

Persistent conditioned place preference to aggression experience in adult male sexually-experienced CD-1 mice

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We recently developed a conditioned place preference (CPP) procedure, commonly used to study rewarding drug effects, to demonstrate that dominant sexually-experienced CD-1 male mice form CPP to contexts previously associated with defeating subordinate male C57BL/6J mice. Here we further characterized conditioned and unconditioned aggression behavior in CD-1 mice. In Exp. 1 we used CD-1 mice that displayed a variable spectrum of unconditioned aggressive behavior toward younger subordinate C57BL/6J intruder mice. We then trained the CD-1 mice in the CPP procedure where one context was intruder-paired, while a different context was not. We then tested for aggression CPP 1 day after training. In Exp. 2, we tested CD-1 mice for aggression CPP 1 day and 18 days after training. In Exp. 3–4, we trained the CD-1 mice to lever-press for palatable food and tested them for footshock punishment-induced suppression of food-reinforced responding. In Exp. 5, we characterized unconditioned aggression in hybrid CD-1 × C57BL/6J D1-Cre or D2-Cre F1 generation crosses. Persistent aggression CPP was observed in CD-1 mice that either immediately attacked C57BL/6J mice during all screening sessions or mice that gradually developed aggressive behavior during the screening phase. In contrast, CD-1 mice that did not attack the C57BL/6J mice during screening did not develop CPP to contexts previously paired with C57BL/6J mice. The aggressive phenotype did not predict resistance to punishment-induced suppression of food-reinforced responding. CD-1 × D1-Cre or D2-Cre F1 transgenic mice showed strong unconditioned aggression. Our study demonstrates that aggression experience causes persistent CPP and introduces transgenic mice for circuit studies of aggression.

Keywords: Aggression, CD-1, conditioned place preference, D1-Cre, D2-Cre, food self-administration, mice, punishment, reward

Received 4 April 2016, revised 23 May 2016 and 12 July 2016, accepted for publication 22 July 2016

Inappropriate aggressive behavior is a common feature of several psychiatric disorders, including drug addiction (Beck *et al.* 2014), autism (Hill *et al.* 2014), depression (Dolenc *et al.* 2015), PTSD (Miles *et al.* 2015), antisocial personality (Anderson & Kiehl 2014) and schizophrenia (Swanson *et al.* 2006). The reasons for the positive relationship between aggressive behavior and psychiatric disorders are unknown. One idea is that in some individuals, aggression toward others is highly rewarding and sustains persistent reward seeking in a manner akin to reward seeking observed with classical rewarding stimuli like palatable food, sex or abused drugs (May 2011; Moran *et al.* 2014). This idea is supported by observations that dominant mice (Fish *et al.* 2002; May & Kennedy 2009) will learn to perform an operant action (lever press or nose-poke) that will give them access to attack a subordinate mouse in a manner analogous to instrumental human aggression-seeking behavior (Elbert *et al.* 2010; Moran *et al.* 2014). Additionally, aggressive behavior in rodents increases nucleus accumbens dopamine levels (Van Erp & Miczek 2000, 2007) and local dopamine receptor blockade decreases aggression-motivated operant responding (Couppis & Kennedy 2008). These findings suggest that nucleus accumbens dopamine, which is known to mediate the rewarding effects of psychostimulant drugs and natural rewards (Wise 2004), also plays a role in aggression reward.

A common method to study the rewarding effects of drugs is the conditioned place preference (CPP; Mucha *et al.* 1982). In this Pavlovian procedure, one distinct context is paired with drug injections while another context is paired with vehicle injections. During a subsequent drug-free test, the laboratory animal chooses between the drug- and the vehicle-paired contexts. An increase in preference for the drug-paired context is commonly regarded as a measure of the drug's rewarding effects [but see Stephens *et al.* (2010) for a critical discussion of learning processes controlling behavior in the CPP procedure].

Based on earlier studies in female Syrian hamsters (Meisel & Joppa 1994) and male OF-1 mice (Martinez *et al.* 1995), we recently adapted a CPP procedure in combination with a social defeat procedure to study aggression reward in CD-1 mice (Golden *et al.* 2016). In this study, we first used the social defeat procedure (Kudryavtseva *et al.* 1991; Miczek &

O'Donnell 1978) to characterize unconditioned aggression of older dominant adult male sexually-experienced CD-1 mice toward younger submissive C57BL/6J male mice during daily sessions. Next, we performed aggression CPP training during which we repeatedly placed the CD-1 mice in both contexts of the CPP apparatus but only introduced a C57BL/6J mouse to one of those contexts, resulting in two distinct contexts: intruder-paired and intruder-unpaired. One day after CPP training, we tested the CD-1 mice for the expression of aggression CPP; during testing, the mice had access to both contexts. We found that CD-1 mice that initially exhibited unconditioned attack of the C57BL/6J mouse during the screening phase (~70% of the mice) developed CPP, while those that did not attack during screening (~30%) did not form a CPP. We also found that projections from basal forebrain (predominately originating from the lateral septum, diagonal band and medial nucleus accumbens shell) to lateral habenula play an important role in modulating this aggression CPP (Golden *et al.* 2016).

Here, we further characterize conditioned and unconditioned aggression behavior in sexually-experienced CD-1 mice. In Exp. 1, we performed a more detailed analysis of the nature of the individual differences in aggression reward by studying CPP in a third group of mice that we term *Variable-aggressive* mice (about 25% of the sample) that initially do not show aggressive behavior but across repeated daily screening sessions become more aggressive. We compared the behavior of these mice to those of *Aggressive* mice (about 50%) that show highly aggressive behavior during all screening sessions and *Non-aggressive* mice (~25%) that do not show aggression. In Exp. 2, we tested all three groups for the persistence of aggression CPP to determine whether the learned behavior will persist for several weeks after CPP training, as is the case with drugs like cocaine and morphine (Mueller & Stewart 2000; Mueller *et al.* 2002). In Exp. 3 and 4, we tested whether aggression CPP is correlated with resistance to punishment-induced suppression of food-reinforced responding, with the prediction that the two behaviors would be correlated, because resistance to punishment has been previously used as a measure of compulsive reward seeking (Deroche-Gamonet *et al.* 2004; Pelloux *et al.* 2007). We assessed resistance to operant punishment (contingent shock) using a procedure we previously used in rats to determine the motivation to seek palatable food, methamphetamine and alcohol during daily sessions in which we gradually increase foot-shock intensity (Krasnova *et al.* 2014; Marchant *et al.* 2013). We performed the punishment procedure after training the CD-1 mice to lever-press for palatable, high-carbohydrate content food pellets. Finally, in Exp. 5, we studied whether the F1 generation offspring of breeding CD-1 with D1- and D2-Cre C57BL/6J transgenic mice would demonstrate robust unconditioned aggression in order to determine the feasibility of using these mice in future studies on pharmacological and circuit mechanisms of conditioned and unconditioned aggression.

Materials and methods

Subjects

We used male ~40g 4–6 month old sexually-experienced CD-1 mice ($n=112$, Charles River Labs, CRL), ~30g C57BL/6J ($n=9$, Jackson Laboratories), ~30g D1-Cre ($n=10$, C57BL/6J background, bred in house), ~30g D2-Cre ($n=8$, C57BL/6J background, bred in house) and ~35g F1 hybrid offspring of CD-1 $\times$ D1-Cre ($n=38$, bred in house) and CD-1 $\times$ D2-Cre ($n=32$, bred in house) as the experimental subjects. We confirmed with CRL animal facility staff that all sexually-experienced CD-1 mice had equal access to receptive females. Specifically, CRL begins pair-housing male CD-1 mice with several females (harem breeding) at PD28, and then continually keeps male CD-1 mice group-housed with the receptive females until they are purchased. Pregnant females are switched with new non-pregnant females, with no break between cycles. Male CD-1 mice that do not successfully breed are removed from the breeding pool and not made available for purchase. We bred the transgenic and F1-hybrid mice by crossing a D1- or D2-Cre male (on C57BL/6J background) with either 2 C57BL/6J or CD-1 females (harem breeding), respectively. We weaned the pups between 3 and 4 weeks after birth and group-housed them. We genotyped and group-housed the mice with their wildtype littermates until 3 weeks before screening at age 12–16 weeks. Then we singly housed each mouse for one night. The day after, under identical conditions, we paired C57BL/6J, transgenic C57BL/6J or F1 hybrids with a sexually-naïve female (C57BL/6J or mixed background) for 20 days. The strain of the paired female mouse was dependent on the availability of sexually-naïve female mice in the Mount Sinai animal colony. We removed the females 3 days before screening. We used non-experimental ~20g 6–12-week old male intruder C57BL/6J (paired with CD-1 and F1 hybrid residents; Jackson Labs) or ~20g BALB/cBy (paired with C57BL/6J and D1-Cre/D2-Cre residents; Jackson Labs) mice for aggression screening and CPP acquisition.

We gave all mice free access to standard food chow and water in all experiments. We singly-housed all experimental mice with enrichment (cotton padding) upon arrival in standard clear polycarbonate cages covered with a stainless steel wire lid and maintained them on a reverse 12-h light/dark cycle (light off at 0800 h). We group-housed the non-experimental C57BL/6J or BALB/cBy intruder mice 4 per cage, under identical housing conditions as the experimental mice. We performed all experiments in accordance with the *Guide for the Care and Use of Laboratory Animals* (8th edition; 2011), under protocols approved by the Animal Care and Use Committee of our Institutes (Icahn School of Medicine at Mount Sinai, Exp. 1 and 5, IRP-NIDA, Exp. 2–4). We excluded 5 CD-1 mice which failed to acquire food self-administration.

Resident-intruder confrontations (screening)

We determined baseline unconditioned aggression in mice by video-recording and analyzing attack behavior in a variant of the resident-intruder task (Kudryavtseva *et al.* 1991; Miczek & O'Donnell 1978; Miczek *et al.* 1984). Each screening session consisted of placing one C57BL/6J or BALB/cBy intruder into the home-cage of a resident mouse, and allowing social interaction to ensue for 5 min (CD-1 residents) or 10 min (C57BL/6J residents) under dim white-light conditions; the intruder mouse was always a novel unfamiliar mouse. We paired CD-1 and F1 hybrid residents with C57BL/6J intruders, and C57BL/6J and transgenic C57BL/6J residents with BALB/cBy intruders for technical and behavioral reasons. Specifically, CD-1 and F1 hybrid mice have lighter fur while C57BL/6J and transgenic C57BL/6J mice have darker fur, and therefore pairing them with an intruder of a notably different fur color allows for an easier discrimination between the resident and intruder mice during behavioral scoring. Additionally, the social defeat literature (Krishnan *et al.* 2007) and our own experience (Golden *et al.* 2011, 2016) indicate that younger, smaller subordinate C57BL/6J intruders do not aggress back on residents. From discussions with other researchers, suggesting that even young CD-1 mice can be relatively aggressive, we chose to use an alternative light-coated intruder strain in conjunction with black-coated C57BL/6J mice. We piloted aggression behavior

in younger, smaller BALB/cBy and found that these mice do not show aggression against older, larger C57BL/6J mice. Based on this observation, we have used them as intruders for resident-intruder testing. We manually recorded the latency to the first attack bout. In the absence of attacks, we scored latency as 300 or 600 seconds (maximum duration of the session). We video recorded all sessions using cameras mounted above the test cages.

We performed resident-intruder assays for either 3 (Exp. 2 and 3) or 10 (Exp. 1) consecutive days. In Exp. 5, we screened all mice for 3 consecutive days, except for the CD-1 × D2-Cre F1 hybrid cohort, which we screened for only 2 days, because of highly aggressive behavior that injured the intruder mice. In Exp. 1, we tested 56 CD-1 mice for unconditioned aggression phenotype on days 1 through 3. We then selected the 14 most aggressive and 14 least aggressive mice for continued screening on days 4 through 10. We classified these mice as either Non-aggressive (failed to attack on any days), Variable-aggressive (failed to attack consistently on days 1 through 3, but acquired aggressive behavior thereafter), and Aggressive (attacked on all days). In Exp. 2 we tested 36 CD-1 mice, and categorized all mice into the same groups: Non-aggressive, Variable-aggressive and Aggressive. In Exp. 5, we tested 60 CD-1 mice, 9 C57BL/6J, 10 D1-Cre, 8 D2-Cre, 38 CD-1 × D1-Cre and 32 CD-1 × D2-Cre.

Aggression CPP

We performed aggression CPP as previously described (Golden et al. 2016). Briefly, we performed place conditioning in custom-built three-chamber CPP boxes with video tracking (CleverSys). Each box consisted of two main chambers separated by a smaller middle chamber, passable via manually removable guillotine-style doors. The two side chambers differed in various contextual features, including visual features (black-and-white stripes vs. uniform gray coloring) and floor design (parallel bars vs. metal grid), while the center chamber had a solid floor with clear Plexiglas siding. We recorded all behaviors from above using color digital video cameras paired with CaptureStar software (CleverSys) under red-light conditions, and analyzed CPP data with TopScanLite behavioral tracking software (CleverSys).

During the CPP pretest and test sessions, we allowed the mice to explore the full three-chambered box for 20 min. For pretest, we analyzed time-spent on either side of the box to determine initial side preference. We used an 'unbiased' CPP procedure (Van Der Kooy 1987) in which we matched the mice in the different groups for their pre-training (term herein pretest) time in the subsequent intruder- and non-intruder-paired side; and within each group, we counterbalanced the sides ('stripe' or 'gray') assigned to the intruder and non-intruder pairing such that the group mean was as close to zero as possible. For pretest and test sessions, we defined CPP score as the difference between the time spent in the intruder-paired side minus the time spent in intruder-unpaired side, such that a positive score indicates preference and a negative score indicates aversion to the intruder-paired side. In Exp. 1, we tested the mice 1 day following CPP training. In Exp. 2, we repeatedly tested the mice on 1 and 18 days after CPP training. We subsequently used the mice from Exp. 2 for food self-administration training and punishment in Exp. 3.

During CPP training, we performed two counterbalanced conditioning sessions per day (10 min each, morning and afternoon) for either 3 (Exp. 1) or 4 (Exp. 2) days. During these sessions, we placed the CD-1 mice in either the intruder-paired side (where we introduced a novel C57BL/6J intruder mouse) or in the intruder-unpaired side (where the CD-1 mice were left alone for 10 min). We scored latency to initial attack during intruder-pairings. If an initially Non-aggressive CD-1 mouse displayed latency to attack behavior comparable to Aggressive mice (repeatedly <300 seconds), we reassigned the mouse to the Aggression phenotype. After observing changes in aggression behavior during CPP training, we reassigned four Non-aggressive CD-1 mice to the Aggression group in Exp. 1.

Food preference testing

Prior to the food self-administration training, we tested all mice for food pellet flavor preference as previously described (Calu et al. 2014). We performed food preference testing in a 42 × 42 × 42 cm³ clear

Plexiglas open field chamber. We directly compared four uniquely flavored 20-mg food pellets [TestDiet; Catalog no. 1811142 – 12.7% fat, 66.7% carbohydrate, 20.6% protein; standard (vanilla flavored), chocolate, banana and bacon]. Following a 1-week acclimation period to the animal housing facility, we exposed the mice to each individual flavor at the onset of the dark cycle in the homecage for 24 h for 4 consecutive days, with the order of flavor exposure counterbalanced across days. On test day, we placed 20 pellets of each unique flavor in each corner of the open field chamber. The mice could explore and consume the pellets for unlimited time under dim light. We ended the test when the mice consumed all 20 pellets of one flavor. We then counted the leftover pellets of each flavor. CD-1 mice displayed preference for standard flavor food pellets (data not shown), which we subsequently used for operant food self-administration training.

Operant self-administration of palatable food

We used standard mouse operant chambers from Med Associates. Each chamber was enclosed in a ventilated sound-attenuating cubicle and illuminated by a house light on the opposite side of the active lever and cue light. We equipped the chambers with two levers located 2.4 cm above the grid floor: one retractable lever (designated as 'active') and one non-retractable lever (designated as 'inactive'). Presses on the retractable active lever resulted in the delivery of a single 20 mg food pellet and a compound tone/light cue, while presses on the stationary inactive lever had no programmed consequences. We positioned the cue light (bright yellow LED) and the speaker (2900 Hz, 20 dB above background) above the active lever. A pellet dispenser delivered food pellets into a receptacle located 1.0 cm above the grid floor. We connected the grid floor to a shock generator (Med Associates) and manually calibrated the current using an amp-meter prior to all punishment sessions.

The training procedure for palatable food self-administration in mice was similar to those used in our previous studies in rats (Caprioli et al. 2015a, 2015b; Krasnova et al. 2014). This phase began 24 h after the final food preference test. We initially gave all mice two 1-h magazine training sessions during which they non-contingently received a 20-mg food pellet every 120 seconds; pellet delivery was paired with a 2-second tone-light cue. Following magazine training, we trained the mice for ten 1-h self-administration sessions under a fixed-ratio-1 (FR-1) 20-second timeout reinforcement schedule (or more formally a fixed-interval 20 reinforcement schedule, FI20). Presses on the active lever resulted in the delivery of a single food pellet paired with a 2-second tone-light cue. After each reinforced lever press, a 20-second timeout period ensued during which the program recorded active lever presses but did not reinforce them. Presses on the inactive lever were recorded but had no programmed consequences.

Punishment-induced suppression of food-reinforced responding

The punishment procedure is based on our previous studies using rats (Marchant et al. 2013, 2014, 2016). In Exp. 3, we exposed three groups (Non-aggressive, $n=10$; Variable aggressive, $n=8$; Aggressive, $n=16$) of CD-1 mice (total $n=34$) to contingent footshock. In Exp. 4, we exposed CD-1 ($n=17$ total) mice to either contingent ($n=8$) or yoked non-contingent ($n=9$) footshock. Mice in the contingent groups received footshock that was triggered by the operant active-lever responding, while those in the non-contingent group received yoked footshock that was independent of their lever-pressing behavior.

The punishment phase consisted of nine daily 1-h food self-administration sessions, containing two sub-phases, using the same FR-1 20-second timeout schedule of reinforcement used during self-administration training. During punishment sessions 1–6, we increased daily the shock intensity at increments of 0.05 mA, starting at 0.05 mA (session 1). During punishment sessions 7–9, we kept the shock intensity constant at 0.35 mA. In Exp. 3 in the contingent punishment group, 50% of reinforced lever-presses resulted in a 0.5-second footshock that was delivered through the grid floor. For Exp. 4, we yoked mice in the non-contingent group to the contingent-condition mice with similar baseline food self-administration responding. When a contingently-punished

mouse received footshock punishment, the program simultaneously delivered the footshock to the yoked mouse in the non-contingent group. For both groups, the punished and unpunished reinforced responses resulted in the 2-second tone-light cue and the delivery of one 20-mg palatable food pellet. We performed Exp. 4 in CD1 mice that were not screened for aggression to confirm that suppression of lever responding in our punishment procedure is not due to non-selective interference with operant responding by footshock exposure (Bouton & Schepers 2015).

Shock sensitivity thresholds

This phase began 24 h following the completion of the punishment phase and consisted of three once-daily test sessions. During each session, we brought the mice to their operant chambers and exposed them to ascending-intensity, non-contingent 1-second footshocks (0.05–0.35 mA, 0.05 mA increments separated by ~60-second). We characterized five different stereotyped behavioral outcomes, in response to the acute footshocks, with the following description: no effect, head orientation towards the grid, paw lifted from the grid, startle and escape jump. Each of these behaviors was given a value of 0–4, respectively, allowing for a quantitative analysis of acute footshock sensitivity (Evans 1961).

Statistical analysis

We analyzed the data with ANOVAs or independent *t*-tests using SPSS (GLM procedure) or Prism. We followed significant main effects and interaction effects ($P < 0.05$) with post-hoc tests (Fisher PLSD). Because our multifactorial ANOVAs yielded multiple main and interaction effects, we only report significant effects that are critical for data interpretation. We indicate results of post-hoc analyses by asterisks in the figures but they are not described in the Results section.

Results

Aggression phenotype stability and aggression CPP 1 day after CPP training (Exp. 1)

The goal of Exp. 1 was to measure the stability of the unconditioned aggression phenotypes and their ability to predict subsequent aggression CPP. We initially observed in a cohort of 56 CD-1 mice that 48% exhibited robust unconditioned aggression (Aggression phenotype), 27% exhibited variable unconditioned aggression (Variable aggression phenotype), and 25% failed to exhibit any aggression (Non-aggressive phenotype) across 3 days of 3-min screening sessions (Fig. 1a,b). From these groups, we randomly selected 14 Non-aggressive and purposely selected the 14 most Aggressive mice for testing phenotype stability, by extending the unconditioned aggression screening to a total of 10 sessions. We observed that all Aggressive mice continued to express robust aggression, quantified as the percent of stable aggression behavior as the dependent variable, while 50% of the Non-aggressive mice switched to the Variable aggression phenotype by session 10 (Fig. 1c). The Log-rank Mantel–Cox test for survival revealed that this trend represents a significant change in the stability curves ($\chi^2 = 9.1$, $P < 0.01$).

For both the Variable-aggressive and Aggressive mice, attack latency decreased across screening sessions (Fig. 1d). We analyzed the attack latency using a repeated measures mixed ANOVA with the between-subject factors of Aggression phenotype (Non-aggressive, Variable-aggressive, Aggressive) and the within-subject factor of Screening session (sessions 1–10). This analysis showed a significant

interaction between the two factors ($F_{18,225} = 7.0$, $P < 0.01$). Post-hoc group differences within each session are shown in Fig. 1d.

We then tested the three aggression phenotypes ($n = 7$ per group; all remaining Non-aggressors, all newly identified Variable-aggressors and seven randomly selected Aggressors) for aggression reward CPP. We found robust expression of aggression CPP 1 day after CPP training in the Variable-aggressive and Aggressive mice. In contrast, the Non-aggressive mice showed conditioned place aversion (CPA) to the intruder-paired context (Fig. 1e, represented heat maps shown in Fig. 1f). We analyzed the data with a repeated measures mixed ANOVA, using the between-subjects factor of Aggression phenotype and the within-subjects factor of CPP test session (pretest, CPP test). This analysis showed a significant interaction between the two factors ($F_{2,18} = 7.8$, $P < 0.01$). A post-hoc one-way ANOVA of CPP Pretest score (Paired side–Unpaired side) showed no significant difference between groups ($P > 0.1$). A post-hoc one-way ANOVA of CPP Test score showed a significant effect of aggression phenotype ($F_{2,18} = 13.5$, $P < 0.01$). Post-hoc group differences within each session are shown in Fig. 1e. Finally, additional characterization of the behavior of the mice from the three groups during the CPP test is provided in Fig. S1a–c, Supporting Information.

Persistence of aggression CPP after training (Exp. 2)

The goal of Exp. 2 was to characterize the persistence of aggression CPP after CPP training. We observed in a cohort of 36 CD-1 mice that 44% exhibited robust unconditioned aggression (Aggressive), 28% exhibited variable unconditioned aggression (Variable aggressive), and 28% failed to exhibit any aggression (Non-aggressive) across the 3 days of 3-min screening sessions (Fig. 2a,b). Based on the results of Exp. 1, we scored attack latency behavior across CPP acquisition sessions. We found that the Aggressive mice consistently attacked with lower latency than the Variable aggressive mice, while the latency of the non-aggressive mice, which rarely attacked and never with a latency below 300 seconds, was very high (Fig. 2c). The repeated measures mixed ANOVA, which included the between-subject factor of aggression phenotype and the within-subject factor of CPP training session, showed a significant effect of aggression phenotype ($F_{6,99} = 2.6$, $P < 0.05$). Post-hoc group differences within each session are shown in Fig. 2c. We next tested the three groups for the expression of aggression CPP 1 day or 18 days after CPP training. We found robust expression of aggression CPP 1 day and 18 days after CPP training in the Variable-aggressive and Aggressive mice but not the Non-aggressive mice (Fig. 2d, representative heat maps shown in Fig. 2f). We analyzed the data with a mixed ANOVA using the between-subjects factor of Aggression phenotype and the within-subjects factor of CPP test session (pretest, day 1 and day 18). This analysis showed a significant interaction between the two factors ($F_{4,66} = 6.5$, $P < 0.01$). A post-hoc one-way ANOVA of CPP Pretest score (Paired side–Unpaired side) showed no significant difference between groups ($P > 0.1$). A post-hoc one-way ANOVA of CPP test within each day showed a significant effect of

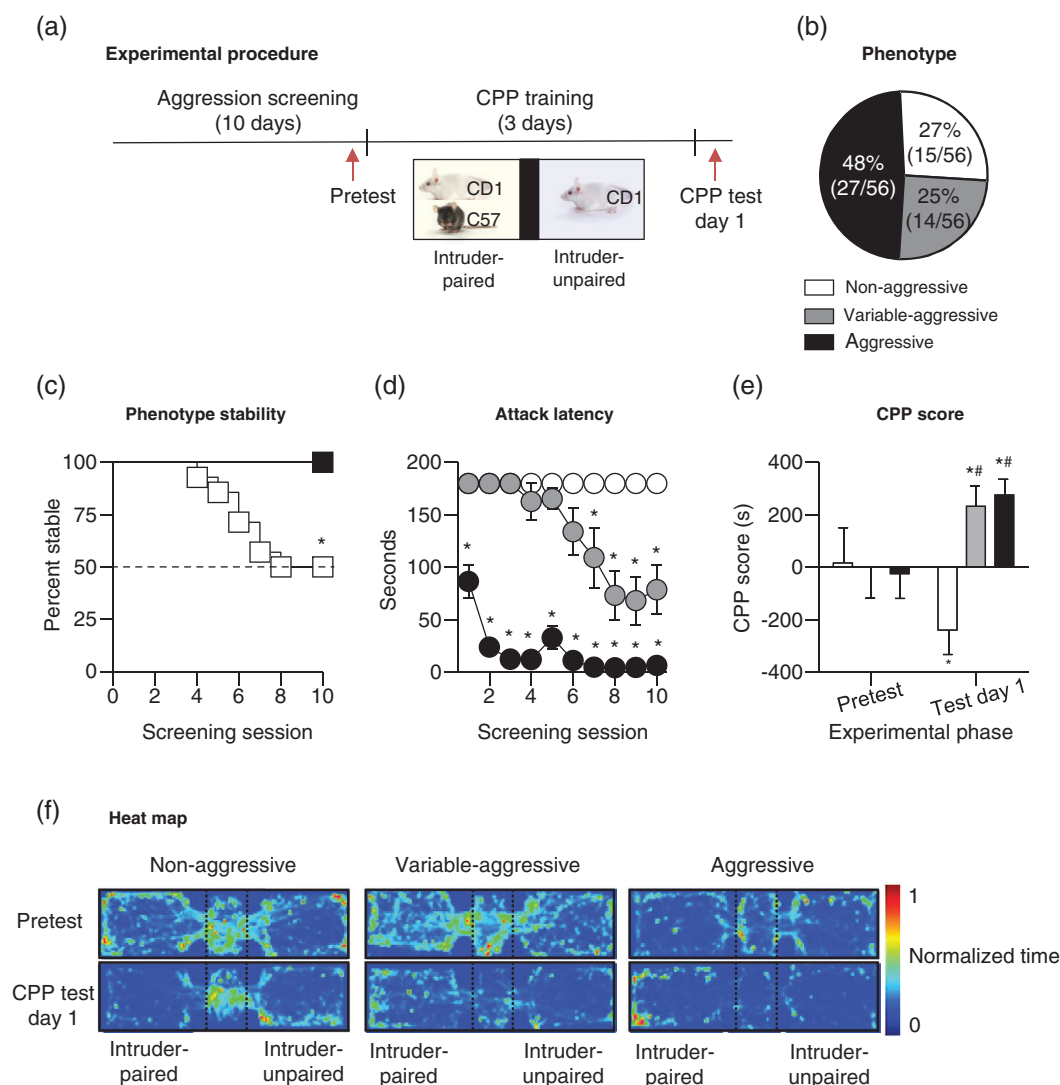


Figure 1: Stability of the aggression CPP phenotype. (a) Experimental time course of the aggression conditioned place preference (CPP) procedure. (b) Aggression phenotype distribution of CD-1 mice ($n=56$) after 3 days of aggression screening (3-min sessions). (c) Stability plot of the Aggressive ($n=14$) and Non-aggressive ($n=14$) phenotypes across 10 screening days (3-min sessions). The 14 most aggressive and 14 least aggressive mice, as determined by the initial 3 screening days, were used from the full cohort. Non-aggressive mice that began to display aggression were subsequently classified as Variable-aggressive. (d) Latency to first attack bout (Non-aggressive, $n=7$; Variable aggressive, $n=7$; Aggressive, $n=14$) across the 10 screening sessions. (e) CPP scores ($n=7$ per group) on Pretest and Day 1 Test sessions (20-min sessions). (f) Representative heat-maps of aggression CPP test behavior. All data are represented as mean $\pm$ SEM. *Different from Non-aggressive during the CPP test session, $P < 0.05$. # Different from Pretest within each group, $P < 0.05$.

aggression phenotype: Day 1 ($F_{2,33} = 14.3$, $P < 0.01$) and Day 18 ($F_{2,33} = 7.6$, $P < 0.01$). Additionally, individual CPP scores on Day 1 and Day 18 were significantly correlated (Fig. 2e; Pearson $r = 0.51$, $P < 0.01$). Finally, the Non-aggressive mice showed an approaching significant trend for CPA to the intruder-paired context on day 1 ($t_9 = 2.2$, $P = 0.056$) but not day 18 ($t_9 = 0.51$, $P = 0.62$). Additional characterization of the behavior of the mice from the three groups during the CPP test and retest is provided in Fig. S1a–c.

Punishment-induced suppression of food-reinforced responding (Exp. 3–4)

The goal of Exp. 4 was to determine if the aggression CPP phenotype can predict sensitivity to punishment-induced suppression of a highly palatable food reward. We trained the same mice used in Exp. 2 for food self-administration; the mice received free access to the regular animal facility food in their homecage. Independent of aggression phenotype, CD-1 mice reliably learned the food self-administration

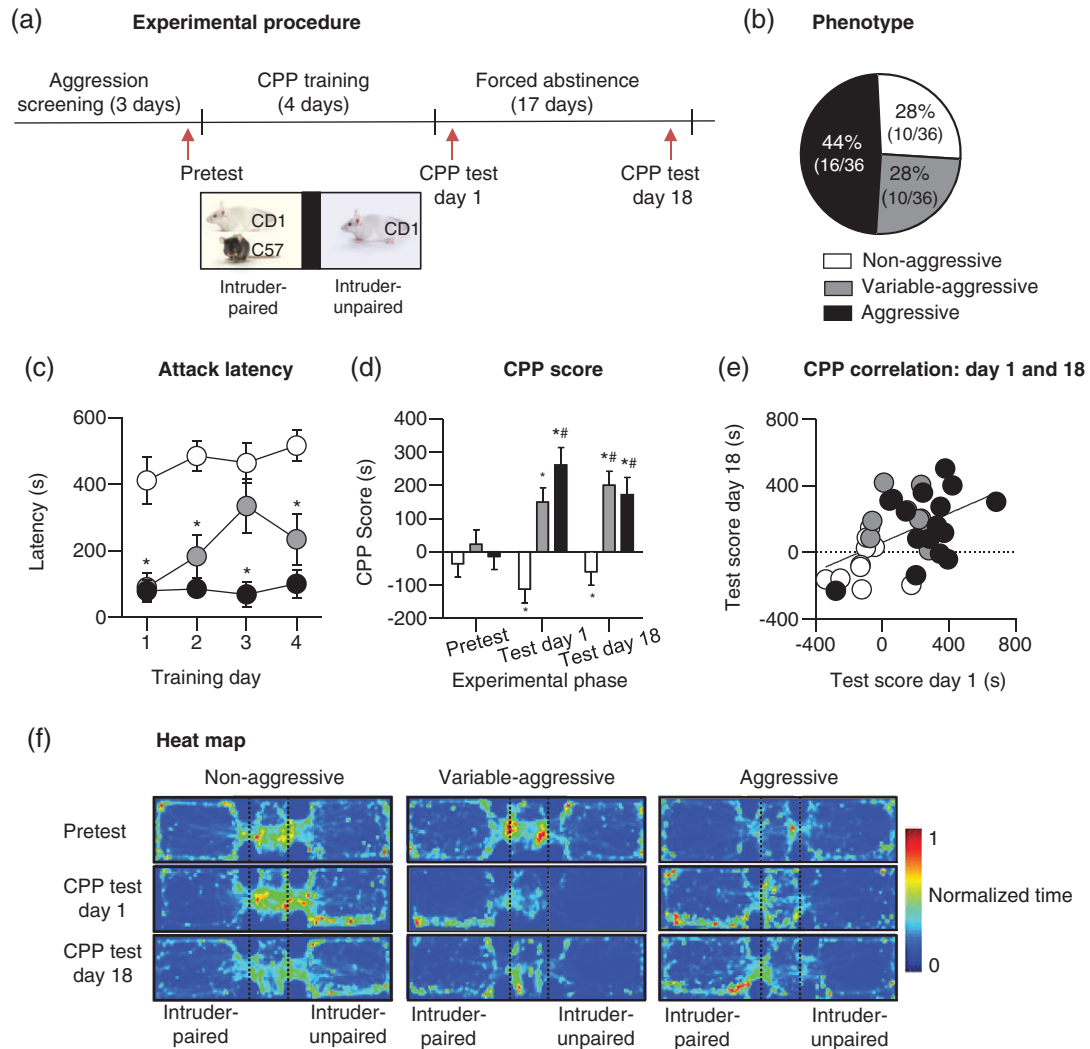


Figure 2: Persistence of the aggression CPP phenotype. (a) Experimental time course of aggression CPP procedure. (b) Aggression phenotype distribution of CD-1 mice ($n=36$) after 3 days of aggression screening (3-min sessions). (c) Latency to first attack bout (Non-aggressive, $n=10$; Variable aggressive, $n=10$; Aggressive, $n=16$) across the four CPP training sessions acquisition (10 min) sessions. (d) CPP scores of the three groups on Pretest, CPP Test Day 1 and CPP Test Day 18 (20-min sessions). (e) Correlation of individual CPP scores from CPP Test Day 1 and Test Day 18 (f) Representative heat-maps of aggression CPP test behavior. All data except for (e) are represented as mean $\pm$ SEM. *Different from Non-aggressive during the CPP test sessions, $P < 0.05$. #Different from Pretest within each group, $P < 0.05$.

task and increased their number of pellets delivered per session across training (Fig. 3a). The mixed ANOVA, which included the between-subjects factor of Aggression phenotype (Non-aggressive, Variable-aggressive, Aggressive) and the within-subjects factor of Training session (1–10), showed a main effect of Training session ($F_{9,279} = 25.2$, $P < 0.01$) but not of Aggressive phenotype or an interaction between the two factors ($P > 0.1$). Analysis of the total number of lever presses during training on the active and inactive levers, which included the between-subjects factor of Aggression phenotype and the within-subjects factors of Training session and Lever (active, inactive), showed a

significant interaction between Training session and Lever ($F_{9,279} = 6.2$, $P < 0.01$) but no effect of Aggression phenotype or interactions between Aggression phenotype and Training day or Lever ($P > 0.1$). Finally, Fig. 3a (right) shows the latency to the first lever press during the training phase.

During the punishment phase, all mice decreased their lever presses for food with increasing shock intensity over days, and there were no aggression phenotype differences in punishment-induced suppression of food-reinforced responding (Fig. 3b; $P > 0.05$). The statistical analysis of the number of food pellets showed a significant effect of Punishment session (sessions 1–7 in which shock level was

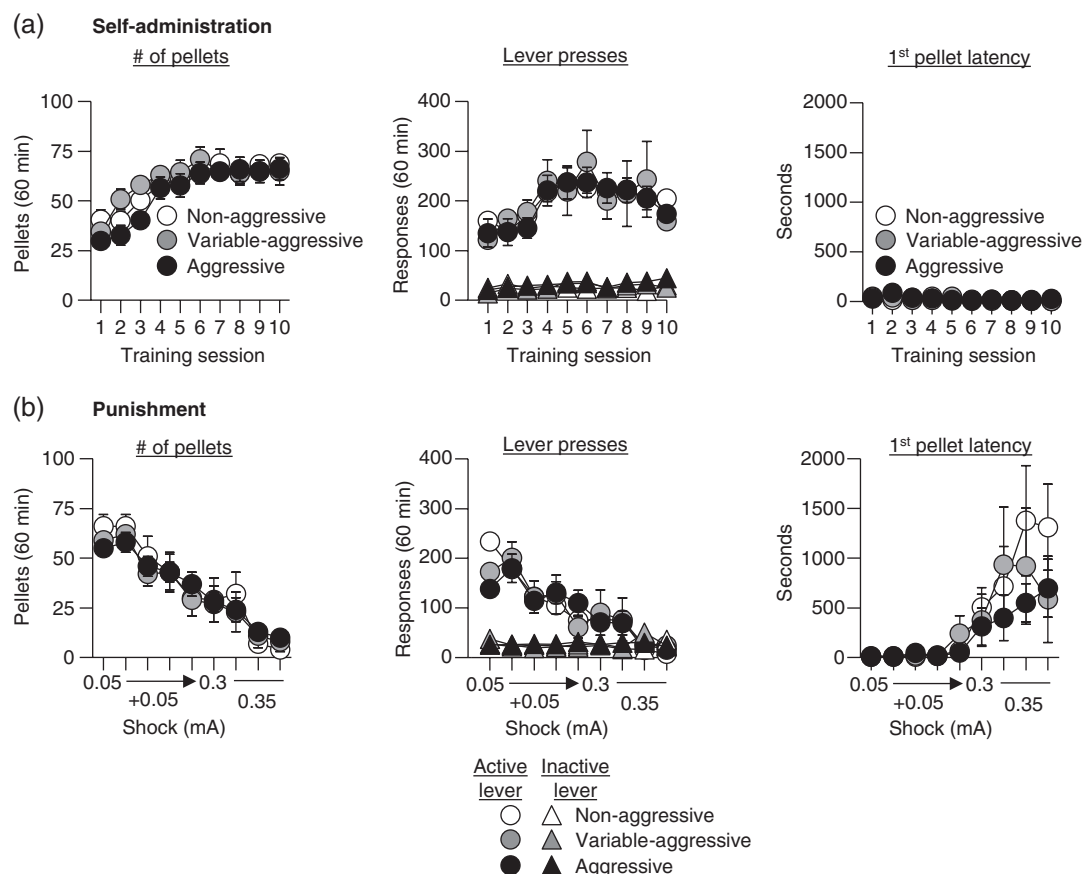


Figure 3: Food self-administration and punishment-induced suppression of food-reinforced responding in Non-aggressive, Variable-aggressive, and Aggressive CD-1 mice. (a) Number of palatable pellets earned (left), total active and inactive lever presses (middle), and latency to first pellet earned in a session (right) in CD-1 ($n=34$) mice over 10 consecutive days (one 1-h session/day) of food self-administration training under a fixed-ratio-1 (FR-1) 20-second timeout reinforcement schedule. (b) Reinforced active lever presses (left), total active and inactive lever presses (middle), and latency to first reinforced active lever press within a session (right) over 9 consecutive days (one 1-h session/day) of punishment. The mice received ascending contingent shock intensities on sessions 1–7 (0.05 mA increase/day), and 0.35 mA on sessions 8–9 on 50% of reinforced active lever presses. All data are represented as mean $\pm$ SEM.

increased) ($F_{6,186} = 24.9$, $P < 0.01$) but no effect of Aggression phenotype nor an interaction between the two factors ($P > 0.1$). The statistical analysis of the number of active and inactive lever presses showed a significant effect of Punishment session ($F_{6,186} = 14.1$, $P < 0.01$) and Punishment session by Lever ($F_{6,186} = 12.9$, $P < 0.01$) but no effect of Aggression phenotype or interactions between the Aggression phenotype and Punishment session or Lever ($P > 0.1$). We also analyzed the latency to the first lever press and this analysis showed a significant effect of Punishment session ($F_{6,186} = 8.2$, $P < 0.01$) but no effect of Aggression phenotype or an interaction between the two factors ($P > 0.1$) (Fig. 3c).

In Exp. 4 we used aggression-naïve food-trained CD-1 mice ($n=17$) to ascertain whether the suppression of lever responding observed in Exp. 3 is dependent on the response-contingency of footshock or on the punishment contingencies. After food self-administration training (Fig. 4a), we divided the mice into contingent (punishment

condition) and non-contingent yoked shock exposure. We found that contingent, but not non-contingent, shock exposure decreased food-reinforced responding (Fig. 4b). The statistical analysis of pellets delivered, which included the between-subjects factor of Shock contingency and the within-subjects factor of Shock intensity, showed a significant interaction between the two factors ($F_{8,120} = 17.8$, $P < 0.01$). The statistical analysis of active and inactive lever presses, which included the between-subjects factor of Shock contingency, and the within-subjects factors of Shock intensity and Lever, showed a significant interaction between the three factors ($F_{6,90} = 10.7$, $P < 0.01$). The analysis of the latency to the first lever press showed a significant interaction between Shock contingency and Shock intensity ($F_{8,120} = 3.2$, $P < 0.01$). These data indicate that under our experimental conditions, suppression of food-reinforced responding is dependent on the contingency of the punishment manipulation and is not driven by non-specific

suppression of lever responding by exposure to a footshock stressor.

Finally, at the end of Exp. 4 we verified that the group differences between the contingent and non-contingent shock conditions are not due to changes in shock sensitivity (Fig. 4c, see *Materials and methods* section for a description of the shock sensitivity measurement procedure).

Unconditioned aggressive behavior in inbred, outbred, and F1 hybrid offspring of CD1 and transgenic C57BL/6J D1-Cre and D2-Cre mice (Exp. 5)

The goal of Exp. 5 was to establish baseline differences in unconditioned aggressive behavior between commonly used inbred transgenic *Cre*-line C57BL/6J mice to determine the feasibility of using these mice in future studies characterizing circuits controlling unconditioned and conditioned aggressive behavior. We first compared 4–6 month-old sexually-experienced male C57BL/6J mice to 4–6 month-old sexually-experienced male CD-1 mice in the resident-intruder screening procedure. We initially observed that C57BL/6J mice exhibited much longer latency to initial attack bout than CD-1 mice (Fig. 5a, left), and therefore extended their screening sessions to 10 min (indicated by blue dashed line) from the standard 5 min (indicated by red dashed line) to account for their lower aggressive behavior. We analyzed the mean attack latency across screening days using the factor of Strain (C57BL/6J, CD-1). This analysis showed significant strain differences ($t_{67} = 8.2$, $P < 0.01$).

This low level of aggression (latency to attack frequently > 10 min) in C57BL/6J is incompatible with the aggression CPP procedure. Therefore, we generated F1 offspring from commonly used D1-Cre and D2-Cre transgenic mice (backcrossed to C57BL/6J mice) crossed with outbred CD-1 mice to determine if the F1 progeny would exhibit aggression levels more similar to C57BL/6J or CD-1 strains. Both F1 CD-1 $\times$ D1-Cre (Fig. 5, middle) and F1 CD-1 $\times$ D2-Cre (Fig. 5, right) mice exhibited aggression levels similar to outbred CD-1 mice, and substantially lower latency to initial attack than the F1 D1-Cre $\times$ C57BL/6J and F1 D2-Cre $\times$ C57BL/6J mice. We analyzed the mean attack latency across screening days using a two-tailed unpaired *t*-test for both F1 hybrids (D1-Cre, CD-1 $\times$ D1-Cre; D2-Cre, CD-1 $\times$ D2-Cre). This analysis showed a significant difference between the original D1-Cre strain vs. the CD-1 $\times$ D1-Cre hybrids ($t_{46} = 9.4$, $P < 0.01$) and D2-Cre strain vs. CD-1 $\times$ D2-Cre hybrids ($t_{38} = 13.8$, $P < 0.01$).

Discussion

We recently developed an experimental procedure that combines the resident-intruder procedure with the CPP procedure to demonstrate that a subset of dominant CD-1 male mice form CPP to a context previously associated with attacking and defeating subordinate C57BL/6J mice (Golden *et al.* 2016). Here we further characterized this aggression CPP phenomenon. We report four main findings. First, after repeated daily exposure to a C57BL/6J intruder mouse, a

subset (~50%) of originally non-aggressive CD-1 mice begin to exhibit unconditioned aggression and subsequently form aggression CPP that is similar to that observed with mice that are highly aggressive during all resident-intruder sessions. These findings support the ‘winner effect’, which suggests that once aggression successfully results in a victorious social encounter, an animal is more likely to continue successful aggressive bouts (Oyegbile & Marler 2005). Second, once an aggression CPP is formed it persists for several weeks. Third, aggression CPP is not associated with sensitivity to punishment-induced suppression of an operant response reinforced with a palatable food reward. Finally, F1 transgenic mice derived from crossing outbred CD1 mice with transgenic inbred D1- and D2-Cre mice show strong unconditioned aggression. Taken together, we conclude that aggression CPP is both stable and persistent, but does not predict resistance to punishment of an operant response maintained by palatable food reward. Additionally, we introduce a simple breeding procedure for generating transgenic D1-Cre and D2-Cre $\times$ CD1 mice for future circuit studies of unconditioned and conditioned aggression.

Appetitive aggression reward in rodents

Our observations that a subset of CD-1 mice form robust and persistent CPP to an intruder-paired context support the notion that aggression acts can be appetitive in nature and are rewarding (May 2011; Moran *et al.* 2014). Our data extend early studies on the formation of aggression CPP in female Syrian hamsters (Meisel & Joppa 1994) or male OF1 mice (Martinez *et al.* 1995) to a context paired with an intruder male. The results of the latter study, however, are difficult to interpret because the aggression CPP response was variable and was only observed when a biased CPP protocol was employed. The notion of appetitive aggression reward is also supported by results from operant conditioning studies in which male mice (Falkner *et al.* 2016; Fish *et al.* 2002; May & Kennedy 2009) perform an operant response reinforced by access to an intruder that can be attacked.

What brain mechanisms control appetitive aggression reward? Seminal work on unconditioned (innate) aggression identified the hypothalamus as a critical brain region underlying aggressive behavior in cats (Glusman & Roizin 1960; Macdonnell & Flynn 1964), rats (Bandler 1969) and non-human primates (Lipp & Hunsperger 1978). Collectively, the midbrain hypothalamic brain regions contributing to aggression have been termed the hypothalamic attack area (HAA), spanning from the mediobasal hypothalamus to the lateral ventromedial nucleus (VMH) (Toth *et al.* 2010). The majority of studies on brain mechanisms of aggression have used variations of the resident-intruder task (Kudryavtseva *et al.* 1991; Miczek & O’donnell 1978). However, from the perspective of learned aggression reward, a limitation of this procedure is that it is unknown whether the observed aggressive behavior toward an intruder is reactive/defense or appetitive (May 2011). Indeed, recent findings on the hypothalamic circuitry underlying aggression suggest that distinct brain circuits control these two forms of aggression: the dorsomedial VMH is implicated in controlling defensive behaviors (Silva *et al.* 2013), while the ventrolateral VMH is

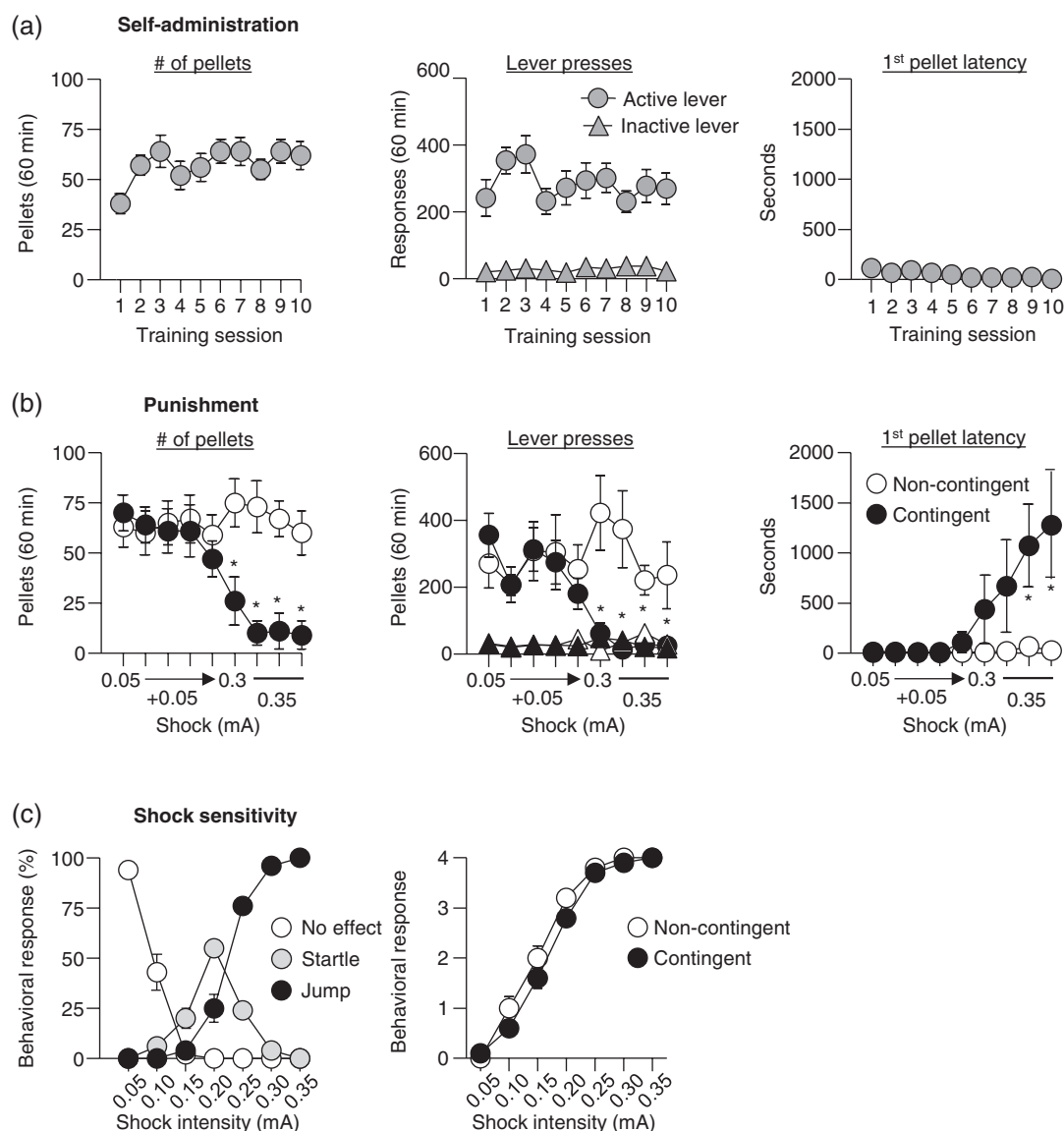


Figure 4: Food self-administration, punishment-induced suppression of food-reinforced responding, and shock sensitivity in CD-1 mice. (a) Number of palatable pellets earned (left), total active and inactive lever presses (middle), and latency to first pellet earned within a session (right) in CD-1 ($n = 17$) mice over 10 consecutive (one 1-h session/day) days of food self-administration training. (b) Reinforced active lever presses (left), total active and inactive lever presses (middle), and latency to first reinforced active lever press within a session (right) over 9 consecutive days (one 1-h session/day) of punishment. The rats received ascending contingent ($n = 8$) or non-contingent ($n = 9$) shock intensities on sessions 1–7 (0.05 mA increase/day) and 0.35 mA on sessions 8–9. (c) (Left) Proportions of stereotyped behavioral response to ascending, unconditioned footshock exposure in CD-1 ($n = 17$) mice. (Right) Baseline behavioral responses (scored as 1–4 in increasing intensity of response) in the previously contingent ($n = 8$) and non-contingent ($n = 9$) groups; data were collected after last day of punishment. All data are represented as mean $\pm$ SEM. *Different from the non-contingent shock group, $P < 0.05$.

implicated in appetitive operant aggression (Falkner *et al.* 2016). There is also evidence that inhibitory basal forebrain projections to the lateral habenula (Golden *et al.* 2016) and nucleus accumbens dopamine D1- and D2-family receptors (Couppis & Kennedy 2008) play a role in appetitive learned aggression reward.

Methodological considerations

Several issues should be considered in interpretation of the present data. One issue is whether our findings in aggressive outbred CD-1 mice generalize to other outbred or inbred strains of mice. We (Golden *et al.* 2016), and others (Couppis & Kennedy 2008; de Almeida & Miczek 2002; Falkner *et al.*

A. Unconditioned attack latency

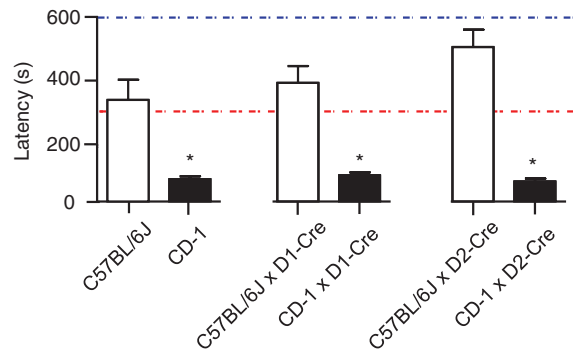


Figure 5: Comparison of unconditioned aggression in inbred C57BL/6J, outbred CD-1 and F1 crossing of C57BL/6J and CD-1 with D1-Cre and D2-Cre mice. (a) Mean latency to first attack bout (C57BL/6J, $n=9$; CD-1, $n=60$; C57BL/6J x D1-Cre, $n=10$; CD-1 x D1-Cre, $n=38$; C57BL/6J x D2-Cre, $n=8$; CD-1 x D2-Cre, $n=32$) across two to three screening sessions. C57BL/6J cohorts were screened for 10 min each day, indicated by the blue line, and CD-1 cohorts were screened for 5 min each day, indicated by the red line (see text). All data are represented as mean $\pm$ SEM. *Significant, $P < 0.05$.

2016; Fish *et al.* 2002; May & Kennedy 2009) have used the CD-1 (or similar CFW) outbred strain, because they are more aggressive than the commonly used inbred C57BL/6J strain. Towards the goal of extending use of the aggression CPP procedure from outbred CD-1 mice to inbred transgenic lines, we examined unconditioned aggression in the resident-intruder task with inbred C57BL/6J mice, and D1- and D2-Cre mice crossed on a C57BL/6J background. However, even with sexual experience, we observed low level of aggressive behavior in these transgenic mice, which is incompatible with the aggression CPP procedure. Inbred mice do exhibit low levels of unconditioned aggression, and with the addition of social instigation procedures (Kudryavtseva *et al.* 2014; Takahashi *et al.* 2015) show escalated aggression levels that are sufficient for investigating the underlying neural mechanisms. However, these procedures generally require weeks of repeated instigated resident-intruder pairings, which have also been shown to induce anxiety (Kudryavtseva *et al.* 2004), making the delineation between appetitive and reactive aggression challenging. Therefore, we generated D1-Cre and D2-Cre transgenic mice crossed on a CD-1 background. In the F1 progeny, we observed levels of unconditioned aggression nearly identical to outbred CD-1 mice, suggesting these hybrid strains may be compatible with the aggression CPP procedure. Future studies will determine if this is the case and open up genetically defined lines of research for studying the neural mechanisms of aggression behaviors. Additionally, the resemblance of unconditioned aggression behavior in the F1 hybrid progeny to CD-1 mice raises the possibility of dominant inheritance pattern of this behavior. The mechanism of inheritance and genes responsible for modulating aggression will be an interesting direction for future studies.

Importantly, even in the CD-1 strain ~25% of the mice did not show aggressive behavior (Non-aggressive phenotype) and ~25% only developed aggression after repeated encounters with an intruder (the Variable aggressive phenotype). Surprisingly, in Exp. 1 and to a lesser degree in Exp. 2, and in our previous study (Golden *et al.* 2016), the Non-aggressive mice developed some level of CPA to the intruder-paired side, suggesting that for this phenotype a forced social interaction with another mouse can be aversive. This observation highlights the importance of screening mice for unconditioned aggression in studies on appetitive aggression reward. These individual differences might account for the variable aggression CPP response observed in the study of Martinez *et al.* (1995) who did not screen the mice for unconditioned aggression in the resident-intruder procedure. The importance of accounting for individual differences in unconditioned aggression is also illustrated in an early study of Tellegen and Horn (1972). These authors showed that unconditioned aggression in different inbred mouse strains was correlated with performance in a T-maze task in which a specific arm of the maze was paired with an intruder mouse that can be attacked. Taken together, in both inbred and outbred strains, individual variability in unconditioned or innate aggression strongly influences the development of learned aggression reward.

Another methodological issue to consider is the interaction between early life experiences in shaping later aggression behaviors. We and others have used 4–6 month-old sexually-experienced mice in aggression-related studies. Previous sexual experience has been shown to promote aggressive behaviors in mice (Levine *et al.* 1965), and therefore may also contribute to aggression reward. Additionally, ~20–30% of ventrolateral VMH neurons that were activated during unconditioned aggression were also activated during mating, suggesting a potential biological basis for the relationship between sexual experience and aggressive behavior (Lin *et al.* 2011). However, the mice we used in the current experiments all had equal continuous access to receptive females from 1 month of age, and were all successful breeders, suggesting that variations in sexual history is not a confounding variable in our study.

Another potential intervening factor is the housing conditions. In this regard, there is evidence that social isolation can escalate aggressive behavior (Da Vanzo *et al.* 1966). However, in our study all CD-1 mice were housed individually after they arrived to our animal facility and therefore, like prior sexual experience, this factor cannot account for the individual differences in aggressive behavior.

Contrary to our prediction, we found that the aggression CPP phenotype did not predict sensitivity to punishment-induced suppression of food-reinforced responding. Recent studies have shown that most rats will voluntarily choose highly palatable food over methamphetamine (Caprioli *et al.* 2015a) and cocaine (Lenoir *et al.* 2007). There is also evidence that rats trained to lever-press for the same palatable food used in our study are more resistant to punishment than rats trained to self-administer methamphetamine under extended daily drug access conditions (Krasnova *et al.* 2014). Thus, it is possible that the high rewarding value of the food under our experimental conditions masked any potential aggression-related individual differences in

punishment-induced suppression of operant responding. A question for future research is whether individual differences in operant aggression self-administration are associated with punishment-induced suppression of the same operant response.

Finally, we used a footshock-based punishment procedure. In rodents, footshock has been shown to elicit unconditioned aggression (Azrin *et al.* 1967; O'Kelly & Steckle 1939) and Pavlovian conditioned aggression responses to a footshock-paired tone (Vernon & Ulrich 1966). Therefore, it is possible that the punishment procedure used in our study masked individual differences in sensitivity by escalating aggression-related behavior. However, work in non-human primates (Azrin 1970; Ulrich *et al.* 1969) and rats (Baenninger & Grossman 1969; Roberts & Blase 1971) have shown that mechanical pain or shock-induced aggression is suppressed by contingent, but not non-contingent, shock punishment. Future studies using non-shock punishments for suppression of operant responding may clarify the potential contribution of general punishment responses from footshock-induced responses, within the context of aggressive behavior.

Concluding remarks

We used our recently developed aggression CPP procedure to study the stability and persistence of aggression CPP and whether individual differences predict resistance to punishment in male CD-1 mice. Our results demonstrate that aggression CPP is both stable and persists for several weeks but does not predict resistance to punishment of an operant response previously maintained by a palatable food reward. These findings extend previous reports on the appetitive nature of aggression in mice and lay a foundation for exploring the neuropharmacological mechanisms underlying persistent aggression reward.

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Acknowledgments

Research was supported by the National Institute on Drug Abuse, Intramural Research Program (Y.S.), PRAT 1F12GM117583-01 (S.A.G.) and NIH grant R01MH090264 (S.J.R.). The authors declare that they do not have any conflicts of interest (financial or otherwise) related to the data presented in this manuscript.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1: Further characterization of aggression CPP/CPA in Exp. 1 and 2. (a) Subtracted CPP (Intruder-paired chamber on Test – Intruder-paired chamber on Pretest). (b) Normalized CPP (Intruder-paired chamber on Test/Intruder-paired chamber on Pretest). (c) Center chamber duration. All data are represented as mean ± SEM. *Different from Non-aggressive during the CPP test sessions, $P < 0.05$.