Compulsive Addiction-like Aggressive Behavior in Mice

Supplementary Information

Content

Supplemental Methods and Materials

Subjects

Our subjects were male ~40 g 4-6-month-old sexually-experienced CD-1 mice (n=146, Charles River Labs, CRL). We confirmed with CRL animal-facility staff that all the sexually experienced CD-1 males had equal access to receptive females. CRL's procedure, referred to as harem breeding, is to pair-house the males with several females from PD28 until purchase. Pregnant females are switched with new non-pregnant females, with no break between cycles. Males that do not successfully breed are removed from the breeding pool and not made available for purchase.

For intruders, we used male 8-12-week-old sexually-naïve subordinate C57BL/6J (C57) mice, primarily due to their well-established ethological characterization as subordinate to CD-1 mice in chronic social defeat stress (1-3). Additionally, aggressive CD-1 residents form a conditioned place preference to C57 intruder-paired contexts where aggression has occurred (4), and this preference persists for several weeks (5), suggesting aggression between these two strains is reinforcing for dominant CD-1 mice.

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) in standard clear-polycarbonate cages covered with stainless-steel wire lids, and we maintained them on a reverse 12-h light/dark cycle (light off at 0800 am). We group-housed the non-experimental C57

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.

Apparatus

We trained and tested all mice in standard Med Associates operant chambers. Each chamber was enclosed in a ventilated sound-attenuating cubicle and illuminated by one of two houselights, each positioned above two retractable levers on opposite sides of the chamber. These two retractable levers were designated “active” and a third non-retractable lever was designated “inactive”; all levers were positioned 2.4 cm above the grid floor. Presses on one active lever (only extended during food self-administration or choice testing) resulted in delivery of 20-mg food pellets and a 2-s light cue (bright yellow LED), while presses on the other, oppositely positioned active lever (only extended during aggression self-administration or choice testing) resulted in the delivery of a subordinate male C57 intruder and a 2-s tone cue (2900 Hz, 20 dB above background). For Exp. 1, we placed the intruder manually within the chamber through the main side panel. For all other experiments, we presented the intruder through an automatic guillotine-style door adjacent to the active lever. We connected the grid floor of each chamber to a shock generator and manually calibrated the current using an ampmeter prior to each punishment session.

To facilitate intruder presentation in Exp. 2 and 3, we attached a custom-made 3D-printed two-level intruder chamber to each operant behavior box; the chamber housed the intruders during self-administration sessions. Each level within the chamber contained one male subordinate C57 intruder, such that the intruder on the lower level was always immediately available to the resident upon successful completion of the operant response. Upon completion of the reinforcement-schedule requirement and presentation of the conditioned tone cue, the automatic guillotine door opened vertically for 10-s and we guided the lower-level intruder into the operant box via a sliding rear wall, which also prevented either the resident or intruder

mouse from moving back into the intruder chamber while the automatic door was open. After the door closed, the second, upper-level intruder was loaded into the emptied lower level through a sliding floorboard in preparation for the next trial. We removed intruder mice from the operant box through the side door of the main operant chamber.

Experimental procedures

Aggression self-administration

The aggression self-administration procedure is based on previous studies (6-10), with some modifications. First, prior to any training sessions for operant access to aggression, we gave the CD-1 residents one 30-min session of food-magazine training and then one or two 60-min sessions of food-self-administration training on a fixed-ratio 1 (FR1) reinforcement schedule. For those sessions, we used a food-paired discriminative house light, a food-paired conditioned stimulus, and an active lever that were all different from those used for aggression self-administration training (see below).

The next day, we gave the CD-1 resident mice three 5-min “magazine” training sessions for access to aggression, separated by 1-h each, in their operant chamber. Each session began with the presentation of the aggression-paired house light followed 10-s later by both a 2-s tone cue and the immediate insertion of a C57 intruder mouse; the house light remained on for the duration of the session to serve as an aggression-paired discriminative stimulus for the resident mouse. Next, we trained the CD-1 mice to self-administer access to an intruder during nine 80-min daily sessions (see specific experiments below), using a discrete-trial design. Each 80-min session included twenty 4-min trials, schematically detailed in Figure 1A. The onset of the trials was signaled by the illumination of the aggression-paired houselight, followed 10-s later by the insertion of the aggression-paired active lever; we allowed the resident CD-1 mice a maximum of 60-s to press the active lever on an FR1 reinforcement schedule before the lever automatically retracted.

Successful lever presses resulted in retraction of the active lever, followed first by a discrete 2-s tone cue and then the opening of the automatic guillotine door, through which an intruder was presented (except for Exp.1, where we put the intruder into the operant chamber manually). The aggression-paired house light remained illuminated for 130-s, such that it terminated 120-s after the insertion of the active lever. We allowed the resident mice access to the intruder either until the first attack bout was initiated or until the house light turned off, at which point we removed the intruder through the main chamber door. We randomized the intruder mice across blocks and days such that no consecutive lever-presses were reinforced with the same intruder. After the termination of the aggression-paired house light, a 110-s inter-trial interval elapsed before the start of the next trial. We recorded the number of successful trials, the latency for active-lever press, the number of inactive-lever presses, and whether a successful trial culminated in an attack bout by the resident. We trained two independent observers to identify attack behavior, using previously operationalized metrics (4, 5).

Food self-administration

The self-administration procedure for food was similar to the one for aggression block (trial)-training, with the following exceptions. First, active lever-presses under the FR1 reinforcement schedule led to the delivery of two 20-mg “preferred” palatable food pellets (TestDiet, Catalogue #1811142, 12.7% fat, 66.7% carbohydrate, and 20.6% protein) (5); pellet deliveries were paired with a 2-s discrete light cue. Second, prior to the trial-design training sessions for food, we gave the mice a 30-min magazine-training session and 1-2 once-daily 1-h sessions in which food was obtainable under an FR1, 20-s timeout reinforcement schedule. For magazine training, we delivered 2 pellets noncontingently every 120-s, paired with a 2-s discrete light cue. The session began with the illumination of the food-paired house light followed 10-s later by the first pellet delivery and the discrete light cue; the food-paired house light remained on for the duration of the session to serve as a discriminative stimulus for the palatable food. At the end of the session, the house light was turned off. For the training sessions with the 1-h FR1, 20-s timeout

schedule, we delivered a single 20-mg food pellet paired with a 2-s discrete light-cue for each reinforced lever press. The sessions began with the illumination of the food-paired house light followed 10-s later by the insertion of the food-paired active lever. The food-paired house light was turned off and active lever retracted at the end of the session. We used the "preferred" TestDiet pellet type, because in preference tests, both mice (5) and rats (11, 12) prefer this pellet over pellets with different compositions and flavors. Furthermore, rats strongly prefer these pellets over intravenous methamphetamine or heroin (13, 14). These pellets allow for efficient acquisition of food self-administration without any food deprivation.

Punishment-induced suppression of aggression self-administration

The punishment procedure is based on our previous studies with palatable food in mice (5), and with palatable food, alcohol, and methamphetamine in rats (15-18). For Exp. 2, after our standard procedures for self-administration of aggression, the punishment phase consisted of ten daily 80-min aggression self-administration sessions over 14 d. We used the same trial design used during the aggression self-administration training (Figure 2A). For each consecutive pair of punishment sessions, we increased the shock intensity by 0.1 mA, starting at 0.1 mA up to 0.4 mA. For the final 4 sessions, we maintained shock intensity at 0.4 mA. During punished self-administration, 50% of the aggression-reinforced lever-presses resulted in a 0.5-s footshock, delivered through the grid floor. Based on the degree of suppression observed in Exp. 2, for Exp. 3 we performed 3 sessions with 0.1 mA, 2 sessions at 0.2 mA, and 2 sessions at 0.25 mA.

Choice-based voluntary (self-imposed) suppression of aggression self-administration

The voluntary abstinence/suppression procedure is based on the discrete-choice task (food versus drug reward) previously developed in rats (13, 19). We conducted the discrete-choice sessions using the same parameters (intruder access, number of palatable food pellets per reward, stimuli associated with the two retractable levers) that we used during the training phase. Each choice trial began with the presentation of the discriminative stimuli for both

palatable food and aggression, followed 10-s later by the insertion of both palatable food- and aggression-paired levers. Mice then had to select one of the two levers. If mice responded within 60-s, they received the reward corresponding with the selected lever; delivery was signaled by the conditioned stimulus for aggression or food (tone or yellow cue light, respectively), the retraction of both levers, and the turning off of the non-selected house light (discriminative stimulus). If a mouse failed to respond on either active lever, both levers were retracted and their associated discriminative stimuli were turned off with no reward delivery. Trial design is shown schematically in Figure 3B.

Test for progressive-ratio reinforcement

We conducted all progressive-ratio tests using the same parameters we used for self-administration training, except for the trial design. Specifically, each progressive-ratio session began with the illumination of the appropriate house light (aggression or food, respectively) and, 10-s later, the extension of the appropriate active lever. When the mouse met the criterion for a reinforced response, the active lever retracted and the 2-s cue was activated, followed by reward delivery. For aggression progressive ratio, the automatic door was opened for 10-s and the intruder was presented. Immediately after the automatic door closed, the active lever was re-extended. We removed the intruder mice immediately after an attack or after 30-s of access. For food progressive ratio, all parameters were identical with the exception that the active lever re-extended 10-s following delivery of food pellets. The progressive-ratio session was terminated if no reinforced responses occurred for 30-min or if a total duration of 2-h had elapsed. During the sessions, we increased the ratio of responses per rewards (food pellets or intruder access, respectively) per the following sequence: 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, etc. (20). The final completed response ratio represents the breakpoint value.

Tests for relapse to aggression seeking during extinction

We tested all mice for relapse to aggression seeking (operationally defined as active-lever responding under extinction conditions) in 30-min test sessions. The aggression-paired house light (discriminative stimulus) signaled the start of the session. The aggression-paired active lever was inserted 10-s later. Active-lever presses caused the delivery of the aggression-paired conditioned cue, with a 10-s timeout period between cue presentations, but no aggression encounter. At the end of the 30-min session, the active lever retracted and the house light was turned off.

Cluster analysis and subject assignment (Exp. 3)

We chose the following 5 behavioral measures for cluster analysis: 1) attacks: mean number of attack trials across the last 4 days of self-administration training; 2) relapse: number of active-lever presses on the first relapse test (either day 1 or day 15); 3) aggression choice: mean number of aggression-trial choices across the last 4 days of choice testing; 4) aggression progressive ratio: mean number of aggression rewards obtained across the 3 days of progressive ratio testing; and 5) resilience ratio: the ratio between the number of successful trials on the last day of punishment (shock level = 0.25 mA) and the first day of punishment (shock level = 0.0 mA), and thus a measure of resistance to punishment. Before clustering the data, we excluded two mice that were more than 2.5 standard deviations/3 median absolute deviations from the 5-dimensional centroid/medoid of the sample, leaving a reduced sample of 58 mice (aggressive, n=41; non-aggressive, n=17).

We then used an SPSS classification procedure, TwoStep clustering, both to determine the number of clusters in the dataset (2 clusters, as indicated by the Bayesian Information Criterion, BIC) and to assign every mouse to one of the 2 clusters. We chose to use relapse and resilience ratio (resistance to punishment) as the variables for two-step clustering because 1) relapse propensity (presumably reflecting persistent craving) and disregard of adverse consequences are cardinal features of addiction, and 2) clustering routines will perform better on small sample sizes with reduced dimensionality (in our case, n=41 observations).

We then validated the initial TwoStep cluster assignments with an independent secondary cluster analysis in MATLAB (MathWorks). We used a hierarchical agglomerative algorithm (Ward's method) on all five behavioral measures and a different criterion (Calinski-Harabasz) to optimize for cluster number. This secondary analysis also determined an optimal partition of 2 clusters, and cluster assignments between TwoStep and hierarchical clustering differed by only one mouse (Normalized Mutual Information (NMI)=0.84, $p<0.0001$ as determined by a null distribution of NMI values from randomized pairs of cluster-membership variables with 10,000 permutations).

To aid in visualization of the cluster analysis results, we presented the data as a principal components projection. For the principal components analysis, we centered and scaled the data to zero mean and unit variance. The components remained unrotated, and the data were projected onto the first three components to enable their visualization in a 3-dimensional space.

Statistical analysis

For statistical analyses other than the cluster analyses, we used ANOVAs or independent t-tests using SPSS (GLM procedure) or Prism. When we obtained significant main effects and interaction effects ($p<0.05$, two-tailed), we followed them up 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 do not describe them in the Results section. We indicate p values for values that are less than 0.001 as $p<0.001$ and provide exact p values for values smaller than 0.05 and greater than 0.001. In Table 1 we provide a complete report of the statistical results.

Table S1. Statistical analysis (SPSS GLM module). Partial Eta^2 = proportion of explained variance. PR: progressive ratio.

Figure number	Factor name	F-value	*p*-value	pEta^2
Figure 1B. Reward trials	Session (within)	$F_{8,200}$=10.3	<0.001*	.29
Figure 1B. Attack trials	Session (within)	$F_{8,200}$=10.1	<0.001*	.29
Figure 1C. Relapse	Abstinence day. (between) Lever (within) Abstinence day x Lever	$F_{1,24}$=1.2 $F_{1,24}$=97.4 $F_{1,24}$=0.9	0.28 <0.001 0.36	.05 .80 .04
Figure 2B. Reward trials	Session (within)	$F_{8,240}$=9.7	<0.001*	0.24
Figure 2B. Attack trials	Session (within)	$F_{8,240}$=6.6	<0.001*	0.18
Figure 2C. Reward trials	Session (within)	$F_{6,180}$=234.1	<0.001*	.89
Figure 2C. Attack trials	Session (within)	$F_{6,180}$=82.3	<0.001*	.733
Figure 2D Cohort 1 Relapse	Cohort 1: Test day (within, 1, 15, 35 Cohort 1: Lever (within) Cohort 1: Lever x Test day (interaction)	$F_{2,30}$=10.1 $F_{1,15}$=5.9 $F_{2,30}$=6.2	<0.001* 0.03* 0.006*	0.40 0.28 0.29
Figure 2D Cohort 2 Relapse	Cohort 2: Test day (within, 15, 35) Cohort 2: Lever (within) Cohort 2: Lever x Test day (interaction)	$F_{1,14}$=29.2 $F_{1,14}$=12.7 $F_{1,14}$=27.3	<0.001* 0.003* 0.001*	0.68 0.48 0.55
Figure 3C. Food rewards	Session (within)	$F_{8,336}$=23.2	<0.001*	.36
Figure 3C. Reward trials	Session (within)	$F_{8,336}$=9.0	<0.001*	.18
Figure 3C. Attack trials	Session(within)	$F_{8,336}$=3.7	<0.001*	.08
Figure 3C. Choice	Reward-type (within) Session (within) Session x Reward type (within)	$F_{1,42}$=260.1 $F_{9,378}$=1.8 $F_{9,378}$=4.3	<0.001* 0.074 <0.001*	.86 .04 .09
Figure 3D. Between-subject Relapse	Lever (within) Abstinence condition (between) Lever x Abstinence condition (interaction)	$F_{1,15}$=63.8 $F_{1,15}$=0.8 $F_{1,15}$=0.7	<0.001* 0.39 0.41	.81 .05 .05
Figure 3D. Within-subject Relapse	Lever (within) Abstinence day (within) Lever x Abstinence day (interaction)	$F_{1,25}$=118.2 $F_{1,25}$=6.8 $F_{1,25}$=6.0	<0.001* 0.015* 0.022*	.83 .21 .19
Figure 3E Progressive ratio	Reward-type (within) Session (within) Reward-type x Session (within)	$F_{1,42}$=14.8 $F_{1,42}$=0.04 $F_{2,84}$=0.3	<0.001* 0.96 0.74	.26 .00 .01
Figure 3F. Punishment	Shock (within)	$F_{7,294}$=30.0	<0.001*	.42
Figure 4F. TwoStep cluster means by measures	4-day attack average Relapse Aggression choice Aggression PR rewards Resilience ratio Food PR	$F_{1,40}$=10.5 $F_{1,40}$=15.8 $F_{1,40}$=6.3 $F_{1,40}$=10.9 $F_{1,40}$=45.8 $F_{1,40}$=0.3	0.002* <0.001* 0.016* 0.002* <0.001* 0.59	.21 .29 .14 .22 .54 .01

Table S2. Comparison of cluster means with univariate ANOVAs (SPSS, GLM Procedure). Asterisks (*) indicate significant between-subjects effects with $p < 0.05$. Red cells indicate features used for clustering in the respective algorithms. PR: progressive ratio; ns: not significant.

Clustering Algorithm							
TwoStep (SPSS)	Original measures					Auxiliary measures	
Typical aggressive (n=30)	**Attacks**	**Relapse**	**Aggression choice**	**Aggression PR**	**Resilience ratio**	Reward trials	Food PR
MEAN SEM	10.3 0.8	128.1 11.7	2.1 0.4	10.6 0.7	0.44 0.05	14.9 0.8	14.2 0.5
Compulsive aggressive (n=11)	**Attacks**	**Relapse**	**Aggression choice**	**Aggression PR**	**Resilience ratio**	Reward trials	Food PR
MEAN SEM	15.1* 0.9	219.6* 20.9	3.7* 0.5	15.1* 0.9	1.03* 0.07	17.3* 0.6	14.8^{ns} 1.1
Hierarchical (MATLAB)	Original measures					Auxiliary measures	
Typical aggressive (n=29)	**Attacks**	**Relapse**	**Aggression choice**	**Aggression PR**	**Resilience ratio**	Reward trials	Food PR
MEAN SEM	10.2 0.8	129.3 12.0	2.0 0.4	10.5 0.8	0.44 0.05	14.8 0.8	14.1 0.5
Compulsive aggressive (n=12)	**Attacks**	**Relapse**	**Aggression choice**	**Aggression PR**	**Resilience ratio**	Reward trials	Food PR
MEAN SEM	15.1* 0.8	209.0* 21.8	3.8* 0.4	15.0* 0.9	1.00* 0.07	17.4* 0.6	15.0^{ns} 1.0

A Eigenvalues

Principal Component	Eigenvalue	% Variance Explained
1	2.32	47.6
2	0.96	19.7
3	0.70	14.4
4	0.54	11.1
5	0.35	7.2

B Scree-plot

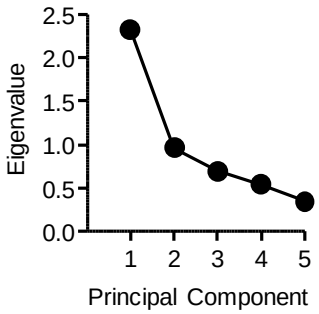

Figure S1. *Eigenvalues and variance of principal components*. (**A**) Table displaying individual eigenvalues and proportions of variance explained by the principal components decomposition of the data (n = 41; data were centered to zero mean and scaled to unit variance prior to PCA). Principal components are sorted in order of decreasing eigenvalue. (**B**) Scree-plot of eigenvalues against their respective principal components.

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