Physiology & Behavior 149 (2015) 107–118

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Individual differences in anticipatory activity to food rewards predict cue-induced appetitive 50-kHz calls in rats

Juan C. Brenes [a,b,c,*], Rainer K.W. Schwarting [c]

[a] Institute for Psychological Research, University of Costa Rica, Rodrigo Facio Campus, 2060 San Pedro, Costa Rica
[b] Neuroscience Research Center, University of Costa Rica, Rodrigo Facio Campus, 2060 San Pedro, Costa Rica
[c] Behavioral Neuroscience, Experimental and Biological Psychology, Philipps-University of Marburg, Gutenbergstr. 18, 35032 Marburg, Germany

HIGHLIGHTS

- Individual differences in anticipatory activity to food predict cue-induced USVs.
- Re-exposition to reward cues elicits USVs and invigorated appetitive behaviors.
- Reward-experienced rats show behavioral cross-tolerance on amphetamine-induced USVs.
- Rats prone to attribute incentive salience to cues respond weakly to DAergic drugs.
- Prone rats still emit USVs to food cues even after being totally sated.

ARTICLE INFO

Article history:
Received 15 July 2014
Received in revised form 13 May 2015
Accepted 15 May 2015
Available online 17 May 2015

Keywords:
Incentive motivation
Pavlovian conditioning
Associative learning
Ultrasonic vocalizations
Dopamine
Amphetamine

ABSTRACT

Reward-related stimuli come to acquire incentive salience through Pavlovian learning and become capable of controlling reward-oriented behaviors. Here, we examined individual differences in anticipatory activity elicited by reward-related cues as indicative of how animals attribute incentive salience to otherwise neutral stimuli. Since adult rats can signal incentive motivation states through ultrasonic vocalizations (USVs) at around 50-kHz, such calls were recorded in food-deprived rats trained to associate cues with food rewards, which were subsequently devalued by satiation. We found that the extent to which animals developed conditioned anticipatory activity to food cues while food deprived determined the level of cue-induced appetitive USVs while sated. Re-exposure to reward cues after a free-testing period reinstated USVs, invigorated reward seeking and consumption, and again, increases in calling occurred only in animals with high levels of cue-induced anticipatory activity. Reward-experienced rats systemically challenged with the catecholamine agonist amphetamine or with the dopamine receptor antagonist flupenthixol showed attenuated responses to these drugs, especially for USVs and in subjects with high levels of cue-induced anticipatory activity. Our results suggest that individuals prone to attribute incentive salience to reward cues showed heightened reward-induced USVs which were reliably expressed over time and persisted despite physiological needs being fulfilled. Also, prone subjects seemed to undergo particular adaptations in their dopaminergic system related with incentive learning. Our findings may have translational relevance in preclinical research modeling compulsive disorders, which may be due to excessive attribution of incentive salience to reward cues, such as overeating, pathological gambling, and drug addiction.

1. Introduction

In Pavlovian experimental preparations, a localizable visual stimulus usually evokes approach and consumption behaviors directed toward the reward cue itself (for review see: [1]), whereas diffuse or non-localizable stimuli such as a tone or a testing context would instead enhance behavioral exploration [2–7]. Both types of non-contingent conditioned responses, although quite consistent, are nevertheless moderated by individual differences [1,8–11]. It has been widely demonstrated that variations in cue-induced conditioned behaviors indicate how animals attribute incentive salience to otherwise neutral stimuli [1,4,9,10,12]. From these conditioned responses, anticipatory activity in the presence of reward-related cues has also traditionally been taken as evidence of incentive motivation [4,5].

Juvenile and adult rats have a complex repertoire of ultrasonic vocalizations (USVs) which differ in their fundamental peak frequencies and in the contexts where they are usually emitted (for review see: [13]). Out of these, high-frequency calls (i.e., 50-kHz calls) are normally

* Corresponding author at: Centro de Investigación en Neurociencias, Universidad de Costa Rica, Post-Office Box ZIP code 2060, San Pedro, Costa Rica.
E-mail address: juan.brenessaenz@ucr.ac.cr (J.C. Brenes).

http://dx.doi.org/10.1016/j.physbeh.2015.05.012
0031-9384/

produced in naturalistic rewarding situations such as mating, and rough-and-tumble play, or triggered by non-naturalistic stimuli such as heterospecific play simulated by tickling [14–16], electrical stimulation of the mesolimbic dopamine (DA) and norepinephrine (NE) pathways [17], or by psychostimulatory drugs like amphetamine and cocaine [12,18–26]. The production of spontaneous [16,27,28], and reward-induced USVs is highly dependent on individual differences [29–33]. It has been consistently shown that reward-induced USVs exhibit great individual variability [25,29,30], which may rely upon differences in the way mesolimbic DAergic and NEergic systems encode information about rewards and their predicting cues [17,29,34,35]. However, the analysis of individual differences has focused on variations in the utterance of 50-kHz calls, especially using the tickling paradigm. The inherently biological background of such inter-individual variability has been demonstrated by breeding rats selectively for their levels of tickling-induced appetitive 50-kHz calls [31,34–37]. At the behavioral and neural levels, high and low callers have been compared based on diverse parameters relevant for reward, positive affect, and social behavior [14,17,31–33,38]. In this regard, subjects with high levels of 50-kHz USVs have been found to show greater reward sensitivity, as indicated by intra-accumbens and systemic amphetamine-increased calling [29,34], higher sensitization to cocaine-induced 50-kHz calls [35], and higher electrical [17] and cocaine self-administration rates [30]. However, the question of whether animals that already differ in their reward-related behaviors also show heightened appetitive 50-kHz calls has not been fully addressed. Efforts have been made toward gauging USV variability by using screening tests of exploratory activity and unconditioned anxiety [16,21,28,39], however not through the use of tests related to learning and motivation. In the present study, therefore, we asked whether individuals with high levels of conditioned anticipatory activity – elicited by food-related cues – show high rates of 50-kHz calls, especially when food rewards were devalued by satiation. We analyzed individual differences in food-deprived rats that had been trained to anticipate food rewards (normal rat chow vs. sweetened condensed milk) under certain cues (experiments 1 to 3), and in rats that had been instrumentally conditioned to access their daily feeding ration by running down a runway maze (in experiment 4). In experiment 5, rats were previously trained in the same Pavlovian conditioning paradigm as in experiments 1 to 3, and after a free-training week, they were re-exposed to food cues in order to evaluate firstly, the ability of reward cues to reinstate calling and secondly, to determine whether preceding individual differences in anticipatory activity still affect rates of USVs. Finally, reward-experienced rats were challenged with the DAergic (and NAergic) agonist amphetamine (experiment 6) or with the DAergic receptor antagonist flupenthixol (experiment 7). In these cases, reward-experienced rats were expected to show a diminished response to the particular effect of each drug, with such an effect indicating the occurrence of behavioral cross-tolerance between Pavlovian incentive learning and DAergic-related drugs [40–42]. Secondly, we asked whether the effects of these DAergic drugs on psychomotor activity and 50-kHz calls vary along with individual differences in anticipatory activity developed during previous incentive training. This assumption arises from evidence suggesting that individual differences in attribution of incentive salience to reward predicting cues are highly dependent on mesolimbic DA activity [8,9,43].

2. General materials and methods

2.1. Subjects

Adult male Wistar rats (Harlan-Winkelmann, Netherlands) served as subjects. In experiment 1, 30 experimentally naïve rats weighing 277–351 g on arrival were used. These rats were used later in experiment 4. Experiment 2 included 24 experimentally naïve rats (weight on arrival: 231–256 g), which also served as subjects in experiments 5 and 7. In experiment 3, 20 experimentally naïve rats (weight on arrival: 240–265 g) were used, which were also the subjects of experiment 6. Upon arrival all animals were housed 4–5 per cage (Macrolon type-IV) in a climate-controlled room with a 12:12 h light–dark schedule (light on at 07:00 h), where they remained undisturbed during one week before testing. Food and water were freely available unless otherwise specified. All procedures were conducted in accordance with the ethical regulations for animal experimentation at the Philipps-University of Marburg. In all experiments, animal order was counterbalanced within and across days and experiments to the fullest extent possible.

2.2. Screening cage test

Rats were screened for their levels of spontaneous USVs as recently described [26]. The test, which was conducted on two consecutive days (5 min each), consisted of recording spontaneous USVs while a given rat explored a clean cage with fresh bedding [16,21,28]. According to the number of 50-kHz calls emitted on both days, experimental groups were counterbalanced without excluding subjects.

2.3. Appetitive cage test

As recently described [44], a given rat was put into a clean cage with bedding, which was then placed on a desk under the microphone, where the recording session immediately started. Two loudspeakers (Avemaster 60 PC stereo system, Germany) connected to a personal computer were placed on either side of the cage. As the conditioned stimulus (CS), a 3-kHz tone (49.2 dB inside the cage) was used. The unconditioned stimulus (UCS) was either normal rat chow (about 20 g) or sweetened condensed milk (10% fat content diluted 1:3 in tap water, Milbona, Germany). For the reward groups, the CS predicted either the start of each daily feeding session (1.5 h access to food per day) or a 30 min-drinking time (milk). Throughout the whole experiment, reward intake took place in the same testing cage used for a given rat. During the first 120 s, animals were left undisturbed ("context" phase), then the CS was presented over another 120 s, subsequently followed by the UCS (food or milk). The overlapping CS–UCS period lasted 30 s once reward intake started. When the tone ended, the animal was allowed to continue consuming the reward for another 60 s before being transported back (in the same testing cage) to the adjacent animal room. A matched control rat was tested simultaneously in a test cage, where it received the same pairing schedule as the matched reward rat, except that food or milk was never delivered there. Afterwards, the pair of control and reward animals was brought back to the animal room and placed on a rack, with controls on odd and reward rats on even rows, so that cages from each group were never side by side. Each control rat remained in its own testing cage while the matched reward rat completed either the 1.5 h-feeding session or 30-min drinking time. At least 3 h after all controls rats had been brought back into their own group cages, namely once the night cycle entered, their 1.5 h-daily feeding session began. In the experiments using milk as reward (2 and 5) all animals were first habituated to the sweetened condensed milk for one week. During this period, control rats had milk in the evening together with their daily food, whereas reward rats had milk in the light period, coinciding exactly with the time of the day during which they would be going to be tested. In experiment 3, both during habituation and testing phases, reward rats had access to their daily food ration only in the testing room, so that the fact of being fed after a 22.5-h FD period was specially linked to this environment. Control rats remained in the testing room during the same time period as reward rats and they were fed only in the animal room hours later.

2.4. Runway maze

The apparatus was a single U-shaped runway maze constructed of black acrylic, which consisted of two arm alleys (50 cm L × 20 cm W × 24 cm H) connected by a 20 cm L corridor. The start box (40 cm L) was equipped with a guillotine door that could be manually lifted from afar using a pulley

cable. The maze was placed on a desk under a microphone held at 45 cm above the center of maze floor. At the distal wall of the goal box, a door was positioned, through which the rat could enter a cage. A second microphone was affixed at 35 cm above the center of the cage floor. The testing area was illuminated by red light (about 10 lx inside the maze) and surrounded by curtains. After handling (see Section 2.6: General procedure), habituation to the runway was begun. This consisted of taking the rats from their home cages and placing them in pairs into the start box of the maze (with the door opened) for about 15 min during three consecutive days. Afterwards, rats had access to their daily food exactly as described in the appetitive cage test. During seven days, starting from the second day of the runway habituation, animals were given a maze habituation session followed by the appetitive cage test procedure. On the next two days, both procedures were combined, that is, single animals were placed into the maze with the cage attached to it (with food for reward rats). The final training took place during 10 consecutive days and consisted of a single daily trial conducted as follows: A given rat was confined to the start box for 120 s, and during the last 60 s, a 3-kHz tone was played, which ended with opening of the door. Afterwards, rats were free to locomote between runway and cage for approximately 4 min. Control rats followed the same procedure but food was never given in the cage. The maze was thoroughly cleaned between trials and subjects with a 0.1% acetic acid solution. USVs were recorded during the entire testing period, since animals often shuttled between runway and cage.

2.5. Behavioral analysis

Locomotion (i.e., the number of cage-halves crossed with three paws, or the number of 20-cm segments crossed in the runway maze), rearing frequency (i.e., the number of upright postures sustained with hind–paws on the floor), eating or drinking times (seconds), and latencies to consume the reward (i.e., time differences between the presentation of food or milk and the first eating or drinking bout, in seconds) were manually scored from videotapes using the EthoLog 2.25 software (University of São Paulo, Institute of Psychology SP, Brazil) as previously described [44]. Fluid intake was determined by weighing bottles before and after testing.

2.6. General procedure

For all experiments, rats were handled for four days (5 min each); afterwards, two consecutive screening cage tests were conducted (see Section 2.2: Screening cage test). Subsequently, animals were counterbalanced into two groups (i.e., control and reward) and put on a 22.5-h food deprivation (FD) schedule by providing free access to their maintenance diet for 1.5 h per day, starting one week before the appetitive cage test or the habituation sessions of the runway maze. During these periods, rats were handled and weighed every other day. Unless otherwise specified, animals were food deprived (FD) from days 1 to 7, and thereafter (days 8–10) they obtained food ad libitum (FAL) in their own home cages.

2.7. Ultrasonic recording and analysis

As previously reported [21,26,28], USVs were monitored with an UltraSoundGate Condenser Microphone (CM16; Avisoft Bioacoustics, Berlin, Germany) and recorded with Avisoft Recorder 2.7 software (sampling rate: 214,285 Hz; format: 16 bit). High resolution spectrograms (frequency resolution: .488 kHz, time resolution: .512 ms) were obtained after a fast Fourier transformation (512 FFT-length, 100% frame, Hamming window, 75% time window overlap), by using the Avisoft SASLabPro 4.38 software. Two experienced observers with an inter-rate reliability over 90% manually counted the USVs off-line from the spectrograms. Exactly as recently described [26], 50-kHz calls were further classified into flat, step-calls, and trills according to their shape and peak frequency (for exemplary sonograms see Figs. 7 and 9). The latter two subtypes were also defined as frequency-modulated (FM) calls. Call subtypes were expressed as percentage of total call number. Since 22-kHz calls were only rarely and non-systematically observed they were omitted from the analysis.

2.8. Statistical analysis

Results are expressed as mean ± SEM. Based upon cumulative rearing levels (i.e., on days 1 to 7) during the context phase (i.e., first 2 min) of the appetitive cage test (in experiments 1 to 3), subjects were categorized as low rearing (LR) and high rearing (HR) rats using the split median method, as previously described [46] (for review see: [45]). This method has long been used in the field of individual differences [9,10,45,47], especially with small samples which limit the use of more sophisticated methods. We restricted the analysis of rearing to the context phase because the highest levels of anticipatory activity and USVs occurred immediately after animals entered the cage (data not shown), and because during the tone phase rearing might have been triggered by the UCS itself and not by the CS cues, since the tone was still played during the CS–UCS overlapping period that lasted 30 s once animals started eating or drinking. In experiment 4 (i.e., runway maze with a baited cage attached to it) rats were classified as high returners (sign-trackers) or low returners (goal-trackers) according to the cumulative number of maze returns back from the baited cage (i.e., on days 1 to 7). In all experiments analyzing individual differences, groups (G: controls, low, and high ranked rats) were compared with one-way ANOVA analyses followed by protected low significant difference (PLSD) *post hoc* tests, when appropriate. In experiment 5, mixed ANOVA analyses with groups (G: control vs. reward) as between-subject factor and testing days (D: days 7, 10, and 17) as within-subjects factor were computed. Bonferroni *post hoc* test was used to adjust multiple within-group comparisons. In experiments 6 (amphetamine) and 7 (flupenthixol) two-way ANOVA analyses with treatments (T: drug vs. vehicle) and groups (G: controls vs. reward rats) were computed. In the latter two experiments the 50-kHz call categories were also analyzed. There, we used mixed two-way ANOVAs with call subtype (C: flat, step-calls, and trills) as a within subject factor and treatments and groups as between subject factors followed by Bonferroni *post hoc* test, when appropriate. In all experiments, linear regression analysis (R^2) between USVs and rearing (e.g., cage test experiments), and between USVs and maze returns (runway experiment) was computed. For all statistical tests, significance was defined as $p < .05$.

3. Experiments 1–3: individual differences in rearing behavior predict cue-induced 50-kHz calls

3.1. Introduction

In these experiments animals were trained to associate cues with food rewards through Pavlovian conditioning. Here we particularly focused on how individuals differ in their ability to attribute incentive salience to otherwise neutral cues indicated by increases in anticipatory activity over FD training. Rearing was chosen since it was the behavioral parameter that consistently increased in anticipation of reward in a recent study [44], and since it seemed to be contingently and topographically related to the way food rewards were delivered (data not shown).

3.2. Methods

Experimental subjects and other procedural details were already described in the General materials and methods section. Briefly, in experiment 1 (reward group = 20, control group = 10) the tone CS signaled the start of each feeding session (i.e., 90 min access to their daily food

ration of normal rat chow), which began in the ultrasonic lab (~2 min) and ended in the animal room. In experiment 2 (reward group = 12, control group = 12), the CS now signaled access to a 30 min-drinking period of sweet condensed milk (~2 min in the cage and the remaining time in the animal room) in the reward group, whereas in the control group it signaled access to tap water. Experiment 3 (reward group = 10, control group = 10) was generally the same as experiment 1 with normal rat chow again used as reward, but contrary to there, both access to reward and the completion of the daily feeding session took place exclusively in the testing room (i.e., 90 min). For all experiments a control rat was tested simultaneously in an adjacent room, where it received the same pairing schedule as the matched reward rat, but it never had accessed to food or milk either in the cage or in the experimental room where testing took place. In experiments 1 and 2, animals were FD on days 1 to 7 and afterwards they obtained FAL in their home cages (days 8 to 10). In experiment 3, only the FD phase was conducted. Based upon cumulative rearing levels (i.e., on days 1 to 7) of the appetitive cage test (in experiments 1 to 3), subjects were categorized as low rearing (LR) and high rearing (HR) rats using the split median method. For all experiments, latencies to approach the rewards, the times spent consuming them, locomotor activity, and USVs were analyzed.

3.3. Results

In experiment 1, animals with low rearing (LR) differed from controls and high rearing (HR) rats (G: $F_{2,27} = 15.20$, $p < .0001$), which did not vary from one another (Fig. 1A). The decreased rearing activity of LR seemed to develop with time, since it was not observable on the first day of testing. In experiment 2, HR rats differed from LR and controls (G: $F_{2,21} = 13.79$, $p < .0001$), which again did not differ from each other (Fig. 2A). In experiment 3, rearing increased over days in both LR and HR subgroups (Fig. 3A) with higher increases in HR rats (D × G: $F_{6,51} = 6.58$, $p < .0001$), which consequently showed higher cumulative rearing levels than LR rats and controls (G: $F_{2,17} = 6.51$, $p < .01$). In all experiments latencies to eat and times spent eating were unaffected by individual differences in rearing behavior (Figs. 1B, 2B, and 3B) (G: all p-values > .05). Similarly, locomotor activity did not differ between LR and HR rats in experiment 1 (LR: 11.76 ± .55, mean ± SEM; HR: 13.18 ± .73; G: $p > .05$), 2 (LR: 16.15 ± 1.69; HR: 16.10 ± .27; G: $p > .05$), and 3 (LR: 10.15 ± .66; HR: 11.75 ± .91; G: $p > .05$). Regarding USVs in experiment 1, LR rats showed less USVs than controls and HR rats (Reward: $R^2 = .248$, $p < .05$; Control: $R^2 = .146$, $p > .05$; G: $F_{2,27} = 4.66$, $p < .05$), which did not differ from each other (Fig. 1C). When subsequently tested under FAL conditions, HR rats emitted more calls than the other groups (Reward: $R^2 = .278$, $p < .05$; Control: $R^2 = .699$, $p < .05$; G: $F_{2,27} = 13.88$, $p < .0001$), which did not differ from each other (Fig. 1C). Interestingly, the effect on appetitive 50-kHz calls was detected even though the previous differences in rearing behavior between LR (12.13 ± 1) and HR (13.43 ± .94) groups vanished out once the salience of the UCS was devalued by FAL (G: $p > .05$). In experiment 2 (Fig. 2C), HR rats now showed more appetitive 50-kHz calls than LR and control rats both during FD (Reward: $R^2 = .473$, $p < .01$; Control: $R^2 = .033$, $p > .05$; G: $F_{2,21} = 8.27$ $p < .002$) and FAL phases (Reward: $R^2 = .359$, $p < .05$; Control: $R^2 = .008$, $p > .05$; G: $F_{2,21} = 5.94$ $p < .01$). Interestingly, in LR rats reward-related cues were ineffective to augment calling over control levels, despite being provided with a high palatable reward (Fig. 2C). Again, differences in calling between LR and HR rats while FAL were still observed even though they no longer differed in rearing (LR: 14.05 ± 1.49, HR: 14.45 ± .64; G: $p > .05$). In experiment 3, increasing the reward density now induced higher rates of USVs and strengthened the association between anticipatory rearing and 50-kHz calls during FD as compared with experiment 1 (Fig. 3C), with HR rats showing consequently significantly more calls than LR and control rats, which did not differ from each other (Reward: $R^2 = .823$, $p < .0001$; Control: $R^2 = .114$, $p > .05$; G: $F_{2,17} = 8.07$, $p < .003$).

3.4. Discussion

In experiments 1 and 2 individual differences in conditioned anticipatory activity developed during FD predicted levels of appetitive 50-kHz calls when FAL. In the second experiment, providing animals with a highly palatable reward while FD, enhanced differences in conditioned anticipatory activity between LR and HR rats. The latter translated into higher rates of cue-induced appetitive 50-kHz calls in HR rats. Remarkably, in experiments 1 and 2 reward devaluation vanished out the differences in anticipatory rearing activity but not in the conditioned affective responses as indicated by the USV levels. The same occurred when, in experiment 3, the density of the food reward was enhanced by providing continued access to food under the same experimental cues. Again, the individual differences between HR and LR rats became larger during the deprivation period, as compared to experiment 1. Differences between LR and HR rats cannot be attributed to differences in psychomotor activity, learning, or motivation to consume the rewards, since locomotion, latencies to approach the rewards, and times spent consuming them did not vary between these subgroups.

4. Experiment 4: individual differences in sign-tracking behavior predict cue-induced 50-kHz calls

4.1. Introduction

When a discrete cue or sign is presented repeatedly in anticipation of a food reward, the cue can become imbued with incentive salience, leading some animals to approach and engage it, a phenomenon known as “sign-tracking” [8,9] (for review see: [1]). In contrast to

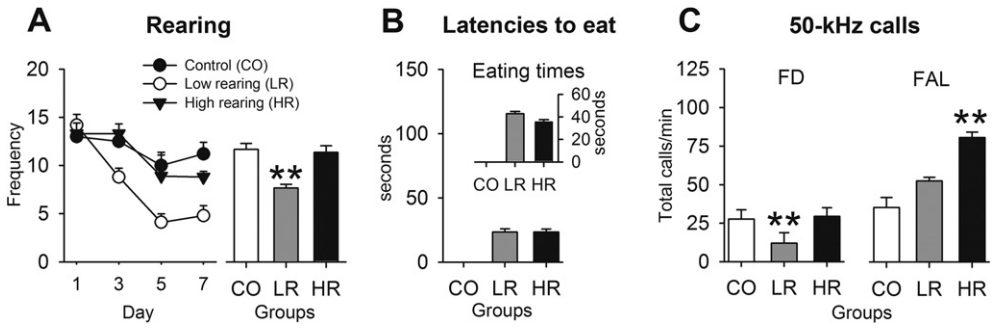

Fig. 1. Experiment 1: Effects of individual differences in anticipatory activity on reward-oriented behaviors and 50-kHz calls of animals trained to associate incentive Pavlovian cues with short access to a low palatable reward (normal rat chow). CO: controls. LR: low rearing. HR: high rearing. A. Rearing behavior. B. Latencies to eat (inset: eating times). C. 50-kHz calls. Animals were first food deprived (FD, days 1–7) and then provided with food ad libitum in their home cages (FAL, days 8–10). Bars represent cumulative values while FD unless otherwise specified. Data are expressed as mean + SEM. **$p < .01$: significant differences compared to the other two groups.

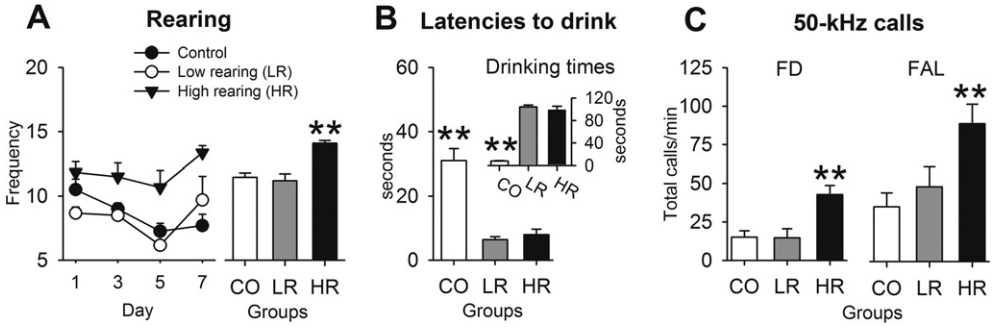

Fig. 2. Experiment 2: Effects of individual differences in anticipatory activity (rearing) on reward-oriented behaviors and 50-kHz calls of animals trained to associate incentive Pavlovian cues with short access to a high palatable reward (sweetened condensed milk). CO: controls. LR: low rearing. HR: high rearing. A. Rearing behavior. B. Latencies to drink (inset: drinking times). C. 50-kHz calls. Animals were first food deprived (FD, days 1–7) and then provided with food ad libitum in their home cages (FAL, days 8–10). Bars represent cumulative values while FD unless otherwise specified. Data are expressed as mean + SEM. $^{**}p < .01$: significant differences compared to the other two groups.

experiments 1–3, here we evaluated individual differences in instrumental behavior of reward animals which were trained to run through a runway maze to access their daily food ration in a cage attached to the end of the goal arm.

4.2. Methods

The same 30 rats used in experiment 1 served as subjects, weighing 361–440 g at the beginning of this experiment, which took place 27 days after the first experiment. Although in the runway maze there was no localizable sign-stimulus specifically paired with the UCS at which attention and behavior could be directed, we took advantage of a pattern that emerged naturally in the runway maze. There, we observed that some animals readily ran down the maze, jumped into the cage and started eating (goal-trackers, GT), whereas others reached the cage (often faster), but before and between eating bouts they repeatedly returned to explore the maze (sign-trackers, ST). This behavior gradually increased over testing days in ST subjects, even though it was unreinforced and opposed to approaching and consuming the food reward, which was only available in the attached cage. Rats were then classified according to the cumulative number of maze returns back from the baited cage while FD (i.e., on days 1 to 7) using the split median method.

4.3. Results

As shown in Fig. 4A, the behavior of returning from the food cage to the runway maze progressively increased over FD days in ST rats ($n = 10$), with GT ($n = 10$) and control rats ($n = 10$) showing about the same number of revisits, which decreased over time there (D × G: $F_{3,81} = 7.22$, $p < .0001$; G: $F_{2,27} = 10.86$, $p < .0001$). Qualitatively, it was furthermore observed that ST rats often nibbled, licked, and sniffed parts of the runway maze (data not shown), a behavioral pattern that eventually extended to the food pellets even while in the FAL phase, albeit rats were totally sated now. Out of the 20 reward rats, 8 subjects (40%) consistently displayed these behaviors and only one of them was ranked as GT ($\chi^2_1 = 7.50$, $p < .01$). In contrast, the latencies to eat (G: $p > .05$) and times spent eating (G: $p > .05$) were about the same in the ST and GT groups (Fig. 4C). In fact, ST rats entered the cage faster than GT and controls (G: $F_{2,27} = 5.71$, $p < .01$) (Fig. 4B), but they did not engage in eating faster than the GT rats (G: $p > .05$), perhaps because they used this extra time to shuttle between maze and cage. Eventually these rats came back to the cage and then spent as much as time eating as the GT rats did (G: $p > .05$). Rearing and locomotion (data not shown) reduced over days in all groups (D: rearing, $F_{3,81} = 102.23$, $p < .0001$; locomotion, $F_{3,81} = 30.46$, $p < .0001$) and at a similar rate (D × G: $p > .05$). Regarding USVs (Fig. 4D), none of the subgroups differed from controls while FD (Reward: $R^2 = .105$, $p > .05$; Control: $R^2 = .-390$, $p > .05$; G: $p > .05$). In the subsequent FAL condition, the animals that had been attracted more by the maze itself during FD (i.e., ST), were those that now called the most (Reward: $R^2 = .317$, $p < .01$; Control: $R^2 = -.112$, $p > .05$; G: $F_{2,27} = 4.98$, $p < .01$) differing from GT and controls, which vocalized at similar rates (Fig. 4D). Finally, since these rats were the same used in experiment 1, we analyzed the concordance of subjects that were ranked as high or low in both experiments (Fig. 5). We found that out of the previous 10 HR rats 6 were now ranked as ST (HR-ST), and from the 10 LR rats 6 became GT (LR-GT). Four subjects per group did not fall into the same categories (UNM: unmatched). When comparing 50-kHz calls in the runway maze among these groups

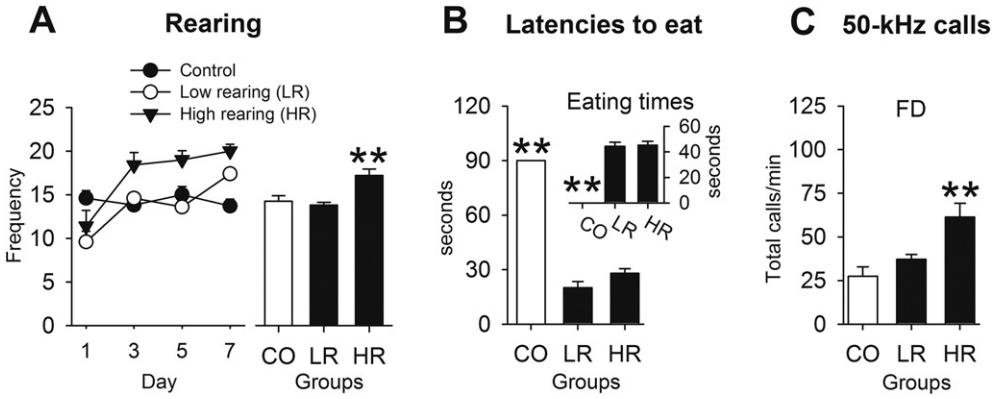

Fig. 3. Experiment 3: Effects of individual differences in anticipatory activity (rearing) on reward-oriented behaviors and 50-kHz calls of animals trained to associate incentive Pavlovian cues with long access to a low palatable reward (normal rat chow). CO: controls. LR: low rearing. HR: high rearing. A. Rearing behavior. B. Latency to eat (inset: eating time). C. 50-kHz calls. Bars represent cumulative values during food deprivation (FD, days 1–7). Data are expressed as mean + SEM. $^{**}p < .01$: significant differences compared to the other two groups.

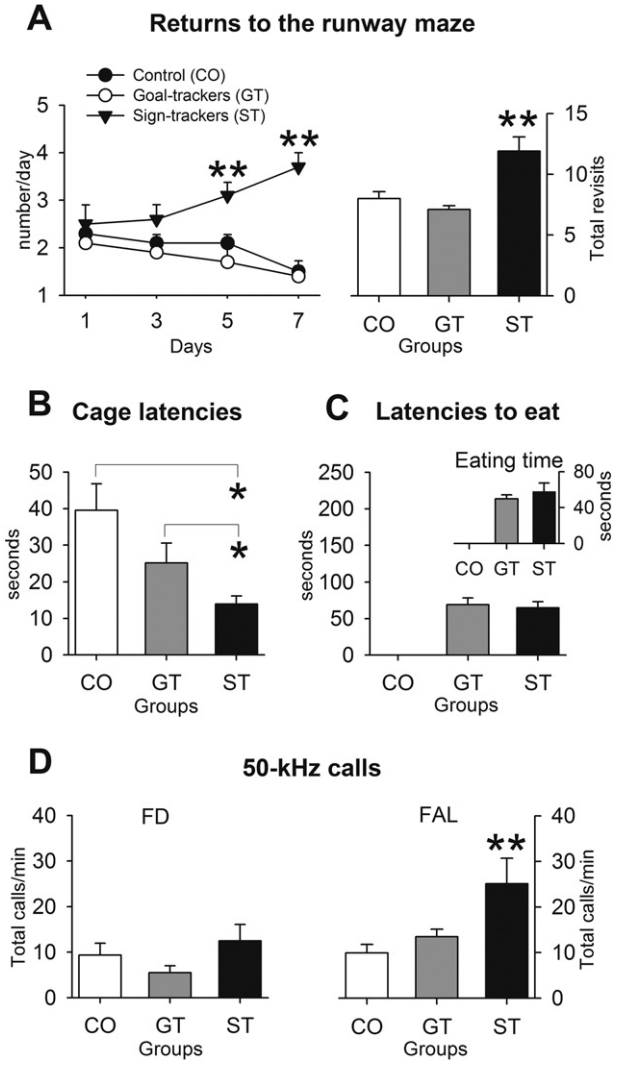

Fig. 4. Experiment 4: Effects of individual differences in sign-tracking behavior (maze returns) on reward-oriented behaviors and 50-kHz calls of animals trained to access their daily feeding ration by running through a runway maze with a baited cage attached to it. CO: controls. GT: goal-trackers (low returners). ST: sign-trackers (high returners). A. Returns to the runway maze made during food deprivation (FD, days 1–7). B. Latencies to enter the cage. C. Latencies to eat (inset: eating time). D. 50-kHz calls. Animals were first food deprived (FD, days 1–7) and then provided with food ad libitum in their home cages (FAL, days 8–10). Bars represent cumulative values on FD unless otherwise specified. Data are expressed as mean + SEM. $^{*}p < .05$: significant differences vs. ST. $^{**}p < .01$: significant differences compared to the other two groups.

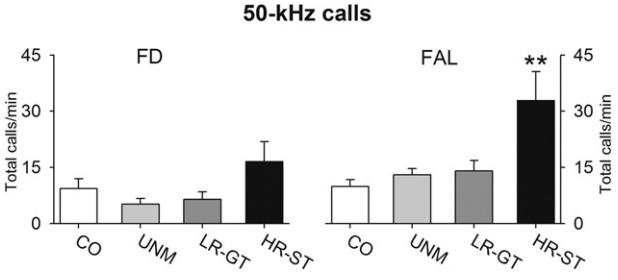

Fig. 5. Experiment 4: Fifty-kHz calls emitted in the runway maze by subjects that had initially been ranked as having low (LR) or high (HR) rearing levels in experiment 1 vs. the same subject that were further classified as being goal-trackers (GT) or sign-trackers (ST) in experiment 4. Out of the 10 HR rats, 6 were ranked as ST (HR-ST), and from the 10 LR rats, 6 were also ranked as GT (LR-GT). Four subjects per group did not fall into the same categories (UNM: unmatched). Controls (CO). Bars represent cumulative values while food deprived (FD) or when food was provided ad libitum in their home cages (FAL). Data are expressed as mean + SEM. $^{**}p < .01$: significant differences compared to the other two groups.

no significant difference was found while FD (Fig. 5), despite HR-ST rats showing descriptively more calls than the other groups (G: $p > .05$). In the FAL condition, call rate in HR-ST rats was now significantly higher than that in all other groups (G: $F_{3,26} = 7.54$, $p < .001$), which called at just about the same rate (Fig. 5).

4.4. Discussion

This experiment supports the notion that individual differences in conditioned anticipatory activity are not restricted to rearing behavior. Inter-individual variability in sign-tracking, therefore, did not derive from constitute traits in exploratory behavior, but to incentive learning. It was clearly demonstrated that when food was not provided from above, reward animals neither developed conditioned rearing, nor showed individual differences in such a parameter, with general exploratory activity rather decreasing over time in all groups. In the runway maze, certain individuals developed a sort of somehow counterintuitive, unreinforced behavior toward the cues predicting access to food, which could not be attributed to deficits in learning and motivation in ST rats, since latencies to eat and times spent eating were about the same between ST and GT rats. As in experiments 1 and 2, the ability of reward-related cues to still induce appetitive 50-kHz calls – even though appetite physiological demands were satisfied – depended on the levels of conditioned anticipatory activity previously developed when rewards were valued. Food-rewarded subjects that did not display sign-tracking behavior while FD, called at equivalent rates as control rats. Regardless of the time elapsed between experiments and the differences in the conditioning procedure, 60% of the rats were systematically ranked as low or high in experiments 1 and 4. Differences in calling became greater in high-ranked rats, whereas low-rankers and unmatched rats showed almost the same call rate as controls did. The latter finding provides evidence for within-subjects stability in attributing incentive salience to reward cues.

5. Experiment 5: re-exposition to reward cues elicited appetitive 50-kHz calls

5.1. Introduction

Here, we asked whether food cues were able to reinstate Pavlovian responding in the form of appetitive 50-kHz calls after a period without exposure to food and food-related cues. Second, we analyzed whether individual differences in anticipatory activity, developed during the acquisition phase of conditioning, were stable enough to still determine utterance of reward-related appetitive 50-kHz calls when re-exposed again to reward cues after a free testing period.

5.2. Methods

The same 24 rats used in experiment 2 served as experimental subjects, with sweetened condensed milk used as reward. As shown in Fig. 6A, before reinstatement animals underwent a 7-day training period on FD, and a 3-day period with FAL. The ability of cues to induce appetitive 50-kHz calls was determined by retesting animals on day 17, that is, 7 and 10 days after the last FAL and FD tests, respectively (Fig. 6A). The latter testing days served to compare the effect of cue-induced reinstatement on day 17. From day 10 to day 17, animals remained undisturbed in their home cages with FAL (Fig. 6A). During this period, they did not experience the rewards or their associated cues. On day 17 and after 24 h of FD, animals were re-exposed to the testing cage.

5.3. Results

On day 17 reward animals approached the milk bottles as fast as they did on days 7 and 10 (Fig. 6B) (D: $p > .05$), and spent as much as the same time drinking as they did before (D: $p > .05$) (Fig. 6C). The

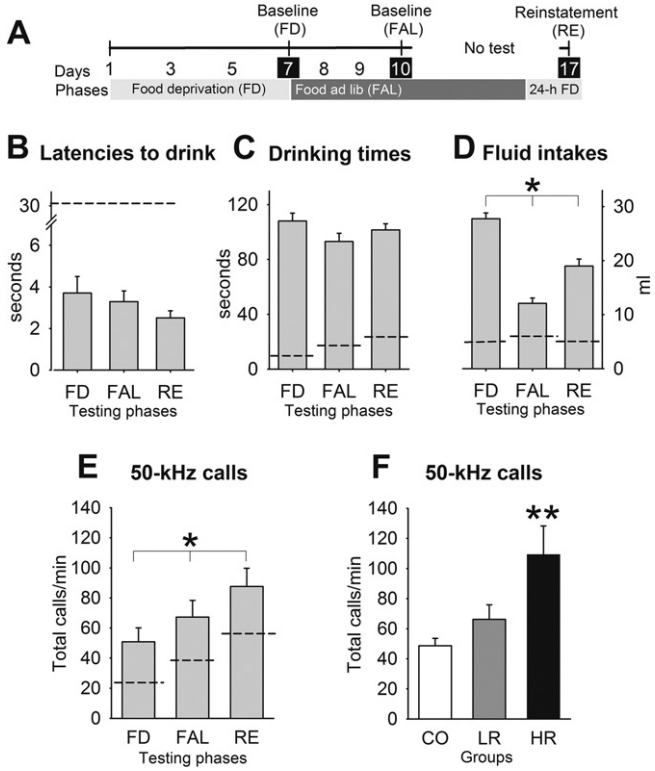

Fig. 6. Experiment 5: Re-exposition to reward cues reinstated the emission of appetitive 50-kHz calls. Cues predicted access to sweetened condensed milk as reward. FD: food deprivation. FAL: food ad libitum. RE: re-exposition. A. Schematic of experiment design. Day 7 and day 10 served as baseline to compare re-exposition on day 17. Twenty-four hours before reinstatement, animals were food deprived. B. Latencies to drink. C. Drinking times. D. Fluid intakes. E. 50-kHz calls between testing phases in reward rats. F. 50-kHz calls between groups. CO: controls. LR: low rearing rats. HR: high rearing rats. Dashed lines indicate the levels of CO group on each parameter. Data are expressed as mean + SEM. $^{*}p < .05$: significant differences among all testing phases. $^{**}p < .01$: significant differences compared to the other two groups.

amount of milk consumed (Fig. 6D), however, was lower than that on the last FD day but higher than that on the last FAL day, one week before reinstatement (D × G: $F_{2,44} = 73.03$, $p < .0001$). For all these parameters, reward rats differed significantly from controls (G: for latency, drinking time, and milk intake: $F_{1,22} = 41.94$, $p < .0001$; $F_{1,22} = 263.80$, $p < .0001$; $F_{1,22} = 235.45$, $p < .0001$). As shown in Fig. 6E, FD and re-exposition to testing cues increased appetitive 50-kHz calls (Fig. 6E) 130% and 172% over their own previous FAL and FD levels, respectively (D: $F_{1,22} = 17.97$, $p < .0001$). Also, calling on day 17 in reward rats showed an elevation of 180% over the level of controls (G: $F_{1,22} = 8.25$, $p < .009$), which showed a dishabituation-like effect in spontaneous USVs when comparing day 7 and day 17 (data not shown).

The analysis of individual differences in rearing behavior was performed exactly as in experiment 2, using the same classification (i.e., based on cumulative rearing during the acquisition phase). Again, we found that locomotor activity did not differ among groups (controls: 14.58 ± .96, LR: 17 ± 2.21 and HR: 13.67 ± 1.31; G: $p > .05$). Rearing behavior was about the same now between LR and HR rats, but controls showed less rearing than LR rats (controls: 18.75 ± 1.01, LR: 24.83 ± 2.82 and HR: 22.67 ± .84; G: $F_{2,21} = 4.35$, $p < .05$). Regarding reward-directed behaviors, LR and HR rats did not differ in the latencies to approach the milk bottles (controls: 26.12 ± 7.1, LR: 2.34 ± .38 and HR: 2.68 ± .59; G: $F_{2,21} = 5.27$, $p < .01$); however, HR rats spent less time drinking than LR animals (controls: 23.91 ± 5.09, LR: 114.17 ± 4.76 and HR: 88.75 ± 1.75; G: $F_{2,21} = 95.36$, $p < .0001$), without affecting the total amount of milk consumed (controls: 5.08 ± .48, LR: 19.5 ± 1.23 and HR: 18.5 ± 2.4; G: $F_{2,21} = 48.95$, $p < .0001$). In these parameters, both reward subgroups differed significantly from controls. As shown in Fig. 6E, reward cues elicited more 50-kHz calls in HR rats than in LR and control conspecifics (Reward: $R^2 = .344$, $p < .05$; Control: $R^2 = .008$, $p > .05$; G: $F_{2,21} = 9.07$, $p < .001$), which did not differ from each other.

5.4. Discussion

In this experiment, cues reinstated Pavlovian responding in the form of anticipatory appetitive 50-kHz calls, but also invigorated reward seeking (i.e., latencies to drink) and consumption (i.e., drinking times and to a lesser extent milk intake). Interestingly, reward cues increased appetitive 50-kHz calls over the previous FD and FAL levels. Since in this experiment animals did not receive extinction trials, the reinstatement test was assessing the ability of cues to retrieve reward representations acquired on previous FD and FAL days. The fact that the last three testing days took place while sated (e.g., FAL days) did not prevent cue-induced calling to occur on reinstatement. On the other hand, individual differences in anticipatory activity – developed during the acquisition phase of conditioning – persisted the time-out period and again, animals with high levels of anticipatory rearing behavior while FD (i.e., days 1 to 7), showed high rates of reward-induced appetitive 50-kHz calls when re-exposed to the testing cues (i.e., day 17).

6. Experiments 6–7: food reward led to behavioral cross-tolerance on amphetamine-induced appetitive 50-kHz calls

6.1. Introduction

Cross-tolerance refers to the expression of a lessened response to a treatment, even though subjects have never experienced it before [41]. Behavioral cross-tolerance has widely been demonstrated among drugs with similar mechanism of action (i.e., cocaine vs. amphetamine [48]), and among drugs and behavioral treatments that recruit similar neurochemical systems (i.e., voluntary exercise attenuating further conditioning for cocaine, morphine, or heroin [40–42,49]). In the current experiment, animals were challenged with the euphorigenic drug amphetamine. This drug strongly induces unconditioned appetitive 50-kHz calls, and these are thought to be indicative of a cathecolamine-dependent positive affective state in rats [20–26]. We anticipated that previous reward experience leads to lessened responses to the psychostimulatory and affective effects of amphetamine. Such a behavioral cross-tolerance between food and amphetamine was expected to be more pronounced in rats with higher levels of anticipatory activity displayed during the acquisition phase. In order to provide additional evidence of the involvement of the DAergic system in food cue-induced appetitive 50-kHz calls, the effects of flupenthixol, an antagonist of DA D1/D2 receptors, were also evaluated.

6.2. Methods

In the following experiments, D-amphetamine and flupenthixol (Sigma St. Louis, MO, USA) were dissolved in vehicle (0.9% NaCl) and administered ip at a dose of 2.5 mg/kg and 0.8 mg/kg, respectively. The doses and schedule of administration were chosen based on previous reports [17,21,26,50]. In experiments 6 and 7 the experimental subjects were the same rats used in experiments 3 and 5, respectively. In both experiments animals were handled and habituated to the injection needle while they continued to being tested for two consecutive days. One day before drug administration, they were injected with vehicle (0.9% NaCl) and this measure was used as a baseline. In experiment 6, animals had already learned to anticipate the delivery of their daily food ration (1.5 h access) in the testing cage from days 1 to 9 (see experiment 3). On the tenth day, D-amphetamine was administered 10 min before the test, which was conducted exactly as in previous training days. In experiment 7, animals continued to be tested after the reinstatement test (e.g., day 17 in experiment 5), and from day 21 onwards

they randomly received either flupenthixol or vehicle 30 min before testing following a Latin square design in which drug-vehicle days were separated by one drug-free testing day.

6.3. Results

As shown in Fig. 7A and B, saline levels of locomotion (G: $p > .05$) and rearing (G: $p > .05$) were about the same between reward and control groups. When given amphetamine, locomotion (T: $F_{1,18} = 20.96$, $p < .0001$) and rearing ($F_{1,18} = 30.74$, $p < .0001$) increased in both groups. These increases, however, were less pronounced in reward rats (locomotion, T × G: $F_{1,18} = 13.65$, $p < .002$; rearing, T × G: $F_{1,18} = 13.93$, $p < .002$) (Fig. 7A and B). Regarding reward consumption under amphetamine, none of the rats even approached the cage grid where the food was delivered (data not shown), which might be attributed to the well-known anorexic effect of this drug [51]. As depicted in Fig. 7C, in saline-treated animals cue-induced 50-kHz calls in reward rats were significantly higher than spontaneous calling in controls (G: $F_{1,18} = 11.56$, $p < .003$). Under amphetamine, calling increased in both groups (T: $F_{1,18} = 45.09$, $p < .0001$), and again, previous reward experience attenuated amphetamine effects, now on 50-kHz calls (T × G: $F_{1,18} = 9.10$, $p < .007$): Relative to saline, increases in 50-kHz calls in reward rats were about 200% lower than in controls (Fig. 7C). In addition to total call number, we further analyzed the 50-kHz call categories (Fig. 7D), since amphetamine has the particular ability to increase the relative number of FM calls, especially the trill subtype, an effect considered as indicative of the strong positive affective state provoked by this drug [24,25]. Under saline, the analysis of the call subtype revealed, as expected, that both groups emitted more flat than step-calls, and trills (Fig. 7D), which did not differ from one another (C: $F_{2,36} = 172.29$, $p < .0001$). Under amphetamine, the relative amount of FM calls increased in both groups (C: $F_{2,36} = 13.90$, $p < .0001$), this increase being less pronounced in reward rats, especially regarding the percentage of trills (C × G: $F_{2,36} = 7.66$, $p < .002$): In controls, trills represented ~52% of total calls, whereas in reward rats trills accounted for only ~30% of total USVs (Fig. 7D).

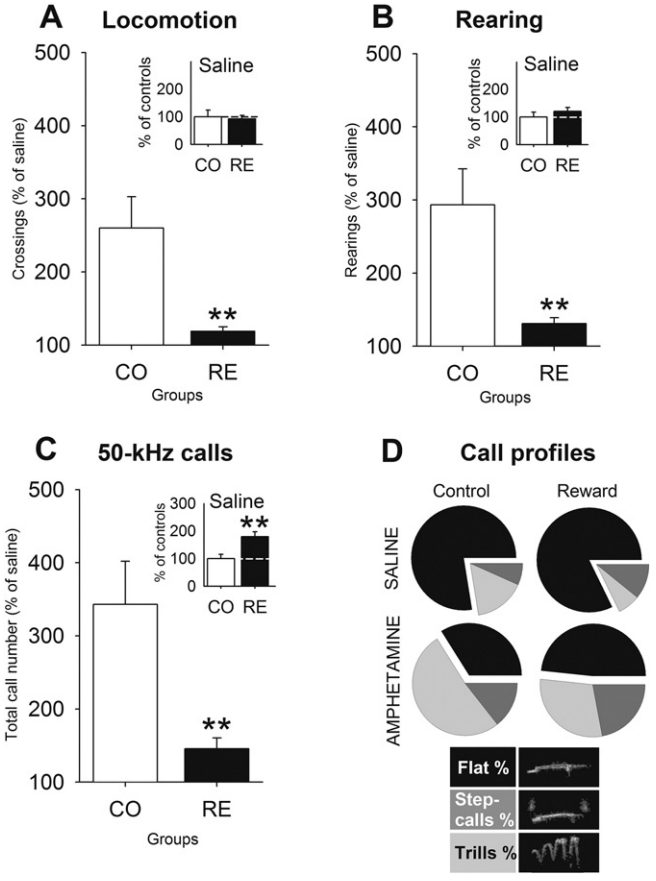

Fig. 7. Experiment 6: Behavioral cross-tolerance between Pavlovian appetitive learning and the stimulatory effects of amphetamine on psychomotor activity (A–B) and ultrasonic vocalizations (C–D). A. Locomotion (inset: locomotion under saline). B. Rearing (inset: rearing under saline). C. 50-kHz calls (inset: 50-kHz call on saline). D. Amphetamine-induced shifts in call profiles. The upper charts show the proportion of calls under saline, and the lower charts show the proportions under amphetamine. Each area represents the number of calls of a given subtype, expressed as the percentage of all 50-kHz calls. Exemplary sonograms of the three call subtypes are shown below. Data are expressed in percentages as mean + SEM. ** $p < .01$: control (CO) vs. reward (RE).

Also, we analyzed whether animals differing in the level of anticipatory activity elicited by reward cues (i.e., rearing behavior during initial training), also differed in their response to amphetamine. To this aim, the same subgroups of LR and HR rats already analyzed in experiment 3 were used here. For rearing (G: $F_{2,17} = 5.27$, $p < .05$) and locomotion (G: $F_{2,17} = 5.18$, $p < .05$), no differences were observed between LR and HR groups, which differed significantly from controls (Fig. 8A and B). In the case of USVs, HR rats showed about 65% less amphetamine-induced 50-kHz calls than LR rats, but the significance level was not reached (G: $p = .051$), perhaps due to the inter-individual variability and the rather small number of subjects per group (Fig. 8C). Despite being not significant in that group, a detailed within-group exploration revealed that the association between rearing behavior and USVs was less pronounced in HR rats ($R^2 = .348$, $p > .05$) than in LR ($R^2 = .540$, $p > .05$) and control counterparts ($R^2 = .572$, $p < .01$). Again, both reward subgroups differed significantly from controls (G: $F_{2,17} = 5.47$, $p < .05$). Since amphetamine mainly affected the trill subtype when including all reward subjects, we analyzed trills between LR and HR rats (Fig. 8D). The regression analysis indicated that there were no associations between rearing behavior and trills calls in controls ($R^2 = .003$, $p > .05$). In LR rats the coefficient was higher yet not significant ($R^2 = .351$, $p > .05$), in contrast with the strong association found in HR rats ($R^2 = .792$, $p < .05$). The averaged percentage of trill calls was significantly lower in HR rats as compared to controls (G: $F_{2,17} = 4.58$, $p < .05$), with LR rats showing no significant differences in relation to these groups (Fig. 8D).

In experiment 7, the latencies to drink (Fig. 9A), which were significantly lower in reward rats, were increased after flupenthixol administration there (G: $F_{1,44} = 7751.43$, $p < .0001$, T × G: $F_{1,44} = 4.20$, $p < .05$). However, the previous significant group differences in drinking times were not affected by the DA antagonist (Fig. 9B) (G: $F_{1,44} = 321.28$, $p < .0001$, T × G: $p > .05$). When treated with saline, 50-kHz calls (G: $F_{1,22} = 5.84$, $p < .05$), rearing (G: $F_{1,22} = 10.16$, $p < .004$), but not locomotion (G: $p > .05$) was significantly higher in reward rats as compared to controls (Fig. 9C–E). Flupenthixol led to an inhibition of locomotion (T: $F_{1,44} = 33.31$, $p < .0001$), rearing (T: $F_{1,44} = 17.29$, $p < .0001$), and USVs (T: $F_{1,44} = 20.97$, $p < .0001$) as compared to vehicle (Fig. 9C–E). Relative to the saline levels, however, locomotion and rearing appeared equally reduced in both groups (G: $p > .05$) (Fig. 9C and D), whereas the reduction in total call number was less pronounced in reward rats (G: $F_{1,22} = 5.01$, $p < .05$; Fig. 9E). On the other hand, the analysis of 50-kHz call subtypes (Fig. 9F) revealed that, as compared to saline levels, flupenthixol increased the percentage of flat calls (G: $F_{1,22} = 8.28$, $p < .009$) and reduced the percentage of FM calls (G: $F_{1,22} = 5.24$, $p < .05$) in control rats (Fig. 9F). In reward rats, conversely, percent increases in flat calls and reductions in FM calls did not reach significance (G: $p > .05$). In addition, the analysis of call subtypes under saline revealed, as expected, that both groups emitted more flat than step-calls and trills (Fig. 9F) (C: $F_{2,44} = 35.01$, $p < .0001$). Under flupenthixol, the relative amount of flat calls increased in both groups (C: $F_{2,44} = 43.83$, $p < .0001$), this increase being slightly more pronounced in controls (81%) than in reward rats (68%) (C × G: $F_{1,22} = 6.92$, $p < .002$). In controls, both step-calls and trills were reduced under flupenthixol, whereas in reward rats the trill subtype was unaffected by the DA antagonist (Fig. 9F).

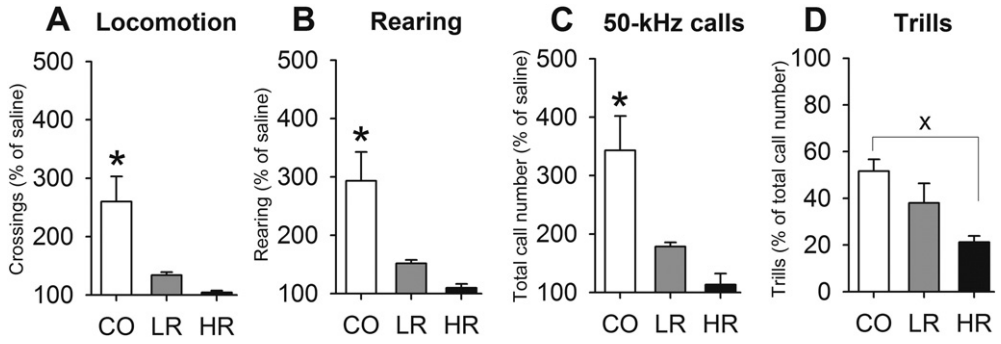

Fig. 8. Experiment 6: Effects of individual differences in anticipatory activity (rearing) on psychomotor activity (A–B) and ultrasonic vocalizations (C–D) induced by amphetamine. CO: controls. LR: low rearing. HR: high rearing. A. Locomotion. B. Rearing. C. 50-kHz calls. D. Amphetamine-induced trills calls. Data are expressed in percentages as mean + SEM. $^{*}p < .05$: significant differences compared to the other two groups. $^{X}p < .05$: significant differences between CO and HR groups.

Finally, the analysis of individual differences was again performed to determine whether the behavioral cross-tolerance of reward experience and flupenthixol varies between LR and HR rats. Here, the same classification based on cumulative rearing displayed during acquisition of conditioning (i.e., experiment 5) was used. We found no significant group differences for locomotion, rearing, and USVs (G: all *p*-values > .05) (Fig. 10A–C). As shown in Fig. 10C, both reward subgroups showed descriptively less inhibition in call rate as compared to controls.

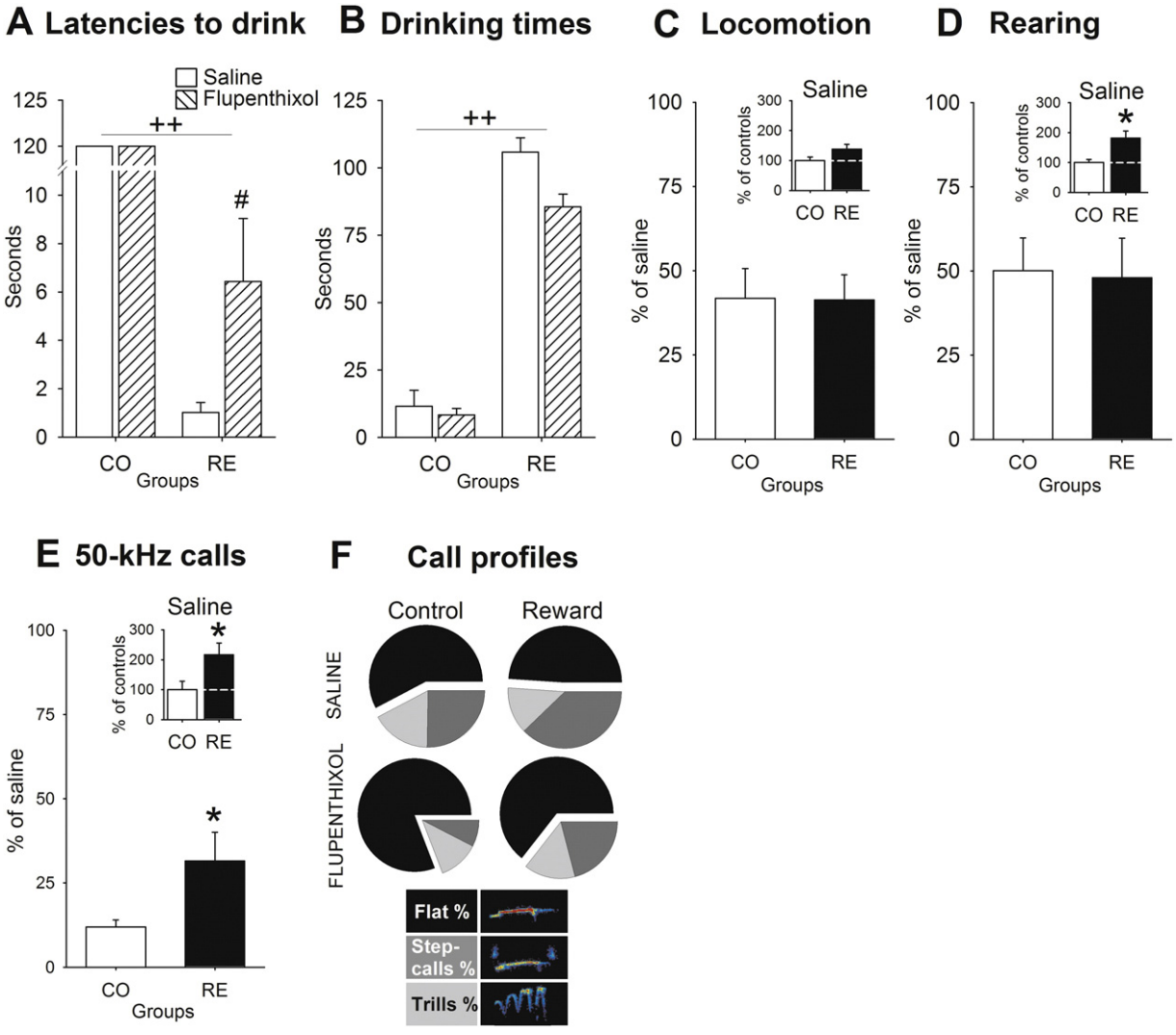

Fig. 9. Experiment 7: Behavioral cross-tolerance between Pavlovian appetitive learning and the inhibitory effects of flupenthixol on reward-oriented behaviors, exploratory activity, and 50-kHz calls. Cues predicted access to sweetened condensed milk as reward. A. Latencies to drink. B. Drinking times. C. Locomotion (inset: locomotion on saline). D. Rearing (inset: rearing on saline). E. 50-kHz calls (inset: 50-kHz calls on saline). F. Flupenthixol-induced shifts in the call profile of different 50-kHz USV subtypes. The upper charts show the proportion of calls under saline, and the lower charts show the proportion of calls affected by flupenthixol. Each area represents the number of calls of a given subtype, expressed as the percentage of all 50-kHz calls. Exemplary sonograms of call subtypes are shown below. Data are expressed in percentages as mean + SEM. $^{++}p < .01$: control (CO) vs. reward (RE). $^{*}p < .05$: CO vs. RE. $^{\#}p < .05$: saline vs. flupenthixol.

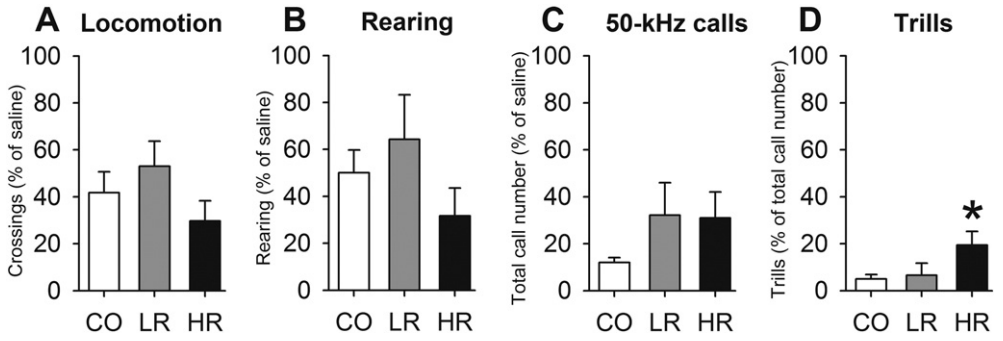

Fig. 10. Experiment 7: Effects of individual differences in anticipatory activity (rearing) on the inhibitory effects of flupenthixol on psychomotor activity (A–B) and USVs (C–D). CO: controls. LR: low rearing. HR: high rearing. A. Locomotion. B. Rearing. C. 50-kHz calls. D. Flupenthixol-reduced trill calls. Data are expressed in percentages as mean + SEM. $^{*}p < .05$: significant differences compared to the other two groups.

For the percentage of trill calls (Fig. 10D), there was an association between such calls and rearing behavior in reward rats ($R^2 = .374$, $p < .05$) but not in controls ($R^2 = .037$, $p > .05$). When comparing all groups, HR rats were found to show significantly less inhibition than both LR and control counterparts (G: $F_{1,21} = 4.02$, $p < .05$), which did not differ from each other.

6.4. Discussion

We found that previous food reward experience dampened the expected psychostimulatory effect of amphetamine both on exploratory activity and 50-kHz calls, with this effect being greater for the latter parameter. When analyzing 50-kHz call categories, the percentage of FM calls, especially the trill subtype, appeared considerably augmented after amphetamine administration in both groups. However, reward rats emitted less trills than controls. When comparing LR and HR rats, no differences in exploratory activity and in the total number of USVs were detected. HR rats, however, were more likely to emit trills but at a lower rate than controls. The administration of flupenthixol led to the opposite effects of amphetamine; that is, flupenthixol reduced exploratory activity and 50-kHz calls, and particularly diminished FM calls by proportionally increasing flat USVs. All these effects, again, were less pronounced in animals with previous Pavlovian conditioning. When analyzing individual differences within the reward group, HR rats showed less inhibition on trill calls.

7. General discussion

The analysis of exploratory activity revealed that rearing behavior appeared consistently conditioned in anticipation of food rewards [2–5]. Rearing was not a mere by-product of general psychomotor arousal induced by experimental manipulations, since locomotion remained unaffected between LR and HR rats across experiments. These individual differences in rearing behavior are consistent with the role attributed to rearing as being indicative of reward-seeking, emotionality, and reactivity to novelty [45,46]. High rearing animals have been found to be more efficient in obtaining and consuming food pellets in a radial-maze [52], and to show earlier behavioral sensitization to systemic nicotine [46]. At the neurochemical level, high rearing rats exhibit enhanced ventral and dorsal striatal DA activity as compared to low rearing counterparts [53]. These individual differences in rearing behavior appear to be quite stable in unselected male outbred Wistar rats (for review see: [45]).

In our current experiments, changes in rearing behavior paralleled those observed in USVs suggesting that they constitute two different dimensions of how attribution of incentive salience can be behaviorally expressed. Individual differences in anticipatory rearing in the cage test and in sign-tracking behavior in the runway maze observed during FD, predicted cue-induced appetitive 50-kHz calls when animals were further tested under FAL. Reducing the salience of the UCS by satiation abolished individual differences in such conditioned anticipatory responses but not in cue-induced appetitive 50-kHz calls, supporting our assumption that affective conditioned responses, such as USVs, can outlast appetitive behaviors driven by impending physiological requirements [44]. In both tests (i.e., cage test and runway maze) the ability of conditioned activity to predict appetitive 50-kHz calls cannot be attributed to constitutional individual differences either in general exploratory activity or in learning and motivation to approach and consume the reward, since locomotion, latencies to consume the reward, times spent eating and drinking did not differ between high and low ranked rats.

Re-exposing animals to the same environmental stimuli that had been previously associated with reward serves to test the ability of cues to trigger reward seeking and affective conditioned responses [11]. Incentive affective representations, in the form of 50-kHz calls, acquired on previous FD and FAL days, persisted after a period without experiencing both the food reward and its related cues. The fact that cue representations were updated while the reward was devalued by satiation (i.e., FAL days) did not prevent cue-induced calling to occur when re-exposed again to the testing environment. Before re-exposition to the cued setting animals were FD for 24 h. The fact of being hungry at the time of testing may have contributed to retrieve the hedonic valence of that particular reward (i.e., sweetened condensed milk) acquired when it was experienced under a state of need. In regard to the latter, there is evidence that physiological state changes can produce unlearned fluctuations or even independent reversals in the ability of a previously learned reward cue to trigger motivation [43,54]. For instance, a learned cue for unpleasantness (i.e., oral infusions of 9% NaCl) can become suddenly desired if the US was made physiologically necessary (e.g., after sodium depletion), despite never having been tested in such a state of depletion [55]. In our experiment reward predictability was unaltered because animals were never tested on extinction. Nevertheless, the time elapsed without being tested seemed to increase incentive motivation as the reward was now more salient. In a similar study, 50-kHz calls elicited by cues predicting access to intravenous cocaine were higher after rats were deprived from cues and cocaine during only two testing days [56]. Thus, in Maier's study [56] and in ours, re-exposition to reward cues after a free-testing period boosted 50-kHz calls. It has been found that reward uncertainty increases the intensity of Pavlovian appetitive motivation toward its predicting cues [57], which may account for the reinstatement effect seen on 50-kHz calls. If this holds true for our experiment, such an incentive motivation reaction should have been greater in individuals prone to attribute incentive salience to testing cues. Our data supported this hypothesis, since rats that had displayed high and prompted levels of conditioned anticipatory activity while FD, were those that still showed high rates of 50-kHz calls when tested again one week later under an appetite physiological state. This evidence, together with

findings of the concordance between HR and ST rats in experiments 1 and 4, suggests that individual differences in incentive learning are consistent within and between different testing conditions and over time.

On the other hand, when the DAergic system was manipulated by means of amphetamine or flupenthixol, reward rats responded as if they had developed a behavioral cross-tolerance to such drugs. It has been found that rats maintained on a high-fat diet become relatively insensitive to amphetamine reward and also fail to acquire lever-press responding for sucrose pellets, showing decreased DA turnover in the nucleus accumbens as well [58]. Other non-food based treatments like environmental enrichment and running-wheel exercise, which are rewarding for rodents, also reduce the psycho-stimulant effects of amphetamine and cocaine [40,42,59] (for review see: [60]). The cross-tolerance effect of a reward experience was also noted on amphetamine-induced increases on the relative number of FM calls and especially on trills, in agreement with reports in which reward-induced FM calls were particularly sensitive to different manipulations of the DAergic system [17,23,24]. As expected, flupenthixol impaired approach responses but not consummatory behavior coinciding with previous results in which this DA antagonist affected the motivation to but not the hedonic valuation of food [3,50,61]. Spontaneous and reward-induced calling was reduced by blocking D1/D2 receptors as previously described [17,24]. However, the ability of flupenthixol to reduce calling was attenuated by previous reward experience. Anticipatory activity, but not 50-kHz calls, was affected to the same extent in both groups suggesting that reward experience particularly affected DAergic mechanisms controlling conditioned affective reward responses (i.e., USVs), rather than general psychomotor activity. Further experiments are required to corroborate these findings. Altogether, these experiments suggest that prolonged Pavlovian incentive learning may have raised brain DA activity, which in turn may have induced a desensitization-like effect by over-stimulating DA receptors [62,63]. There is evidence indicating that DA levels appear augmented in the nucleus accumbens of animals with high Pavlovian conditioned responses [63]. In addition, rat and human studies have shown that food and other rewarding stimuli, which raise DA activity, down-regulate DA receptors [62,64,65] (for review see: [66]).

Regarding individual differences, HR rats showed a reduced percentage of amphetamine-induced trill calls as compared to controls, whereas HR rats treated with flupenthixol showed less inhibition in calling than both LR and control rats. The effect of both drugs supports the notion of trills as being the most consistent USV subtype signaling catecholamine-induced euphoric states [18,24–26]. Even though the experiments differed in training schedules and food rewards used, they led to somewhat coherent results suggesting that animals prone to attribute incentive salience to reward cues undergo particular adaptations in the mesolimbic DAergic system [9]. Regarding total call number and psychomotor activity, however, no differences between LR and HR emerged, perhaps because the doses used in our experiments were too high to discriminate between LR and HR rats. If this holds true, then these doses would have masked the effects of amphetamine on broader behavioral categories such as total 50-kHz calls, or locomotion, and rearing.

8. Conclusion

In summary, individuals prone to attribute incentive salience to reward cues, indicated by high levels of either rearing activity or sign-tracking behavior, showed heightened reward-induced affective responses, namely 50-kHz calls. When re-exposing rats to reward cues after a non-testing period, USVs were elicited even at higher rates than before, especially in prone subjects. USVs appeared reliably expressed over time and persisted despite appetite physiological needs were fulfilled. Interestingly, USVs were still elicited by reward cues even though reward-oriented behaviors and exploratory activity were drastically weakened by reward devaluation. Also, prone subjects seemed to undergo particular adaptations in their DAergic system related with incentive learning as indicated by the effects of DAergic drugs. Our findings may have translational potential, since in some individuals excessive attribution of incentive salience to reward cues may lead to compulsive behavior disorders such as overeating, pathological gambling, and drug addiction.

Acknowledgments

We thank Natalie Heyse for assistance with data collection and analysis. This work was supported by the grant Schw 559/10-1 from the Deutsche Forschungsgemeinschaft. JCB was funded by the Deutscher Akademischer Austauschdienst (DAAD, Germany), and the University of Costa Rica.

References

[1] T.E. Robinson, L.M. Yager, E.S. Cogan, B.T. Saunders, On the motivational properties of reward cues: individual differences, Neuropharmacology 76 (2014) 450–459.
[2] M.F. Barbano, M. Cador, Various aspects of feeding behavior can be partially dissociated in the rat by the incentive properties of food and the physiological state, Behav. Neurosci. 119 (2005) 1244–1253.
[3] M.F. Barbano, M. Cador, Differential regulation of the consummatory, motivational and anticipatory aspects of feeding behavior by dopaminergic and opioidergic drugs, Neuropsychopharmacology 31 (2006) 1371–1381.
[4] D. Bindra, How adaptive behavior is produced: a perceptual-motivation alternative to response reinforcement, Behav. Brain Sci. 1 (1978) 41–91.
[5] Holland PC. Origins of behavior in Pavlovian conditioning. In: Medin DL, editor. The Psychology of Learning and Motivation (vol. 18), San Diego: Academic; 1984, p. 129–74.
[6] H.M. Jenkins, B.R. Moore, The form of the auto-shaped response with food or water reinforcers, J. Exp. Anal. Behav. 20 (1973) 163–181.
[7] R.A. Rescorla, Behavioral studies of Pavlovian conditioning, Annu. Rev. Neurosci. 11 (1988) 329–352.
[8] S.B. Flagel, S.J. Watson, T.E. Robinson, H. Akil, Individual differences in the propensity to approach signals vs goals promote different adaptations in the dopamine system of rats, Psychopharmacology 191 (2007) 599–607.
[9] S.B. Flagel, J.J. Clark, T.E. Robinson, L. Mayo, A. Czuj, I. Willuhn, et al., A selective role for dopamine in stimulus-reward learning, Nature 469 (2011) 53–57.
[10] T.E. Robinson, S.B. Flagel, Dissociating the predictive and incentive motivational properties of reward-related cues through the study of individual differences, Biol. Psychiatry 65 (2009) 869–873.
[11] L.M. Yager, T.E. Robinson, Cue-induced reinstatement of food seeking in rats that differ in their propensity to attribute incentive salience to food cues, Behav. Brain Res. 214 (2010) 30–34.
[12] P.J. Meyer, S.T. Ma, T.E. Robinson, A cocaine cue is more preferred and evokes more frequency-modulated 50-kHz ultrasonic vocalizations in rats prone to attribute incentive salience to a food cue, Psychopharmacology 219 (2012) 999–1009.
[13] M. Wöhr, R.K.W. Schwarting, Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation, Cell Tissue Res. 254 (2013) 81–97.
[14] J. Burgdorf, R.A. Kroes, J.R. Moskal, J.G. Pfaus, S.M. Brudzynski, J. Panksepp, Ultrasonic vocalizations of rats (*Rattus norvegicus*) during mating, play, and aggression: behavioral concomitants, relationship to reward, and self-administration of playback, J. Comp. Psychol. 122 (2008) 357–367.
[15] J. Burgdorf, J. Panksepp, Tickling induces reward in adolescent rats, Physiol. Behav. 72 (2001) 167–173.
[16] R.K.W. Schwarting, N. Jegan, M. Wöhr, Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats, Behav. Brain Res. 182 (2007) 208–222.
[17] J. Burgdorf, P.L. Wood, R. Kroes, J.R. Moskal, J. Panksepp, Neurobiology of 50-kHz ultrasonic vocalizations in rats: electrode mapping, lesion, and pharmacological studies, Behav. Brain Res. 182 (2007) 274–283.
[18] A.M. Ahrens, S.T. Ma, E.Y. Maier, C.L. Duvauchelle, T. Schallert, Repeated intravenous amphetamine exposure: rapid and persistent sensitization of 50-kHz ultrasonic trill calls in rats, Behav. Brain Res. 197 (2009) 205–209.
[19] D.J. Barker, D.H. Root, S. Ma, S. Jha, L. Megehee, A.P. Pawlak, et al., Dose-dependent differences in short ultrasonic vocalizations emitted by rats during cocaine self-administration, Psychopharmacology 211 (2010) 435–442.
[20] J. Burgdorf, B. Knutson, J. Panksepp, S. Ikemoto, Nucleus accumbens amphetamine microinjections unconditionally elicit 50 kHz ultrasonic vocalizations in rats, Behav. Neurosci. 115 (2001) 940–944.
[21] C. Natusch, R.K.W. Schwarting, Using bedding in a test environment critically affects 50-kHz ultrasonic vocalizations in laboratory rats, Pharmacol. Biochem. Behav. 96 (2010) 251–259.
[22] N. Simola, S. Fenu, G. Costa, A. Pinna, A. Plumitallo, M. Morelli, Pharmacological characterization of 50-kHz ultrasonic vocalizations in rats: comparison of the effects of different psychoactive drugs and relevance in drug-induced reward, Neuropharmacology 63 (2012) 224–234.

[23] A.J. Wintink, S.M. Brudzynski, The related roles of dopamine and glutamate in the initiation of 50-kHz ultrasonic calls in adult rats, Pharmacol. Biochem. Behav. 70 (2001) 317–323.
[24] J.M. Wright, M.R. Dobosiewicz, P.B. Clarke, The role of dopaminergic transmission through D1-like and D2-like receptors in amphetamine-induced rat ultrasonic vocalizations, Psychopharmacology 225 (2013) 853–868.
[25] J.M. Wright, J.C. Gourdon, P.B.S. Clarke, Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context, Psychopharmacology 211 (2010) 1–13.
[26] M. Pereira, R. Andreatini, R.K.W. Schwarting, J.C. Brenes, Amphetamine-induced appetitive 50-kHz calls in rats: a genuine affective marker of mania? Psychopharmacology 231 (2014) 2567–2577.
[27] S.M. Brudzynski, A. Pniak, Social contacts and production of 50-kHz short ultrasonic calls in adult tats, J. Comp. Psychol. 116 (2002) 73–82.
[28] M. Wöhr, B. Houx, R.K.W. Schwarting, B. Spruijt, Effects of experience and context on 50-kHz vocalizations in rats, Physiol. Behav. 93 (2008) 766–776.
[29] A.M. Ahrens, C.W. Nobile, L.E. Page, E.Y. Maier, C.L. Duvauchelle, T. Schallert, Individual differences in the conditioned and unconditioned rat 50-kHz ultrasonic vocalizations elicited by repeated amphetamine exposure, Psychopharmacology 229 (2013) 687–700.
[30] J.R. Browning, D.A. Browning, A.O. Maxwell, Y. Dong, H.T. Jansen, J. Panksepp, et al., Positive affective vocalizations during cocaine and sucrose self-administration: a model for spontaneous drug desire in rats, Neuropharmacology 61 (2011) 268–275.
[31] J. Burgdorf, J. Panksepp, S.M. Brudzynski, R. Kroes, J.R. Moskal, Breeding for 50-kHz positive affective vocalization in rats, Behav. Genet. 35 (2005) 67–72.
[32] T. Mällo, D. Matrov, L. Herm, K. Kõiv, M. Eller, A. Rinken, et al., Tickling-induced 50-kHz ultrasonic vocalization is individually stable and predicts behaviour in tests of anxiety and depression in rats, Behav. Brain Res. 184 (2007) 57–71.
[33] R. Rygula, H. Pluta, P. Popik, Laughing rats are optimistic, PLoS One 7 (12) (2012) e51959.
[34] S.M. Brudzynski, M. Silkstone, M. Komadoski, K. Scullion, S. Duffus, J. Burgdorf, et al., Effects of intraaccumbens amphetamine on production of 50 kHz vocalizations in three lines of selectively bred long-Evans rats, Behav. Brain Res. 217 (2011) 32–40.
[35] P. Mu, T. Fuchs, D.B. Saal, B.A. Sorg, Y. Dong, J. Panksepp, Repeated cocaine exposure induces sensitization of ultrasonic vocalization in rats, Neurosci. Lett. 453 (2009) 31–35.
[36] J. Burgdorf, J. Panksepp, S.M. Brudzynski, M.C. Beinfeld, H.C. Cromwell, R.A. Kroes, et al., The effects of selective breeding for differential rates of 50-kHz ultrasonic vocalizations on emotional behaviour in rats, Dev. Psychobiol. 51 (2009) 34–46.
[37] K.M. Harmon, J. Burgdorf, J.R. Moskal, R.A. Kroes, J. Panksepp, H.C. Cromwell, Rats selectively bred for low levels of 50 kHz ultrasonic vocalizations exhibit alterations in early social motivation, Dev. Psychobiol. 50 (2008) 322–331.
[38] M. Wöhr, M. Kehl, A. Borta, A. Schänzer, R.K.W. Schwarting, G.U. Höglinger, New insights into the relationship of neurogenesis and affect: tickling induces hippocampal cell proliferation in rats emitting appetitive 50-kHz ultrasonic vocalizations, Neuroscience 163 (2009) 1024–1030.
[39] A. Borta, M. Wöhr, R.K.W. Schwarting, Rat ultrasonic vocalization in aversively motivated situations and the role of individual differences in anxiety-related behaviour, Behav. Brain Res. 166 (2006) 271–280.
[40] K.P. Cosgrove, R.G. Hunter, M.E. Carroll, Wheel-running attenuates intravenous cocaine self-administration in rats: sex differences, Pharmacol. Biochem. Behav. 73 (2002) 663–671.
[41] B.T. Lett, V.L. Grant, M.T. Koh, G. Flynn, Prior experience with wheel running produces cross-tolerance to the rewarding effect of morphine, Pharmacol. Biochem. Behav. 72 (2002) 101–105.
[42] M.A. Smith, K.T. Schmidt, J.C. Iordanou, M.L. Mustroph, Aerobic exercise decreases the positive-reinforcing effects of cocaine, Drug Alcohol Depend. 98 (2008) 129–135.
[43] K.C. Berridge, From prediction error to incentive salience: mesolimbic computation of reward motivation, Eur. J. Neurosci. 35 (2012) 1124–1143.
[44] J.C. Brenes, R.K.W. Schwarting, Attribution and expression of incentive salience are differentially signaled by ultrasonic vocalizations in rats, PLoS One 9 (7) (2014) e102414.
[45] C.R. Pawlak, Y.J. Ho, R.K.W. Schwarting, Animal models of human psychopathology based on individual differences in novelty-seeking and anxiety, Neurosci. Biobehav. Rev. 32 (2008) 1544–1568.
[46] C.R. Pawlak, R.K.W. Schwarting, Repeated nicotine treatment in rats with high versus low rearing activity: analyses of behavioural sensitisation and place preference, Psychopharmacology 178 (2005) 440–450.
[47] P.V. Piazza, J.M. Deminière, M. Le Moal, H. Simon, Factors that predict individual vulnerability to amphetamine self administration, Science 245 (1989) 1511–1513.
[48] W.L. Woolverton, D. Kandel, C.R. Schuster, Tolerance and cross-tolerance to cocaine and D-amphetamine, J. Pharmacol. Exp. Ther. 205 (1978) 525–535.
[49] Smith MA, Pitts EG. Wheel running decreases the positive reinforcing effects of heroin. Pharmacol. Rep. 2012;64:960–64.
[50] S.R. Laviolette, K. Nader, D. van der Kooy, Motivational state determines the functional role of the mesolimbic dopamine system in the mediation of opiate reward processes, Behav. Brain Res. 129 (2002) 17–29.
[51] J.E. Blundell, C.J. Latham, E. Moniz, R.A. McArthur, P.J. Rogers, Structural analysis of the actions of amphetamine and fenfluramine on food intake and feeding behaviour in animals and in man, Curr. Med. Res. Opin. 6 (1979) 34–54.
[52] J. Görisch, R.K.W. Schwarting, Wistar rats with high versus low rearing activity differ in radial maze performance, Neurobiol. Learn. Mem. 86 (2006) 175–187.
[53] C.M. Thiel, C.P. Müller, J.P. Huston, R.K.W. Schwarting, High versus low reactivity to a novel environment: behavioural, pharmacological and neurochemical assessments, Neuroscience 93 (1999) 243–251.
[54] Berridge KC. Reward learning: reinforcement, incentives, and expectations. In: Medin DL, editor. Psychology of Learning and Motivation (vol. 40), New York: Academic Press; 2001, p. 223–78.
[55] M.J.F. Robinson, K.C. Berridge, Instant transformation of learned repulsion into motivational "wanting", Curr. Biol. 23 (2013) 282–289.
[56] E.Y. Maier, A.M. Ahrens, S.T. Ma, T. Schallert, C.L. Duvauchelle, Cocaine deprivation effect: cue abstinence over weekends boosts anticipatory 50-kHz ultrasonic vocalizations in rats, Behav. Brain Res. 214 (2010) 75–79.
[57] P. Anselme, M.J.F. Robinson, K.C. Berridge, Reward uncertainty enhances incentive salience attribution as sign-tracking, Behav. Brain Res. 238 (2013) 53–61.
[58] J.F. Davis, A.L. Tracy, J.D. Schurdak, M.H. Tschop, J.W. Lipton, D.J. Clegg, et al., Exposure to elevated levels of dietary fat attenuates psychostimulant reward and mesolimbic dopamine turnover in the rat, Behav. Neurosci. 122 (2008) 1257–1263.
[59] M.E. Cain, M.G. Mersmann, M.J. Gill, S.T. Pittenger, Dose-dependent effects of differential rearing on amphetamine-induced hyperactivity, Behav. Pharmacol. 23 (2012) 744–753.
[60] Solinas M, Thiriet N, Chauvet C, Jaber M. Prevention and treatment of drug addiction by environmental enrichment. Prog. Neurobiol. 2010;92:572–92.
[61] S. Ikemoto, J. Panksepp, Dissociations between appetitive and consummatory responses by pharmacological manipulations of reward-relevant brain regions, Behav. Neurosci. 110 (1996) 331–345.
[62] P.H. Roseboom, M.E. Gnegy, Acute in vivo amphetamine produces a homologous desensitization of dopamine receptor coupled adenylate cyclase activities and decreases agonist binding to the DI site, Mol. Pharmacol. 34 (1988) 148–156.
[63] A. Tomie, A.S. Aguado, L.A. Pohorecky, D. Benjamin, Individual differences in Pavlovian autoshaping of lever pressing in rats predict stress-induced corticosterone release and mesolimbic levels of monoamines, Pharmacol. Biochem. Behav. 65 (2000) 509–517.
[64] P.M. Johnson, P.J. Kenny, Dopamine D2 receptors in addiction-like reward dysfunction and compulsive eating in obese rats, Nat. Neurosci. 13 (2010) 635–641.
[65] E.P. Noble, Addiction and its reward process through polymorphisms of the D2 dopamine receptor gene: a review, Eur. Psychiatry 15 (2000) 79–89.
[66] N.D. Volkow, G.J. Wang, D. Tomasi, R.D. Baler, The addictive dimensionality of obesity, Biol. Psychiatry 73 (2013) 811–818.