ANOVA revealed an effect of block ($F(2, 48) = 4.582$, $p = 0.0151$) and housing ($F(1, 24) = 18.08$, $p = 0.0003$), but not drug treatment ($F(1, 24) = 2.115$, $p = 0.1588$). However, it also revealed an interaction of block and housing ($F(1, 24) = 8.962$, $p = 0.0063$, but no other interactions (block x treatment $(2, 44) = 2.019$, $p = 0.1449$); block x housing ($F(2, 48) =$

0.3076, p = 0.7367); Block x treatment x housing (F(2, 44) = 1.528, p = 0.2282)). Post hoc comparisons showed that group-housed animals have higher average entries per block as compared to single-housed animals. Diazepam-treated, group-housed animals have greater average number of food hopper entries in *Block 3* than following saline treatment and both saline and diazepam-treated, single-housed animals (Saline (Group) - Block 3 vs. Diazepam (Group) - Block 3, # $p < 0.05$; Saline (Isolation) - Block 3 vs. Diazepam (Group) - Block 3, ## $p < 0.01$); Diazepam (Isolation) - Block 3 vs. Diazepam (Group) - Block 3, ## $p < 0.01$). Diazepam-treated, group-housed animals also have greater entries within *Block 1* compared to diazepam-treated, single-housed animals (Diazepam (Group) - Block 1 vs. Diazepam (Isolation) - Block 1, ### $p < 0.001$)**.**

To determine whether another anxiolytic could reverse PRT, we next tested propranolol, a non-selective β-adrenergic receptor antagonist with anxiolytic properties in humans and mice[32–35]. Here, a repeated measures 2-way ANOVA (block x drug treatment) examining average nosepoke latency among group-housed animals revealed a significant effect of block (F(2, 22) = 12.88, p = 0.0002) and an interaction of drug treatment and block (F(2, 22) = 7.431, p = 0.0034). Post hoc comparisons revealed that saline treatment has no effect on the increased latency driven by increasing probability of shock (Saline - Block 1 vs. Saline - Block 3, **** $p < 0.0001$) while propranolol (10 mg/kg) treatment reduced the average latency to nosepoke in *Block 3* as compared to saline treatment (## $p < 0.01$) **(Figure 4a)**. Further, a repeated measures 2-way ANOVA (block x drug treatment) examining average nosepoke latency among single-housed animals revealed a significant effect of block (F(2, 22) = 9.992, p = 0.0007), but no effect of treatment or interaction between factors. Post hoc multiple comparisons revealed a statistically significant increase in nosepoke latency for saline-treated mice (p = 0.0019), but no significant increase in nosepoke latency among propranolol-treated mice (p = 0.5644) **(Figure 4b)**. These effects were also observed in animals' average latency to retrieve the reward as a repeated measures 2-way ANOVA (block x drug treatment) among group-housed animals revealed a significant effect of block (F(2, 22) = 12.37, p = 0.0002), but no interaction of drug treatment and block (F(2, 22) = 3.101, p = 0.0634). However, in this group post hoc comparisons revealed that saline-treated animals have increased reward retrieval latency (Saline - Block 1 vs. Saline - Block 3, ** $p < 0.0001$) while propranolol-treated animals show no significant change in reward retrieval latency (Propranolol - Block 1 vs. Propranolol - Block 3 (p = 0.2768) **(Figure 4c)**. Further, a repeated measures 2-way ANOVA (block x drug treatment) examining average reward retrieval latency among single-housed animals revealed a significant effect of block (F(2, 24) = 22.60, $p < 0.0001$) and interaction between block and treatment factors (F(2, 24) = 4.193, p = 0.0274). Post

hoc multiple comparisons revealed a statistically significant increase in nosepoke latency for saline-treated mice ($p < 0.0001$), while propranolol treatment reduced the average reward retrieval latency in *Block 3* as compared to saline treatment (# $p = 0.0251$) **(Figure 4d)**. These data suggest a role for β-adrenergic receptor signaling during PRT that can be suppressed to minimize action-punishment contingencies.

To determine whether PRT testing alters locomotor activity across blocks as a proxy for shock-induced freezing, we used video recording and analysis (**Figure 5a)**. A one-way ANOVA revealed mice to have greater movement velocity during *Block 1* as compared to *Block 2* ($p < 0.0001$) and *Block 3* $p < 0.0001$), but no significant difference in immobility time (**Figure 5b-c**). This is likely due to the nature of the assay as the animal likely experiences a time of increased activity due to the novelty of handling and being placed in the box. We then interpret that activity is reduced as the animal spends more time engaged in the task. Further, a two-way, repeated measures ANOVA examining the percentage of time near the food hopper or nosepoke port revealed a significant effect of zone location ($F(1, 11) = 148.6$, $p < 0.0001$) with posthoc comparisons showing animals were observed spending a greater percentage of time near the food hopper compared to the nosepoke port in all block regardless of shock probability ($p < 0.0001$) (**Figure 5d**). This location preference is also likely due to the nature of the assay such that animals retrieve the pellet from the food hopper and remain nearby to ingest the pellet. Lastly, a two-way, repeated measures ANOVA examining hopper and nosepoke zone activity revealed a main effect of block ($F (1.742, 19.16) = 17.05$, $p < 0.0001$) where mice tend to reduce average number of entries (per minute) into the zones near the hopper ($p = 0.0107$) and the nosepoke port ($p = 0.003$) (**Figure 5e**).

Finally, to better understand the construct and content validity of PRT, we continued to test animals under a typical control treatment condition (saline injection, i.p.). Despite this administration procedure having a small impact on stress responsivity, this procedure does not impact or influence the effect of PRT testing (**Figure 6a-b, see also Figure 3a-d**). Probabilistic punishment significantly increased the trial-to-trial variability in nosepoke latency for group-housed ($F4, 33 = 2.874$, $p < 0.05$) and single-housed mice ($F4, 33 = 5.907$, $p < 0.01$) **(Figure 6d-e)**. In contrast, the no-shock control session, when animals performed the same number of trials and blocks in the absence of punishment, the mean nosepoke latency and the variance nosepoke latency did not differ across blocks **(Figure 6f)**. To test whether the increase in nosepoke latency reflected learning of action-punishment contingency or a general consequence of unpredictable shock, we compared nosepoke latency of early trials in *Block 2* and *3*, before the delivery of the 1st shock. Mice displayed a subtle increase in nosepoke latency in *Block 2* and *3* however, this

increase was not significant for group-housed mice (repeated measures one-way ANOVA $F(1.334, 13.34) = 1.685$, $p = 0.2213$). In contrast, this increase was significant in *Block 3* before the first shock ($F(1.064, 14.89) = 7.794$, $p < 0.05$) in single-housed mice **(Figure 6g)** suggesting that single housing could influence learning of action-punishment contingencies from minimal previous experience.

Discussion: In this study we have translated an important rat behavioral task into mice and demonstrated its utility as a novel murine model for assessing repeatable anxiolytic-sensitive cautionary behavior. Importantly, this model's task-oriented design allows the interrogation of behavioral reinforcement and passive avoidance, both important behavioral measures to more accurately quantify murine behavioral phenotypes related to negative affective disorders such as Generalized Anxiety Disorder. Furthermore, underlying neural circuit dynamics can be probed at neurophysiological timescales with this approach[11]. Critically, this test can be repeated multiple times under multiple conditions enabling long-term studies, increasing within-subject power, and reducing the overall number of animals needed to test. In addition to porting this assay to both sexes of mice, our study design included evaluating the effects of chronic isolation in adulthood. This approach allowed us to further determine whether this model would detect any anxiety- or motivation-related effects of adult single housing, a condition sometimes necessitated in behavioral and circuit neuroscience. Although animals had success in completing the task following modifications, many mice did not consume all dispensed pellets (**Supplemental Figure 3g**). Alas, this number of rewards (15 per block) was the minimum possible, given that the probability of shock needed for *Block 2* resulted in only one shock. To avoid this restriction, future studies could use liquid rewards (e.g., sucrose solutions) and a lickometer-based sipper where access could be limited. This could enable experiments to have more flexibility for changing probabilities and number of trials per block. This could help minimize any satiety or motivational constraints and maintain responding and consumption throughout the entirety of the task. However, the explicit goal of this study was to maintain validity in translating this task to mice with as little deviance from the original rat model as possible. Further, we tested an additional shock intensity, 0.1 mA, along with the previously demonstrated 0.3 mA shock. Here we found that, as with previous rat studies[11,36,37], mice only exhibit higher latency to act on the operant nosepoke port following cue presentations under conditions of probabilistic punishment. This effect was most prominent for sessions using 0.1 mA shock in both group- and single-housed animals **(Figure 2d)**. However, despite not having any significant difference in nosepoke latency for group and single-housed mice, 0.3 mA shock blocks had a significant effect on reward retrieval latency

in both group and single-housed mice in *Block 3* **(Figure 2e)** and increased the likelihood of animals not completing the task (**Supplementary Figure 1f**). Given this striking discrepancy in behavioral outcome, we selected 0.1 mA shock intensity for subsequent anxiolytic treatments (**Figure 3 & Figure 4**).

Importantly, as demonstrated in Park and Moghaddam's initial study[11], diazepam treatment abolishes the behavioral response to increased punishment probability. Previous studies have demonstrated diazepam as an anxiolytic in C57BL/6 mice[38]. However, a recent study has suggested that these effects are largely driven by sedation in open field and elevated plus maze testing[39]. Despite this conflicting evidence, our data do not suggest any sedative effect given the animals' propensity to continue to perform the task and, importantly, do so even more efficiently. For both group- and single-housed animals, we observed an increase in nosepoke latency and reward latency with saline treatment, while diazepam treatment reduced these changes in response latency (**Figure 3a-d**). These data suggest that increased latency in responding is indicative of an anxiogenic state given that diazepam only reduces nosepoke latency during probabilistic punishment in *Block 3*. Importantly, we did not observe any effect of animal housing or sex on the canonical anxiety-related behavioral assessments of exploratory approach-avoidance, open field test and elevate plus maze (**Supplemental Figure 2a,b,e,f**), despite previous work reporting reduced anxiety-like behavior in female rodents[31,40]. However, in our hands social isolation increased distance traveled in both OFT and EPM testing, contrary to other results[31]. Furthermore, we observed differential effects of shock intensity on nosepoke latency in male mice that were dependent on housing, that had no influence in female mice. Group-housed male mice had significantly higher nosepoke latency during probabilistic punishment for 0.1 mA shock, but nosepoke latency during 0.3 mA shock blocks did not reach significance. In contrast, single-housed male mice had significantly higher nosepoke latency during probabilistic punishment for 0.3 mA shock, with nosepoke latency during 0.1 mA shock showing no significant increase (**Supplementary Figure 5a**). This indicates a discrepancy in social isolation's effect on the sensitivity to shock intensity for male mice in this specific model. Punishment risk increased nosepoke latency in female mice, but shock intensity and housing had no significant influence (**Supplementary Figure 5b**). These data suggest that female mice may have minimal sensitivity to adult social isolation and changes in shock intensity as assessed in this assay. This aligns with other research that has suggested sex plays a role in active and passive avoidance tasks. Previous work has demonstrated that female mice have lower avoidance rates in an active avoidance test, but have significantly higher avoidance latencies in a passive avoidance task[41]. However, PRT testing in females rats showed greater latency in

Blocks 2 and *3*, while male rats showed no difference in responding, contrasting previously described PRT behaviors in male rats[11,37]. Despite these conflicting data, we propose that the effects observed in our experiments are not at odds with previous study, but inform potential differences in implementing this assay in different species[5,12].

Further, isolated mice had lower nosepoke latencies, generally (see **Figure 6a-b**), with less variance in responding during probabilistic punishment. As with rats[11], changes in nosepoke latency during probabilistic punishment blocks parallels changes in the variance of response latencies within those blocks in mice (i.e. as the probability of punishment increases, the mean and variance for nosepoke latency for the block increases; **Figure 6c-e**), where responding during *Block 3* shows the greatest variance among the highest mean responding. This increased variance could be characterized as a measure of "hesitancy" or "caution" during this reward seeking behavior. Indeed, a recent study found that mice delayed their action for a signaled reward when a new, unsignaled rule that punished the action was added[42]. However, the interpretation from that study suggested that this "caution" is not due to an anxiogenic state as it is unaffected by diazepam administration[42]. This discrepancy is likely due to multiple different factors: apparatus (shuttle box vs. operant box), animal state (water-restricted vs. food-restricted), reward (water vs. sweetened pellet), and the probability of punishment (1.00 vs 0.13). Our model examined this change in signal-response latency during a period of low probability of unsignaled punishment, while the other model relied on either explicit unsignaled action-contingent punishment or punishment without contingency. Given these differences, it is difficult to make direct comparisons of the "caution" demonstrated in these rodent behavioral models. Furthermore, recent work from the Moghaddam lab expanded on their previous findings to determine how diazepam affects this change in behavior[36]. Here they found that exposure to conflicting reward and punishment contingencies caused ventral tegmental area (VTA) and dorsomedial prefrontal cortex (dmPFC) neurons to have modified activity in the time period before the action and subsequent reward periods, but did not change neuronal responses to punishment. Interestingly, treatment with diazepam potentiated VTA response to action and enhanced its correlated activity with the dmPFC, but still did not influence the neural response to the punishment. Thee authors propose that the encoding information related to the action, but not punishment, is critical to adapting behavior to punishment risk contingencies and results in an anxiogenic state in rats[36]. Overall, increased punishment risk likely disrupts goal-oriented neuronal activity, while diazepam administration enhances coordination of activity within this neurocircuitry to refocus this activity to influence and maintain goal-directed actions.

The main purpose of this study was to establish a model of anxiety-related behavior in mice that was analogous to the experience of risk uncertainty demonstrated in human populations [16,43,44]. Typical neuroscience studies of the neural underpinnings of anxiety assess exploratory behaviors through exposure to ambiguous or novel contexts[10,12]. Many of these tests can only be performed once per animal as the novelty of the environment is critical to the interpretation of the behavior. While these approaches has been incredibly valuable for understanding cell type-selective the mechanisms of acute fear and anxiety states[32,33,45–55], these assays do not explicitly model more complex situations where these negative affective states can develop from learning that certain goal-directed actions have some risk of an aversive outcome. Alternatively, here we use uncertain punishment contingencies during action execution to study this unique behavior. These types of conflict produce self-reported elevations in anxiety in humans and have been validated with anxiolytic treatment with benzodiazepines in rodents and non-human primates[56–58]. Recent procedures assessing reward and punishment learning simultaneously have further indicated that learning of punishment contingencies may be critical to understanding behavioral and neuronal changes related to anxiety[11,36,37,59,60]. Here we present a complementary assay to examine the learning of these contingencies that has significant potential for the study of anxiety-like behavior in mice. Overall, these experiments demonstrate that effective implementation of the Punishment Risk Task (PRT) model is possible in mice and, with careful adaptation, mostly parallels the behavioral outcomes that have been seen in rat studies. We hope that this detailed study will enable wider adoption of this repeatable murine model for the preclinical study of anxiety-related behaviors.

Methods:

Subjects

Male and female C57BL/6J mice 3-6 months of age were bred locally in barrier facility in a different building. Upon weaning, all animals were group-housed. Following transfer to the experimental facility and 1 week of habituation, mice were separated into singly-housed cages or kept in their original group-housed cage. After 4 weeks of isolated or grouped housing, mice were tested for canonical behavioral phenotypes in the open field test (OFT) and elevated plus maze (EPM). One week later, mice were weighed and placed under a restricted diet to maintain 90% of free feeding bodyweight. After one week, animals were trained for the punishment risk task (PRT). Mice had free access to water. Mice were maintained on a 12:12-hr light/dark cycle (lights on at 7:00 AM). all procedures were approved by the Animal Care and Use Committee of Washington University and conformed to US National Institutes of Health guidelines.

Animal Handling

During experimental housing conditions, animals were gently handled weekly (e.g., picked up by the base of the tail and placed on a scale). During PRT training and testing, mice were handled daily. Prior to any drug administration experiment, mice were briefly restrained by hand and lightly stroked on the abdomen by the experimenter once a day for three days.

Behavior

Open Field Test

OFT testing was performed in a sound attenuated behavior testing room as previously described[32,33]. Lighting was stabilized at ~12 lux. Animals were allowed to habituate to the room for 1 hour prior to testing. Each animal was individually placed into a 2500 cm^2 enclosure for 20 minutes. The center of the apparatus was defined as a square equal to 50% of the total area. Activity was video recorded via a Google Pixel 3 XL and videos analyzed using Ethovision XT 13 (Noldus Information Technology, Wageningen, The Netherlands).

Elevated Plus Maze

EPM testing was performed within a sound attenuated behavior testing room. Lighting was stabilized at ~12 lux. Animals were allowed to habituate to the room for 1 hour prior to testing. Each animal was individually placed into a plus-shaped platform for 15 minutes. The apparatus is comprised of two open arms (25 x 5 x 0.5 cm) across from each other and perpendicular to two closed arms (25 x 5 x 16 cm) with a center platform (5 x 5 x 0.5 cm). The apparatus is 50 cm above the floor. Activity was video recorded via a Google Pixel 3 XL and videos analyzed using Ethovision XT 13 (Noldus Information Technology, Wageningen, The Netherlands).

Punishment Risk Task

The Punishment Risk Task (PRT) was performed within sound-attenuated boxes (Med Associates Inc., Fairfax, VT). Mice were trained to nose poke in response to a pre-assigned auditory cue inside the associated cue port to receive a sugar pellet (Dustless Precision Pellet - F0071, Bio-Serv, Flemington, NJ) at the port located on the opposite side of the chamber on a fixed-ratio schedule of one. The fixed ratio remained at one throughout all training and test sessions. Specifically, each mouse received one sweetened pellet after every completed nose poke entry. Completion of nose poke entry was determined by disruption of an infrared activity monitor located

inside the nose poke port. Each mouse completed 10 training sessions consisting of 45 trials in 60 minutes prior to recording session. After training sessions, mice will undergo a no-shock control test session, throughout which they will perform 45 trials of nose pokes divided into three blocks separated by 2 minutes of darkness and in the absence of punishment. Then, mice were trained with the task consisting of three blocks with varying degrees of action-punishment contingency, contingencies being 0, 0.066, or 0.133. The punishment employed in this task is electrical foot shock (0.1 mA or 0.3 mA, 300 ms). Each block consisted of 15 trials with the associated contingency value assigned to each block in ascending probable order. That is, in block 1 there was a 0% chance of foot shock, in block 2 there was a 6.66% chance of foot shock, and in block 3 there was a 13.33% chance of foot shock. Each block was separated by 2 minutes of darkness for the animal to be able to discern a change in block. In addition, the first shock trial of block 2 and the two shock trials of block 3 were randomly selected from the initial trials of each block. In block 2, mice were randomly assigned to received one shock upon nosepoke number 2 or 3. In block 3 mice were randomly assigned to received one foot shock upon nosepoke number 2 or 3, then a second foot shock upon nosepoke number 6, 7, 8, or 9. All training and test sessions were terminated if not completed in 180 minutes, and only data from completed sessions were analyzed. The number of mice and session that were terminated was recorded. We did not observe habituation to the foot shock as evidenced by lack of behavioral changes across sessions. Animals that did not reach training criteria or complete the test session within 180 minutes were excluded. Throughout all complete sessions, nosepoke latency and reward latency was recorded. Nosepoke latency refers to time between auditory/light cue onset and completed nose poke and reward latency refers to time between reward delivery and reward retrieval. PRT locomotor activity was captured via a Google Pixel 3 XL and videos analyzed using Ethovision XT 13 (Noldus Information Technology, Wageningen, The Netherlands).

Drug Treatment

Diazepam Treatment

Diazepam (Sigma-Aldrich, St. Louis, MO, USA) was diluted in 0.9% physiological saline and prepared on treatment days. Experimental conditions included three separate test sessions, each consisting of three blocks. Each mouse received an intraperitoneal (i.p) administration of saline for the first and last session and diazepam administration (2 mg/kg, i.p.) for the second test session. Following treatment administration, each mouse was immediately returned to their homecage for 10 minutes prior to PRT. Each test session was separated by three days.

Propranolol Treatment

Propranolol (Tocris, Bristol, UK) was diluted in 0.9% physiological saline and prepared on treatment days. Experimental conditions included three separate test sessions, each consisting of three blocks. Each mouse received an intraperitoneal (i.p) administration of saline for the first and last session and propranolol administration (10 mg/kg, i.p.) for the second test session. Following treatment administration, each mouse was immediately returned to their homecage for 10 minutes prior to PRT. Each test session was separated by three days.

Data Analysis & Statistics

All data collected via Med-Associates Operant Conditioning hardware and software and were averaged and expressed as mean ± SEM. Statistical significance was taken as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, as determined by two-way repeated measures ANOVA or three-way repeated measures ANOVA followed by Tukey's post hoc tests as appropriate. Group sizes for approach-avoidance measures were n = 21 (group-housed) and n = 20 (singly-housed). Group sizes for PRT testing were n = 12 (group-housed) and n = 15 (singly-housed). Statistical analyses were performed in GraphPad Prism 9.0 (Graphpad, La Jolla, CA)

Acknowledgements: We thank the other members of the Al-Hasani and McCall labs, particularly Marwa O. Mikati, Justin Woods, and Makenzie R. Norris, for helpful feedback on this project. This work was supported by the National Institutes of Health (R01NS117899, J.G.M.), the Brain & Behavior Research Foundation (NARSAD YI – 28565, J.G.M.), and the Rita Allen Foundation with help from the Open Philanthropy Project (J.G.M.). We would like to acknowledge biorender.com for cartoons in Figure 2.

Author contributions: K.E.P. and J.G.M conceived the project and designed the detailed experimental protocols. K.E.P. and S.C.H. performed the mouse experiments. K.E.P., J.S.A., S.C.H., and J.G.M performed the investigation and analyzed the data. K.E.P., and J.G.M wrote the paper. J.G.M acquired funding. J.G.M. provided research supervision and led overall project administration. All authors discussed the results and contributed to revision of the manuscript.

Conflict of Interest: The authors declare no conflicts of interest.

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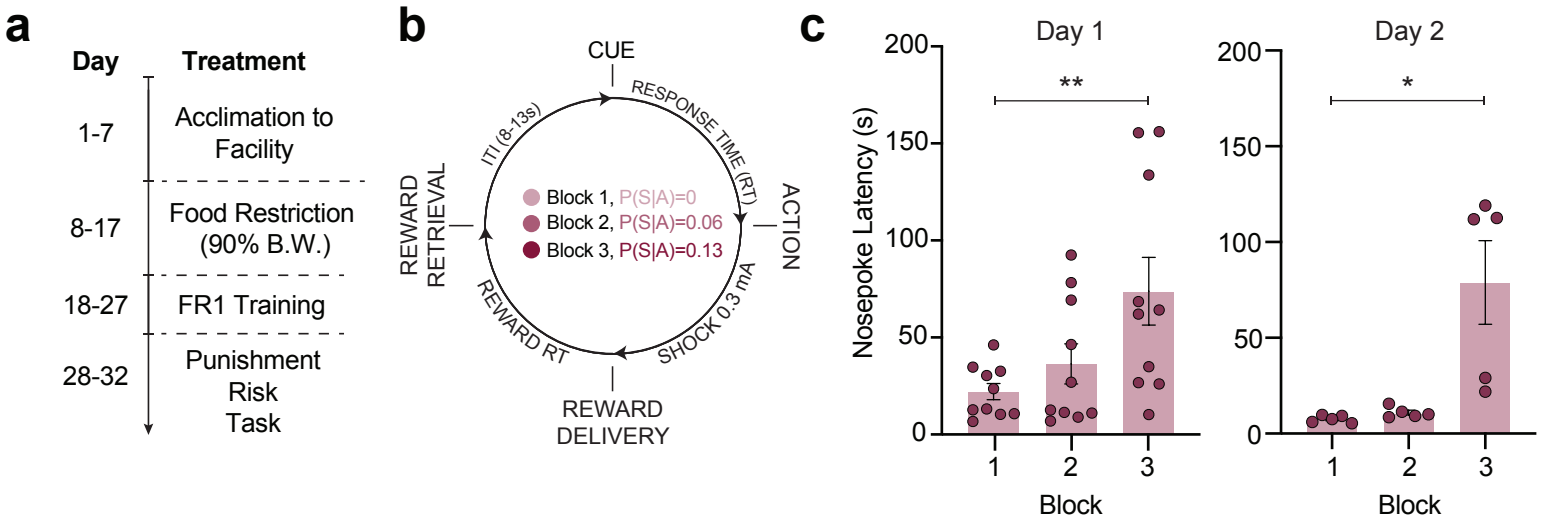

a
Day
Treatment
1-7
Acclimation to Facility
8-17
Food Restriction (90% B.W.)
18-27
FR1 Training
28-32
Punishment Risk Task
b
CUE
RESPONSE TIME (RT)
ACTION
SHOCK 0.3 mA
REWARD DELIVERY
REWARD RT
REWARD RETRIEVAL
ITI (8-13s)
Block 1, P(S|A)=0
Block 2, P(S|A)=0.06
Block 3, P(S|A)=0.13
c
Day 1
Day 2
Nosepoke Latency (s)
200
150
100
50
0
**
*
1
2
3
Block

Figure 1: Initial study testing the Punishment Risk Task in male and female mice. (a) Calendar of behavioral testing **(b)** Diagram depicting specifics of behavioral task. **(c)** Average nosepoke latency increases as probability of risk increases and is repeatable behavioral test (Day 1: main effect of block, ** $p = 0.0051$; Day 2: main effect of block, * $p = 0.033$.

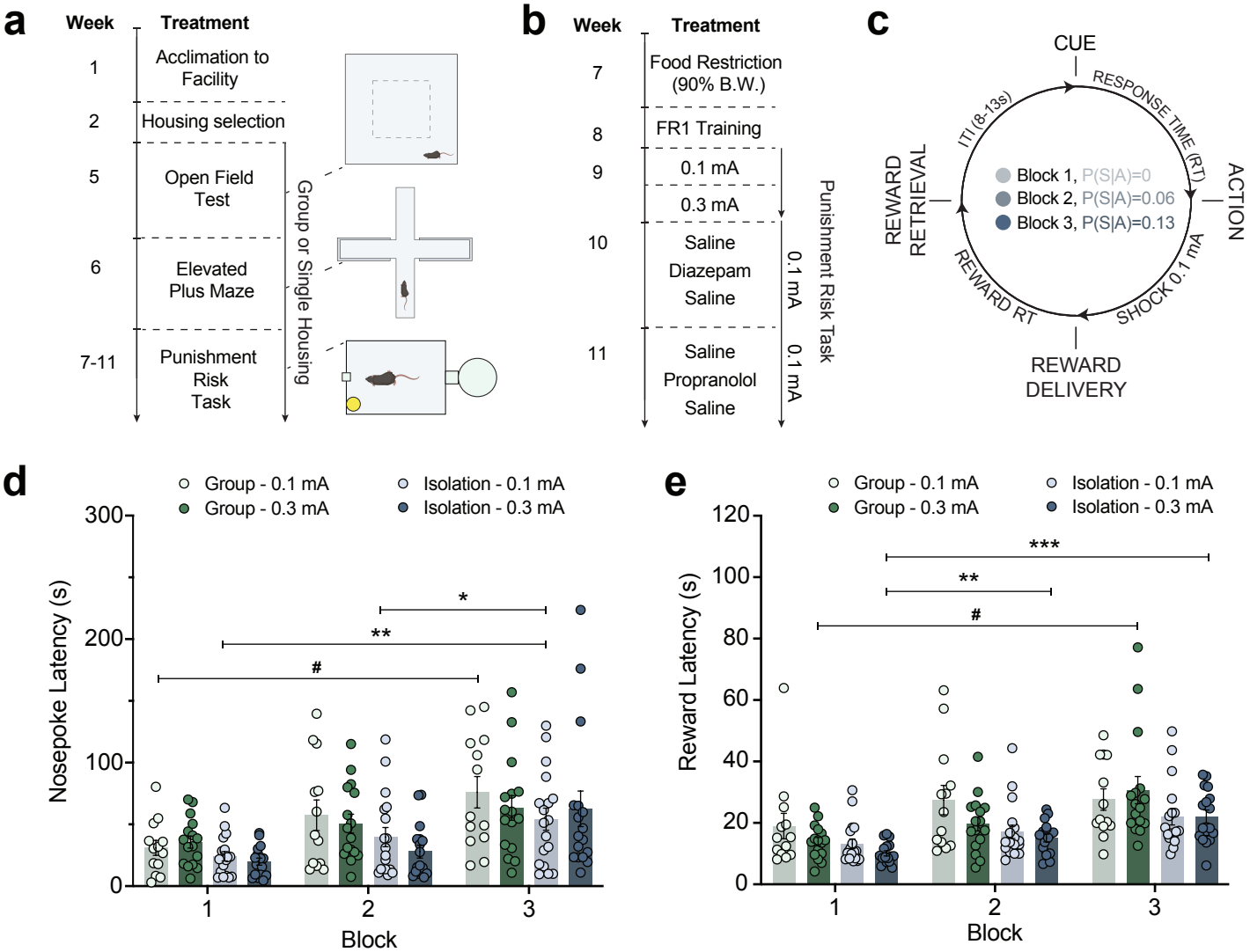

a
Week
Treatment
1
Acclimation to Facility
2
Housing selection
5
Open Field Test
6
Elevated Plus Maze
7-11
Punishment Risk Task
Group or Single Housing
b
Week
Treatment
7
Food Restriction (90% B.W.)
8
FR1 Training
9
0.1 mA
0.3 mA
10
Saline
Diazepam
Saline
11
Saline
Propranolol
Saline
0.1 mA
0.1 mA
Punishment Risk Task
c
CUE
RESPONSE TIME (RT)
ACTION
SHOCK 0.1 mA
REWARD DELIVERY
REWARD RT
REWARD RETRIEVAL
ITI (8-13s)
Block 1, P(S|A)=0
Block 2, P(S|A)=0.06
Block 3, P(S|A)=0.13
d
Group - 0.1 mA
Group - 0.3 mA
Isolation - 0.1 mA
Isolation - 0.3 mA
Nosepoke Latency (s)
Block
e
Group - 0.1 mA
Group - 0.3 mA
Isolation - 0.1 mA
Isolation - 0.3 mA
Reward Latency (s)
Block

Figure 2: Punishment risk increases caution during goal-directed actions. (a) Cartoon depicting the calendar of social isolation and behavioral testing. **(b)** Calendar for Punishment Risk Task (PRT) training and test. **(c)** Diagram depicting specifics of behavioral task (ITI: Intertrial Interval, RT: Reaction Time, P(S|A): Probability of Shock). **(d)** Increasing probability of punishment increases average latency to nosepoke following cue presentation in both group-housed and single-housed animals regardless of shock intensity (0.1 or 0.3 mA) with post hoc comparisons (Group: 0.1mA - Block 1 vs 0.1mA - Block 3, # $p < 0.05$; Isolation: 0.1mA - Block 1 vs 0.1mA - Block 3, ** $p < 0.01$; Isolation: 0.1mA - Block 2 vs 0.1mA - Block 3, * $p < 0.05$. **(e)** Increasing probability of punishment increases average latency to retrieve pellet in both group-housed and single-housed animals regardless of shock intensity, post hoc comparisons (Group: 0.3mA - Block 1 vs 0.3mA - Block 3, # $p < 0.05$; Isolation: 0.3mA - Block 1 vs 0.3mA - Block 2, ** $p < 0.01$; Isolation: 0.3mA - Block 1 vs 0.3mA - Block 3, *** $p < 0.001$).

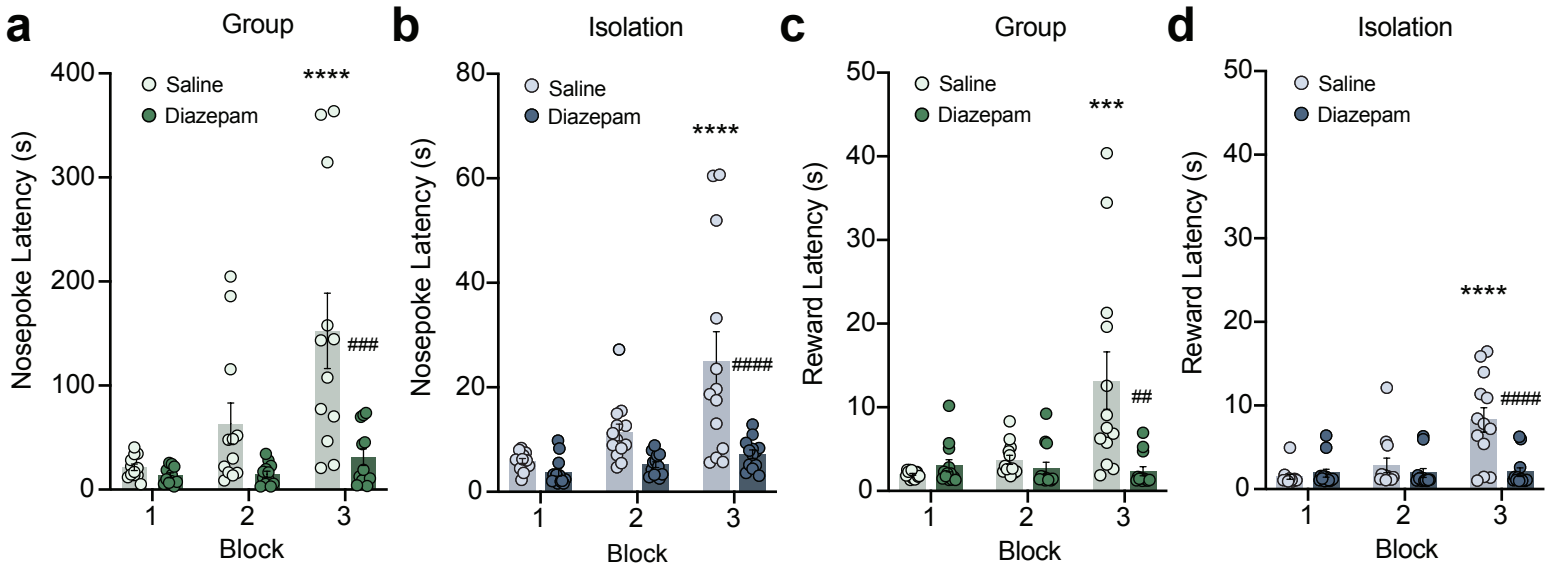

a
Group
Saline
Diazepam
Nosepoke Latency (s)
400
300
200
100
0
****
###
1
2
3
Block
b
Isolation
Saline
Diazepam
Nosepoke Latency (s)
80
60
40
20
0
****
####
1
2
3
Block
c
Group
Saline
Diazepam
Reward Latency (s)
50
40
30
20
10
0
***
##
1
2
3
Block
d
Isolation
Saline
Diazepam
Reward Latency (s)
50
40
30
20
10
0
****
####
1
2
3
Block

Figure 3: Diazepam reduces punishment risk-associated response latency during goal-directed actions (a) Diazepam (2 mg/kg) treatment reduces average latency to nosepoke among group-housed animals as compared to saline treatment; Saline - Block 1 vs Saline - Block 3, **** $p < 0.0001$; Saline - Block 3 vs. Diazepam - Block 3, #### $p < 0.0001$ **(b)** Diazepam (2mg/kg) treatment reduces average latency to nosepoke among single-housed animals as compared to saline treatment; Saline - Block 1 vs Saline - Block 3, **** $p < 0.0001$; Saline - Block 3 vs. Diazepam - Block 3, #### $p < 0.001$. Note the change in scale on y-axes between **a** & **b. (c)** Diazepam (2 mg/kg) treatment reduces average latency to retrieve reward pellet among group-housed animals as compared to saline treatment; Saline - Block 1 vs Saline - Block 3, *** $p < 0.001$; Block 3 - Saline vs. Diazepam - Block 3, ## $p < 0.01$. **(d)** Diazepam (2 mg/kg) treatment reduces average latency to retrieve reward pellet among single-housed animals as compared to saline treatment; Saline - Block 1 vs Saline - Block 3, ** $p < 0.01$; Saline - Block 3 vs. Diazepam - Block 3, # $p < 0.05$.

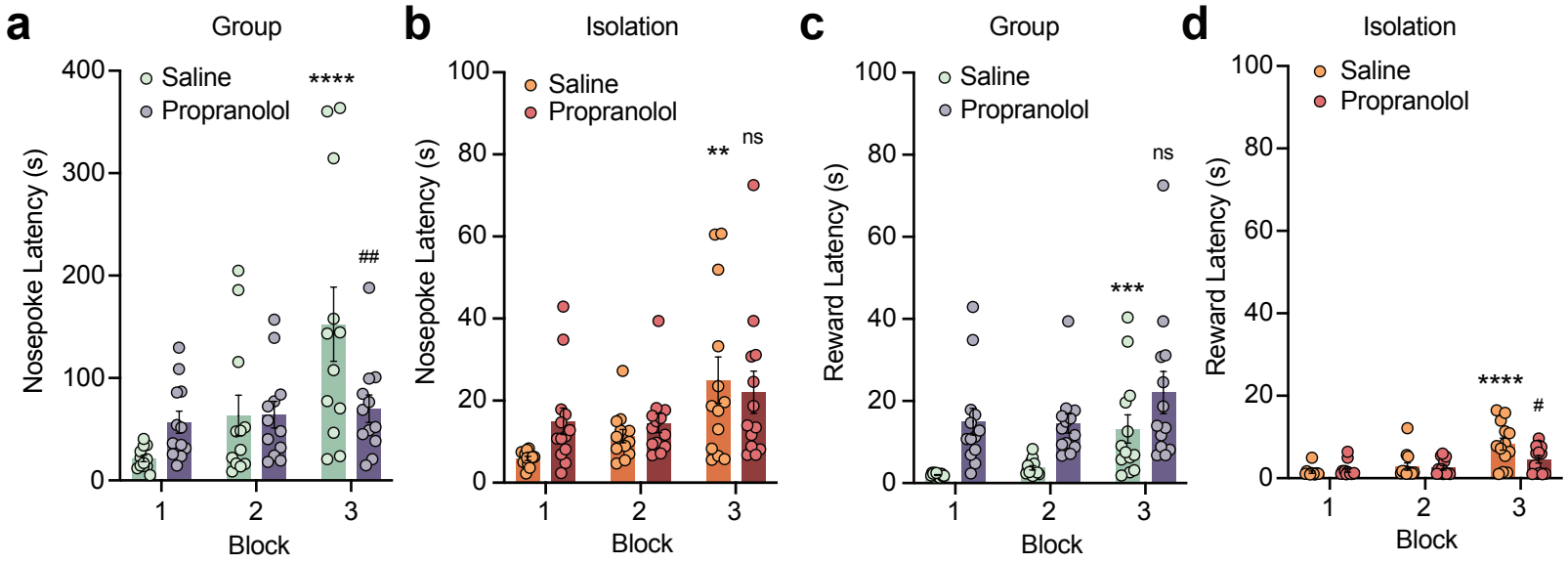

a
Group
Saline
Propranolol
Nosepoke Latency (s)
400
300
200
100
0
****
##
1
2
3
Block
b
Isolation
Saline
Propranolol
Nosepoke Latency (s)
100
80
60
40
20
0
**
ns
1
2
3
Block
c
Group
Saline
Propranolol
Reward Latency (s)
100
80
60
40
20
0
***
ns
1
2
3
Block
d
Isolation
Saline
Propranolol
Reward Latency (s)
100
80
60
40
20
0
****
#
1
2
3
Block

Figure 4: Propranolol prevents increased latency to nosepoke following increased probability of punishment in group and single-housed animals (a) Propranolol (10 mg/kg) treatment reduces average latency to nosepoke among group-housed animals as compared to saline treatment; Saline - Block 1 vs Saline - Block 3, **** $p < 0.0001$; Saline - Block 3 vs. Propranolol - Block 3, ## $p < 0.01$. Note the change in scale on y-axes between **a** and **b. (b)** Propranolol (10 mg/kg) treatment prevents increased average latency to nosepoke in single-housed animals as compared to saline treatment; Saline - Block 1 vs. Saline - Block 3, ** $p < 0.01$; Propranolol - Block 1 vs Propranolol - Block 3, ns, $p = 0.56$. **(c)** Propranolol (10 mg/kg) treatment has no effect on average latency to retrieve reward pellet among group-housed animals as compared to saline treatment; Saline - Block 1 vs Saline - Block 3, *** $p < 0.001$. d) Propranolol (10 mg/kg) treatment prevents increased average latency to retrieve reward pellet in single-housed animals as compared to saline treatment; Saline - Block 1 vs. Saline - Block 3, **** $p < 0.0001$; Propranolol – Block 1 vs Propranolol – Block 3, #, $p = 0.051$.

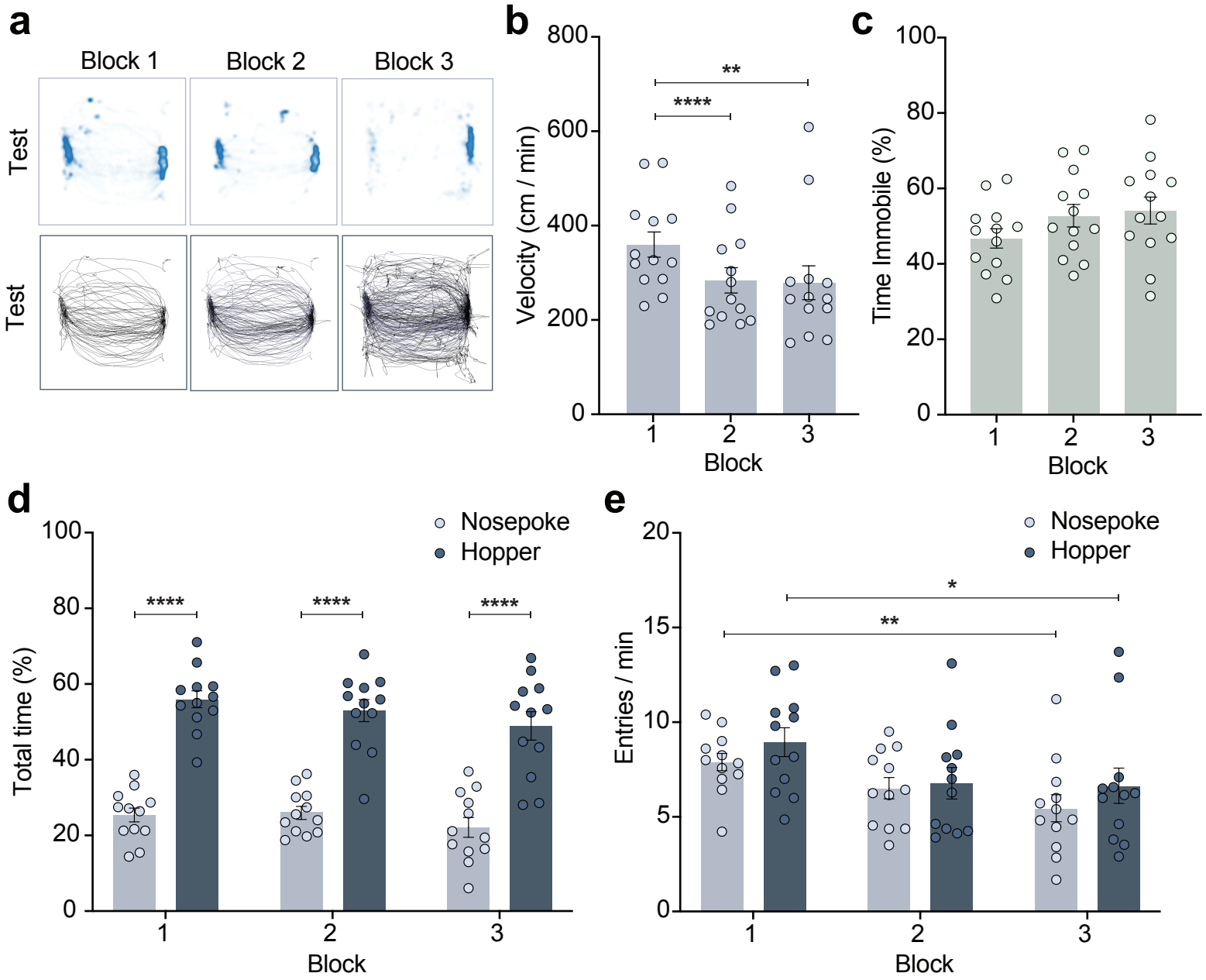

a
Block 1
Block 2
Block 3
Test
Test
b
Velocity (cm / min)
800
600
400
200
0
**
****
1
2
3
Block
c
Time Immobile (%)
100
80
60
40
20
0
1
2
3
Block
d
Total time (%)
100
80
60
40
20
0
Nosepoke
Hopper
****
****
****
1
2
3
Block
e
Entries / min
20
15
10
5
0
Nosepoke
Hopper
*
**
1
2
3
Block

Figure 5: Punishment risk and task related activity behaviors. (a) Heatmaps and traces for each block during PRT. **(b)** Average velocity reduces throughout test session (Block 1 vs Block 2, **** $p < 0.0001$; Block 1 vs Block 3, **** $p < 0.01$). **(c)** Punishment risk does not affect time spent immobile in single-housed animals (ns, $p > 0.99$). **(d)** Regardless of block, animals spend a greater percentage of session in hopper zone compared to nosepoke zone (Block 1-Nosepoke vs Block 1-Hopper, **** $p < 0.0001$; (Block 2-Nosepoke vs Block 2-Hopper, **** $p < 0.0001$; Block 3-Nosepoke vs Block 3-Hopper, **** $p < 0.0001$). **(e)** Average zone entries per minute reduces throughout test session (Block 1 Nosepoke vs Block 3 Nosepoke, **** $p < 0.0001$; Block 1-Hopper vs Block 3-Hopper, **** $p < 0.01$).

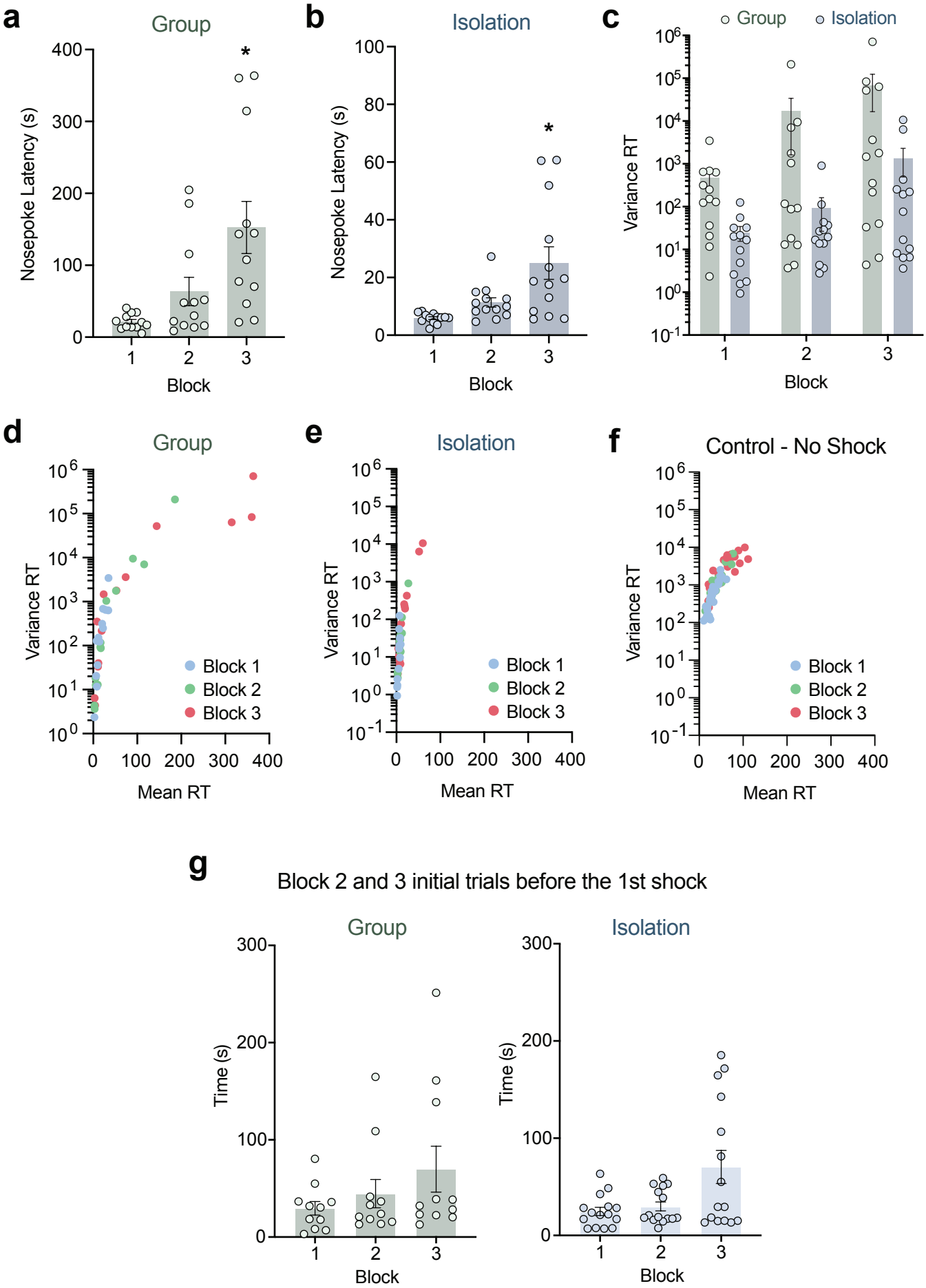
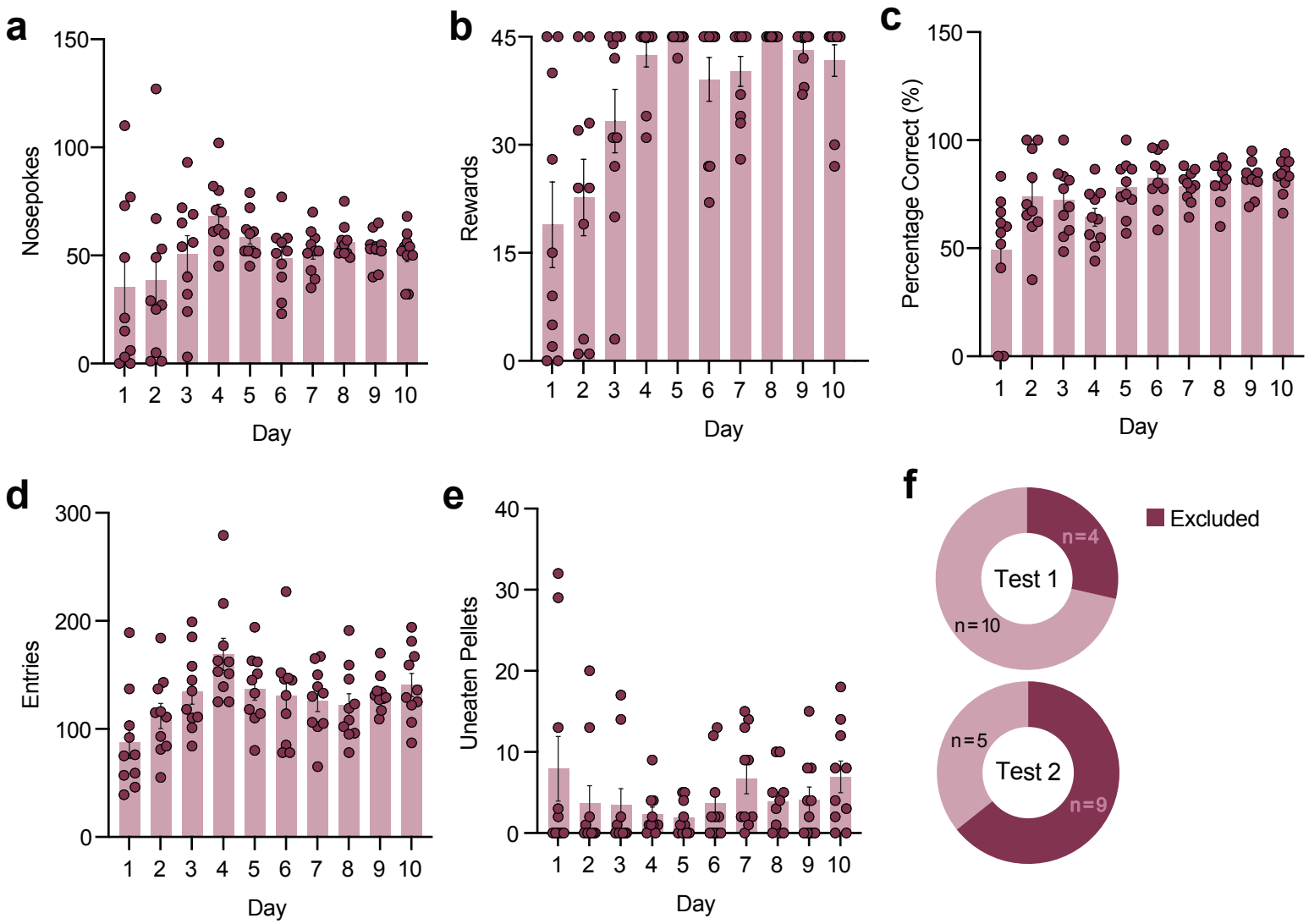
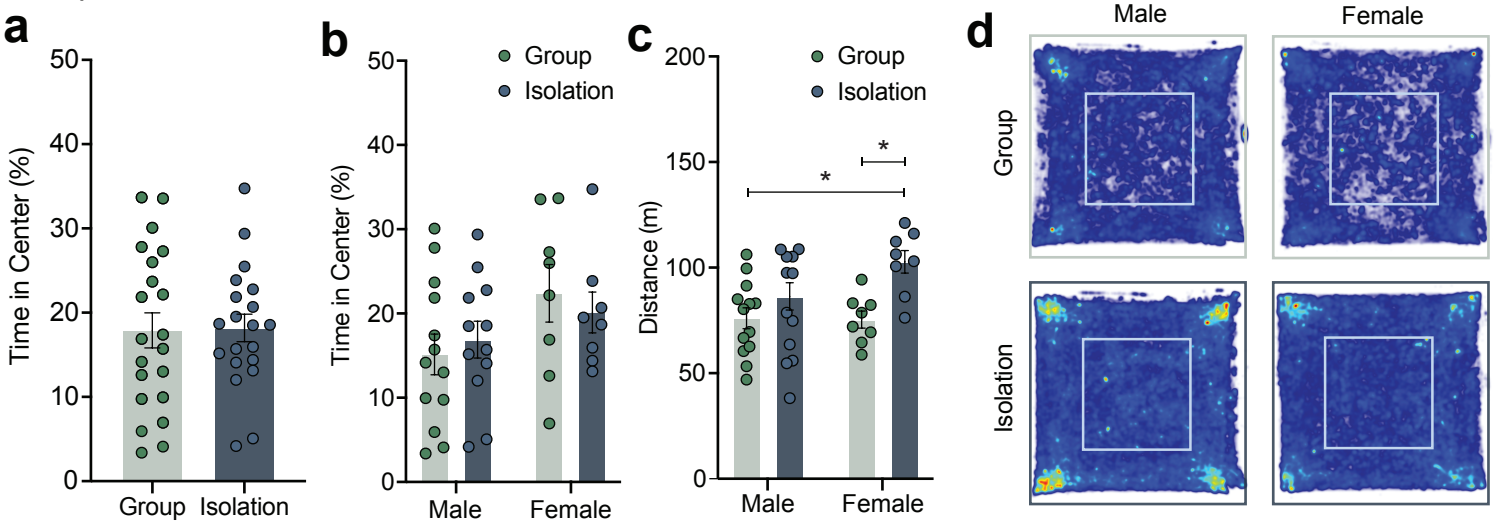

a
Group
Nosepoke Latency (s)
Block
b
Isolation
Nosepoke Latency (s)
Block
c
Group
Isolation
Variance RT
Block
d
Group
Variance RT
Mean RT
Block 1
Block 2
Block 3
e
Isolation
Variance RT
Mean RT
Block 1
Block 2
Block 3
f
Control - No Shock
Variance RT
Mean RT
Block 1
Block 2
Block 3
g
Block 2 and 3 initial trials before the 1st shock
Group
Time (s)
Block
Isolation
Time (s)
Block

Figure 6: Punishment risk increases variance for trial-to-trial nosepoke latency. (a) Increased average nosepoke latency with increasing punishment risk following saline treatment among group-housed animals (Block 1 vs Block 3, * $p < 0.05$) and **(b)** single-housed animals (Block 1 vs Block 3, * $p < 0.05$). Note change in scale for y-axes in **a** and **b**. **(c)** Average trial-to-trial variance for each block in group and single-housed animals. **(d)** Colored dots represent individual animals' variance in nosepoke latency across trials in different blocks as a function of mean nosepoke latency for group and single-housed animals. **(e)** Individual mean and variance in nosepoke latency of all animals across trials (combined group and isolation animals) and **(f)** no-shock control session for all animals. To examine whether animals predicted upcoming increase in punishment risk based on learning from previous sessions, we compared the nosepoke latency of the initial trials of Block 2 and 3 prior to the 1st shock in each block for **(g)** group and single-housed animals separately and **(h)** all animals combined. Asterisks indicate a significant increase from the Block 1 average nosepoke latency (* $p<0.05$).

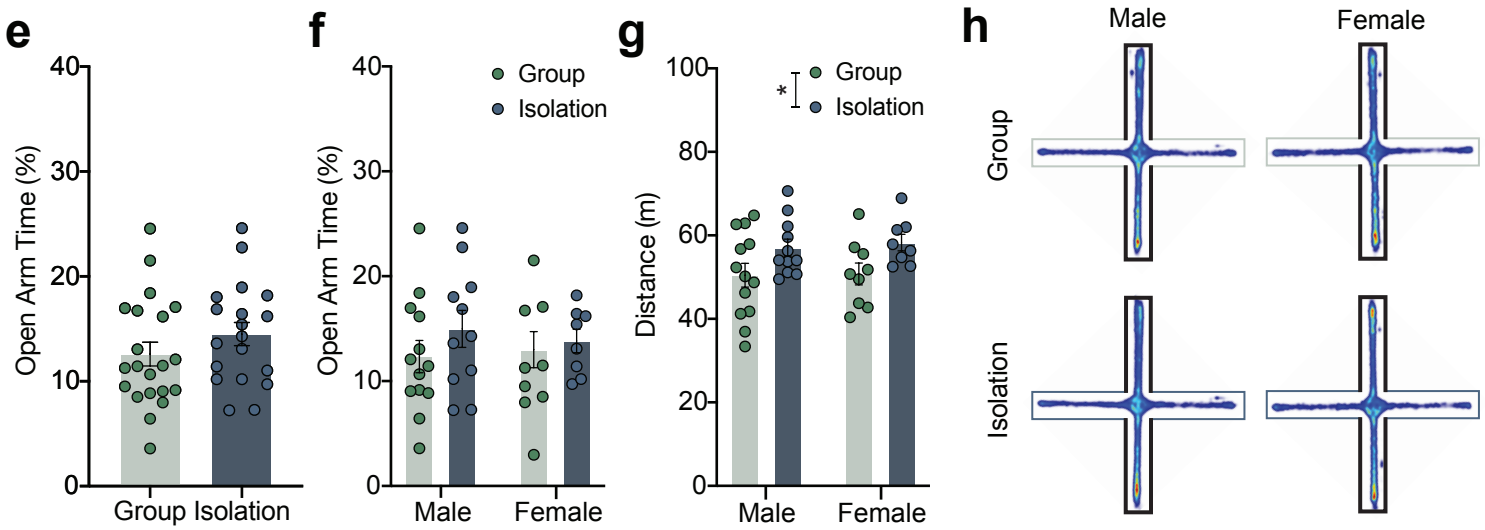

a
Nosepokes
Day
b
Rewards
Day
c
Percentage Correct (%)
Day
d
Entries
Day
e
Uneaten Pellets
Day
f
Excluded
Test 1
n=4
n=10
Test 2
n=5
n=9

Supplemental Figure 1: Animals learn cue-action contingency in FR1 training (a) Average nosepokes during training days (1-10). **(b)** Percentage of correct nosepokes (Rewarded nosepoke / total nosepokes) during training days (1-10). **(c)** Average number of pellet hopper entries during training days (1-10). **(d)** Average number of rewards received during training days (1-10). **(e)** Average number of uneaten pellets left over during training days (1-10). **(f)** Percentage of animals excluded from analysis due to incomplete PRT test (Top: Test Day 1, Bottom: Test Day 2).

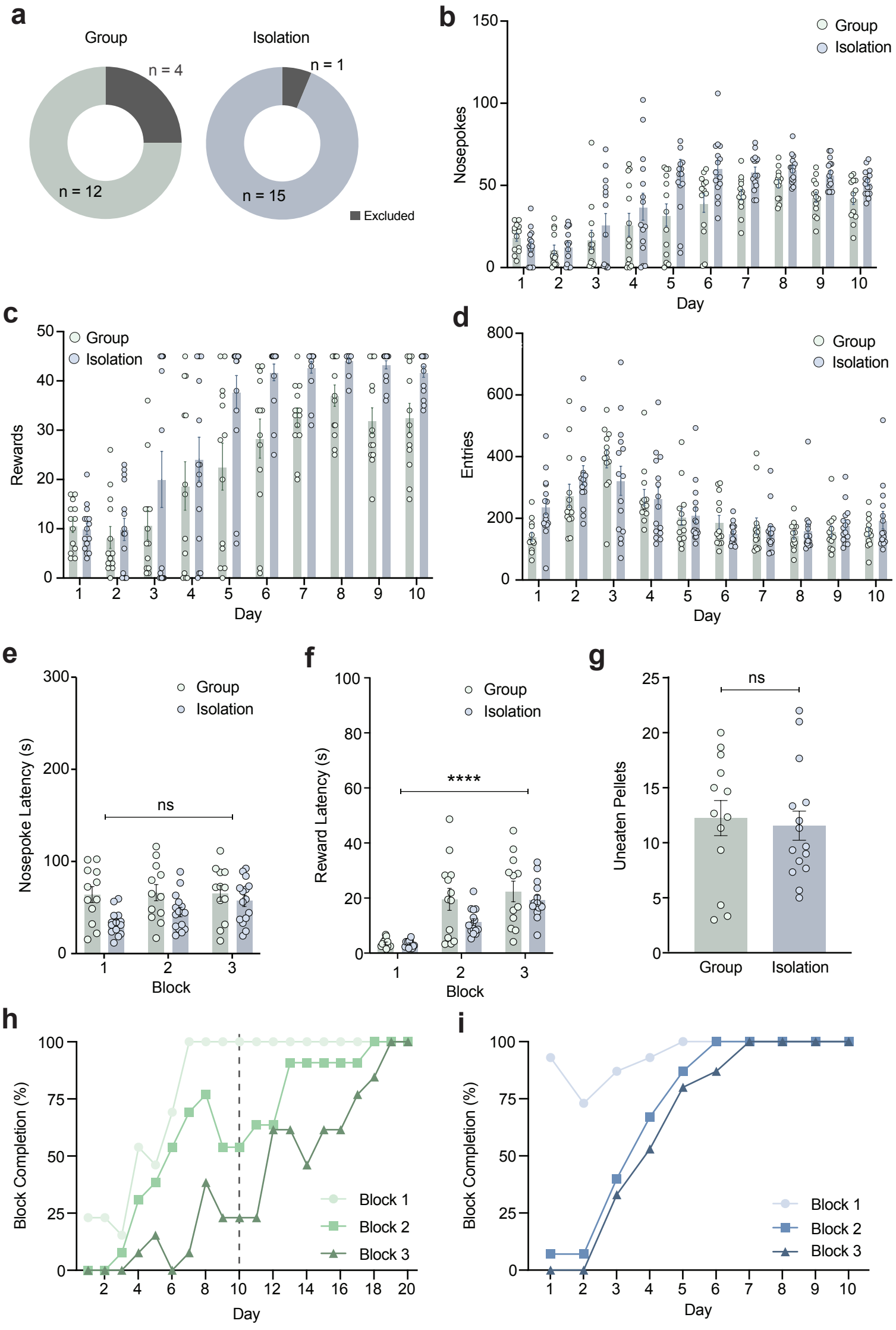
Open field Test



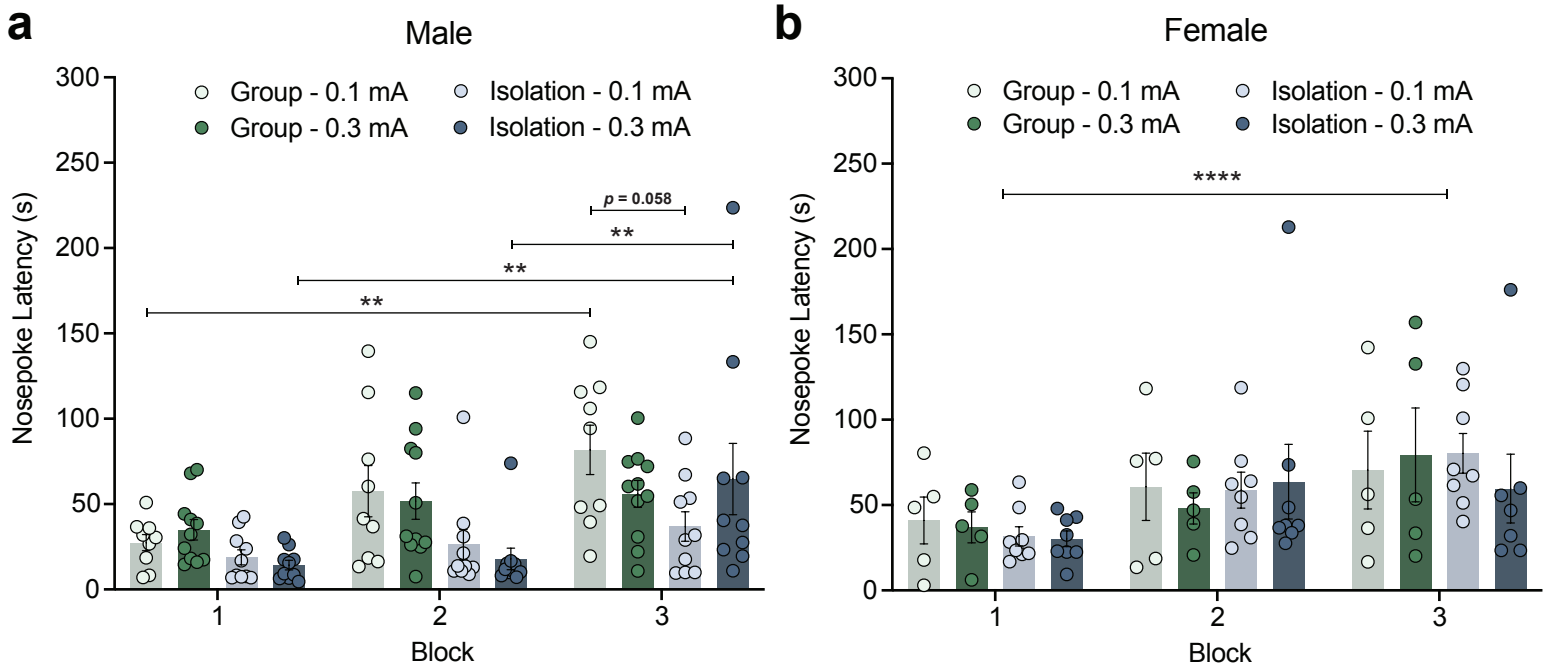
Elevated Plus Maze



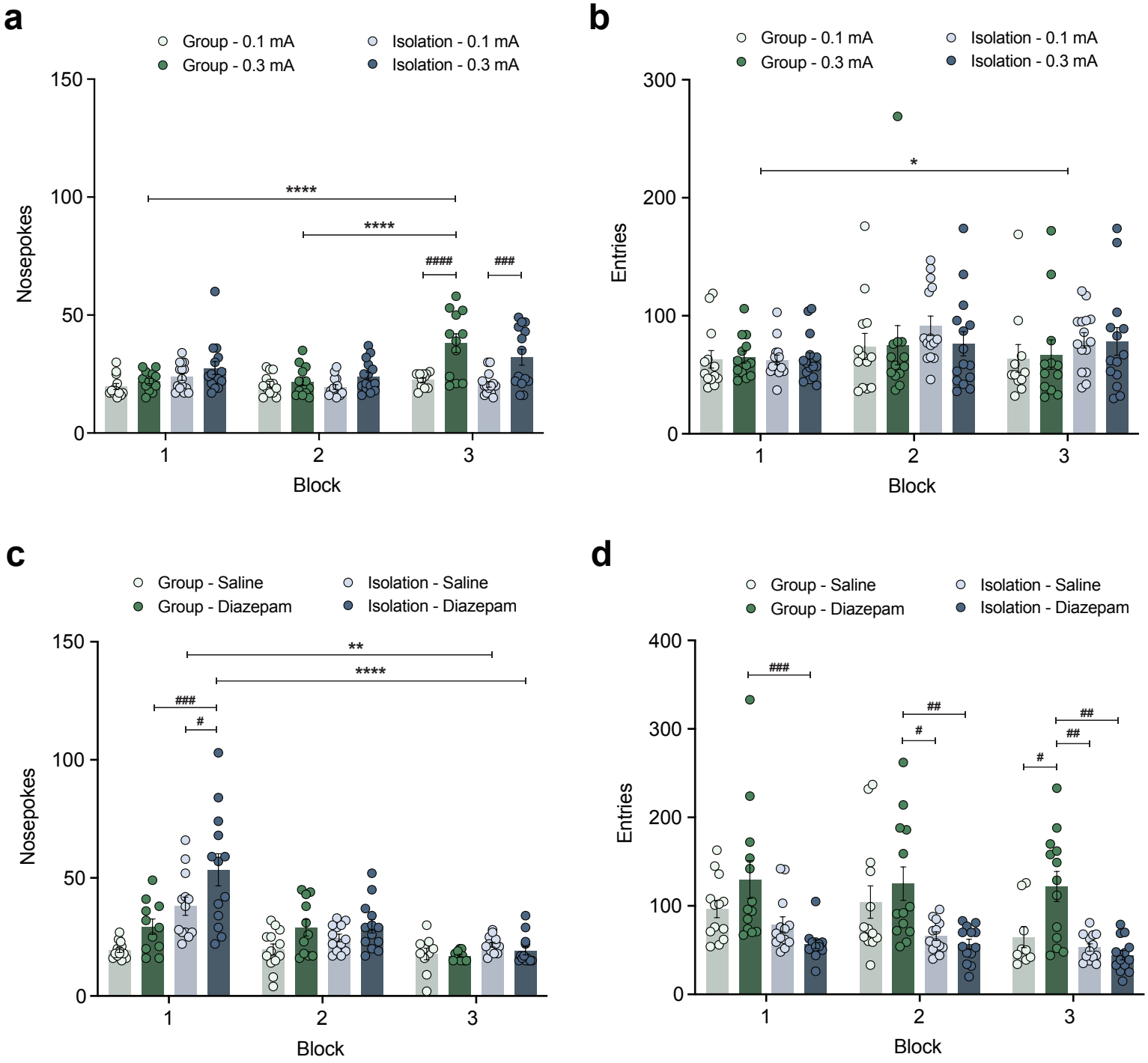
Supplemental Figure 2: Adult social isolation has minimal effect on canonical exploratory behaviors. (a) Adult social isolation does not affect center zone exploration in open field test. **(b)** Male and female mice do not differ in the percentage of time spent exploring the center zone of open field test, regardless of housing. **(c)** Single-housed female mice have greater total distance traveled in the open field test compared to group-housed male mice ($p < 0.05$) and group-housed female mice ($p < 0.05$). **(d)** Heat maps depicting average time spent in open field test for group and single-housed mice. **(e)** Social isolation does not affect open arm exploration in elevated plus maze. **(f)** Male and female mice do not differ in the percentage of time spent exploring the open arms of elevated plus maze. **(g)** Isolation housing significantly increases total distance traveled in the elevated plus maze (Group vs Isolation, * $p < 0.05$), but male and female mice are not different within these groups. **(h)** Heat maps depicting average time spent in elevated plus maze for group and single-housed mice.


a
Group
Isolation
n = 4
n = 12
n = 1
n = 15
Excluded
b
Group
Isolation
Nosepokes
Day
c
Group
Isolation
Rewards
Day
d
Group
Isolation
Entries
Day
e
Group
Isolation
ns
Nosepoke Latency (s)
Block
f
Group
Isolation
****
Reward Latency (s)
Block
g
ns
Uneaten Pellets
Group
Isolation
h
Block 1
Block 2
Block 3
Block Completion (%)
Day
i
Block 1
Block 2
Block 3
Block Completion (%)
Day

Supplementary Figure 3: Punishment risk task training in group and single-housed mice. **(a)** Pie chart displaying total number of animals that did not successfully complete PRT training and were excluded from further analysis. **(b)** Average nosepokes during training days (1-10) in group and single-housed mice. **(c)** Average number of rewards received during training days (1-10). **(d)** Average number of pellet hopper entries during training days (1-10). **(e)** Animals do not display any significant change in average nosepoke latency during "no shock" test day, $p = 0.069$. **(f)** Animals have increased average reward retrieval latency during no shock test day, Block 1 vs Block 3, $p < 0.0001$. **(g)** There is no significant difference in the average number of uneaten pellets left over during no shock test day (Group vs. Isolation, t=0.3332, df=24.44, $p = 0.7418$) **(h)** Percentage completion of blocks of rewards during training for group-housed mice. Dashed line indicates day single-housed animals completed training. **(i)** Percentage completion of blocks of rewards during training for group-housed mice.


a
Male
Group - 0.1 mA
Group - 0.3 mA
Isolation - 0.1 mA
Isolation - 0.3 mA
Nosepoke Latency (s)
300
250
200
150
100
50
0
p = 0.058
**
**
**
1
2
3
Block
b
Female
Group - 0.1 mA
Group - 0.3 mA
Isolation - 0.1 mA
Isolation - 0.3 mA
Nosepoke Latency (s)
300
250
200
150
100
50
0
****
1
2
3
Block

Supplementary Figure 4: Adult social isolation differentially affects latency to nosepoke depending on shock intensity and sex (a) Social isolation differentially affects latency to nosepoke in male mice exposed to increasing shock intensity (Group (0.1 mA) - Block 1 vs. Group (0.1 mA) - Block 3, ** $p < 0.01$; Isolation (0.3 mA) - Block 1 vs. Isolation (0.3 mA) - Block 3, ** $p < 0.01$; ** $p < 0.01$; Isolation (0.3 mA) - Block 2 vs. Isolation (0.3 mA) - Block 3, ** $p < 0.01$; Group (0.1 mA) - Block 3 vs. Isolation (0.1 mA) - Block 3. **(b)** Female mice display increased latency to nosepoke as punishment risk increases regardless of housing, but do not respond differently to changes to shock intensity ($p < 0.0001$, Block 1 vs. Block 3).


a
Group - 0.1 mA
Group - 0.3 mA
Isolation - 0.1 mA
Isolation - 0.3 mA
Nosepokes
Block
****
****
####
###
b
Entries
*
c
Group - Saline
Group - Diazepam
Isolation - Saline
Isolation - Diazepam
**
****
###
#
d
###
##
#

Supplementary Figure 5: Secondary punishment risk task behaviors are differentially affected by shock intensity and diazepam treatment (a) Greater intensity shock (0.3 mA) increases the average number of total nosepokes in Block 3 for both group-housed animals (Group - Block 1 vs. Group - Block 3, **** $p < 0.0001$; Group - Block 2 vs. Group - Block 3, **** $p < 0.0001$; Group (0.1 mA) - Block 3 vs. Group (0.3 mA) - Block 3, #### $p < 0.0001$; and single-housed animals (Isolation (0.1 mA) - Block 3 vs. Isolation (0.3 mA) - Block 3, #### $p < 0.001$). **(b)** Intensity of shock does not affect average number of pellet hopper entries for group- or single-housed animals. **(c)** Diazepam (2 mg/kg) treatment differentially affects average number of nosepokes per block for single-housed animals. Diazepam treatment increases average nosepokes for single-housed animals in Block 1 (Saline - Block 1 vs Diazepam - Block 1, # $p < 0.05$) as compared to saline treatment and as compared to group-housed animals (Diazepam (Group) - Block 1 vs Diazepam (Isolation) - Block 1, ### $p < 0.001$. Single-housed animals have greater average nosepokes in Block 1 following saline and Diazepam treatment (Saline - Block 1 vs. Saline - Block 3, ** $p < 0.01$; Diazepam - Block 1 vs Diazepam - Block 3, **** $p < 0.0001$). **(d)** Group-housed animals have higher average hopper entries per block as compared to single-housed animals. Diazepam treated group-housed animals have greater average number of food hopper entries in Block 3 (Saline (Group) - Block 3 vs. Diazepam (Group) - Block 3, # $p < 0.05$; Saline (Isolation) - Block 3 vs. Diazepam (Group) - Block 3, ## $p < 0.01$); Diazepam (Isolation) - Block 3 vs. Diazepam (Group) - Block 3, ## $p < 0.01$).